MEGALOGLYCOLIPIDS – UNUSUALLY COMPLEX GLYCOSPHINGOLIPIDS OF HUMAN ERYTHROCYTE MEMBRANE WITH A, B, H AND I BLOOD GROUP SPECIFICITY

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1. Introduction

Recentlv we have described a new form of water soluble antigens of the human erythrocyte membrane with A, B, H, and I blood group activity [1, 2]. The preparations of the antigens comprised 90% carbohydrate, 7% amino acids and about 2% sphingosine. The composition of the carbohydrate portion of the antigens resembled that of blood group active glycolipids [3–5] i.e. fucose, galactose, *N*-acetylglucosamine, and glucose were present while *N*-acetylgalactosamine was encountered only in the preparations with A blood group activity [1]. However the content of *N*-acetylglucosamine was unusually high (30%) while that of glucose was unusually low (2%). In the absence of detergents these materials formed large molecular aggregates in aqueous solutions. We concluded that the antigens were either glycoproteins or the glycospingolipids of unusual complexity with 30–50 sugar residues per mole of ceramide. Presently we report a new evidence in favor of the glycolipid nature of these substances.

2. Materials and methods

The procedure for the isolation of the water soluble antigens with H and B blood group specificity was similar to that described previously [1]. The only modification was the substitution of QAE Sephadex A-25 for DEAE Sephadex in the last two column separations. Hexose, hexosamine, fucose, sialic acid and glucose were determined as in the previous publication [1]. Sphingosine was estimated by the method of Lauter and Trams [7], and fatty acids according to Laurell and Tibbling [8]. Gas chromatography of neutral sugars was performed by the method of Bhattia et al. [9] employing a JEOL JGC-1100 gas chromatograph and flame ionization detector. Silylation reagent was prepared according to Carter and Gaver [10]. Column (4 mm × 2 m) contained 10% SE30 coated on chromosorb Q (30–60 mesh). The temperature was kept at 130°C for 10 min then increased by 1°C per min up to 200°C. Amino acids were determined in a Technicon instrument equipped for a single column analysis. Samples were hydrolysed with 6 M HCL at 100°C for 24 hr. The acid was removed by evaporation in vacuo. Analytical gel filtration was performed on a column packed with Sepharose 6B in 0.02% sodium azide. The column (1.5 × 75 cm) was calibrated with dextrans of known molecular weight. The H and B blood group activites were determined by the hemagglutination inhibition technique [1] employing *Ulex europaeus* extracts and normal anti-B serum respectively. The I blood group activity was estimated by the same technique but at 4°C. The anti-I autoimmune sera were provided by Dr. W. Dzierżkowa-Borodej. Reference standards of H and B active glycoproteins from cysts were obtained from Professor W. T. J. Morgan. Standards of glycolipids/lactosylceramide, globoside and H-II glycolipid/ were isolated from erythrocytes by Dr. A. Piasek and Ms. H. Miller-Podraza of this laboratory.

3. Results

The composition of the water soluble antigens is given in table 1. Generally the analytical values resembled these reported in the earlier publication [1] except for the amino acid content of the H-active preparation. The low levels of amino acids in this preparation resulted probably from a more efficient removal of contaminating protein by QAE-Sephadex, which was used instead of the previously employed DEAE-Sephadex. The H and B blood group activities of the antigens were similar to those displayed by cyst glycoproteins. The two antigens were subjected to alkaline degradation by two procedures (see tables 2 and 3). In procedure I the *N*-acetylglucosamine – asparagine linkages should be spared [11]. On the other hand procedure II should effect the cleavage of *N*-glycoside bonds [12]. As could be expected the harsher procedure II brought about a more extensive degradation of the protein portions of the studied preparations. However, the amino acid composition of the degraded antigens did not change (for the sake of brevity only the values for aspartic acid and, serine and threonine are listed in tables 1, 2 and 3). Sugars and sphingosine contents of the antigens were unaffected by alkali. Also the H, B, and I blood group activities were largely recovered after the alkali treated materials were *N*-acetylated.

The H active material (10 mg) was peracetylated with 1 ml of pyridine–acetic anhydride 1:2 at 90°C overnight [14].

