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Original Article

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Glycan Recognition

Characterization of sheep erythrocyte glycosphingolipids recognized by human anti-Forssman antibodies

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Abstract

The FORS histo-blood group system is the most recently discovered carbohydrate-based human blood group system. FORS is a rare blood group system, and most individuals have naturally occurring anti-FORS1 antibodies in plasma. Screening for anti-FORS1 antibodies is often done by hemagglutination assays using FORS1-expressing sheep erythrocytes, since FORS1-positive human erythrocytes are most often not available. Here, we have characterized the non-acid glycosphingolipids from sheep erythrocytes and isolated subfractions, with mass spectrometry, binding of antibodies and lectins, and by enzymatic hydrolysis. This demonstrated the presence of Forssman and Galili pentaosylceramides, and a Galili heptaosylceramide. Two complex glycosphingolipids recognized by human anti-FORS1 antibodies were characterized as a Forssman neolacto hybrid hexaosylceramide (GalNAcα3GalNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer) and a Forssman Galili hybrid heptaosylceramide (GalNAcα3GalNAcβ3Galα3Galβ4GlcNAcβ3Galβ4Glcβ1Cer). These are novel glycosphingolipid structures, and to our knowledge, the first case of an elongated Galili antigen. Thus, the anti-Forssman antibodies in human serum bind not only to the classical Forssman pentaosylceramide (GalNAcα3GalNAcβ3Galα4Galβ4Glcβ1Cer), but also when the GalNAcα3GalNAcβ3 sequence is presented on a neolacto core chain and even on a Galili carbohydrate sequence.

Key words: anti-Forssman antibodies, Forssman antigen, glycosphingolipid characterization, mass spectrometry, sheep erythrocyte glycosphingolipids

Introduction

There are currently 38 human blood groups systems recognized by the International Society of Blood Transfusion (Storry et al. 2019). Seven of these are based on carbohydrate antigens, as e.g. the ABO, Lewis, P_1P_K, I, and GLOB systems. The most recent member in the group of carbohydrate-based blood groups systems is the FORS system (Storry et al. 2014).

Until recently, humans were considered to be a Forssman antigen-negative species without a functional Forssman synthase, and having naturally occurring anti-Forssman (anti-FORS1) antibodies in plasma. However, it has now been demonstrated that the rare individuals of the A_{pae} phenotype express the Forssman antigen (GalNAcα3Galβ3Galα4Galβ4Glcβ1Cer) on their erythrocytes (Svensson et al. 2013). In these individuals, the gene *GBGT1*, coding for the Forssman synthase (α-1,3-N-acetylgalactosaminyltransferase, EC 2.4.1.88), encodes an arginine to glutamine change at residue 296, and transfection experiments showed that Agr296Gln reactivates the human Forssman synthase. Accordingly, the FORS system was recognized as a novel blood group system.

The worldwide frequency of the FORS system remains to be determined, although it is most likely a rare blood group system. This was confirmed in a recent study where sheep erythrocytes were used to search for anti-FORS1 antibodies in plasma samples from 800 individuals. Only one individual negative for anti-FORS1 antibodies was found (Jesus et al. 2018). Likewise, the FORS-encoding alleles (c.887G>A) was not found among 2504 individuals, when next-generation sequencing results were extracted from the 1000 Genomes Project (Hult et al. 2018).

The majority of human sera contain natural anti-FORS1 antibodies, of both IgM and IgG classes (Strokan et al. 1998), making these antibodies substantial transfusion and transplantation barriers, able to induce intravascular hemolysis of FORS-positive erythrocytes. The anti-FORS1 antibodies can also be involved in perinatal hemolytic disease (Young et al. 1979; Svensson et al. 2013; Hult and Olsson 2017).

Genotyping the *GBGT1* gene and seeking the substitution of arginine to glutamine at codon 296 is the most reliable way to characterize a donor/patient as FORS positive. Detection of the presence of anti-FORS1 antibodies by hemagglutination assays using sheep erythrocytes may also be used (Jesus et al. 2018). However, the agglutination of sheep erythrocytes by human serum may also be due to anti-Gal antibodies directed towards the Galili antigen (Galα3Galβ4GlcNAc-), which is present on glycolipids and glycoproteins of non-primate mammals and New World monkeys (Galili et al., 1984; Galili 2013).

The aim of the present study was to characterize the non-acid glycosphingolipids of sheep erythrocytes, with particular interest in components recognized by human anti-Forssman antibodies and anti-Gal antibodies. There are a few reports with characterization of sheep erythrocyte glycosphingolipids from the 1970s and 1980s (Fraser and Mallette, 1974; Momoi and Yamakawa 1978; Koizumi et al. 1988). The presence of minor N-acetylglucosamine-containing non-acid glycosphingolipids in sheep erythrocytes was reported by Momoi and Yamakawa (1978), but, apart from neolactotetraosylceramide, the structure of these glycosphingolipids was not elucidated. Koizumi et al. (1988) have reported that anti-Forssman antibodies, in addition to the Forssman pentaosylceramide, recognize two unknown glycosphingolipids in sheep erythrocytes. However, a thorough characterization of sheep erythrocyte glycosphingolipids with the methods available today has not been done. Here, non-acid glycosphingolipids from sheep erythrocytes, and isolated subfractions, were characterized with mass spectrometry, binding of antibodies and lectins, and by enzymatic hydrolysis. This demonstrated the presence of Forssman and Galili (Galα3Galβ4GlcNAcβ3Galβ4Glcβ1Cer) pentaosylceramides, and a Galili heptaosylceramide (Galα3Galβ4GlcNAcβ3Galβ4GlcNAcβ3-Galβ4Glcβ1Cer). Furthermore, two novel glycosphingolipids with a terminal Forssman epitope: a Forssman neolacto hybrid hexaosylceramide (GalNAcα3GalNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer) and a Galili pentaosylceramide elongated with a terminal Forssman determinant (GalNAcα3GalNAcβ3Galα3Galβ4GlcNAcβ3Galβ4G-lcβ1Cer) were characterized.

Results

Separation of the total non-acid glycosphingolipids from sheep erythrocytes

In order to characterize the non-acid glycosphingolipids of sheep erythrocytes, a total non-acid glycosphingolipid fraction (50 mg) was first separated by chromatography on a silica gel column. The stepwise separations of the sheep erythrocyte non-acid glycosphingolipids, and the analyzes done at each step, are summarized in Figure 1A. The first separation gave one fraction containing compounds migrating in the mono- to pentaosylceramide region (Fraction SE-1; Figure 1A and B, lane 2), one fraction with compounds migrating as tri- to pentaosylceramides (Fraction SE-2; Figure 1B, lane 3) and other fraction containing mainly pentaosylceramides and more slow-migrating glycosphingolipids (Fraction SE-3; Figure 1B, lane 4). Binding of human serum antibodies to a compound co-migrating with reference Forssman glycosphingolipid (GalNAcα3Galβ3Galα4Galβ4Glcβ1Cer) (Figure 1C, lane 5) in the three sheep erythrocyte fractions was obtained (Figure 1C, lanes 2–4). In the third fraction, there was also a distinct binding to two compounds migrating below the Forssman reference (Figure 1C, lane 4).

The fraction containing mainly pentaosylceramides and slow-migrating glycosphingolipids (fraction SE-3) was separated on an Iatrobeads column, and the fractions obtained were pooled according to their mobility on thin-layer chromatograms, giving eight glycosphingolipid-containing fractions, which were denoted fractions SE-I–SE-VIII. Fraction SE-I contained compounds migrating as tri- and tetraosylceramides on thin-layer chromatograms, and this fraction was not further characterized. Fractions SE-II–SE-VIII are shown in Figure 2A, lanes 1–7. These fractions were characterized by LC-ESI/MS and by binding of antibodies and lectins.

Characterization of fractions SE-II–SE-VIII

Mass Spectrometry. The subfractions from sheep erythrocytes were digested with endoglycoceramidase, and the oligosaccharides obtained were analyzed by LC-ESI/MS on porous graphitized carbon columns. This gives resolution of isomeric oligosaccharides, and the carbohydrate sequence can be deduced from series of C-type fragment ions obtained by MS^2 (Chai et al. 2001; Karlsson et al. 2010). Furthermore, MS^2 spectra of oligosaccharides with a Hex or HexNAc substituted at C-4 have diagnostic cross-ring ^{0,2}A-type fragment ions (^{0,2}A and ^{0,2}A-H_2O), which allow differentiation of linkage positions. Thus, such ^{0,2}A-type fragment ions are found in MS^2 spectra of oligosaccharides with globo (Galα4Gal) or type-2 (Galβ4GlcNAc) core structures, but are absent in the spectra

