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Blood Group A Determinants with Mono- and Difucosyl Type 1 Chain in Human Erythrocyte Membranes†

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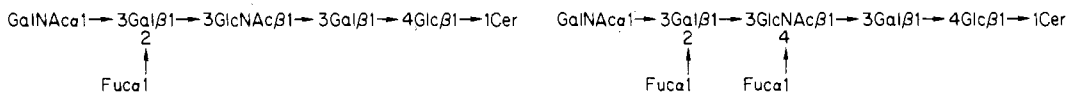
ABSTRACT: Application of a monoclonal antibody defining monofucosyl type 1 chain A (AH21) revealed the presence of a glycolipid having the same thin-layer chromatography mobility as A^a but showing a clear reactivity with AH21. This glycolipid was detectable in Le^{a-b-} erythrocytes but not in Le^{a+b-} or Le^{a-b+} erythrocytes. Another monoclonal antibody defining difucosyl type 1 chain A (HH3) detected the presence of a glycolipid component reacting with this antibody in Le^{a-b+} erythrocytes but not in Le^{a+b-} or Le^{a-b-} erythrocytes. The component defined by monoclonal antibody AH21 and that defined by HH3 were isolated and characterized by ^1H NMR spectrometry and methylation analysis as having the structures



The ^1H NMR spectra of these glycolipid antigens were characterized by resonances for anomeric protons that are identical with those of glycolipids with type 1 chain previously isolated but distinctively different from those of type 2 chain analogues. Resonances reflecting ceramide composition are characteristic for these antigens from human erythrocytes and are distinguishable from those of the same antigen from other sources.

Four types of blood group A glycolipids, A^a, A^b, A^c, and A^d, which differ in the length and branching status of their carrier carbohydrate chains, have been isolated and characterized from human erythrocyte membranes. These components carry the same blood group A determinant (GalNAcα1→3-[Fucα1→2]Gal), and their carrier carbohydrates are mainly of type 2 chain structure (Galβ1→4GlcNAcβ1→3Gal) [see, for review, Hakomori (1981)]. Other major carriers of blood group ABH antigens in human erythrocytes are band 3 and band 4.5 glycoproteins, and the majority of their carbohydrates (lactosaminoglycans) also consist of type 2 chain (Fukuda et al., 1979; Finne, 1980). In addition, A_1 erythrocytes contain a trace quantity of globo-A (GalNAcα1→3[Fucα1→2]-Galβ1→3GalNAcβ1→3Galα1→4Galβ1→4Glcβ1→1Cer) (Clausen et al., 1984) and a relatively large quantity of the repetitive A determinant (GalNAcα1→3[Fucα1→2]Galβ1→-3GalNAcα1→3[Fucα1→2]Galβ1→4GlcNAcβ1→3Galβ1→-R) (type 3 chain; Clausen et al., 1985), both of which are absent in A_2 erythrocytes. Blood group Lewis antigens (Le^a and Le^b) of erythrocytes are not synthesized in hematopoietic

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tissues or mature erythrocytes but are acquired from serum (Sneath & Sneath, 1955). They have been identified as glycosphingolipids and found to be transferred into erythrocytes from serum lipoproteins (Marcus & Cass, 1969).

Blood group ABH antigens isolated from gastrointestinal and ovarial cyst mucin have been characterized as having predominantly type 1 chain and minor quantities of type 2 chain [see, for review, Watkins (1980)]. A branched megalosaccharide structure having type 1 chain on one branched side chain and type 2 chain on the other side chain has been proposed (Lloyd & Kabat, 1968). Type 1 chain A determinants, either mono- or difucosyl, have been suggested to be specifically expressed on A_1 erythrocytes and thus to constitute the structural basis for a qualitative A_1–A_2 distinction (Lloyd & Kabat, 1968; Moreno et al., 1979; Kisailus & Kabat, 1978). Blood group A determinants with difucosyl type 1 chain (ALe^b) and difucosyl type 2 chain (ALe^y) have been isolated from hog gastric mucosa (Slomiany & Slomiany, 1975) and from dog and human intestine (McKibbin et al., 1982). However, our knowledge of the distribution of these A variants in various tissues and cells is highly fragmentary. Recently, we established a monoclonal antibody (AH21) directed to monofucosyl type 1 chain A determinant (Abe et al., 1984) that did not cross-react with monofucosyl type 2 chain A, difucosyl type 2 chain A, or difucosyl type 1 chain A (H. Clausen and S. Hakomori, unpublished data). We also established another monoclonal antibody (HH3) directed to difucosyl type 1 chain A, which did not cross-react with monofucosyl type 1 chain A or mono- or difucosyl type 2 chain A (H. Clausen, J. M. McKibbin, and S. Hakomori, unpublished data). This paper describes the application of these reagents to study the distribution of type 1 chain A in human erythrocytes in relation to blood group Lewis status. The presence of a ceramide hexasaccharide having monofucosyl type 1 chain A exclusively in blood group Le^{a-b-} erythrocytes and the presence of a ceramide heptasaccharide having difucosyl type 1 chain in Le^{a-b+} erythrocytes were found. These glycolipids have been isolated from pooled human erythrocytes and characterized by ^1H NMR spectroscopy and methylation analysis.

