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 neo-lacto 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 (GalNAcα3GalNAcβ3Galβ4Glcβ1Cer), but also when the GalNAcα3GalNAcβ3 sequence is presented on a neo-lacto 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, P1, PK, 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 Apha phenotype express the Forssman antigen (GalNAca3Galβ3Galα4Galα4Glcβ1Cer) 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 allele (c.887G>A) was not found among 2304 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-acetylgalactosamine-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 (Galβ3Galα4GlcNAcβ3Galα4Glcβ1Cer) pentaosylceramides, and a Galili heptaosylceramide (Galβ3Galα4GlcNAcβ3Galα4GlcNAcβ3-Galβ3Galα4Glcβ1Cer). Furthermore, two novel glycosphingolipids with a terminal Forssman epitope: a Forssman neolacto hybrid hexaosylceramide (GalNAca3Galα4GlcNAcβ3Galα4GlcNAcβ3Galα4Glcβ1Cer) and a Galili pentaosylceramide elongated with a terminal Forssman determinant (GalNAca3Galα4GlcNAcβ3Galα4Glcβ1Cer) 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 (GalNAca3Galβ3Galα4Glcβ1Cer) (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 Iatro beads 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- to 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 MS2 (Chai et al. 2001; Karlsson et al. 2010). Furthermore, MS3 spectra of oligosaccharides with a Hex or HexNAc substituted at C-4 have diagnostic cross-ring β,α-type fragment ions (β,α-A and β,α-A-H2O), which allow differentiation of linkage positions. Thus, such β,α-type fragment ions are found in MS3 spectra of oligosaccharides with globo (Galα4Gal) or type-2 (Galβ4GlcNAc) core structures, but are absent in the spectra...
Sheep erythrocyte Forssman-like glycosphingolipids

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 (GalNAc\(^\beta\)3Gal\(^\alpha\)3Gal\(^\beta\)3Gal\(^\alpha\)4Gal\(^\beta\)4Glc\(^\beta\)1Cer), 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 (Gal\(^\alpha\)3Gal) or type-1 (Gal\(^\beta\)3GlcNAc) 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-HexNAc-Hex-Hex sequence. Cross-ring \(^2\)A-type fragments are diagnostic for carbohydrates substituted at C-4 (Chai et al. 2001; Karlsson et al. 2010), and, thus the \(^2\)A\(_3\) fragment ion at \(m/z\) 443 and the \(^2\)A\(_3\)-H\(_2\)O fragment ion at \(m/z\) 425 demonstrated a substitution of the HexNAc at C-4, i.e. a type-2 chain, while the \(^2\)A\(_5\) ion at \(m/z\) 808 and the \(^2\)A\(_5\)-H\(_2\)O 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 Gal\(^\alpha\)3Gal\(^\beta\)4GlcNAc-terminated pentasaccharide, i.e. a Galili pentasaccharide (Gal\(^\alpha\)3Gal\(^\beta\)4GlcNAc\(^\beta\)3Gal\(^\beta\)4Glc).

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 \(^2\)A\(_4\) fragment ion at \(m/z\) 687 demonstrating 4-substitution of the internal Hex. Taken together, this indicated a FORS pentasaccharide (GalNAc\(^\alpha\)3GalNAc\(^\beta\)3Gal\(^\alpha\)4Gal\(^\beta\)4Glc) and a FORS pentasaccharide (Gal\(^\alpha\)3Gal\(^\beta\)4GlcNAc\(^\beta\)3Gal\(^\beta\)4Glc).

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-HexNAc-Hex-Hex-Hex sequence. The prominent \(^2\)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 (GalNAc\(^\alpha\)3GalNAc\(^\beta\)3Gal\(^\beta\)4GlcNAc\(^\beta\)3Gal\(^\beta\)4Glc).

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 \((\text{GalNAc}_\alpha 3\text{GalNAc}_\beta 3\text{Gal}_\alpha 4\text{Gal}_\beta 4\text{Glc}_\beta 1\text{Cer})\), 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, MS² 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. MS² of the ion at \(m/z\) 1112 again identified the Forssman neolacto hybrid (Figure 4B).

The MS² 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 heptasaccharide. The \(0^2A_3\) fragment ion at \(m/z\) 443 indicated a Hex-Hex-HexNAc terminal with substitution of the HexNAc at C-4, while the \(0^2A_5\) fragment ion at \(m/z\) 808, along with the \(0^2A_3\)-H₂O fragment ion at \(m/z\) 790, demonstrated the 4-substitution of the internal HexNAc. Taken together, this indicated a Hex-Hex-HexNAc-Hex-HexNAc-Hex-Hex sequence, were present in the MS² spectrum (Figure 4D). The \(0^2A_4\) 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-HexNAc-Hex-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 MS² 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 pentao-lysylceramide 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 Galβ 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) MS² of the ion at m/z 868 (retention time 24.4 min). (C) MS² of the ion at m/z 909 (retention time 16.1 min). (D) MS² 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 MS² 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) MS² of the ion at m/z 1112 (retention time 24.8 min). (C) MS² of the ion at m/z 1233 (retention time 29.8 min). (D) MS² 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 MS² 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).
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 (Galα3Galβ4GlcNAcβ3Galβ4Glcβ1Cer). 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 heptasaccharide with a terminal Forssman epitope (Galα3Galβ4GlcNAcβ3Galβ4Glc).