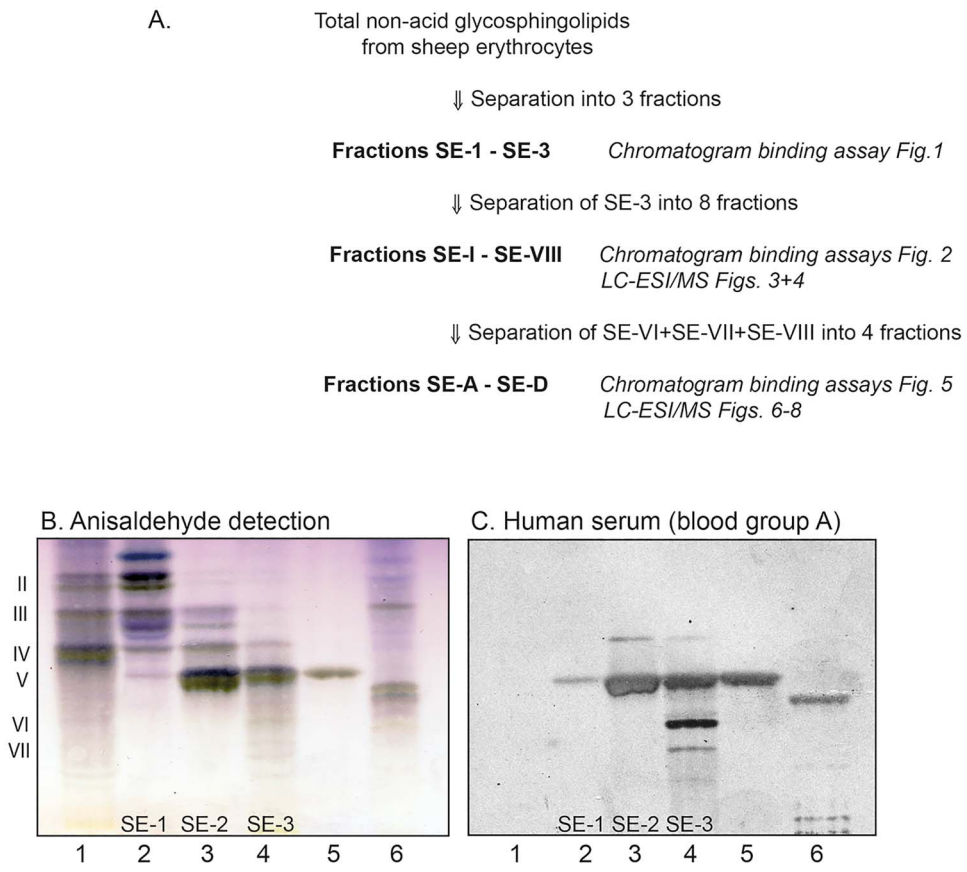

Fig. 1. Glycosphingolipid subfractions isolated from sheep erythrocytes and binding of human serum antibodies. Thin-layer chromatogram after detection with anisaldehyde (**A**) and autoradiogram obtained by binding of human serum antibodies from a blood group A individual (**B**), followed by autoradiography for 12 h, as described under *Materials and methods*. The solvent system used was chloroform/methanol/water (60:35:8, by volume). The lanes were: lane 1, reference non-acid glycosphingolipids of human erythrocytes blood group AB, 40 μg; lane 2, glycosphingolipid subfraction SE-1 isolated from sheep erythrocytes, 4 μg; lane 3, glycosphingolipid subfraction SE-2 isolated from sheep erythrocytes, 4 μg; lane 4, glycosphingolipid subfraction SE-3 isolated from sheep erythrocytes, 4 μg; lane 5, reference Forssman pentaosylceramide (GalNAcα3GalNAcβ3Galα4Galβ4Glcβ1Cer), 4 μg; lane 6, reference non-acid glycosphingolipids of rabbit erythrocytes, 40 μg. The Roman numbers to the left of (**B**) indicate the number of carbohydrate units in the bands. This figure is available in black and white in print and in color at *Glycobiology* online.

obtained from oligosaccharides with isoglobo (Galα3Gal) or type-1 (Galβ3GlcNAc) core chains. Comparison of retention times and MS^2 spectra of oligosaccharides from reference glycosphingolipids was also used for the identification of oligosaccharides.

Globotri, globotetra, neolactotetra and FORS oligosaccharides were identified by LC-ESI/MS of fraction SE-II (data not shown).

Fractions SE-III, IV and V. LC-ESI/MS of the oligosaccharides obtained from fraction SE-III (Figure 3A) gave a base peak chromatogram with three molecular ions at *m/z* 868, *m/z* 909 and *m/z* 1112, respectively. The ion at *m/z* 909 eluted as double peaks at 16.1 and 16.8 min, the ion at *m/z* 1112 eluted at 24.2 min, while the ion at *m/z* 868 eluted as double peaks at 24.4 and 25.1 min. MS^2 of the molecular ion at *m/z* 868 (Figure 3B) gave a series of C ions (C_2 at *m/z* 341, C_3 at *m/z* 544, and C_4 at *m/z* 706) identifying a Hex-Hex-HexNAc-Hex-Hex sequence. Cross-ring ^{0,2}A-type fragments are diagnostic for carbohydrates substituted at C-4 (Chai et al. 2001; Karlsson et al. 2010), and, thus the $^{0,2}A_3$ fragment ion at *m/z* 443 and the $^{0,2}A_3$-H_2O fragment ion at *m/z* 425 demonstrated a substitution of the HexNAc at C-4, i.e. a type-2 chain, while the $^{0,2}A_5$ ion at *m/z* 808 and the $^{0,2}A_5$-H_2O ion at *m/z* 790 were derived from a cross-ring cleavage of the 4-substituted Glc of the lactose unit at the reducing end. These spectral features allowed the identification of a Galα3Galβ4GlcNAc-terminated pentasaccharide, i.e. a Galili pentasaccharide (Galα3Galβ4GlcNAcβ3Galβ4Glc).

The MS^2 spectrum of the molecular ion at *m/z* 909 (Figure 3C) had a series of C-type ions (C_2 at *m/z* 423, C_3 at *m/z* 585 and C_4 at *m/z* 747), demonstrating a HexNAc-HexNAc-Hex-Hex-Hex pentasaccharide. In addition, there was a $^{0,2}A_4$ fragment ion at *m/z* 687 demonstrating 4-substitution of the internal Hex. Taken together, this indicated a FORS pentasaccharide (GalNAcα3GalNAcβ3Galα4Galβ4Glc).

A molecular ion at *m/z* 1112 corresponds to a hexasaccharide with three HexNAc and three Hex. Here, the MS^2 spectrum obtained (Figure 3D) had a series of C-type fragment ions at *m/z* 423 (C_2), *m/z* 585 (C_3), *m/z* 788 (C_4) and *m/z* 950 (C_5), demonstrating a HexNAc-HexNAc-Hex-HexNAc-Hex-Hex sequence. The prominent $^{0,2}A_4$ fragment ion at *m/z* 687 demonstrated a 4-substitution of the internal HexNAc, i.e. a type-2 chain. Collectively this allowed a tentative identification of a saccharide with a FORS terminal and a type-2 core chain, i.e. a Forssman neolacto hybrid (GalNAcα3GalNAcβ3Galβ4GlcNAcβ3Galβ4Glc).

The ion at *m/z* 1112 was the predominant molecular ion in the base peak chromatograms from fractions SE-IV and SE-V, and also

