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STRUCTURES OF THE EIGHT- TO NINE-SUGAR GLYCOLIPIDS OF HUMAN BLOOD GROUP A ERYTHROCYTES*

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ABSTRACT

Two glycolipid fractions, isolated in 1975 from blood group A_1 erythrocytes and shown on the basis of direct-inlet mass spectrometry to contain eight- and nine-sugar A-type sequences, have been reinvestigated by fast-atom-bombardment mass spectrometry and overlay analysis with selected monoclonal anti-A antibodies. The presence of three separate glycolipids was concluded, consistent with a common paragloboside backbone [β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-D-Glc] and a typical erythrocyte ceramide component (sphingosine, and 22-, 23-, 24-, and 25-carbon nonhydroxy fatty acids). It is proposed that they carry A determinants based on Type 1 [β-D-Gal*p*-(1→3)-β-D-Glc*p*NAc], Type 2 [β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc], and Type 3 [β-D-Gal*p*-(1→3)-α-D-Gal*p*NAc] chains, respectively. The Type 1 (eight sugars) and Type 3 (nine sugars) glycolipids appeared in mixtures of both the native and the acetylated form. The existence of Type 1 glycolipid, which appears to be a genuine erythrocyte glycolipid as concluded from the ceramide composition, had been predicted earlier by other workers.

INTRODUCTION

Human erythrocytes are by far the most studied cells for their immunology, especially blood group serology[1]. In recent years, studies of the chemical basis of blood group reactivities have intensified, mostly those of glycolipids[2]. A problem of particular interest that has fascinated many scientists is the distinction[2,3] between subgroups A_1 and A_2. Conflicting views on the chemical basis of this distinction were based on a quantitative or a qualitative difference between the determinants. Early studies by Watkins and Morgan[4] suggested a less efficient GalNActransferase for the A_2 than for the A_1 determinant, which would be responsible for the lower number of blood group A determinants and the higher number of precursor blood

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group H determinants on A_2 as compared to A_1 erythrocytes. This view was supported by many later contributions. On the basis of detailed immunochemical studies with A_1 and A_2 glycoproteins, the other hypothesis presented a qualitative difference as well[5,6]. It proposed that A_1 individuals express A determinants on the basis of both Type-1 [β-D-Gal*p*-(1→3)-β-D-Glc*p*NAc] and Type-2 [β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc] chains, whereas A_2 individuals lack Type-1 chains. However, it has been recognized for a long time that erythrocyte glycolipids have Type-2 chains only[2]. Recent attempts to identify Type-1 chains with the help of a specific anti-A Type 1 monoclonal antibody, AH21, resulted in the isolation, in minor proportions, of a six-sugar glycolipid having a ceramide composition typical of blood plasma and not of erythrocyte glycolipids; this suggested an uptake from the plasma and not an original erythrocyte component[7]. A recent proposal for explaining the difference between A_1 and A_2 was based on the discovery of a blood group A determinant having a Type-3 chain[8], which is a repetitive A determinant (**1**). This is present in A_1 erythrocytes only whereas A_2 erythrocytes carry the immediate precursor (**2**), which is the H determinant based on the Type-3 chain[9,10].

Isolated erythrocyte glycolipids have been used by us as model substances for mass spectrometry[11,12] and n.m.r. spectroscopy[13–15]. The results of a particular chromatographic fraction of pooled blood group A_1 erythrocytes were presented earlier by one of us[16]. They included aspects on Type-1 chain compounds in erythrocytes and blood plasma. As a dedication to Professor Walter T. J. Morgan, who initiated the work just summarized, the material investigated earlier[16] has been reinvestigated by two methods that were not available at the time of the first presentation; namely f.a.b. mass spectrometry, and overlay analysis on t.l.c. with monoclonal antibodies.

RESULTS

Separation of non-acid glycolipids of pooled A_1 erythrocytes resulted in two fractions (Fig. 1) which were well separated on t.l.c. as acetylated derivatives, but which had identical migration as nonderivatized samples. The deacetylated samples were used for overlay analysis with monoclonal antibodies and for f.a.b. mass spectrometry. Both permethylated and lithium aluminium hydride-reduced, permethylated derivatives were used for e.i. mass spectrometry and ^{1}H-n.m.r. spectroscopy.

