Forssman penta- and tetraglycosylceramide are xenoantigens of ostrich kidney and liver

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The heterophile antigens Galα1→3Gal and N-glycolyneuraminic acid are the major obstacle to grafting mammal organs, especially from pig, to man. Lack of expression of these common xenoantigens by birds has raised interest in ostrich as a potential organ donor for xenotransplantation. Glycosphingolipids of ostrich liver and kidney were investigated for their carbohydrate determinants. Both organs were found similar in their glycolipid composition with three major families, mono-, di-, and pentaglycosylceramide. The pentaglycosylceramide was characterized as the Forssman antigen. In both organs, the ceramide portion was highly hydroxylated with prevalence of α-hydroxylated fatty acids, C18 phytosphingosine in kidney and C18 sphingosine in liver Forssman glycolipid. These data indicate that hydroxylation of kidney glycosphingolipids, which is found in mammals, has been maintained since the divergence of birds from other vertebrates. Characterization of a minor glycolipid as a Forssman tetraglycosylceramide built on the galabiosylceramide core indicates that the Forssman tetraglycosylceramide also exists in vivo. Its precursors, galactosyl- and galabiosylceramide, were characterized in kidney and liver. The Forssman antigen is the third heterophile antigen against which man raises natural antibodies. Its localization in the vascular endothelium and connective tissue makes ostrich an unpromising organ or cell donor for xenotransplantation to man.

Key words: Forssman glycolipids/ostrich/xenotransplantation/electrospray-ion trap mass spectrometry/¹H NMR

Results

Thin-layer chromatography

Thin-layer chromatography of ostrich kidney neutral glycolipids displayed a very simple pattern compared to porcine kidney neutral glycolipids, with only three glycolipids in sufficient quantity to be quantified by sphingosine assay (Figure 1A, lane 2). GL-1 accounted for 63% (8 µmol per kidney), GL-2 for 25% (5 µmol per kidney), and GL-5 for 11.5% (1.5 µmol per kidney) of the total sphingosine content of the kidney glycolipid sample. The tri- and tetrahexasylceramides, major neutral glycolipids of porcine kidney (Figure 1A, lane 1), were hardly detectable. GL-4 accounted for less than 0.5% of the sphingosine content of the glycolipid sample. The chromatography profile of ostrich liver neutral glycolipids displayed the same three major glycolipids as kidney (Figure 1A, lane 3), with a more important contribution of GL-5 (30%, 7 µmol per liver), and a smaller contribution of GL-1 (50%, 11.7 µmol per liver) and GL-2 (17%, 4 µmol per liver) to the total sphingosine content of the glycolipid fraction. HPTLC-immunostaining was performed with antibodies against antigens of the human ABO blood group system, the xenogenic Galα1→3Gal epitope and the Forssman glycolipid. Only the anti-Forssman antibody was reactive with ostrich liver and kidney neutral glycolipids, at the level of GL-5 (Figure 1B, lanes 4–5). Neutral glycolipids were purified by preparative HPTLC and submitted to electrospray-ion trap mass spectro-
molecular ions $[M + Na]^+$. The MS spectrum of kidney GL-5 glycolipids in the positive ion mode are sodium adducts of ions obtained by electrospray-ion trap MS analysis of native structural characterization.

886 contained C18 phytosphingosine and three Hex (Figure 2, upper panel). The major ceramide species displayed a series of molecular ions consistent with theoretical values for an oligosaccharide chain containing two HexNAc and displayed a prevalent molecular ion (although less intense. In contrast, the MS spectrum of liver GL-5 contained C18, 22 fatty acids (m/z 1452.5), d18:1/h22:0 (m/z 1552.9), d18:1/n24:0 (m/z 1564.8), and d18:1/h23:0 (m/z 1566.9) were minor. Signals for unsaturated fatty acids were observed for d18:1/h24:1 (m/z 1578.8) and d18:1/n24:1 (m/z 1562.8).

