FOUR ANTIGENIC VARIANTS OF BLOOD GROUP A GLYCOLIPID: EXAMPLES OF HIGHLY COMPLEX, BRANCHED CHAIN GLYCOLIPID OF ANIMAL CELL MEMBRANE*

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Summary - Four variants of blood group A-active glycolipid (A^a,A^b,A^c,A^d) have been isolated from lipid extract of human erythrocyte membrane. A^a is a ceramide hexasaccharide, and A^b is a ceramide octasaccharide; these two variants each contain a straight carbohydrate chain with the same A-determinant group, but differ in chain lengths. The A^c variant is a mixture of ceramide deca- to hendecasaccharide characterized by the presence of two different carbohydrate chains cojointed with a branching structure at the galactosyl residue, which is attached to glycosylceramide. The variant A^d is a mixture of ceramide dodeca- to tettareskaidecasaccharide with branchings and highly complex structure.

Blood group ABH glycolipids have been characterized by the presence of a common carbohydrate residue which consists of galactose, N-acetylglucosamine, and glucose, and which is attached to a ceramide (1,2). Two A-active peaks were separated on Silica gel chromatography (3); three polymorphic forms ("variants") of blood group A glycolipid were separated, each carrying the same A-specificity (1,2,4), although the differences in their composition and structures were not known. This paper describes the thorough fractionation and isolation of human erythrocyte glycolipids which gave four distinctive variants of blood group A glycosphingolipid. A preliminary view of the structures of these variants is also presented.

EXPERIMENTAL PROCEDURE

The glycosphingolipid fraction of membranes of blood group A_1 erythrocytes, sorted by Dolichos biflorus agglutinin, was prepared by 90% ethanol extraction,

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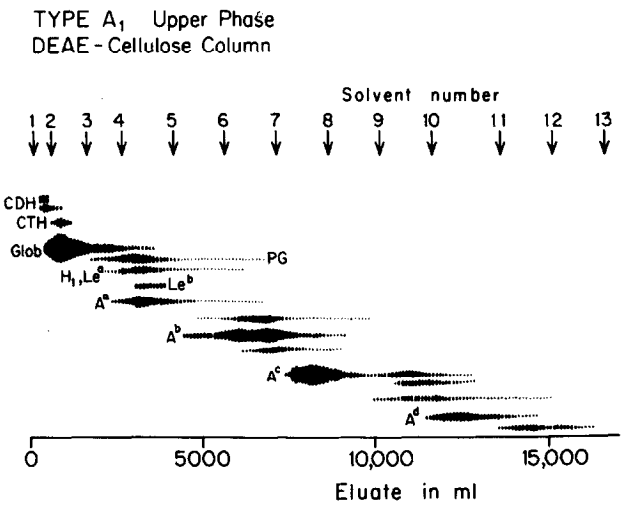

Figure 1.

Separation pattern of long-chain neutral glycolipids by chromatographies on DEAE-cellulose and thin-layer of Silica gel H.

Column size 2.5x30 cm. Solvent numbers indicated by chloroform-methanol-water mixtures of the following proportions: 1) 90:20:0.2, 2) 87.5:12.5:0.2, 3) 85:15:0.2, 4) 80:20:0.2, 5) 77.5:22.5:0.2, 6) 75:25:0.2, 7) 70:30:0.2, 8) 67.5:32.5:0.4, 9) 65:35:0.4, 10) 60:40:0.5, 11) 57.5:42.5:0.5, 12) 55:45:0.5, 13) 0:100:0. Each fraction (50 ml) eluted from DEAE-cellulose column was concentrated to dryness, and the residue was dissolved in 1 ml of chloroform-methanol, spotted on Silica gel H plate, and developed with chloroform-methanol-water (60:35:8). Spots were revealed with 0.2% orcinol in 2 M sulfuric acid. Thin-layer chromatography patterns of every second fraction are shown in this figure.

CDH: lactosylceramide; CTH: Galα1→4Galβ1→4Glc→Cer; Glob: Globoside(GalNAcβ1→4Galα1→4Galβ1→4Glc→Cer); PG: paragloboside (Galβ1→4GlcNAcβ1→3Galβ1→4Glc→Cer: Siddiqui & Hakomori, unpublished); H,Le^a,Le^b: glycolipids with those blood group activities (Stellner & Hakomori, unpublished); A^a,A^b,A^c,A^d: variants of A-active glycolipids. The majority of CDH, CTH, globosides, and PG was found in the lower phase.