The f.a.b. mass spectra of the two fractions are shown in Fig. 2 and the interpretation of the fragmentation is given in Scheme 1. Fraction 1 (upper spectrum) showed a pattern in accordance with a single chain, eight-sugar, A-Type glycolipid (A-8) producing molecular-weight ions at *m/z* 2052 for a molecule with sphingosine and a 24:0 nonhydroxy fatty acid. There were also homologues at *m/z* 2024 (22:0), 2038 (23:0), and 2066 (25:0), and also unsaturated 24:1 (better visible in the fragment peaks at *m/z* 649, 810, 972, 1175, 1337, and 1540). The sequence of the sugars was easily interpreted from the fragments containing the lipid part and a

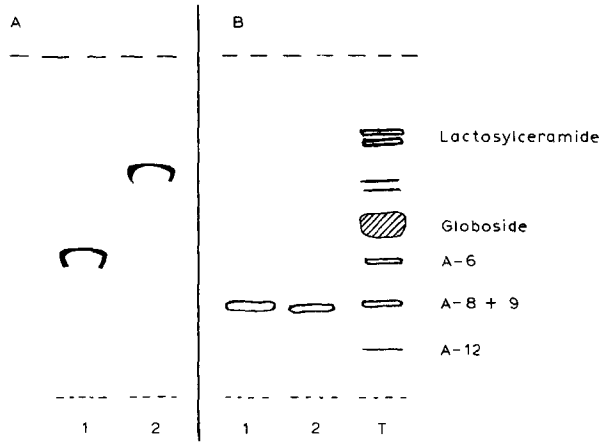


Fig. 1. Schematic representation of thin-layer chromatographic behaviour of selected glycolipid fractions: (A) Acetylated Fractions 1 and 2 developed in 93:7 (v/v) chloroform–methanol. (B) Non-derivatized Fractions 1 and 2 and a total (T) non-acid glycolipid fraction of A_1 erythrocytes developed in 60:35:8 (v/v) chloroform–methanol–water. Indications to the right show the number of sugars of A-active glycolipids[2].