The solvent was evaporated under the stream of nitrogen with several additions of toluene. The residue was dissolved in 1 ml of chloroform–methanol–water 65:30:8 solvent mixture and chromatographed on a 1.5 X 10 cm column packed with silicic acid. The column was then eluted with 100 ml of the same solvent mixture and fractions 3 ml in volume were

Table 1
The composition of the B and H antigens

	B (%)	H (%)
Galactose	44.0	47.0
Glucose	4.0	4.2
Fucose	9.0	12.0
Glucosamine	28.0	30.0
Sphingosine	3.2	3.5
Fatty acids	ca: 2.0	ca: 2.0
Sialic acid	4.0	2.0
Amino acids (total)	6.7	2.5
Aspartic acid	1.4	0.33
Serine	0.52	0.38
Threonine	0.61	0.18

Table 2
Alkaline degradation of B substance by procedure I

Component	Recovery in non-dialysable fraction %	Recovery in dialysable fraction %
Hexose	89	4
Fucose	92	5
Glucose	90	4
Glucosamine	88	–
Spingosine	95	Trace
Amino acids (total)	35	–
Aspartic acid	21	–
Serine	35	–
Threonine	25	–
B activity	100	3
I activity	100	3

B substance (20 mg) was hydrolysed in 2.5 M NaOH for 2.5 hr at 85°C and then neutralised with 2.0 M HCL. The material was N-acetylated [20] and dialysed for 10 days.

Table 3
Alkaline degradation of B and H substances by procedure II
Recovery in non-dialysable fraction (%)

Component	Globoside	B substance	H substance
Hexose	85	88	82
Fucose	–	98	102
Glucose	–	87	81
Spingosine	95	89	85
Amino acids (total)	–	5	9
Aspartic acid	–	4	8
Serine	–	4	11
Threonine	–	4	9
B activity	–	100	–
H activity	–	–	100
I activity	–	100	100

The materials were separately hydrolysed in 1 M NaOH for 6 hr at 100°C. After neutralisation the samples were N-acetylated [20] and dialysed for 48 hr.

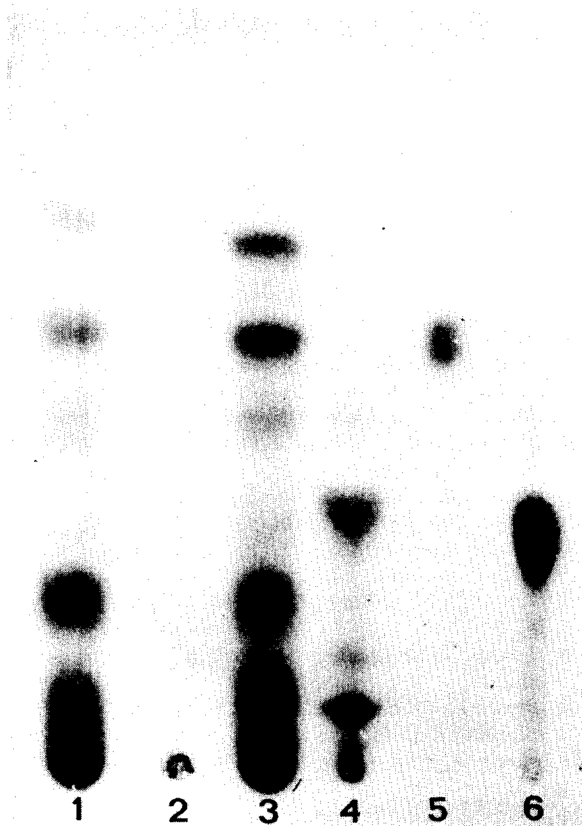

Fig. 1. Partial acid hydrolysis of H blood group substance. The hydrolysis was carried out in 1 M HCL in water-methanol 1:1 solvent mixture for 4 min at 100°C. The acid was removed by dialysis. Hydrolysis products were chromatographed on silica gel H employing chloroform–methanol–water 65:30:8 solvent mixture. Spots were stained with 0.5% orcinal in 5% H_2SO_4 in ethanol at 105°C. 1, hydrolytic products of H substance. 2, intact H substance. 3, hydrolytic products of crude mixture of H-active glycolipids (including H-II glycolipid) from human erythrocytes. 4, crude mixture of H active glycolipids. 5, lactosylceramide. 6, globoside.

collected. Hexose (67% of the total) was recovered in fractions 3 – 10, the remaining fractions being free of detectable hexose. The pooled fractions 3–10 were dried at reduced pressure, the residue was dissolved in dry methanol and deacetylated with sodium methoxide [5]. The material was then analysed for sugars, amino acids, shingosine and blood group activity: Glc 2, sphingosine 1, Gal 22, GlcNAc 14, Fuc 6.3. In the original preparation the molar ratios were Glc 2.5, sphingosine 1.0, Gal 22, GlcNAc 14, Fuc 7.