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followed by precipitation at -20°C and fractionation by organic solvents (1,5). The sphingolipid fraction (10 g) was dissolved in 1 liter of chloroform-methanol (2:1); insoluble particles were rejected by filtration through "Celite" and partitioned with water repeatedly according to the Svennerholm modification of Folch's method (6). The aqueous extract was concentrated, dialyzed and lyophilized. The residue was then fractionated on DEAE-cellulose according to the modified method of Rouser et al (7). The solvent systems for elution are described in the legend to Figure 1. Glycolipids eluted were determined by thin-

layer chromatography on Silica gel H, and the pattern of elutions from the DEAE-cellulose column are recorded in Figure 1. Gangliosides and hematosides were retained on the DEAE-column and were not eluted by the solvents that eluted all neutral glycolipids including variants of A-glycolipid. The glycolipids were further separated into components by thin-layer chromatography on Silica gel H after acetylation by the method previously described (1,2). Each component was eluted and deacetylated in chloroform-methanol-0.5% sodium methoxide (2:1:0.6) at room temperature for 20 minutes. The free glycolipids thus separated and recovered were essentially homogeneous on thin-layer chromatography, having been freed from sialosylglycolipids.

Carbohydrate composition was determined by gas chromatography according to the modified Sawardeker method after hydrolysis with 0.5 N H_2SO_4 in 90% acetic acid (8). Blood group A activity was determined by inhibition of A hemagglutination, by the use of anti-A serum with 4 hemagglutination doses and by a specific precipitin reaction with Dolichos biflorus on double diffusion gel. A part of the Dolichos agglutinin used in this study was donated by Dr. Marilynn Etzler (University of California, Davis)(9). Methylation was carried out in dimethylsulfoxide, sodium hydride and methyliodide (10). The permethylated glycolipid (0.5-1 mg) was subjected to acetolysis in 0.3 ml of 95% acetic acid containing 0.5 N sulfuric acid at 80°C for 24 hours, followed by hydrolysis after addition of 0.3 ml water. The partially methylated alditol acetates and hexosaminitol acetates were prepared by the procedures described (8,11,12). They were eventually identified by their retention time on gas chromatography (3% ECNSS[1] column) and by their mass spectra (11,12) obtained by Finnigan gas chromatography-mass spectrometer "Peak Identifier" with a quadrupole separator.

RESULTS AND DISCUSSION

The carbohydrate composition of four variants of A-glycolipid (A^a,A^b,A^c,A^d) indicated that A^b contained more GlcNAc and Gal than A^a, that A^c had more GlcNAc and Gal than A^b, and that A^d had more GlcNAc than A^c, although the ratio of

1. cyanoethylsilicone-ethyleneglycolsuccinate

Fuc/Glc/GalNAc for all variants was nearly constant (1:1:1 or 1.5:1:1.5). Migration rate on Silica gel H with chloroform-methanol-water (60:35:8) of A^a,A^b, A^c, and A^d against that of globoside (R-globoside value)(1) was 0.75, 0.50, 0.35 and 0.15, respectively. All of the four variants showed strong precipitin with Dolichos biflorus agglutinin; 0.1-0.5 mg of A^a and A^b variants and 0.05-0.1 mg of A^c and A^d variants equally inhibit A-hemagglutination caused by 4 hemagglutination doses of anti-A serum. A^c variant showed a precipitin with Ulex europeus. A^a should be a ceramide hexasaccharide having a ratio of GlcNAc/Gal/GalNAc/Glc/Fuc 1:2:1:1:1. A^b should be a ceramide octasaccharide, showing a ratio of GlcNAc/Gal/GalNAc/Glc/Fuc 2:3:1:1:1. A^c should be a mixture of ceramide deca- to hendecasaccharide with an average ratio of GlcNAc/Gal/GalNAc/Glc/Fuc 3:4:1.5:1:1.5. A^d should be a mixture of ceramide dodeca- to tettareskaidecasaccharide, showing an average ratio of GlcNAc/Gal/GalNAc/Glc/Fuc 5:3:1.5:1:1.5. The results of methylation analysis of neutral sugars and aminosugars of these variants A^a, A^b,A^c, and A^d is shown in Table 1.

Based on sugar analysis, Smith degradation, and the results of methylation study, it is assumed that A^a should be a ceramide hexasaccharide and that A^b should be a ceramide octasaccharide, both having a straight carbohydrate chain with different lengths, the structures of which were postulated as seen in Figure 2.