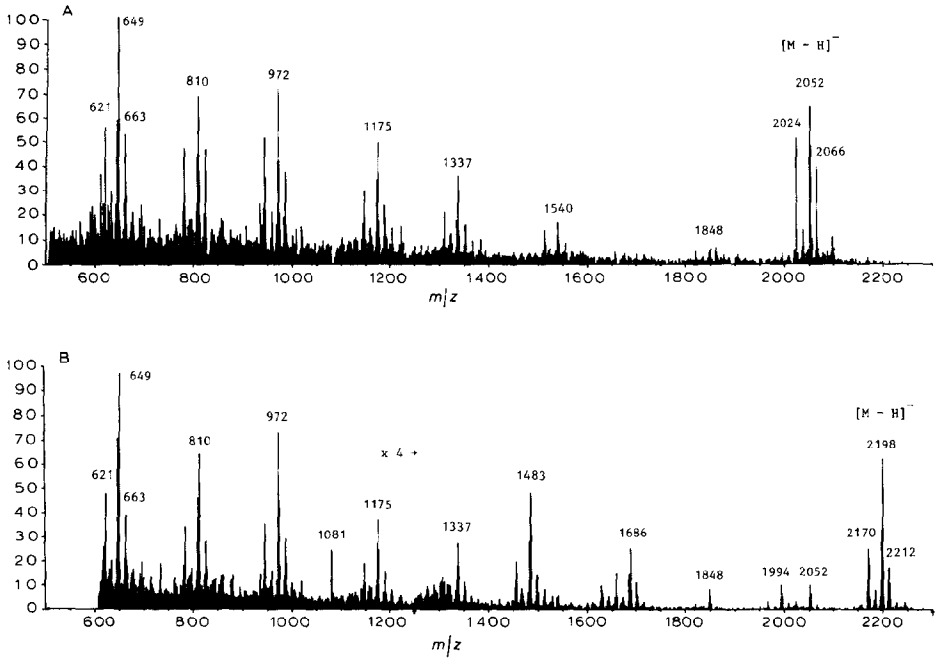
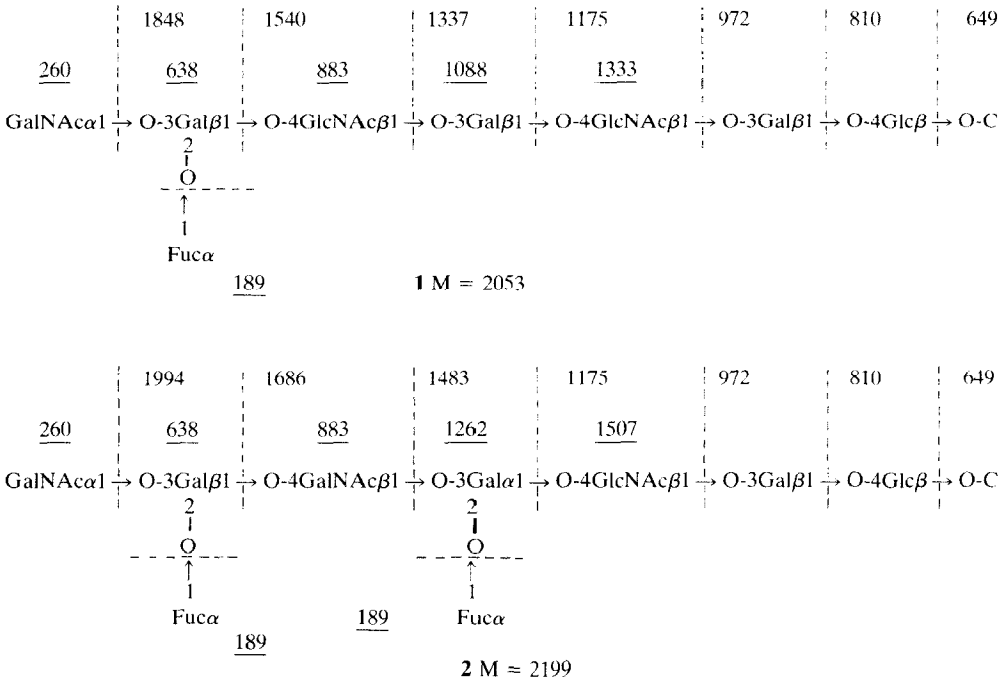


Fig. 2. F.a.b. mass spectra of Fraction 1 (A) and Fraction 2 (B) in nonderivatized form and recorded in the negative-ion mode.



Scheme 1. Structures **1** and **2** for the interpretation of mass spectra of Figs. 2 and 3. The detailed structures correspond to the glycolipid interpreted to make up Fraction 1 (**1**) and to the major glycolipid of Fraction 2 (**2**), as determined by Hakomori and assoc.[2,8]. The structure of the minor eight-sugar glycolipid present in Fraction 2 differs from **1** by having a Type 1 chain [β-D-Gal*p*-(1→3)-D-GlcNAc] instead of a Type-2 chain [β-D-Gal*p*-(1→4)-D-GlcNAc] at the A determinant (a Type 1–Type 2 instead of a Type 2–Type 2 sequence). The underlined mass numbers refer to the permethylated derivatives and e.i. mass spectrum (Fig. 3), and the nonunderlined numbers to nonderivatized glycolipids and f.a.b. mass spectrum (Fig. 2). The real masses of the fragments indicated with, *e.g.*, 1848 and 1994, are in fact 1849 and 1995, respectively. The f.a.b. fragments (Fig. 2) are, however, 1848 (1849 − 1) and 1994 (1995 − 1), respectively, and the recorded masses have been given in the Scheme.