Molecular ions were submitted to collision-induced dissociation that gave rise to ions resulting from the cleavage of the glycosidic bonds. According to the nomenclature established by Domon and Costello (1996), fragments containing the nonreduc-

**Mass spectrometry analysis**

Ions obtained by electrospray-ion trap MS analysis of native glycolipids in the positive ion mode are sodium adducts of molecular ions $[M + Na]^+$. The MS spectrum of kidney GL-5 displayed a series of molecular ions consistent with theoretical values for an oligosaccharide chain containing two HexNAc and three Hex (Figure 2, upper panel). The major ceramide species contained C18 phytosphingosine and $\alpha$-hydroxylated C22-23-24 fatty acids (m/z 1570.8, 1584.9, 1598.9, respectively), and nonhydroxylated C24 fatty acid with C18 sphingosine (m/z 1564.9). Glycolipid ions for ceramide with unsaturated fatty acid were also observed for d18:1/h24:1 (m/z 1563.0), d18:1/h24:1 (m/z 1578.7) and n18:0/h24:1 (m/z 1597.0). The ions for the ceramide species with C18 sphingosine and nonhydroxylated C16,22 fatty acids (m/z 1452.6 and 1537.0) and $\alpha$-hydroxylated C16,22 fatty acids (m/z 1468.5 and 1553.0) were unambiguous, although less intense. In contrast, the MS spectrum of liver GL-5 displayed a prevalent molecular ion (m/z 1580.8) corresponding to the ceramide species d18:1/h24:0. Ions for the ceramide species d18:1/h16:0 (m/z 1452.5), d18:1/h22:0 (m/z 1552.9), d18:1/n24:0 (m/z 1564.8), and d18:1/h23:0 (m/z 1566.9) were minor. Signals for unsaturated fatty acids were observed for d18:1/h24:1 (m/z 1578.8) and d18:1/n24:1 (m/z 1562.8).

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**Fig. 1.** HPTLC of neutral glycosphingolipids. (A) Neutral glycosphingolipids of pig kidney cortex (lane 1), ostrich kidney (lane 2), and ostrich liver (lane 3) after chemical visualization with phenol/sulfuric acid. (B) Immunostaining with anti-Forssman monoclonal antibody of neutral glycolipids of ostrich liver (lane 4) and kidney (lane 5), and purified GL-4 from ostrich kidney (lane 6). Chromatography was developed in chloroform/methanol/water (60:35:8). Numbers in the right and left margins indicate the number of carbohydrate residues. GL-4 Glycosphingolipid. F. Forssman glycolipid.
homologous to the major \( Y'' \) ion generated by collision-induced dissociation of peptides (Biemann, 1992). The complementary fatty acid ion, homologous to the B type of peptide ions, was not observed. In accordance with the fragmentation scheme, the \( Y_1 \) ion obtained by fragmentation of the F-4 molecular ion \( m/z \) 1418.7 (Figure 4) yielded a \( \psi_1 \) ion at \( m/z \) 484.1 (Figure 5, upper panel), consistent with C18 sphingosine. Fragmentation of the \( Y_2 \) ion also yielded psychosine type ions. For example, MS\(^2\) of the \( Y_2 \) ion \( m/z \) 1030.6, generated from the kidney F-5 molecular ion \( m/z \) 1598.9 (Figure 2), gave rise to the \( Y_1 \) ion \( m/z \) 868.6, the dihexosyl ions \( b_2 \) and \( c_2 \) and two ions interpreted as \( \psi \) ions containing C18 phytosphingosine (Figure 5, MS\(^3\)). The ion \( m/z \) 664.3 was interpreted as the dihexosyl-phytosphingosine \( \psi_2 \) ion, and the ion \( m/z \) 502.2 was interpreted as the monohexosyl-phytosphingosine \( \psi_1 \) ion. The same \( \psi \) ions, indicating C18 phytosphingosine, were obtained after MS\(^3\) of F-5 molecular ions \( m/z \) 1584.9 and 1570.8, whereas MS\(^3\) of the minor molecular ion \( m/z \) 1468.5 yielded \( \psi \) ions \( m/z \) 646.3 (\( \psi_2 \)) and 484.1 (\( \psi_1 \)) indicating C18 sphingosine. In contrast, fragmentation of the molecular ions containing nonhydroxylated fatty acids yielded the \( Z_0 \) ceramide ion without \( \psi \) ions (Figure 3, MS\(^3\)).