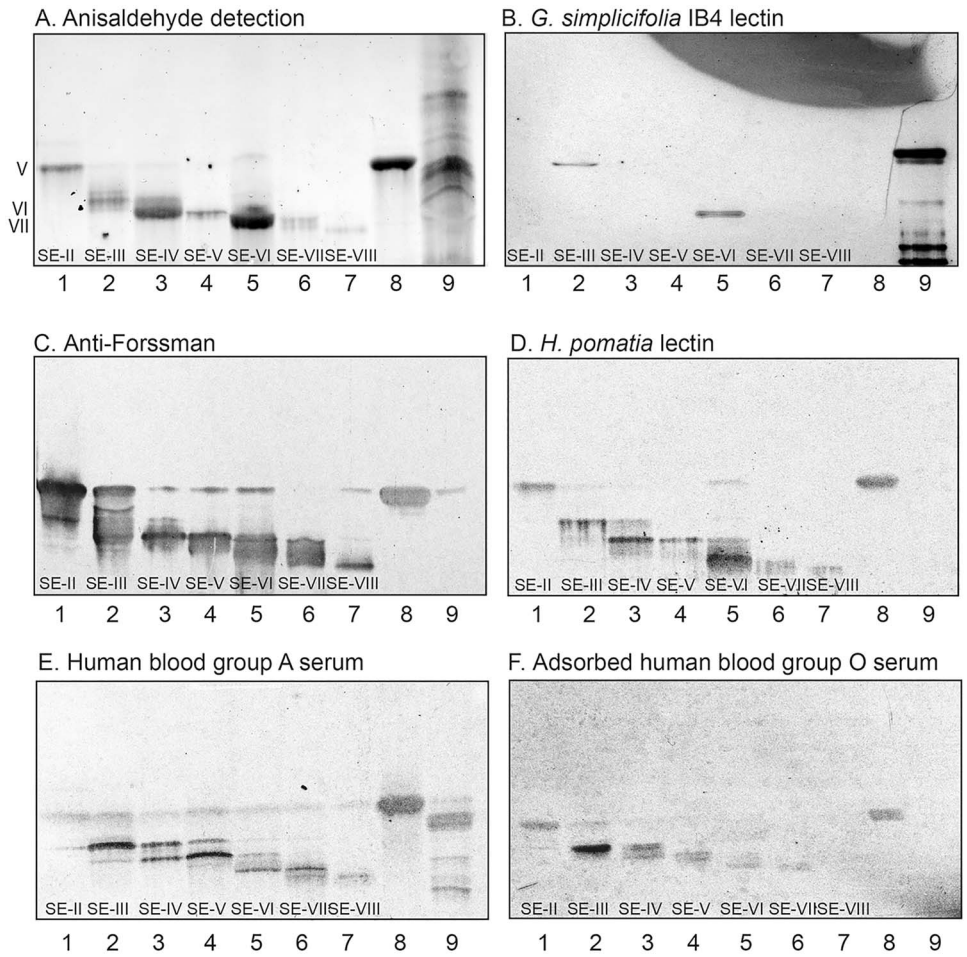

Fig. 2. Binding of human serum antibodies, anti-Forssman antibodies and lectins to subfractions from sheep erythrocyte glycosphingolipids. Thin-layer chromatogram after detection with anisaldehyde (**A**), and autoradiograms obtained by binding of *G. simplicifolia* IB4 lectin (**B**), monoclonal anti-Forssman antibodies (**C**), *H. pomatia* lectin (**D**), human serum antibodies from a blood group A individual (**E**), and human serum antibodies from a blood group O individual depleted of anti-Gal antibodies (**F**), followed by autoradiography for 12 h, as described under "Materials and methods". The solvent system used was chloroform/methanol/water (60:35:8, by volume). The lanes were: lanes 1–7, glycosphingolipid subfractions SE-II–SE-VIII isolated from sheep erythrocytes, 4 μg/lane; lane 8, reference Forssman pentaosylceramide (GalNAcα3GalNAcβ3Galα4Galβ4Glcβ1Cer), 4 μg; lane 9, reference non-acid glycosphingolipids of rabbit erythrocytes, 40 μg. The Roman numbers to the left of (**A**) indicate the number of carbohydrate units in the bands.

here, MS^2 identified a HexNAc-HexNAc-Hex-4HexNAc-Hex-4Hex saccharide (data not shown).

Fractions SE-VI, VII and VIII. The base peak chromatogram obtained by LC-ESI/MS of the oligosaccharides derived from fraction SE-VI (Figure 4A) had three molecular ions at *m/z* 1112, *m/z* 1233 and *m/z* 1274, respectively. MS^2 of the ion at *m/z* 1112 again identified the Forssman neolacto hybrid (Figure 4B).

The MS^2 spectrum of the molecular ion at *m/z* 1233 (Figure 4C) had a number of C type fragment ions (C_4 at *m/z* 706, C_5 at *m/z* 909 and C_6 at *m/z* 1071) in line with a Hex-Hex-HexNAc-Hex-HexNAc-Hex-4Hex heptasaccharide. The $^{0,2}A_3$ fragment ion at *m/z* 443 indicated a Hex-Hex-HexNAc terminal with substitution of the HexNAc at C-4, while the $^{0,2}A_5$ fragment ion at *m/z* 808, along with the $^{0,2}A_5$-H_2O fragment ion at *m/z* 790, demonstrated the 4-substitution of the other internal HexNAc. Taken together, this indicated a Hex-Hex-4HexNAc-Hex-4HexNAc-Hex-4Hex heptasaccharide, most likely a Galili heptasaccharide (Galα3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glc).

A molecular ion at *m/z* 1274 corresponds to a heptasaccharide with three HexNAc and four Hex. Here, a number of B- and C-type fragment ions at *m/z* 405 (B_2), *m/z* 585 (C_3), *m/z* 747 (C_4), *m/z* 950 (C_5) and *m/z* 1112 (C_6), demonstrating a HexNAc-HexNAc-Hex-Hex-HexNAc-Hex-Hex sequence, were present in the MS^2 spectrum (Figure 4D). The $^{0,2}A_5$ fragment ion at *m/z* 849 demonstrated the 4-substitution of the internal HexNAc. Taken together, this gave a tentative identification a heptasaccharide with HexNAc-HexNAc-Hex-Hex-4HexNAc-Hex-4Hex sequence.

The base peak chromatograms from LC-ESI/MS of fractions SE-VII and SE-VIII were very weak and did not allow MS^2 characterization.

Chromatogram Binding Assays. To substantiate the data from mass spectrometry, the binding of a number of carbohydrate recognizing ligands (Table I) to the glycosphingolipid fractions from sheep erythrocytes was thereafter examined (Figure 2). Total non-acid glycosphingolipids from sheep erythrocytes, which have Galili pentaosylceramide and larger glycosphingolipids with Galili epitope (Hanfland et al. 1988), were used as a control for the binding of human serum anti-Gal antibodies.

The binding of the Galα binding *Griffonia simplicifolia* IB4 lectin (Hayes and Goldstein 1974) to fractions SE-III and SE-VI (Figure 2B,

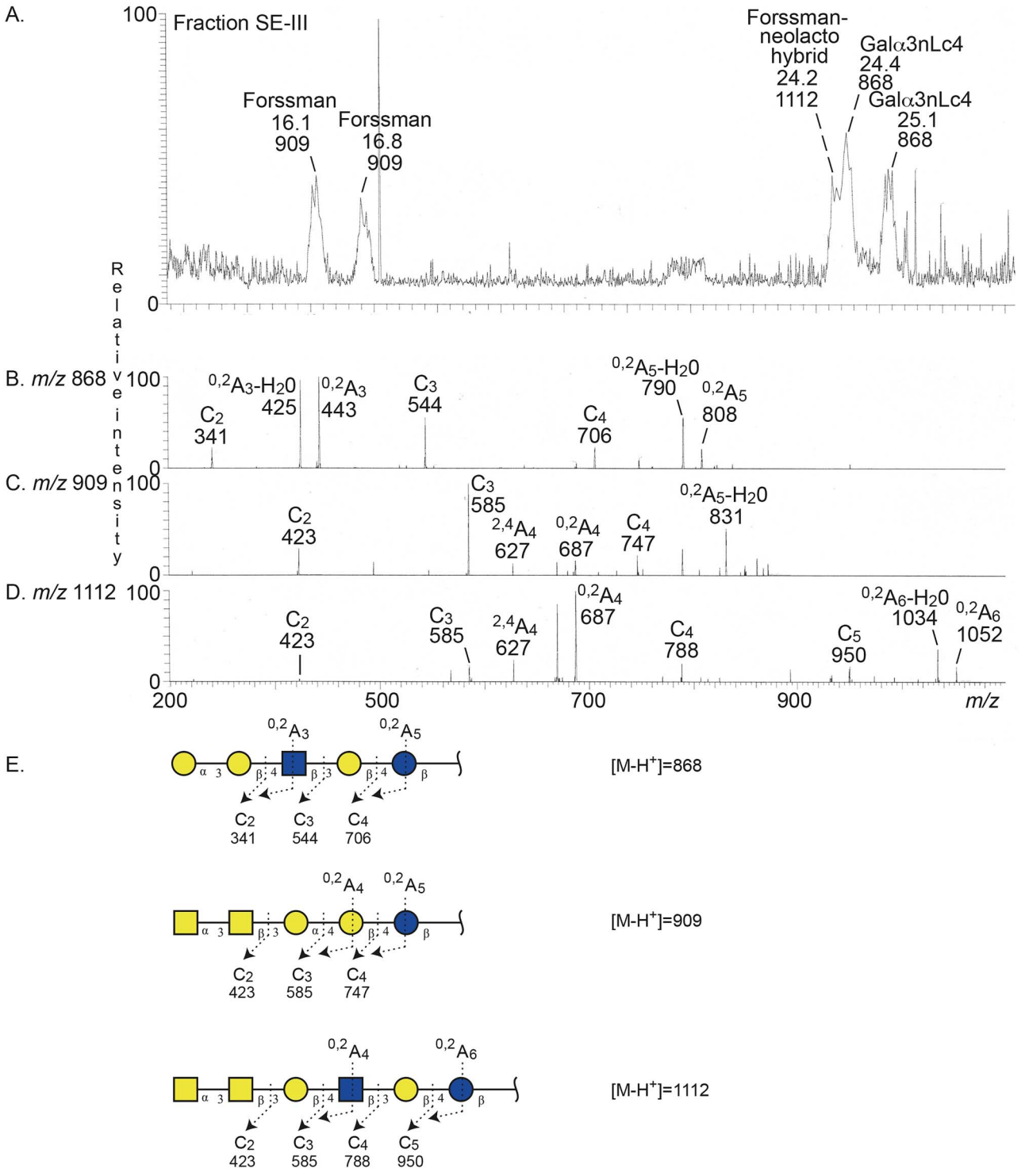

Fig. 3. LC-ESI/MS of the oligosaccharides obtained by digestion of subfraction SE-III from sheep erythrocytes with *Rhodococcus* endoglycoceramidase II. (**A**) Base peak chromatogram from LC-ESI/MS of the oligosaccharides derived from fraction SE-III. (**B**) MS^2 of the ion at *m/z* 868 (retention time 24.4 min). (**C**) MS^2 of the ion at *m/z* 909 (retention time 16.1 min). (**D**) MS^2 of the ion at *m/z* 1112 (retention time 24.2 min). (**F**) Interpretation formulas showing the deduced oligosaccharide sequences. See Table II for oligosaccharide structures. The identification of oligosaccharides was based on their retention times, determined molecular masses and subsequent MS^2 sequencing, and on the basis of knowledge of glycosphingolipid biosynthetic pathways. The proposed structures are depicted using the Symbol Nomenclature for Glycomics (SNFG) (Varki et al. 2015; Neelamegham et al. 2019), and nomenclature of fragments defined by Domon and Costello (1988).