successively longer carbohydrate chain. Thus, the sugar residues linked to the ceramide residue are successively an hexose, an hexose, an acetamidodeoxyhexose, an hexose, an acetamidodeoxyhexose, an hexose with a fucose branch, and an acetamidodeoxyhexose residue. The distribution of the fatty acids is well observed for all the sequence ions. This result is in accordance with the known behaviour of nonderivatized glycolipids examined under the present conditions[17]. The f.a.b. mass spectrum of Fraction 2 (Fig. 2B) showed molecular ions at *m/z* 2198, in accordance with an additional fucose residue, as compared to Fraction 1, and with a very similar ceramide distribution (sphingosine combined with mainly 22:0, 23:0, 24:0, and 25:0 nonhydroxy fatty acids). The sugar-sequence ions clearly indicated the following sugar residues from the ceramide end: an hexose, an hexose, an acetamidodeoxyhexose, an hexose with a fucose branch, an acetamidodeoxyhexose, an hexose with a fucose branch, and an acetamidodeoxyhexose residue. In addition to

peaks for this nine-sugar compound (A-9), peaks were seen, in the upper half of the spectrum, that are specific for the eight-sugar sequence (Fig. 2A). These were at *m/z* 2052 (molecular-weight ion), 1848, and 1337. In common for both the eight- and nine-sugar compounds were ions at *m/z* 649 (ceramide), 810, 972, and 1175, which identify the three-sugar sequence next to the ceramide residue (Scheme 1).

In conclusion, Fraction 1 contained an eight-sugar A-Type glycolipid (**1**, Scheme 1), and Fraction 2 had, as a major component, a nine-sugar A-Type glycolipid (**2**, Scheme 1) with a fucose group linked within the chain, identical to the Type-3 glycolipids[8-10]. In addition, Fraction 2 contained a smaller proportion of an eight-sugar glycolipid having a sequence identical with, and a ceramide composition very similar to that of Fraction 1.

Further support for the sequences postulated was obtained by the e.i. mass spectra of permethylated (Fig. 3 and not reproduced) and lithium aluminum hydride-reduced, permethylated derivatives[11,12] (not reproduced). For Fraction 2 (Fig. 4), the evidence was clear for two different terminal sequences (Scheme 1). The terminal sugars were acetamidodeoxyhexose [*m/z* 260 and 228 ($-CH_3OH$)] and fucose groups (*m/z* 189 and 157). The terminal tri- and tetra-saccharide sequences correspond to *m/z* 638 and 883 (851), respectively. The peak at *m/z* 1262 corresponds to the terminal hexasaccharide sequence of the major nine-sugar compound (bottom structure of Scheme 1), and the peak at *m/z* 1088 to the terminal pentasaccharide sequence of the minor eight-sugar compound (**1**, Scheme 1). Further indication that these two peaks belonged to separate glycolipids was that the peak at *m/z* 1088 was relatively more intense at lower temperature, as com-

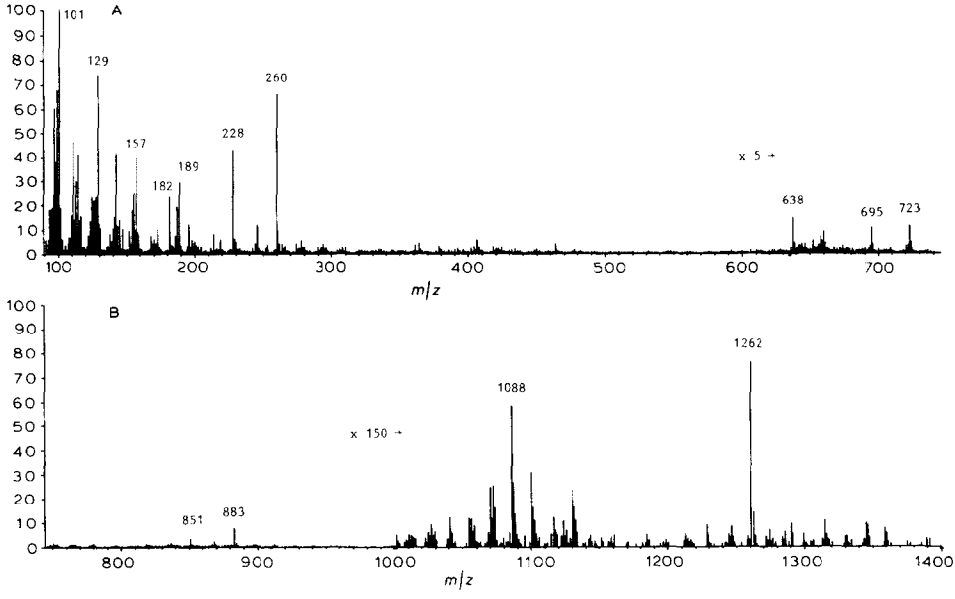


Fig. 3. E.i. mass spectrum of permethylated Fraction 2.