The MS spectrum of liver GL-4 mainly displayed molecular ions for a globoside with a ceramide portion containing C18 sphingosine and \( n_{16} \):0 (\( m/z \) 1250), \( n_{18} \):0 (\( m/z \) 1278.1), \( n_{22} \):0 (\( m/z \) 1333.8), \( n_{24} \):0 (\( m/z \) 1361.8), and \( h_{24} \):0 (\( m/z \) 1377.8). Molecular ions assignable to the Forssman tetracyglycosylceramide could be

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**Fig. 2.** Electrospray-ion trap MS spectra of native Forssman-5 purified from ostrich kidney and liver. The masses indicated are the experimental values of monoisotopic masses [M+Na\(^+\)]. d, \( d_{18} \):1 (sphingosine); t, \( t_{18} \):0 (phytosphingosine).
detected. Their contribution to the spectrum was minor, consistent with the lack of reactivity of liver GL-4 with the anti-Forssman antibody.

The molecular ions for kidney GL-2 were consistent with a dihexosyl carbohydrate chain. The prevalent ceramides contained C18 sphingosine linked to nonhydroxylated C16 fatty acid (m/z 884.5) and α-hydroxylated C22, 23, and 24 fatty acids (m/z 984.5, 998.4, and 1012.4, respectively). The molecular ions for kidney GL-1 indicated a monohexosyl carbohydrate chain. Ceramides contained mainly α-hydroxylated C22, 23, and 24 fatty acids either linked to C18 sphingosine (m/z 822.6, 836.8, 850.8, respectively), or C18 phytosphingosine (m/z 840.7, 854.8, 868.7, respectively).

**Methylation analysis**
Characterization of individual sugars and their linkages was obtained by methylation analysis (Figure 6). The GC profile of the partially methylated alditol acetates of F-5 (Figure 6, lower...
Panel) was consistent with a Forssman carbohydrate pentasaccharide with peaks for 3,4,6-tri-\(O\)-Me-GalNAcMe (GalNAc1\(\rightarrow\)), 4,6-di-\(O\)-Me-GalNAcMe (\(\rightarrow\)3GalNAc1\(\rightarrow\)), 2,4,6-tri-\(O\)-Me-Gal (\(\rightarrow\)3Gal1\(\rightarrow\)), 2,3,6-tri-\(O\)-Me-Gal (\(\rightarrow\)4Gal\(\rightarrow\)1), and 2,3,6-tri-\(O\)-Me-Glc (\(\rightarrow\)4Glc1\(\rightarrow\)). The GC profile of F-4 displayed the characteristic peaks consistent with a Forssman tetrasaccharide GalNAc1\(\rightarrow\)3GalNAc1\(\rightarrow\)3Gal\(\rightarrow\)4Gal1. In addition there was a peak of 2,3,6-trimethylglucitol, of lesser intensity than the trimethylgalactitol peaks. It was interpreted as arising from globoside, detected by mass spectrometry.

The GC profile of GL-2 yielded peaks for 2,3,6-tri-\(O\)-Me-Gal, 2,3,6-tri-\(O\)-Me-Glc, and 2,3,4,6-tetra-\(O\)-Me-Gal. Their relative

Fig. 4. Electrospray-ion trap MS of native Forssman-4 purified from ostrich kidney (upper panel) and collision-induced dissociation of the molecular ion m/z 1418.7 (d18:1/h24:0 ceramide) (lower panel). The masses indicated are the experimental values of monoisotopic masses [M+Na\(^+\)], d, d18:1 (sphingosine); t, t18:0 (phytosphingosine). The arrow in the MS spectrum (upper panel) marks the peak of the molecular ion m/z 1361.8 of globoside (d18:1/n24:0 ceramide).
Fig. 5. MS^4 collision-induced dissociation of the molecular ion m/z 1418.7 (d18:1/h24:0 ceramide) of kidney F-4 (upper panel) and MS^3 dissociation of the molecular ion m/z 1598.9 (t18:0/h24:0 ceramide) of kidney F-5 (lower panel).

intensities were consistent with an equal contribution of galabiose and lactosylceramide in kidney, and a 1:4 ratio in liver. The GC profile of GL-1 yielded peaks for 2,3,4,6-tetra-O-Me-Gal and 2,3,4,6-tetra-O-Me-Glc, indicating the presence of galactosyl and glucosylceramide in a 1:2 ratio in kidney, and the reverse in liver.