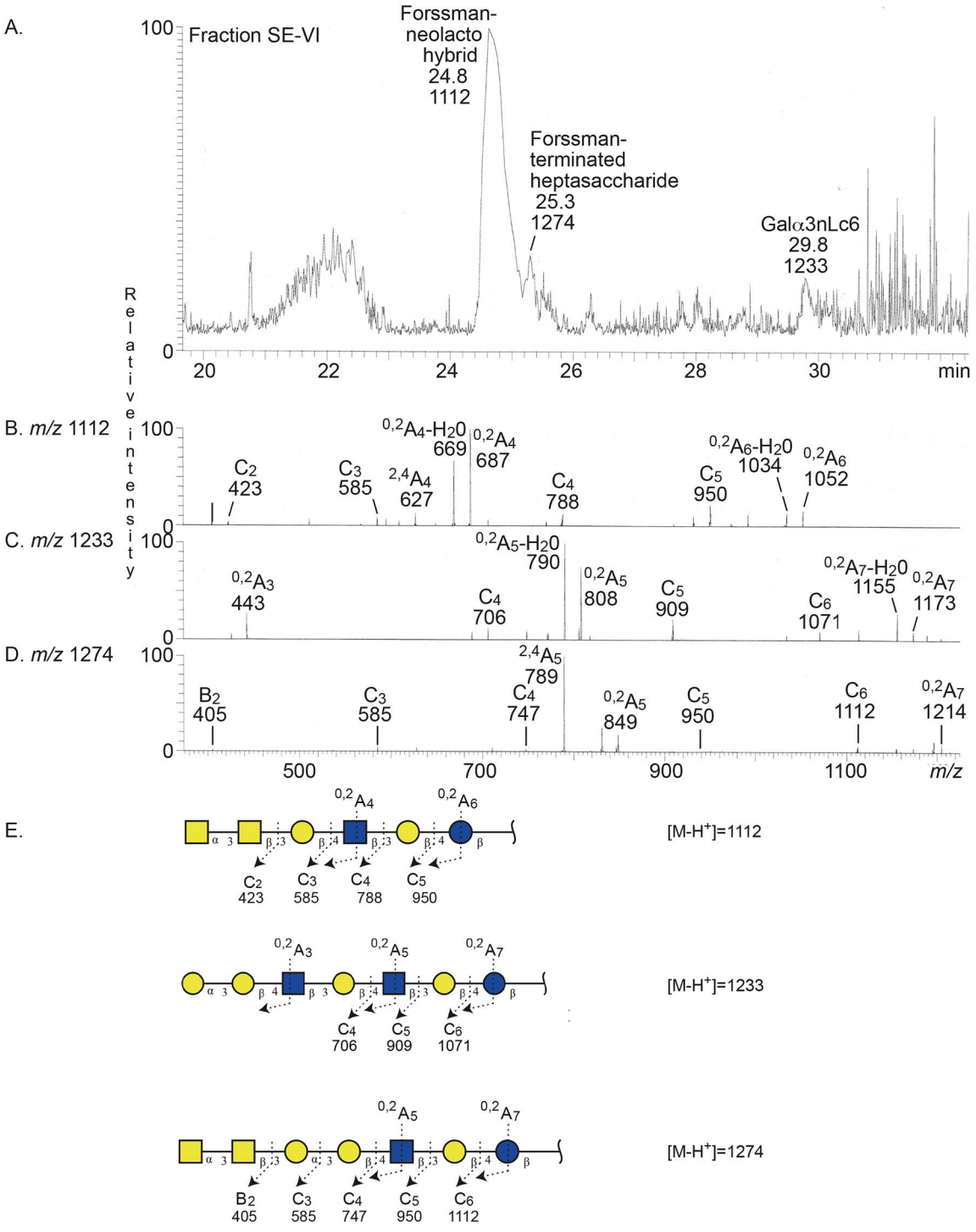

Fig. 4. LC-ESI/MS of the oligosaccharides obtained by digestion of subfraction SE-VI from sheep erythrocytes with *Rhodococcus* endoglycoceramidase II. (**A**) Base peak chromatogram from LC-ESI/MS of the oligosaccharides derived from fraction SE-VI. (**B**) MS^2 of the ion at *m/z* 1112 (retention time 24.8 min). (**C**) MS^2 of the ion at *m/z* 1233 (retention time 29.8 min). (**D**) MS^2 of the ion at *m/z* 1274 (retention time 25.4 min). (**E**) Interpretation formulas showing the deduced oligosaccharide sequences. See Table II for oligosaccharide structures. The identification of oligosaccharides was based on their retention times, determined molecular masses and subsequent MS^2 sequencing, and on the basis of knowledge of glycosphingolipid biosynthetic pathways. The proposed structures are depicted using the Symbol Nomenclature for Glycomics (SNFG) (Varki et al. 2015; Neelamegham et al. 2019) and nomenclature of fragments defined by Domon and Costello (1988).

Table I. Carbohydrate binding ligands used in chromatogram binding assays

Ligand	Manufacturer	Binding specificity
H. pomatia lectin	Sigma-Aldrich	GalNAcα
Griffonia simplicifolia IB4 lectin	Advanced Targeting Systems	Galα
Anti-Forssman	ATCC	Galα3Galβ4GlcNAcβ3Galβ4Glc (Forssman antigen)
Human serum (blood group A)	–	Galα3(Fucα2)Gal (blood group B antigen) Galα3Galβ4GlcNAc (Galili antigen) Galα3Galβ4GlcNAcβ3Galβ4Glc (Forssman antigen)
Adsorbed human serum (blood group O)	–	GalNAcα3(Fucα2)Gal (blood group A antigen) Galα3(Fucα2)Gal (blood group B antigen) Galα3Galβ4GlcNAcβ3Galβ4Glc (Forssman antigen)

lanes 2 and 5) was in line with the presence of a Galili pentasaccharide (Galα3Galβ4GlcNAcβ3Galβ4Glc) in fraction SE-III and a Galili heptasaccharide (Galα3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glc) in fraction SE-VI.

The monoclonal anti-Forssman antibodies recognized compounds co-migrating with the reference Forssman glycosphingolipid in fractions SE-II and SE-III (Figure 2C, lanes 1 and 2), in agreement with the presence of Forssman in these fractions. Binding to more slow-migrating compounds in fractions SE-III–SE-V was also obtained (Figure 2C, lanes 3–5), supporting the presence of the Forssman neolacto hybrid hexaosylceramide (GalNAcα3GalNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer). The different migration of the binding compounds in these fractions is most likely due to a variant ceramide composition. Finally, the anti-Forssman antibodies also bound to a slow-migrating glycosphingolipid in fraction SE-VI (Figure 2C, lane 6), in line with the presence of the HexNAc-HexNAc-Hex-Hex-4HexNAc-Hex-4Hex heptaosylceramide with a terminal Forssman epitope (GalNAcα3GalNAc).

Binding of the GalNAcα-binding *Helix pomatia* lectins (Sanchez et al. 2006) and human serum antibodies to compounds co-migrating with the glycosphingolipids recognized by the monoclonal anti-Forssman antibodies was also obtained (Figure 2D–F). Binding of human serum antibodies still occurred after the adsorption of anti-Gal antibodies (Figure 2F). The adsorbed serum bound to the reference Forssman pentaosylceramide (Figure 2F, lane 8), but did not bind to the Galili pentaosylceramide or larger Galili epitope carrying glycosphingolipids from sheep erythrocytes (Figure 2F, lane 9), in line with the removal of the anti-Gal antibodies by the adsorption.

Characterization of fractions SE-A–SE-D

In an attempt to further characterize the complex minor glycosphingolipids of sheep erythrocytes, fractions SE-VI–SE-VIII were thereafter pooled, and further separated by Iatrobeads column chromatography, giving four glycosphingolipid-containing fractions (denoted fractions SE-A–SE-D) after pooling (Figure 5A, lanes 1–4). All four fractions contained a slow-migrating glycosphingolipid recognized by the monoclonal anti-Forssman antibodies and by human serum antibodies (Figure 5B and C, lanes 1–4). However, there was no binding of the Galα3-recognizing *G. simplicifolia* IB4 lectin to these fractions (Figure 5D, lanes 1–4).

The four fractions were digested with endoglycoceramidase, and the oligosaccharides obtained were analyzed by LC-ESI/MS. Thereafter, the oligosaccharides were reduced and re-analyzed by LC-ESI/MS. Thereby, a neolacto tetrasaccharide, Le^a, H type-1 and H type-2 pentasaccharides, and Le^b and Le^y hexasaccharides were identified in fraction SE-A (Supplementary Figures S1 and S2). The major molecular ion of fractions SE-B, SE-C and SE-D was at *m/z* 911 (reduced form; exemplified in Figure 6A), and here MS^2 gave identification of the Forssman pentasaccharide as above. These three fractions also had molecular ions at *m/z* 708, *m/z* 1073, *m/z* 1114 and *m/z* 1235 (reduced form), and here a neolacto tetrasaccharide (*m/z* 708), a neolacto hexasaccharide (*m/z* 1073), the Forssman neolacto hybrid (*m/z* 1114) and the Galili heptasaccharide (*m/z* 1235) were again identified by MS^2 (data not shown). The three fractions also had a molecular ion at *m/z* 1276. MS^2 of this ion (Figure 6B) gave a Y ion series (Y_3 at *m/z* 546, Y_4 at *m/z* 708, Y_5 at *m/z* 870 and Y_6 at *m/z* 1073), which along with the B_2 ion at *m/z* 405, the B_3 ion at *m/z* 567 and B_5 ion at *m/z* 932 again gave the identification of a HexNAc-HexNAc-Hex-Hex-4HexNAc-Hex-4Hex heptasaccharide.