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, Lea, H type-1 and H type-2 pentasaccharides, and Leb and Leα hexaosylceramides 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 MS² 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 hexaosylceramide (m/z 1073), the Forssman neolacto hybrid (m/z 1114) and the Galili heptasaccharide (m/z 1235) were again identified by MS² (data not shown). The three fractions also had a molecular ion at m/z 1276. MS² of this ion (Figure 6B) gave a Y ion series (Y3 at m/z 546, Y4 at m/z 708, Y5 at m/z 870 and Y6 at m/z 1073), which along with the B2 ion at m/z 405, the B3 ion at m/z 567 and B5 ion at m/z 932 again gave the identification of a HexNAc-HexNAc-Hex-HexNAc-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 (Galα3Galβ4Glc).

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 hexaosylceramide) and m/z 1276 (HexNAc-HexNAc-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). MS² of the ion at m/z 708 at retention time 10.4 min identified a globo tetrasaccharide, and MS² of the ion at m/z 911 at retention time 18.6 min demonstrated HexNAc-Hex-4HexNAc-Hex-4Hex heptasaccharide (data not shown).

The MS² spectrum of the ion at m/z 1073 at retention time 18.2 min (Figure 8A) had a series of Y ions (Y3 at m/z 546, Y4 at m/z 708 and Y5 at m/z 870) and a series of B ions (B2 at m/z 364, B3 at m/z 526, B4 at m/z 729 and B5 at m/z 891). The 2,4-A4 ion at m/z 586 and the 3,2-A4-H2O ion at m/z 628 demonstrated a 4-substitution.

### 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)   |
| Adsorbed human serum (blood group O) | –                           | Galα3(Fucα2)Gal (blood group A antigen)   |

Ligand Manufacturer Binding specificity

| 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)   |
| Adsorbed human serum (blood group O) | –                           | Galα3(Fucα2)Gal (blood group A antigen)   |

Ligand Manufacturer Binding specificity

| 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)   |
| Adsorbed human serum (blood group O) | –                           | Galα3(Fucα2)Gal (blood group A antigen)   |
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 Y5 ion at m/z 870 and the Z5 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 Y5 ion at m/z 911 and a Z5 ion at m/z 893, caused by loss of a terminal Hex. The features of this spectrum gave a tentative identification of a Hex-4HexNAc-Hex-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-Hex 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 (Galα₃Galβ4GlcNAcβ3Galβ4Glc/β1Cer) from rabbit erythrocytes (data not shown), and upon MS^3 of this ion (Figure 8C), a spectrum with high similarity to the MS^2 spectrum of reference Galili pentaosaccharide (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-FORS 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 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 heptaoisylceramide among the non-acid
Sheep erythrocyte Forssman-like glycosphingolipids

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) MS² 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 MS² 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                      | GalNAcβ3GalNAcβ3Galα4Galβ4Glc                 |
| Le⁺ penta                     | Galβ3(Fucα4)GlcNAcβ3Galβ4Glc                  |
| H type-1 penta                | Fucα2Galβ3GlcNAcβ3Galβ4Glc                   |
| H type-2 penta                | Fucα2Galβ4GlcNAcβ3Galβ4Glc                   |
| Galili penta/Galα3nLc4        | Galβ3Galβ4GlcNAcβ3Galβ4Glc                   |
| Neolactohexa                  | Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glc            |
| Le⁺ hexa                      | Fucα2Galβ3(Fucα4)GlcNAcβ3Galβ4Glc             |
| Le⁺ hexa                      | Fucα2Galβ4(Fucα3)GlcNAcβ3Galβ4Glc             |
| Forssman neolacto             | GalNAcβ3GalNAcβ3Galβ4GlcNAcβ3Galβ4Glc         |
| Galili hepta/Galα3nLc6        | Galβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glc       |
| Forssman Galili               | GalNAcβ3GalNAcβ3Galβ3Galβ4GlcNAcβ3Galβ4Glc    |
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 (GalNAca3GalNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer) and a Forssman Galili hybrid heptaosylceramide (GalNAca3GalNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer). 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 (GalNAca3GalNAcβ3Galβ4Glcβ1Cer), but also when the GalNAca3GalNAcβ3 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 (Kociolek 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. 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. MS² from LC-ESI/MS of the reduced oligosaccharides from fraction SE-D after enzymatic digestion. (A) MS² 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) MS² 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) MS² 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) MS² 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 MS² 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).
purgations 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 1H-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 diethylhether/n-hexane (1:5, by volume) containing 0.5% (w/v) polysorbylemetalrate (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% NaN3 (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 125I-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 × 104 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 125I-labeled rabbit anti-mouse antibodies (DakoCytomation Norden A/S; Z0239) diluted to 2 × 106 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 125I-labeled H. pomatia lectin (Sigma-Aldrich; L3382) and 125I-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 1.

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 μU 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 NaBH4 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 MS² scans of the three most abundant ions in each scan (two microscans, maximum 100 ms, target value of 10,000). The threshold for MS² 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 MS² 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 μL chicken liver α-N-acetylgalactosaminidase (Prozyme/Agilent; GKX5001) at 37°C overnight, and thereafter with 50 μL 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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