^1H NMR spectroscopy
The 400 MHz ^1H NMR spectrum of F-5 displayed two α-anomeric and three β-anomeric proton signals (Figure 7, Table I). It compared well with the spectrum of the Forssman glycolipid from equine kidney obtained by Dabrowski et al. at 338 K (65°C). The strong R1-b doublet of doublets at 4.091 p.p.m., occurring with very low signals for the trans-vinyl protons R-4 (5.382 p.p.m.) and R-5 (5.558 p.p.m.) of sphingosine, indicated that phytosphingosine was the major long chain base. The low resonance between 2.0 and 2.2 p.p.m. for nFA-2 was consistent with predominance of α-hydroxylated fatty acids, with some degree of insaturation indicated by the cis-vinyl and cis-allyl resonances (5.325 p.p.m. and 1.997 p.p.m., respectively). The presence of the trihydroxybase and α-hydroxylated fatty acids was responsible for the downfield shift of the β-Glc H-1 resonance (4.229 p.p.m.) compared to that of F-5 from sheep erythrocytes (4.183 p.p.m.), the ceramide of which was d18:1/n24:0 and only a small amount of d18:1/h24:0 (mass spectrum not shown). The β-Glc H-1 resonance of liver F-5 which contained α-hydroxylated fatty acids with mainly shingosine was slightly less shifted toward higher frequency (4.213 p.p.m.).
Table I. Anomeric proton resonances (p.p.m.) of Forssman-4 and Forssman-5 glycolipids in DMSO at 55°C

<table>
<thead>
<tr>
<th>Forssman-5</th>
<th>V</th>
<th>IV</th>
<th>III</th>
<th>II</th>
<th>I</th>
<th>Reference</th>
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<tr>
<td>GalNAcα→3GalNAcβ1→3Galβ1→4Galβ1→4Glcβ1</td>
<td>4.74</td>
<td>4.56</td>
<td>4.82</td>
<td>4.27</td>
<td>4.17</td>
<td>Equine kidney, Dabrowski et al., 1980a</td>
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<tr>
<td></td>
<td>4.765</td>
<td>4.615</td>
<td>4.389</td>
<td>4.290</td>
<td>4.229</td>
<td>Sheep erythrocytes, this work</td>
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<tr>
<td></td>
<td>4.765</td>
<td>4.594</td>
<td>4.834</td>
<td>4.283</td>
<td>4.213</td>
<td>Kidney, this work</td>
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<tr>
<td></td>
<td>4.765</td>
<td>4.630</td>
<td>4.866</td>
<td>4.132</td>
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GalNAcα→3GalNAcβ1→3Galβ1→4Galβ1

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<tr>
<th>Forssman-4</th>
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<td>4.866</td>
<td>4.132</td>
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<td>Kidney, this work</td>
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α Obtained at 65°C.

The anomic region of the ¹H NMR spectrum of kidney F-4 displayed a series of four signals consistent with a Forssman-4 oligosaccharide chain, GalNAcα→GalNAcβ→Galα→Galβ (Figure 7, Table I). The signal of the βGal I anomeric proton was assigned by comparison with the signal for the same residue in the galabiosylceramide Galα→Galβ→Cer present in kidney GL-2 (not shown). A series of low intensity α-anomeric signals was interpreted as resonances for the globoside carbohydrate chain (Figure 7, F-4 spectrum, arrows). H-1 signals for β-Glc (4.231 p.p.m.), β-Gal (4.297 p.p.m.), and β-GalNAc (4.568 p.p.m.) were individualized, whereas the H-1 signal for the globoside α-Gal was assigned to a signal at 4.848 p.p.m. enlarging the tall α-Gal signal of F-4 (4.866 p.p.m.). The presence of strong R-4 and R-5 signals indicated that sphingosine was the major sphingoid base. The lack of resonance for nFA-2 was consistent with a high degree of fatty acid α-hydroxylation.

Tissue localization of the Forssman antigen

Positive immunofluorescent reactions of kidney and liver with the anti-Forssman antibody were observed on cryostat sections only, whereas conventional paraffin-embedded sections did not react with the antibody. Immunofluorescence was detected at the same location in ostrich as in guinea pig, in the connective tissue, vascular endothelial cells, the midsegment piece and collecting ducts of the ostrich nephron corresponding to the renal distal tubules and collecting ducts of guinea pig kidney, and the liver Kupffer cells.