An internal Hex-Hex carbohydrate sequence is very unusual. We speculated that this was a Galili pentasaccharide (Galα3Galβ4GlcNAcβ3Galβ4Glc) with a terminal Forssman epitope (GalNAcα3GalNAcβ3Galα3Galβ4GlcNAcβ3Galβ4Glc).

To substantiate this speculation, the reduced oligosaccharides from fraction SE-D were first digested with α-N-acetylgalactosaminidase, and thereafter with β-N-hexosaminidase, and the resulting oligosaccharides from the two enzymatic digestions were analyzed by LC-ESI/MS (Figure 7B and C) and compared with the untreated oligosaccharides (Figure 7A) from the fraction SE-D. Upon treatment with α-N-acetylgalactosaminidase (Figure 7B), terminal GalNAcα (203 Da) was removed from *m/z* 911 (Forssman pentasaccharide), *m/z* 1114 (Forssman neolacto hexasaccharide) and *m/z* 1276 (HexNAc-HexNAc-Hex-Hex-4HexNAc-Hex-4Hex heptasaccharide), resulting in novel molecular ions at *m/z* 708 (911-203; retention time 10.4 min), *m/z* 911 (1114-203; retention time 18.6 min) and *m/z* 1073 (1276-203, retention time 18.2 min). MS^2 of the ion at *m/z* 708 at retention time 10.4 min identified a globo tetrasaccharide, and MS^2 of the ion at *m/z* 911 at retention time 18.6 min demonstrated HexNAc-Hex-4HexNAc-Hex-4Hex pentasaccharide (data not shown).

The MS^2 spectrum of the ion at *m/z* 1073 at retention time 18.2 min (Figure 8A) had a series of Y ions (Y_3 at *m/z* 546, Y_4 at *m/z* 708 and Y_5 at *m/z* 870) and a series of B ions (B_2 at *m/z* 364, B_3 at *m/z* 526, B_4 at *m/z* 729 and B_5 at *m/z* 891). The $^{2,4}A_4$ ion at *m/z* 586 and the $^{0,2}A_4$-H_2O ion at *m/z* 628 demonstrated a 4-substitution

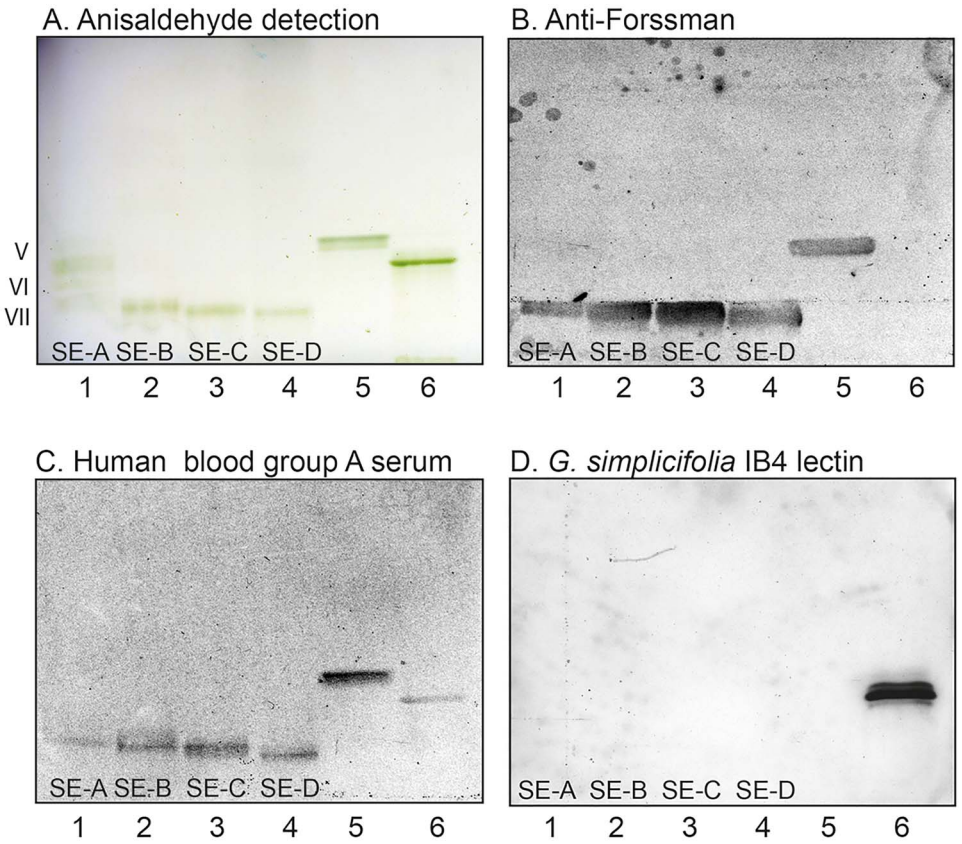

Fig. 5. Binding of anti-Forssman antibodies, human serum antibodies and lectins to subfractions from sheep erythrocyte glycosphingolipids. Thin-layer chromatogram after detection with anisaldehyde (**A**), and autoradiograms obtained by binding of anti-Forssman antibodies (**B**), human serum antibodies from a blood group A individual (**C**) and *G. simplicifolia* IB4 lectin (**D**), followed by autoradiography for 12 h, as described under *Materials and methods*. The solvent system used was chloroform/methanol/water (60:35:8, by volume). The lanes were: lanes 1–4, glycosphingolipid subfractions SE-A–SE-D isolated from sheep erythrocytes, 4 μg/lane; lane 5, reference Forssman pentaosylceramide (GalNAcα3GalNAcβ3Galα4Galβ4Glcβ1Cer), 4 μg; lane 6, Galili pentaosylceramide (Galα3Galβ4GlcNAcβ3Galβ4Glcβ1Cer), 4 μg. The Roman numbers to the left of (**A**) indicate the number of carbohydrate units in the bands.

of the internal HexNAc (Chai et al. 2001; Karlsson et al. 2010). Taken together, this allowed the identification of a hexasaccharide with HexNAc-Hex-Hex-4HexNAc-Hex-4Hex sequence. The most important ions in this spectrum were the Y_5 ion at *m/z* 870 and the Z_5 ion at *m/z* 852, which were due to loss of terminal HexNAc. The MS^2 spectrum obtained of the ion at *m/z* 1073 at retention time 27.8 min (Figure 8B) was very similar, but had a Y_5 ion at *m/z* 911 and a Z_5 ion at *m/z* 893, caused by loss of a terminal Hex. The features of this spectrum gave a tentative identification of a Hex-HexNAc-Hex-4HexNAc-Hex-Hex saccharide.

Further digestion with β-N-hexosaminidase (Figure 7C) resulted in one more loss of 203 Da, and *m/z* 708 was converted to *m/z* 505, *m/z* 911 to *m/z* 708 and *m/z* 1073 to *m/z* 870. Here, MS^2 of the ion at *m/z* 505 at retention time 9.4 min identified a globo trisaccharide, and MS^2 of the ion at *m/z* 708 at retention time 15.4 min demonstrated Hex-4HexNAc-Hex-4Hex tetrasaccharide, i.e. a neolacto tetrasaccharide (data not shown).

The ion at *m/z* 870 eluted at the same retention time as the reduced pentasaccharide obtained from reference Galili pentaosylceramide (Galα3Galβ4GlcNAcβ3Galβ4Glcβ1Cer) from rabbit erythrocytes (data not shown), and upon MS^2 of this ion (Figure 8C), a spectrum with high similarity to the MS^2 spectrum of reference Galili pentasaccharide (Figure 8D) was obtained.

Thus, the sequential hydrolysis with α-N-acetylgalactosaminidase and β-N-hexosaminidase demonstrated a Galili pentasaccharide elongated with a terminal Forssman determinant, i.e. a GalNAcα3GalNAcβ3Galα3Galβ4GlcNAcβ3Galβ4Glc heptasaccharide.

The oligosaccharides derived from sheep erythrocyte glycosphingolipids and identified by LC-ESI/MS are summarized in Table II.

Discussion

The recently characterized FORS system is a rare blood group system, and only a few FORS-positive individuals have so far been identified (Svensson et al. 2013). Thus, naturally occurring anti-FORS1 antibodies are present in the plasma of most people. These antibodies can hemolyze FORS1-expressing erythrocytes in the presence of complement in vitro (Svensson et al. 2013), indicating that anti-FORS1 may cause intravascular hemolysis if FORS1-positive erythrocytes are used for transfusion.