pared to that at *m/z* 1262, which corresponds to the expected difference in distillation in the ion source for the two glycolipids. In the spectrum of permethylated Fraction 1 (not reproduced), the sequence ions were obtained in accordance with structure **1** (Scheme 1). Also, the peak at *m/z* 182 was almost the base peak, an indication of Type-2 chains in the molecules. In the spectrum of Fraction 2 (Fig. 3), this peak was much less intense. The spectrum of permethylated-reduced Fraction 2 showed peaks in agreement with a nine-sugar glycolipid (**2**) being a major part of this mixture[11,12]. One may note that the peaks at *m/z* 695 and 723 of permethylated Fraction 2 (Fig. 3) may be diagnostic for an A determinant based on the structure β-D-Gal*p*-(1→3)-D-GalNAc, since they have so far been found only in spectra of compounds containing A Type 3 or A Type 4 determinants (A based on a globopentaosylceramide structure).

The region of anomeric signals in the ^{1}H-n.m.r. spectra of permethylated and permethylated-reduced Fraction 1 have been published elsewhere[14] and clearly indicated a blood group A determinant and two Type-2 sequences (no downfield shift for Type-1 signals after reduction[14,15]). The ^{1}H-n.m.r. spectrum of permethylated-reduced Fraction 2 (not reproduced) showed two overlapping signals for α-L-fucopyranosyl groups and two separate signals for 2-acetamido-2-deoxy-α-D-galactopyranosyl groups (one signal almost identical with that for Fraction 1).

Overlay analysis with monoclonal antibodies for various fractions separated by t.l.c. gave important information (not reproduced). The anti-A antibody of broad specificity (A581) stained both Fractions 1 and 2, as well as total nonacid glycolipids of both A_1 and A_2 individuals in the region of 6, 8–9, 12, and more sugars (compare Fig. 1), although the stains of A_2 samples were much weaker. The anti-A Type-1 (AH21) and anti-A Type 3 (Vinas) both reacted with Fraction 2 but not with Fraction 1. They also showed the staining of glycolipids of A_1 and A_2 individuals in the region of 8–9, 12, and more sugars but not in the six-sugar region. However, the binding was apparently much weaker to the bands of the A_2 samples.

DISCUSSION

Two glycolipid fractions were isolated during 1975 from A_1 erythrocytes and shown by mass spectrometry[11,12] to contain an eight-sugar glycolipid (Fraction 1) and a mixture of nine-sugar (major) and eight-sugar glycolipids (Fraction 2), all having blood group A determinants. They were also analysed by ^{1}H-n.m.r. spectroscopy[14]. The original conclusion (based on some indications from degradation data) was that the eight-sugar glycolipids differed with respect to chain type (1 or 2). The two fractions have now been reinvestigated by f.a.b. mass spectrometry and overlay analysis with specific monoclonal antibodies in order to verify the original conclusions.

The structure of the blood group A-active, eight-sugar glycolipid of Fraction 1 was found to contain two Type-2 chains (repeated *N*-acetyllactosamine residues, see **1**, Scheme 1) on the basis of mass spectrometry[11,12], ^{1}H-n.m.r. analysis[14], and

degradation (unpublished). Therefore, this structure is identical with that reported earlier by Hakomori and assoc. (see ref. 2). It was earlier concluded that the major nine-sugar glycolipid of Fraction 2 has a difucosyl sequence based on mass spectrometry[11,12], but this proposal was wrong with respect to the position of the second fucosyl group, which had been proposed to be of the Le^b or Y type. The present f.a.b.m. spectrum (Fig. 2), in combination with the e.i.m. spectrum (Fig. 3) definitely localized this fucosyl group (Scheme 1), indicating that this glycolipid is of the Type-3 A glycolipid recently reported[8]. This conclusion is supported by the reactivity of Fraction 2 with the anti-A Type 3 antibody, which does not cross-react with A Type 1. Furthermore, the ^{1}H-n.m.r. spectrum of this fraction showed four signals for the α configuration.