Discussion

The neutral glycolipid composition of ostrich kidney and liver lacked the major neutral glycolipids expressed in porcine kidney (Figure 1): globotriaosyl- and globotetraosylceramide (globoside), lactoseries glycolipids carrying the xenogenic afucoB (Jalali-Araghi and Macher, 1994; Samuelsson et al., 1994) and GalLe⁵ determinants (Bouhours et al., 1997), and the A/H histo-groups (Samuelsson et al., 1994). The lack of reactivity of ostrich kidney and liver glycolipids with the anti-Galα→3Gal antibody was in agreement with a previous study of other ostrich tissues by immunofluorescence with the lectin *Griffonia simplicifolia* IB4 (Taniguchi et al., 1996), indicating that ostrich, like chicken (Oriol et al., 1996), does not express the Galα→3Gal xeno-epitope. The interest of the present study was to find that ostrich expresses another xenoantigen, the Forssman pentaglycosylceramide (F-5) GalNAcα→3GalNAcβ1→3Galβ1→4Galβ1→4Glcβ1→Cer. F-5 has been first characterized by mass spectrometry in horse spleen (Sidiqui and Hakomori, 1971; Stellner et al., 1973) and kidney (Karlsson et al., 1974), and with ³¹P NMR spectroscopy in sheep erythrocytes (Dabrowski et al., 1980). Pig, which is a Forssman-negative species, expresses large amounts of GloCer and GloβCer found in ostrich tissues indicate that elongation of
Fig. 7. Proton NMR spectra of ostrich kidney and liver and sheep erythrocyte Forssman-5, and ostrich kidney Forssman-4, obtained at 400 MHz and 55°C in DMSO referenced to internal Me₄Si. The spectra shown correspond to the downfield region, olefinic methine (R-5, R-4, cis-vinyl) and anomeric oligosaccharide protons, and the upfield region for the α-carbonyl (nFA-2), allylic, alkyl (R-6) and acetamido methyl (NAc) protons. In the spectrum of ostrich kidney F-4, arrows indicate anomeric protons of globoside oligosaccharide chain at 4.231 p.p.m. (β-Glc), 4.297 p.p.m. (β-Gal), 4.568 p.p.m. (β-GalNAc) and 4.848 p.p.m. (α-Gal).

globoside into Forssman pentaglycosylceramide is very efficient (Figure 8). Another finding of the present study was the expression of a Forssman tetraglycosylceramide which was fully characterized in ostrich kidney, despite its small contribution. This unusual glycolipid has been previously found in hamster fibroblast NIL cells (Gahmberg and Hakomori, 1975). It is the first report of its occurrence in a living animal tissue. Mass spectrometry was essential for identification in ostrich kidney and
liver of this minor glycolipid, which was detected by the anti-Forssman antibody only in the purified tetraglycosylceramide fraction of kidney glycolipids. The MS spectrum of native molecules was already indicative of the presence of two N-acetylated hexoses and two hexoses. The MS/MS capability of the ion trap made clear that a dihexosylceramide was elongated with a di-N-acetylhexosamine. This initial finding led to the full characterization of the Forssman tetraglycosylceramide (F-4) with a di-N-acetylhexosamine. It was found that ostrich kidney and liver contain, in addition to glucosyl- and lactosylceramide, significant amounts of Galβ1→Cer and Galα1→4Galβ1→Cer, which can be considered as precursors of the synthesis of F-4.

Mass spectrometry and 1H NMR spectroscopy analyses gave concordant data for a high degree of ceramide hydroxylation. α-hydroxylated fatty acids were prevalent in Forssman glycolipids of both kidney and liver. Fragmentation of the amide bond of α-hydroxylated fatty acid-containing mono- and dihexosylceramide, as molecular or daughter ions, yielded psychosine type fragments characteristic of the sphingoid base. Only C18 sphingoid bases were found. Liver F-5 only contained the dihydroxylated monounaturated base sphingosine, whereas the trihydroxylated saturated base phytosphingosine was prominent in kidney F-5. The difference in base composition of the Forssman pentaglycosylceramides was consistent with their distinct tissue origins. Differential tissue expression of sphingoid bases in glycolipids makes ostrich similar to mammals. For example, the Forssman pentaglycosylceramide of the mouse small intestine, which only contains sphingosine (Gustavsson et al., 1996), has been localized in the nonepithelial tissue whereas the major epithelial glycolipid, asialo-GM1, contains phytosphingosine (Umezaki et al., 1989). The sphingoid base composition of kidney F-4 was different from that of kidney F-5, with sphingosine as the major base. This finding might indicate that kidney F-4 and F-5 are synthesized in different cell types. Phytosphingosine has been described in kidney glycolipids in man (Karlsson and Martensson, 1968), cow (Karlsson et al., 1973), horse (Karlsson et al., 1974), and pig (Holgersson et al., 1990). The present work indicates that expression of phytosphingosine in kidney glycolipids has been consistently maintained in species at least since the divergence of birds from the other vertebrates.