The amino acids decreased to less than 1% by weight. The recovery of blood group H and I activity amount to about 70%. The aggregate forming tendency of the material as determined by gel filtration was not changed after acetylation and deacetylation in spite of a very low content of amino acids. Thus this property of the antigens does not result from uneven distribution of oligosaccharide chains along the peptide backbone which is thought to be responsible for the aggregation of the major sialoglycoprotein of erythrocyte membrane [23].

The H-active antigen was subjected to partial acid hydrolysis. The fragments which were released were found to be soluble in chloroform–methanol solvent mixtures. When separated on this layer of silicic acid they displayed a pattern similar to that shown by the hydrolytic products of a complex H-II glycolipid [5] (see fig. 1).

4. Discussion

The results indicate that protein is not an essential component of the water soluble antigens of erythrocytes. By alkali degradation at 100°C the amino acid content of H- and B-active antigens could be reduced to 0.3% and 0.2% respectively. Also when the H-active antigens were acetylated and purified as the acetate, its amino acid decreased to less than 1%. Moreover the amino acids of the residual peptide in the alkali degraded material did not show any change in composition. For instance, an enrichment in aspartate was to be expected if the water soluble antigens were glycoproteins or glycopeptides in which carbohydrate and protein portions were linked through glycosylamine bond involving *N*-acetyl-glucosamine and asparagine residues. On the other hand the assumption that the antigens are glycolipids fits all experimental data. A remarkable stability of the carbohydrate and sphingosine residues of these substances towards hot alkali is just what is expected of glycolipids. The fact that the alkali treated antigens retain their tendency towards aggregate formation as indicated by analytical gel filtration implies that this property is the result of the presence of sphingosine and not of amino acids. A strong support for a

glycolipid nature of the water soluble antigens comes from partial acid hydrolysis of these materials. This treatment resulted in the liberation of smaller fragments which were soluble in chloroform–methanol–water solvent mixtures and migrated on thin layers of silicic acid as typical glycolipids. Therefore we conclude that the water soluble antigens of erythrocytes with A, B, H and I blood group activity are unusually complex glycolipids which contain about 20–40 sugar residues per mole ceramide, which is in accordance with our earlier estimate [1]. Glycolipids of such unusual complexity have not been so far found in nature. We propose to designate them megaloglycolipids. The length of sugar chains was estimated from the sphingosine content. However the content of glucose was twice as much as could be expected when only a single glucose residue was present per one oligosaccharide chain. The higher content of glucose might result from the presence of trace amounts of impurities, especially as the materials were fractionated several times on poly-glucose-containing chromatographic adsorbents. This point obviously needs clarification because on the basis of glucose content the number of carbohydrate residues in the megaloglycolipids would amount to 20.

The content of megaloglycolipids in erythrocyte membrane is small and yields of only 15–20 mg can be obtained from 1 kg of dried stroma which corresponds to about 50 l of blood. Yet these antigens are responsible a large part of A, B, H and I blood group activity of the aqueous extracts obtained by treatment of erythrocyte membrane with butanol-water solvent mixture [1, 2]. Therefore the earlier assumptions that erythrocytes comprise substantial amounts of blood group active glycoproteins with A, B, H and I blood group specificity may need revision [6, 15–19].

Likewise a low blood group A, B, H and I blood group specificity as displayed by the preparation of the major erythrocyte membrane sialoglycoprotein [21] may result from a contamination with the megaloglycolipids. However, the presence of some amount of A, B, H activity in erythrocyte glycoproteins cannot be entirely excluded as indicated by the recent results of Fukuda and Osawa [22]. The latter authors reported H activity in an erythrocyte sialoglycoprotein preparation which was free of detectable sphingosine.

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