The difference in structure of the two eight-sugar glycolipids separated into Fractions 1 and 2 is of major interest. The two fractions were isolated by preparative t.l.c. as acetylated derivatives but could not be separated in the nonderivatized form (Fig. 1); this separation is analogous to that leading to the isolation of A Type 3 glycolipid[8]. Although old silica gel was used for this t.l.c. separation, our experience was that chloroform–methanol is a better medium for separation of these derivatives than dichloroethane–acetone–water[8] (less sharp bands but better resolution). The bands, well separated under these conditions, excluded the minor eight-sugar glycolipid of Fraction 2 from Fraction 1. Also, the relative mobilities on t.l.c. (see Fig. 1) could produce contamination of Fraction 1 by Fraction 2 through trailing, and not the reverse. There is no evidence, however, of the major nine-sugar glycolipid in Fraction 1. As concluded from the f.a.b. mass spectra (Fig. 2), the ceramide compositions of the three glycolipids were very similar (sphingosine and 22, 23, 24, and 25 nonhydroxy fatty acids, typical of erythrocyte glycolipids). Therefore, the difference in structure between the two eight-sugar glycolipids resides in the sugar part. As the anti-A Type 1 antibody (AH21) bound to Fraction 2 but not to Fraction 1, and did not cross-react with A Type 3 (ref. 8) (which is the major compound of Fraction 2), a reasonable conclusion is that the eight-sugar glycolipid of Fraction 2 has a structure identical with that of **1** (Scheme 1), but with a Type-1 chain next to the A determinant. It is also known that compounds with Type-1 sequences move faster on t.l.c. than those with Type-2 sequences as acetylated derivatives, although they may travel identically as nonderivatized compounds[7]. Therefore, the compound having the proposed Type-1–Type-2 eight-sugar sequence of Fraction 2 is expected to be separated from the compound having Type-2–Type-2 eight-sugar sequence of Fraction 1 (see Scheme 1). However, our present experience with the two eight- and the nine-sugar glycolipids of Fraction 2 did not allow a practical separation to obtain pure compounds for a conclusive analysis.

Overlay of antibodies on thin-layer chromatograms with total non-acid glycolipids, isolated from single individuals having different blood groups, showed that only glycolipids from blood group A individuals bound the three antibodies used. The anti-A Type 1 (AH21) did not react with the six-sugar fraction (Fig. 1), but, as

expected from the reactivity with Fraction 2, it bound to the eight-nine sugar fraction, and with still more slow-moving bands. This indicated that Type 1 chains exist also as blood group A determinants on glycolipids of greater complexity than the eight-sugar glycolipid reported here. When comparing glycolipids from A_1 and A_2 individuals, they apparently both bind AH21 to the same fractions, but the A_2 glycolipids were much weaker. We have no explanation for the absence of reactivity in the six-sugar fraction where an A Type 1 glycolipid, proposed to be of plasma origin would be present[7]. Also, our result on the binding to compounds having more than six sugar residues is at variance with reported data[7], although we used the same antibody. Therefore, one should be careful in the interpretation of data from overlay analysis, where subtle differences in technical conditions (including the plastic coating) may exist in separate laboratories. Therefore, the present data are only suggestive, and the results will have to be carefully repeated and extended.

The prediction of Kabat and assoc.[5,6] on the existence of Type-1-based blood group A determinants on human erythrocytes may, thus, prove to be correct, although the relevance for the A_1 and A_2 subgroup problem is still an open question, in view of the recent results on Type-3-based determinants[8–10]. The Type-1-based A-8 compound of the present work has a typical erythrocyte ceramide residue, and not the more complex ceramide residue known for blood plasma[18,19]. Therefore, it is unlikely that this glycolipid had been taken up from plasma, as has been demonstrated for, *e.g.*, Lewis glycolipids (for an actual discussion, see ref. 19). Furthermore, it appears that glycolipids having shorter-chain and 2-hydroxy fatty acids are selectively taken up from plasma[20], but there is no indication for the presence of these compounds in the fraction investigated. However, a relation of the expression of this glycolipid to the secretor status[1] should be analysed, since the secretor gene has been proposed to code for an (1→2)-α-L-fucosyltransferase with specificity for a Type-1 chain[21] (see also ref. 10).