The minimum Forssman determinant GalNAcα1→3GalNAcβ1→R resembles the afucoB determinant Galα1→3Galβ1→R. It is synthesized from UDP-GalNAc and globoside by an α3-GalNAc transferase, the cDNA of which has been isolated from a canine kidney cell line (Haslam and Baenziger, 1996). The Forssman transferase has been found very similar to the murine α3-Gal transferase synthesizing the afucoB epitope (35% amino acid sequence similarity) and the human α3-GalNAc/Gal transferase synthesizing the A/B blood group determinants (42% amino acid sequence similarity). Therefore, from structural and genetic standpoints, the Forssman antigen is analogous to the histo-group A/B and afucoB determinants. However, A/B and afucoB determinants are built on lactoseries glycolipids and glycoproteins, whereas Forssman antigens are exclusively glycosphingolipids of the globo series or galaseries, as demonstrated here.

It has been proposed that the genes for the histo-group transferases arise by duplication and subsequent divergence of an ancestral gene (Yamamoto et al., 1991). In human, the afucoB determinant is not expressed, but two inactivated genes for the α3-galactosyltransferase are present, a pseudogen on chromosome 12 and the remnant of the ancestral gene in the vicinity of the A/B/O locus on chromosome 9 (Joziasse et al., 1991). Most mammals express the A/O histo group and the afucoB determinants. By contrast, the distribution of the Forssman antigen does not follow a clear evolutionary pathway. Among mammals, guinea pig, mouse, hamster, dog, cat, sheep, horse, and cow express Forssman antigens (Forssman, 1911; Tanaka and Leduc, 1956). Rabbit, pig and rat are nonexpressing. The same erratic expression is found in birds: goose and pigeon are negative whereas chicken is Forssman positive (Tanaka and Leduc, 1956; Kitamoto et al., 1980). Humans have been considered Forssman-negative, primarily because the Forssman antigen has not been found in erythrocytes, in concordance with the presence in human sera of natural antibodies against the widespread Forssman antigen (Young et al., 1979). However, all human sera are not equivalent as to their anti-Forssman reactivity which displays a great variability (Young et al., 1979). The Forssman antigen has
been described in human gastric mucosa (Hakomori et al., 1977), lung (Yoda et al., 1980), and kidney (Breimer, 1985) in some individuals. Concordance between low levels of anti-Forssman antibodies and expression of the Forssman antigen has been noticed in the case of gastric tumors (Hakomori et al., 1977), but the relationship remains unclear for normal individuals.

In the present study, lack of Forssman reactivity of paraffin-embedded sections made unlikely the expression of the Forssman determinant in ostrich glycoproteins, whereas its expression in ostrich glycosphingolipids was supported by the Forssman reactivity of cryosections. The anti-Forssman antibody stained blood vessels in kidney and liver cryosections, demonstrating that in ostrich, as in chicken (Kitamoto et al., 1980) and in guinea pig, the Forssman antigens are present in the endothelium and connective tissue. Therefore, the death of cultured ostrich endothelial cells observed after incubation with native human serum (Taniguchi et al., 1996) could be explained by natural anti-Forssman-dependent complement mediated attack. In conclusion, besides physiologic barriers and anatomic differences which may prevent the transplantation of ostrich organs to man (Taniguchi et al., 1996), ostrich is not a better organ or cell donor than pig with regards to natural antibody-mediated hyperacute rejection, because of the endothelial expression of the common Forssman xenoepitope.

Materials and methods

Purification of glycosphingolipids

Kidneys (150 g each) and liver (650 g) were collected after exsanguination, cut into small pieces and lyophilized. Lipids were extracted from lyophilized materials by successive incubations at 70°C first in methanol and then three times in the mixture chloroform/methanol (1:2; 4 ml/g) (Bouhours et al., 1992)). Glycosphingolipids were isolated from acetylated lipids by Florisil column chromatography (Saito and Hakomori, 1971). Neutral glycolipids were separated from acid glycolipids by DEAE-Sephadex A-25 (acetaform) column chromatography (Ueno et al., 1978).