Since human-FORS1-positive erythrocytes most often are not available, hemagglutination assays using FORS1-expressing sheep erythrocytes are often used for the screening of anti-FORS1 antibodies. The initial purpose of this study was to investigate if glycosphingolipids with the Galili epitope are present in sheep erythrocytes, since hemagglutination by binding of anti-Gal antibodies might influence the results of such studies. The presence of a Galili pentaosylceramide and a Galili heptaosylceramide among the non-acid

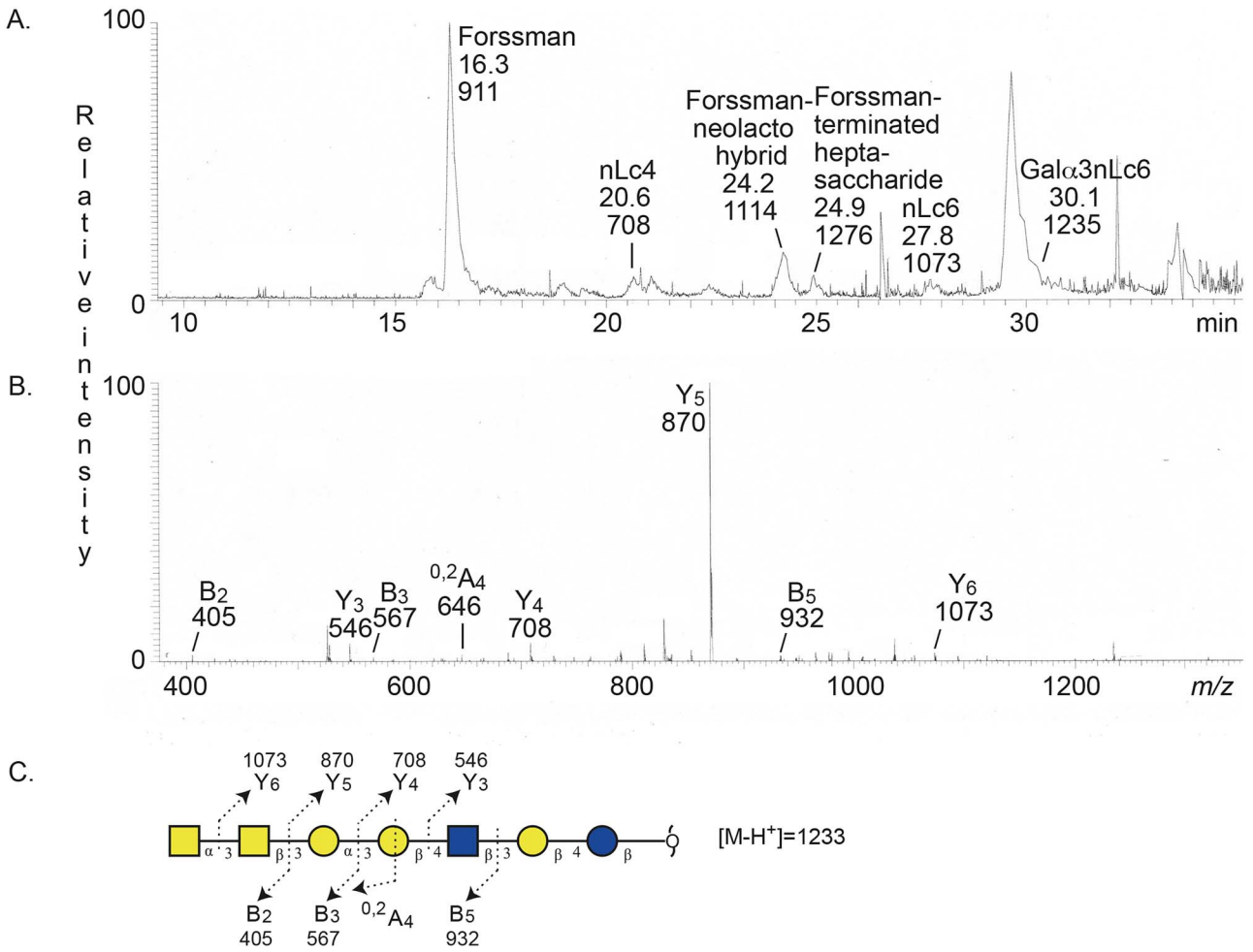

Fig. 6. LC-ESI/MS of the reduced oligosaccharides obtained by digestion of fraction SE-D from sheep erythrocytes with *Rhodococcus* endoglycoceramidase II. (**A**) Base peak chromatogram from LC-ESI/MS of the reduced oligosaccharides obtained by digestion of fraction SE-D. (**B**) MS^2 of the ion at *m*/z 1276 (retention time 24.9 min). (**C**) Interpretation formula. See Table II for oligosaccharide structure. The identification of oligosaccharides was based on their retention times, determined by molecular masses and subsequent MS^2 sequencing, and on the basis of knowledge of glycosphingolipid biosynthetic pathways. The proposed structures are depicted using the Symbol Nomenclature for Glycomics (SNFG) (Varki et al. 2015; Neelamegham et al. 2019), and nomenclature of fragments defined by Domon and Costello (1988).

Table II. Oligosaccharides derived from sheep erythrocyte glycosphingolipids identified by LC-ESI/MS

Trivial name	Structure
Globotri	Galα4Galβ4Glc
Globotetra	GalNAcβ3Galα4Galβ4Glc
Neolactotetra	Galβ4GlcNAcβ3Galβ4Glc
Forssman	GalNAcα3GalNAcβ3Galα4Galβ4Glc
Le^a penta	Galβ3(Fucα4)GlcNAcβ3Galβ4Glc
H type-1 penta	Fucα2Galβ3GlcNAcβ3Galβ4Glc
H type-2 penta	Fucα2Galβ4GlcNAcβ3Galβ4Glc
Galili penta/Galα3nLc4	Galα3Galβ4GlcNAcβ3Galβ4Glc
Neolactohexa	Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glc
Le^b hexa	Fucα2Galβ3(Fucα4)GlcNAcβ3Galβ4Glc
Le^y hexa	Fucα2Galβ4(Fucα3)GlcNAcβ3Galβ4Glc
Forssman neolacto	GalNAcα3GalNAcβ3Galβ4GlcNAcβ3Galβ4Glc
Galili hepta/Galα3nLc6	Galα3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glc
Forssman Galili	GalNAcα3GalNAcβ3Galα3Galβ4GlcNAcβ3Galβ4Glc