EXPERIMENTAL

Methods. — ^{1}H-N.m.r. spectroscopy of permethylated and $LiAlH_4$-reduced permethylated derivatives was done as described[13–15]. Fast-atom-bombardment mass spectrometry was performed as described[17] with a ZAB-HF spectrometer equipped with a VG 11-250 data system (VG Analytical Ltd, Manchester, U.K.). The matrix was triethanolamine and the sample (10 μg) was dissolved in methanol (2 μL). Xenon atoms (8 keV), a f.a.b. gun current of 1 mA, and an 8-kV acceleration were used. Electron-ionization mass spectrometry of derivatized glycolipids was performed as described[11,12,22]. The sample (5 μg) was distilled off in the ion source between 305 and 360°, and the spectrum recorded at 320°. The electron energy was 70 eV, ion current 0.2 mA, and acceleration voltage 8 kV. The masses were calibrated against Fomblin (Dow Chemicals). In the spectra reproduced, absolute masses are given, *e.g.*, the mass at *m/z* 1088 of Fig. 3 is 1087.565 and is given as 1088, and not as 1087 (nominal mass).

Immunological overlay analysis. — Anti-A antibody of broad specificity (A581) was purchased from Dakopatts AS, Denmark. The anti-A Type 1 monoclonal antibody (AH21) was kindly provided by Dr. S. Hakomori and its specificity has been described[7,8,23]. The anti-A Type-3 monoclonal antibody (Vinas) was kindly provided by Dr. R. Oriol (see ref. 24). The conditions for overlay analysis on a thin-layer chromatogram have been described elsewhere[25].

Isolation of glycolipids. — Human blood was obtained from the Blood Bank, Sahlgren's Hospital, Göteborg, and it was typed according to routine transfusion strategy. Blood from single individuals (one transfusion unit) was obtained fresh, but pooled blood from A_1 individuals was outdated blood. Erythrocyte membranes were prepared by toluene flotation[18], and a total non-acid glycolipid fraction was isolated as described[26]. The total fraction from pooled erythrocytes was subfractionated on silicic acid columns; the silic acid was a sedimented fraction[27] with a particle size <45 μm (Mallinckrodt Chem. Works, St. Louis, MO, U.S.A.). Elution was done stepwise with increasing amounts of methanol in chloroform. The homogeneous fraction containing 8–9-sugar, glycolipids, obtained after repeated chromatography, was acetylated[26] and subjected to preparative t.l.c. on Silica Gel G (Merck, Darmstadt, F.R.G.), spread on 20 × 20 cm glass plates with an estimated layer thickness of 0.1 mm, in 93:7 (v/v) chloroform–methanol. Two well separated bands, detected with I_2 vapour had R_F ~0.45 (Fraction 1) and 0.70 (Fraction 2). Each band was scraped off and transferred to a silicic acid column (0.5 g), packed in pure chloroform. After elution with chloroform (10 mL), the acetylated glycolipid was eluted with 2:1 (v/v) chloroform–methanol (10 mL). This preparative procedure was repeated once. Analytical t.l.c. showed that the two fractions were homogeneous and not contaminated with each other. Each fraction was deacetylated with toluene (0.5 mL), methanol (0.5 mL) and 0.2M KOH in methanol (1 mL) for 30 min. After addition of acetic acid (a few drops), the samples were dialysed for 4 days against running tap water and evaporated. The weights obtained for this preparation were 1.7 mg for Fraction 1 and 3.3 mg for Fraction 2. Analytical t.l.c. using 25:25:4 (v/v) chloroform–methanol–water as solvent and 4-methoxybenzaldehyde as detection reagent[27] showed Fractions 1 and 2 as single, homogeneous bands with similar mobilities.

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