Thin layer chromatography

Neutral glycolipids were chromatographed on HPTLC silica gel 60 aluminum plates (Merck) developed in chloroform/methanol/water (60:35:8). Immunostaining of chromatograms was done as described previously (Bouhours et al., 1987) with murine anti-A (NaM87-1F6, Centre Régional de Transfusion Sanguine, Nantes, France), anti-type 2 H (MR-3-517, Institut National de la Transfusion Sanguine, Paris, France), anti-Le
\^b/type 1 H (Wistar Institute), anti-B (Dako, Denmark) monoclonal antibodies, rat anti-Forssman monoclonal antibody (M1/22.25.8.HL, ATCC TIB 121), and chicken anti-GalT1-3Gal polyclonal antibody (Bouhours et al., 1998). Murine monoclonal antibodies were detected with sheep biotinylated anti-mouse immunoglobulins, and rat monoclonal antibody with rabbit biotinylated anti-rat immunoglobulins, labeled with streptavidin–horseradish peroxidase (HRP) conjugate. Chicken polyclonal antibody was detected with HRP-labeled rabbit anti-chicken antibodies. Visualization was obtained by chemiluminescence with the ECL Western blotting system (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 a 0.05% solution of primulin in aceton/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).

Quantitative measurements

Quantities of glycolipids were determined by measurement of sphingosine content according to a procedure described earlier (Bouhours and Glickman, 1976), either in glycolipid mixtures, or in suspensions of silica gel containing individual glycolipids scraped off the chromatogram of 40 nmol of glycolipid solution on a 2 cm streak, after visualization with primuline.

Methylation analysis

Purified glycolipids were permethylated by the method of Ciucanu and Kerek (1984). The permethylated glycolipids were submitted to acetylation, reduction and acetylation (Yang and Hakomori, 1971). Gas chromatography of the partially methylated alditol acetates was done on a 25 m x 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\(^{-1}\), and then up to 250°C at a rate of 5°C min\(^{-1}\).

Electrospray-ion trap mass spectrometry

Analyses were performed on a HP-Bruker ESQUIRE-LC mass spectrometer (Bruker Franzen, Bremen, Germany) equipped with an atmospheric pressure electrospray ionization source. Samples dissolved in ethyl acetate/methanol (1:1) were introduced in the electrospray source with a syringe pump at 2 µl/min. High voltages of the end plate and capillary were set at 3.5 and 4 kV, respectively. The nebulizer pressure was 1 psi. The drying nitrogen flow was 1 l/min and its temperature was 250°C. Capillary exit voltage was set at 350 V. Voltages of skimmer 1 and 2 were set at 80 V and 25 V, respectively. Spectra were acquired on the positive mode with the standard scanning range and a resolution of 0.6 m/z. For MS/MS operation, isolation windows were set in order to contain the three main isotopic molecular mass peaks, and fragmentation energy was set at the exact value that was necessary for the disappearance of the isolated ions. Spectra were averaged on 10–30 trap cycles and recorded as profile spectra. They were processed with the version 1.5c of the Bruker Data Analysis software.

\(^{1}H\)-NMR spectroscopy

Each native glycolipid was equilibrated three times in deuterated methanol. The deuterated glycolipid was dissolved in 0.5 ml of Me\(_2\)SO-\(_d_6\) containing 2% D\(_2\)O. 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.

Histochemistry

Kidney and liver samples from an ostrich and a guinea pig (a known Forssman-positive mammal) were split in two parts: (1)
one part was fixed in formalin 4%, paraffin-embedded, cut at 4 μm and the sections were rehydrated by routine histology techniques; (2) the second part was embedded in Tissue-Tek C for 4 min. Both types of tissue sections were incubated with rat anti-Forssman monoclonal antibody (diluted 1 to 2) for 60 min in a wet chamber. After washing (3 x 5 min), the sections were incubated for 60 min with fluorescein-labeled rabbit anti-rat lg (diluted 1 to 20) (Dako, Denmark), washed again and mounted with 10 μl of Mowiol ( Hoechst, Germany) under coverslides (Candelier et al., 1993). The fluorescence of stained sections was observed in an epifluorescence microscope (Aristoplan, Leitz-Leika, Germany).

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Abbreviations

GL, glycosphingolipid; F, Forssman glycolipid; MS, mass spectrometry; GC, gas chromatography; HPTLC, high performance thin layer chromatography; Me₂SO-d₆, hexadeuterated dimethyl sulphoxide; DMSO, dimethyl sulphoxide.

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