In striking contrast to these two variants, the hydrolysates of methylated A^c and A^d were characterized by the presence of 2,4-di-O-methylgalactitol, which was absent in the hydrolysates of methylated A^a and A^b. The variants A^c and A^d should therefore have a branching with 1→3 and 1→6 Gal. These two carbohydrate chains are attached by branching to a common glycosylceramide (see Figure 2). This structure was further supported by degradation studies which will be published later. A similar branching with the structure GlcNAcl→3(GlcNAcl→6)Gal has been well-established in blood group glycoproteins by Kabat (14) and in some milk oligosaccharide by Kobata and Ginsburg (15). A^d could be a mixture of ceramide dodeca- to tettareskaidecasaccharide with a branching structure.

TABLE I

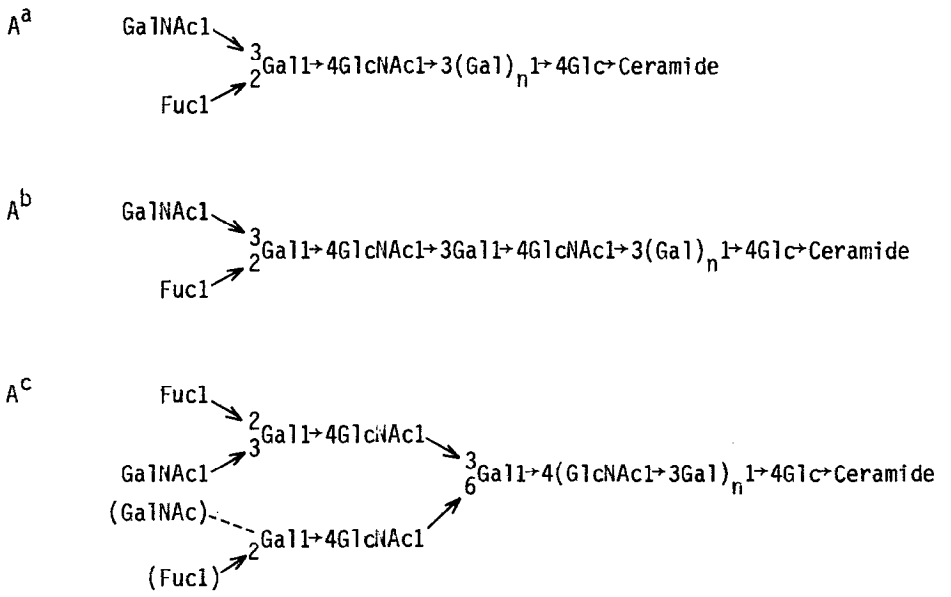
Methylated Sugars Found in the Hydrolysates of Four Permethylated Variants of Blood Group A Glycolipid

Methylated Sugars Identified as Acetates	Fraction of Variants			
	A^{a}*	A^{b}*	A^{c}†	A^{d}†
4,6-di-O-methylgalactitol	0.97	1.2	0.8	1.3
2,3,4-tri-O-methylfucositol	1.03	1.0	1.6	3.2
2,4,6-tri-O-methylgalactitol	2.3ǂ	3.2ǂ	3.3ǂ	1.6
2,3,6-tri-O-methylglucitol	1.2	1.1	1.1	1.3
2,3,4,6-tetra-O-methylgalactitol	0.2	0.2	0.8	0.3
2,4-di-O-methylgalactitol	0.0	0.0	1.0	1.0
3,6-di-O-methyl-2-deoxy-2-N-methylacetamidoglucitol§¶	1.5¶	2.3¶	4.1¶	3.0¶
3,4,6-tri-O-methyl-2-deoxy-2-N-methylacetamidogalactitol§	1.0	1.0	1.6	0.8