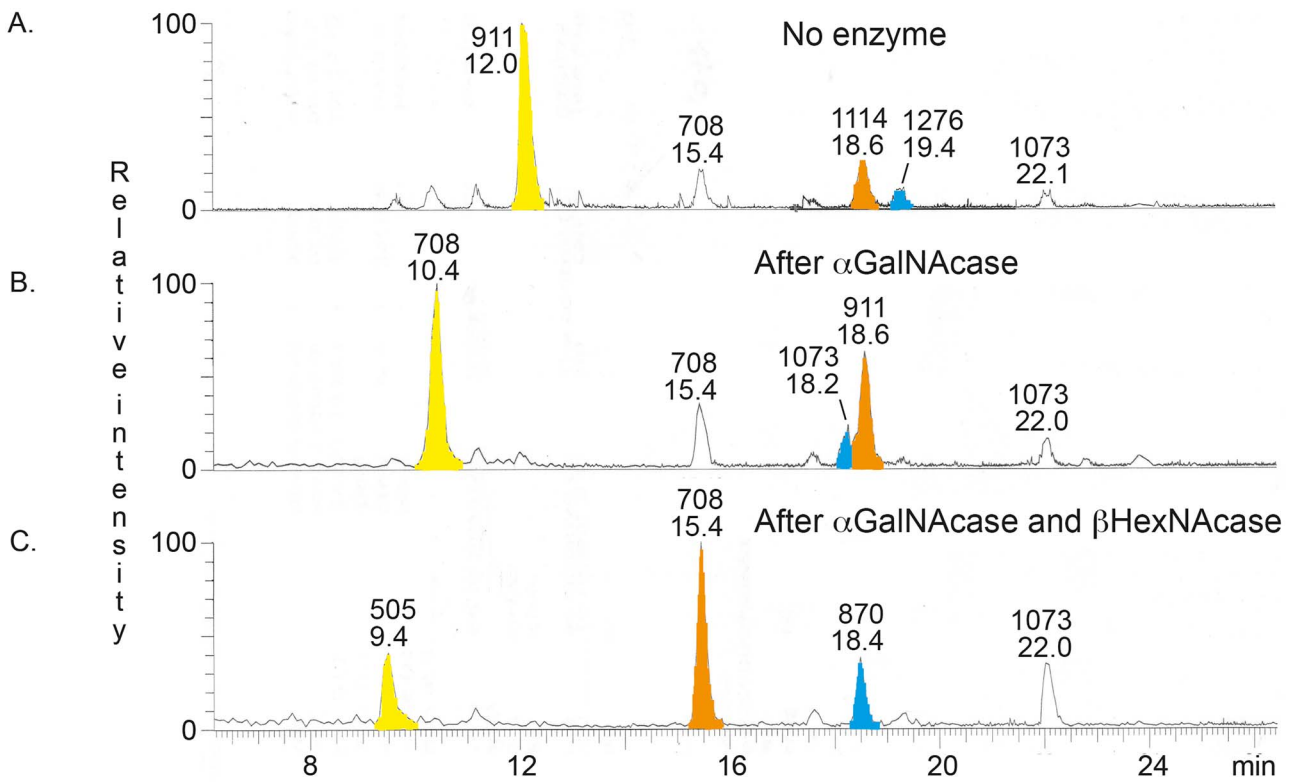

Fig. 7. Enzymatic digestion. (**A**) Base peak chromatogram from LC-ESI/MS of the reduced oligosaccharides obtained by digestion of fraction SE-D with *Rhodococcus* endoglycoceramidase II. (**B**) Base peak chromatogram from LC-ESI/MS of the reduced oligosaccharides from fraction SE-D after digestion with α-N-acetylgalactosaminidase. (**C**) Base peak chromatogram from LC-ESI/MS of the reduced oligosaccharides from fraction SE-D after digestion with both α-N-acetylgalactosaminidase and β-N-acetylhexosaminidase. The colors denote parent and related product glycans. Treatment with α-N-acetylgalactosaminidase (**B**) gave removal of terminal GalNAcα (203 Da) from *m/z* 911 (Forssman pentasaccharide; yellow), *m/z* 1114 (Forssman neolacto hexasaccharide; orange) and *m/z* 1276 (HexNAc-HexNAc-Hex-Hex-4HexNAc-Hex-4Hex heptasaccharide; blue), resulting in novel molecular ions at *m/z* 708 at retention time 10.4 min (yellow), *m/z* 911 at retention time 18.6 min (orange), and *m/z* 1073 at retention time 18.2 min (blue). Further digestion with β-N-hexosaminidase (**C**) gave one more loss of 203 Da, and *m/z* 708 was converted to *m/z* 505 (yellow), *m/z* 911 to *m/z* 708 (orange) and *m/z* 1073 to *m/z* 870 (blue).

glycosphingolipids of sheep erythrocytes was demonstrated by LC-ESI/MS and binding of *G. simplicifolia* IB4 lectin. Thus, removal of anti-Gal antibodies might facilitate the interpretation of the results when searching for anti-FORS1 antibodies by hemagglutination assays using sheep erythrocytes.

However, removal of anti-Gal antibodies by adsorption is a cumbersome procedure, and not suitable for large-scale screenings. An alternative approach for screening for anti-FORS1 antibodies is to introduce synthetic FORS1 glycolipids in the erythrocyte membrane of human blood group O cells, using for example the function-spacer-lipid (FSL) Kode constructs (KODE Technology) (Frame et al. 2007).

During the course of experiments, we found that among the non-acid glycosphingolipids from sheep erythrocytes there were also compounds migrating below the pentaosylceramide region, which were recognized by human serum antibodies. Binding to these compounds also occurred after adsorption of anti-Gal antibodies. After partial purification, these binding-active glycosphingolipids were characterized as a Forssman neolacto hybrid hexaosylceramide (GalNAcα3GalNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer) and a Forssman Galili hybrid heptaosylceramide (GalNAcα3GalNAcβ3Galα3Galβ4GlcNAcβ3Galβ4Glcβ1Cer). These are novel glycosphingolipid structures and the first characterization of complex glycosphingolipids larger than pentaosylceramide carrying the Forssman disaccharide. Furthermore, to our knowledge, this is the first characterization of an elongated Galili antigen.

Conclusion

Thus, the anti-Forssman antibodies in human serum bind not only to the classical Forssman pentaosylceramide (GalNAcα3GalNAcβ3Galα4Galβ4Glcβ1Cer), but also when the GalNAcα3GalNAcβ3 sequence is presented on a neolacto core chain and even on a Galili carbohydrate sequence.

Materials and methods

Glycosphingolipid preparations

The isolation of total acid and total non-acid glycosphingolipids from sheep erythrocytes (5.2 L of sheep blood) was done as described (Falk et al. 1981). First, the blood was centrifuged and the plasma removed. The cells were washed five times with one volume saline, and thereafter cell membranes were prepared by toluene flotation (Koicielak et al. 1973). The membranes were thereafter extracted two times with methanol and one time with chloroform and methanol (2:1 by volume). The extract was subjected to mild alkaline hydrolysis and dialysis, followed by the separation on a silicic acid column. Acid and non-acid glycosphingolipid fractions were obtained by chromatography on a DEAE-cellulose column. In order to separate the non-acid glycosphingolipids from alkali-stable phospholipids, the non-acid fractions were acetylated and separated on a second silicic acid column, followed by deacetylation and dialysis. Final

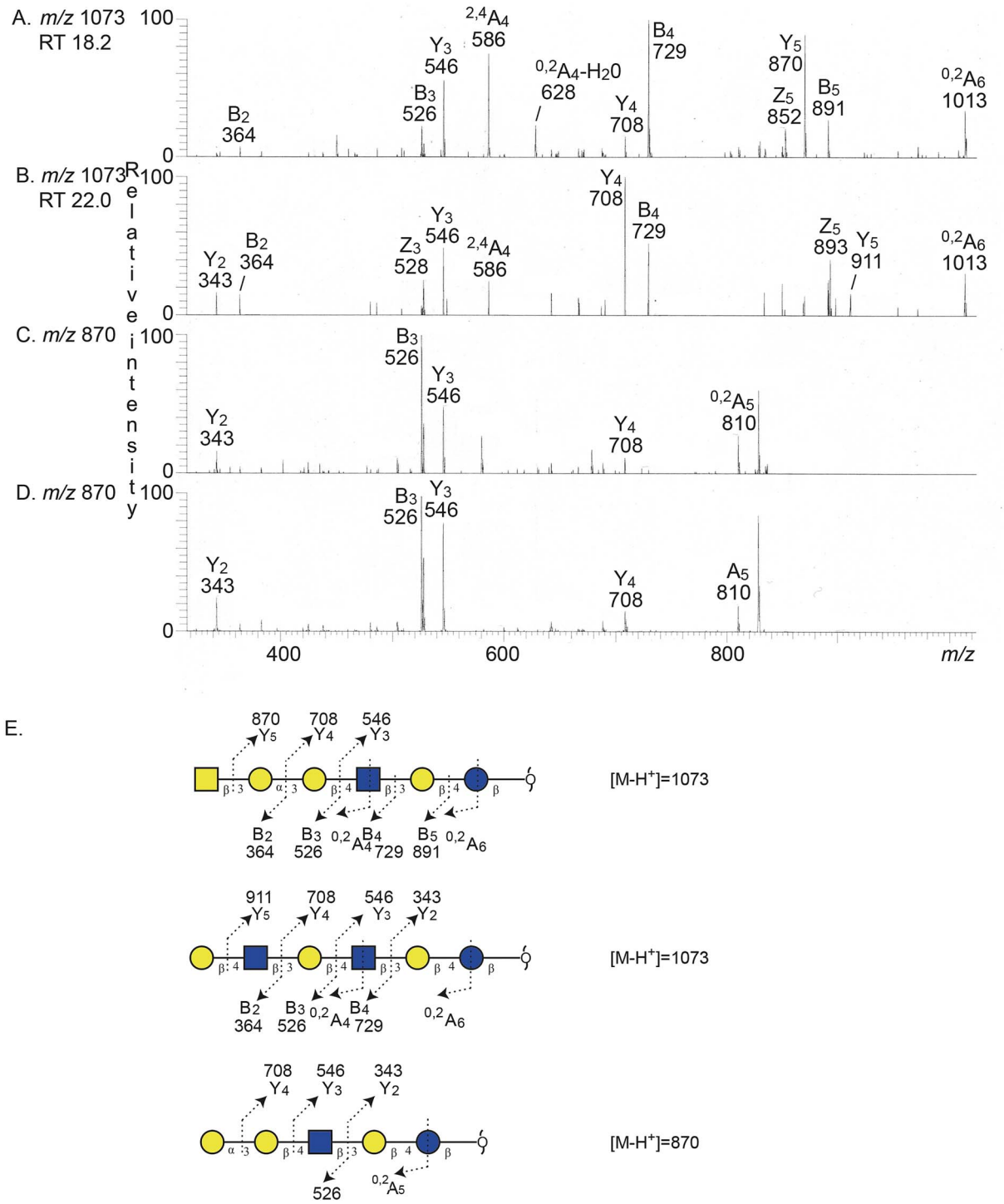

Fig. 8. MS^2 from LC-ESI/MS of the reduced oligosaccharides from fraction SE-D after enzymatic digestion. (**A**) MS^2 of the ion at *m/z* 1073 (retention time 18.2 min) from LC-ESI/MS of the reduced oligosaccharides from fraction SE-D after digestion with α-N-acetylgalactosaminidase (Figure 7B). (**B**) MS^2 of the ion at *m/z* 1073 (retention time 22.1 min) from LC-ESI/MS of the reduced oligosaccharides from fraction SE-D after digestion with α-N-acetylgalactosaminidase (Figure 7B). (**C**) MS^2 of the ion at *m/z* 870 (retention time 18.4 min) from LC-ESI/MS of the reduced oligosaccharides from fraction SE-D after digestion with both α-N-acetylgalactosaminidase and β-N-acetylhexosaminidase (Figure 7C). (**D**) MS^2 of the ion at *m/z* 870 (retention time 18.4 min) from LC-ESI/MS of the reduced oligosaccharides from reference Galili pentaosylceramide (Galα3Galβ4GlcNAcβ3Galβ4Glcβ1Cer) from rabbit erythrocytes. The identification of oligosaccharides was based on their retention times, determined molecular masses and subsequent MS^2 sequencing, and on the basis of knowledge of glycosphingolipid biosynthetic pathways. The proposed structures are depicted using the Symbol Nomenclature for Glycomics (SNFG) (Varki et al. 2015; Neelamegham et al. 2019), and nomenclature of fragments defined by Domon and Costello (1988).

purifications were done by chromatographies on DEAE-cellulose and silicic acid columns. Thereby, 80 mg of total non-acid glycosphingolipids were obtained.