*The ratio of methylated sugar was calculated as 3,4,6-tri-O-methyl-2-deoxy-2-N-methylacetamidogalactitol 1.0. † These two variants gave 2,4-di-O-methylgalactitol, indicating a branching structure. The ratio of methylated sugar was calculated as 2,4-di-O-methylgalactitol 1.0. ǂ The excess proportion of 2,4,6-tri-O-methylgalactitol does not agree with the monosaccharide ratio given by direct sugar analysis on gas chromatography. This could be caused by the relatively low yield of other methylated sugars and by a partial volatilization of some methylated sugars (e.g. trimethylfucositol), which could have occurred during derivarization of sugars. The molar ratio of partially methylated sugars in the hydrolysates of permethylated complex carbohydrates does not always agree with the quantitative composition of monosaccharides. The lower yield of methylated glucitol occurred as this sugar is directly bound to ceramide; the lower yield of 2-deoxy-2-N-methylamidohexitol could occur as the glycosidic linkage of this sugar is highly resistant to hydrolysis. A lower yield of 4,6- or 2,4-di-O-methylgalactitol could also be expected , as this galactose residue derives from a branching point to which aminosugars are linked. Possibility of contamination with glycolipids having higher contents of (1→3)Gal cannot be ruled out, although this would contradict the results of sugar analysis. § Aminosugars identified as derivatives of N-acetyl, N-methyl-hexosaminitol (2-deoxy-2-N-methylacetamido-hexitol (12,13). ¶ Recovery of di-O-methyl-aminosugar is not quantitative; 4,6-di-O-methyl-2-deoxy-2-N-methylacetamidoglucitol was not detected (details of aminosugar analysis to be published later). A small amount of 2,3,4,6-tetra-O-methylgalactitol in A^{a},A^{b}, and A^{d} is probably due to the presence of a contaminating glycolipid with galactosyl residue as non-reducing terminal. The relatively large amount of 2,3,4,6-tetra-O-methylgalactitol in A^{c} suggests that some of the branched carbohydrate chains might have galactosyl residue as non-reducing terminal.

The variants A^{a} and A^{b} are blood group A-specific and no other activity was detected in these variants. The variant A^{c} showed, however, reactivity not

Figure 2.



A^d Similar to A^c but with excessive GlcNAc and with an additional branching structure.

Possible differences in structures of "variants" of blood group A glycolipid.

The number of sugar residues corresponding to the structure $(Gal)_n$ in A^a and A^b and $(GlcNAcl\rightarrow 3Gal)_n$ in A^c has not yet been determined. The excess molar proportion of 2,4,6-tri-O-methylgalactitol found in the hydrolysate of permethylated A^a,A^b, and A^c (see Table 1), however, indicates that n could be 1 to 2 for A^a and A^b and 1 to 3 for A^c. Assignment of ceramide hexasaccharide to structure A^a (i.e., n=1) was verified by interconversivenesss between A^a and H-active ceramide pentasaccharide, whose structure was recently established (Stellner, Watanabe, and Hakomori, unpublished). The variant A^c is a mixture of glycolipids with or without the terminal sugar residues as shown in parentheses. Methylation analysis indicates that some terminals of branched chain are Gal, some are Fucl→2, and only a small part is in completed form, GalNAc→(Fuc→)Gal. The structures of A^a and A^b were supported in an analogy of H-active glycolipid (Stellner, Watanabe. and Hakomori, unpublished), and a long chain Le^b glycolipid (17). A similar sugar sequence as in A^a structure was postulated by Iseki on the basis of enzymatic hydrolysis (18).

only with blood group A reagents, but also with the H-reagent of Ulex europeus. Whether the H-active glycolipid is present in these fractions as contaminants or one of the branched carbohydrate chains carries H-active terminal has not

been decided. The latter possibility is more plausible in view of the sugar analysis and methylation study.

The following points are of special interest: 1) The same A-determinant group, GalNAcα1→3(Fucl→2)Gal, was carried by carbohydrate chains of differing sizes and with differing structural complexities. 2) Analogous to blood group glycoprotein (14), variants A^c and A^d had a branching structure at the galactosyl residue through 1→3 and 1→6 Gal structure. 3) Irrespective of the complexity of molecules (chain length or branching) only one A-determinant group might be present in these variants. The variant A^d is highly complex, including two carbohydrate chains attached by branching structures; nevertheless, still only one of the terminals seemed to carry the specific A-determinant. This would provide a ceramide with multispecific functions.

Such a highly complex glycosphingolipid with a branching structure or with a long carbohydrate chain (more than eight) could be widely distributed among various cell membranes, although they are an extreme minority (1-2 mg derived from 500 g of packed cell membrane). Nevertheless, as receptor site or as membrane antigen, the activity of these "minority glycolipids", when they are organized in membrane, is much higher than that of "abundant glycolipids", such as lactosylceramide, globoside, gangliosides, and hematosides (2,16).

Separation of glycolipids into components with single carbohydrate chain becomes increasingly difficult as the chain length increases, and separation into components is impossible by the existing techniques when the sugar residue becomes more than ten. It is possible, therefore, that variants A^c and A^d are mixtures of glycolipids with slightly different structures; those structures given in Figure 2 are therefore only tentative. Further study is needed for complete understanding of this new molecular species of membrane.

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