Part of the total non-acid glycosphingolipid fraction from sheep erythrocytes (50 mg) was separated into three fractions by chromatography on a silica gel column eluted with increasing amounts of methanol in chloroform. Thereby, three glycosphingolipid containing fractions were obtained (denoted fractions SE-1 (6.7 mg), SE-2 (20 mg) and SE-3 (20 mg)).

After initial tests for binding of human serum antibodies (see below), fraction SE-3 was separated on a 5-g Iatrobeads (Iatrobeads 6RS-8060; Iatron Laboratories, Tokyo) column eluted with chloroform:methanol:water (60:35:8, by volume), 20 × 1 mL. The fractions were analyzed by thin-layer chromatography and pooled according to their mobility on thin-layer chromatograms. Thereby, eight glycosphingolipid-containing fractions were obtained (denoted fractions SE-I–SE-VIII). These fractions were characterized by LC-ESI/MS and by binding of carbohydrate-recognizing ligands.

Thereafter, fractions SE-VI–SE-VIII were pooled and further separated on a 2-g Iatrobeads column eluted with chloroform:methanol:water (60:35:8, by volume), 40 × 0.5 mL. After pooling, four glycosphingolipid-containing fractions were obtained (denoted fractions SE-A–SE-D).

Reference glycosphingolipids

Total acid and non-acid glycosphingolipid fractions were isolated as described (Karlsson 1987). Individual glycosphingolipids were isolated by repeated chromatography on silicic acid columns and by HPLC, and identified by mass spectrometry (Samuelsson et al. 1990; Karlsson et al. 2010) and ^{1}H-NMR spectroscopy (Koerner Jr et al. 1983).

Thin-layer chromatography

Thin-layer chromatography was done on aluminum- or glass-backed silica gel 60 high-performance thin-layer chromatography plates (Merck, Germany; 105641/105547). Glycosphingolipid mixtures (40 μg) or pure glycosphingolipids (4 μg) were applied to the plates and developed with chloroform/methanol/water 60:35:8 (by volume). Chemical detection was done with anisaldehyde (Waldi 1962).

Adsorption of anti-Gal antibodies

Adsorption of anti-Gal antibodies (Mayr 2005) was performed by adding human plasma to rabbit erythrocytes in the proportion of 2:1 (by volume). After 1 h incubation at room temperature, the tubes were centrifuged. Thereafter, the plasma was collected and tested for hemagglutination of rabbit erythrocytes. The procedure was repeated until no reaction was observed.

Chromatogram binding assays

Binding of human serum antibodies to glycosphingolipids on thin-layer chromatograms was done as previously reported (Zhou et al. 2004). Dried chromatograms were dipped for 1 min in diethylether/n-hexane (1:5, by volume) containing 0.5% (w/v) polyisobutylmethacrylate (Sigma-Aldrich; 181544). After drying, the chromatograms were soaked in phosphate-buffered saline, pH 7.3 (PBS) containing 2% bovine serum albumin (w/v), 0.1% NaN_3 (w/v) and 0.1% Tween 20 (by volume) (PBS/BSA/Tween) for 2 h at room temperature. The chromatograms were subsequently covered with human serum diluted 1:10 in PBS/BSA/Tween, and incubated for 2 h at room temperature. After washing with PBS, there followed a second 2 h incubation with ^{125}I-labeled (labeled by the IODO-GEN method according to the manufacturer's instructions (Pierce/Thermo Scientific) goat anti-human IgG/IgM/IgA (H-L) antibodies (Invitrogen/Thermo Scientific; 31128) diluted to 2 × 10^6 cpm/mL in PBS/BSA/Tween. Finally, the plates were washed six times with PBS. Dried chromatograms were autoradiographed for 12 h using XAR-5 X-ray films (Carestream; 8941114).

Binding assays with monoclonal anti-Forssman antibodies were done using hybridoma supernatants (ATCC; M1/22.25.8HL) diluted 1:100 in PBS/BSA/Tween and incubated for 2 h at room temperature. The chromatograms were washed with PBS, and then incubated with ^{125}I-labeled rabbit anti-mouse antibodies (DakoCytomation Norden A/S; Z0259) diluted to 2 × 10^6 cpm/mL in PBS/BSA/Tween. After washing with PBS and drying, the chromatograms were autoradiographed for 12 h with XAR-5 X-ray films.

Binding of ^{125}I-labeled *H. pomatia* lectin (Sigma-Aldrich; L3382) and ^{125}I-labeled *G. simplicifolia* IB4 lectin (Advanced Targeting Systems; PR-02) to glycosphingolipids on thin-layer chromatograms was done as described (Teneberg et al. 1994).

The binding specificities of the ligands used in the chromatogram binding experiments are given in Table I.

Endoglycoceramidase digestion and LC-ESI/MS

Endoglycoceramidase II from *Rhodococcus* spp. (Takara Bio Europe AB; 4460) was used for the hydrolysis of the non-acid glycosphingolipids (Karlsson et al. 2010). The glycosphingolipids (50 μg) were resuspended in 100 μL of 0.05 M sodium acetate buffer, pH 5.0, containing 120 μg of sodium cholate, and sonicated briefly. Thereafter, 1 mU of enzyme was added, and the mixture was incubated at 37°C for 48 h. The reaction was stopped by the addition of chloroform/methanol/water to the final proportions 8:4:3 (by volume). The oligosaccharide-containing upper phase thus obtained was separated from detergent on a Sep-Pak QMA cartridge (Waters; WAT020545). The eluent containing the oligosaccharides was dried under nitrogen and under vacuum.

Selected oligosaccharide samples were reduced by adding 20 μL of 200 mM $NaBH_4$ in 50 mM KOH to the sample and incubating at 50°C for 2 h (Karlsson et al. 2010). The samples were then acidified by adding 10 μL of glacial acetic acid, and the oligosaccharides were desalted by the cation exchange chromatography, and thereafter evaporated to dryness.

The glycosphingolipid-derived oligosaccharides were resuspended in 50 μL of water and analyzed by LC-ESI/MS as described (Karlsson et al. 2010). The oligosaccharides were separated on a column (100 × 0.25 mm) packed in-house with 5 μm porous graphite particles (Hypercarb, Thermo-Hypersil, Runcorn, UK). An autosampler, HTC-PAL (CTC Analytics AG, Zwingen, Switzerland) equipped with a cheminert valve (0.25 mm bore) and a 2 μL loop, was used for sample injection. An Agilent 1100 binary pump (Agilent technologies, Palo Alto, CA) delivered a flow of 250 μL/min, which was split down in an 1/16″ microvolume-T (0.15 mm bore) (Vici AG International, Schenkon, Switzerland) by a 50 cm × 50 μm i.d. fused silica capillary before the injector of the autosampler, allowing approximately 3–5 μL/min through the column. The oligosaccharides (3 μL) were injected on to the column and eluted with an acetonitrile gradient (A: 10 mM ammonium bicarbonate; B: 10 mM ammonium bicarbonate in 80% acetonitrile). The gradient (0–45% B) was eluted for 46 min, followed by a wash step with 100% B and equilibration of the column for 24 min. A 30 cm × 50 μm i.d. fused silica capillary was used as transfer line to the ion source.

The saccharides were analyzed in negative ion mode on an LTQ linear quadrupole ion trap mass spectrometer (Thermo Electron, San José, CA). The IonMax standard ESI source on the LTQ mass spectrometer was equipped with a stainless steel needle kept at −3.5 kV. Compressed air was used as a nebulizer gas. The heated capillary was kept at 270°C, and the capillary voltage was −50 kV. Full scan (*m/z* 380–2000, 2 microscans, maximum 100 ms, target value of 30,000) was performed, followed by data-dependent MS^2 scans of the three most abundant ions in each scan (two microscans, maximum 100 ms, target value of 10,000). The threshold for MS^2 was set to 500 counts. Normalized collision energy was 35%, and an isolation window of 3 u, an activation $q = 0.25$ and an activation time of 30 ms, was used. Selected fractions were also analyzed at *m/z* 1300–2000. Data acquisition and processing were conducted with Xcalibur software (Version 2.0.7).

Manual assignment of glycan sequences was done on the basis of knowledge of glycosphingolipid biosynthetic pathways, with the assistance of the Glycoworkbench tool (Version 2.1), and by comparison of retention times and MS^2 spectra of oligosaccharides from reference glycosphingolipids (Karlsson et al. 2010).

Enzymatic hydrolysis

The reduced oligosaccharides from fraction SE-D were dissolved in 20 µL of 100 mM sodium citrate phosphate buffer (pH 5.0) and first digested with 1.5 mU chicken liver α-N-acetylgalactosaminidase (Prozyme/Agilent; GKX5001) at 37°C overnight, and thereafter with 50 mU Jack bean β-N-acetylhexosaminidase (Prozyme/Agilent; GKX5003) at 37°C overnight. The samples were cleaned up with Hypersep Hypercarb (Thermo Scientific) according to the manufacturer's instructions.

Supplementary data

Supplementary data for this article are available online at http://glycob.oxfordjournals.org/.

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Conflict of interest statement

The authors declare no conflict of interest.

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