Materials and Methods

Isolation and Purification of Glycolipids. For purification of glycolipids, pooled outdated human whole blood was lysed in ice-cold tap water containing 0.2% acetic acid, and membranes were prepared by continuous centrifugation on a Sharpless centrifuge and extracted with 2-propanol–hexane–water (55:25:20 v/v/v). The "upper phase glycolipid fraction" was prepared as previously described (Kannagi et al., 1982b). The neutral glycolipid fraction was separated by DEAE-Sephadex (A-25) chromatography (Yu & Ledeen, 1972), and the upper neutral glycolipid fraction was fractionated by low-pressure high-performance liquid chromatography (HPLC)[1] on a 1 × 50 cm column of Iatrobeads 6RS-8060 (60-μm particles; Iatron Chemical Co., Tokyo, Japan) (Kannagi et al., 1982a,b, 1983b). The elution was programmed from 55:40:5 (v/v/v) to 55:20:25 (v/v/v) 2-propanol–hexane–water during 200 min with a flow rate of 3 mL/min. Each 6-mL fraction was collected on a fraction collector (total of 600 mL of eluate collected over 100 fractions). Each fraction was analyzed by high-performance thin-layer chromatography (HPTLC) in a solvent of chloroform–methanol–water (56:38:10 v/v/v). The fraction that contained a glycolipid with monofucosyl type 1 chain A (type 1 chain A^a) and the fraction that contained a glycolipid with difucosyl type 1 chain A (ALe^b) were eluted in fractions 45–52 and fractions 53–55, respectively (Figure 1). Further purification of each glycolipid fraction was performed by HPLC on a 0.4 × 50 cm column of Iatrobeads 6RS-8010 (10-μm particles) with a shallow gradient elution, i.e., for purification of A^a 2-propanol–hexane–water from 55:40:5 (v/v/v) to 55:30:15 (v/v/v) and for purification of ALe^b 2-propanol–hexane–water from 55:38:7 (v/v/v) to 55:30:15 (v/v/v). The HPLC-purified fractions were further purified as acetates by preparative HPTLC on a Baker plate (J. T. Baker Chemical Co., Phillipsburg, NJ) in the solvent described in the legends for Figures 2 and 3.

The reactivity of each fraction separated on HPLC with various monoclonal antibodies was determined by solid-phase radioimmunoassay and TLC immunostaining (Magnani et al., 1980). The fractions showing a positive reactivity with mo-

[1] Abbreviations: HPLC, high-performance liquid chromatography; HPTLC, high-performance thin-layer chromatography; ^1H NMR, proton nuclear magnetic resonance; GC–MS, gas chromatography–mass spectrometry.

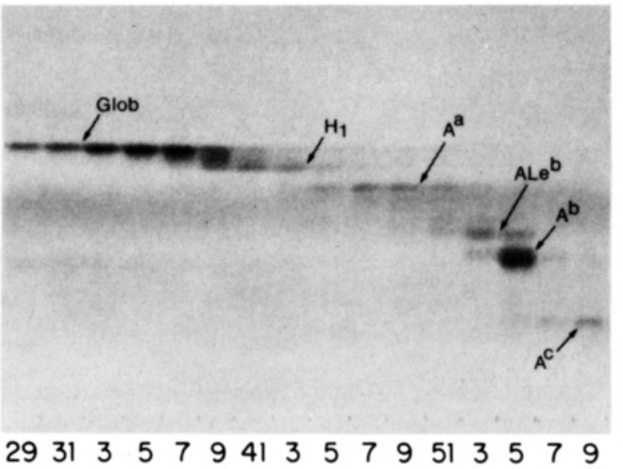


FIGURE 1: TLC pattern of neutral glycolipids from human type A blood separated through low-pressure HPLC as described under Materials and Methods. Plate was developed in chloroform–methanol–water (50:40:10) and visualized with orcinol–H_2SO_4. Lane numbers indicate fraction numbers as collected. Fractions 45–52 and 53–55, containing A^a and ALe^b, respectively, were pooled and further purified. Glob, globoside; H_1, A^b, A^c, see Hakomori (1981).

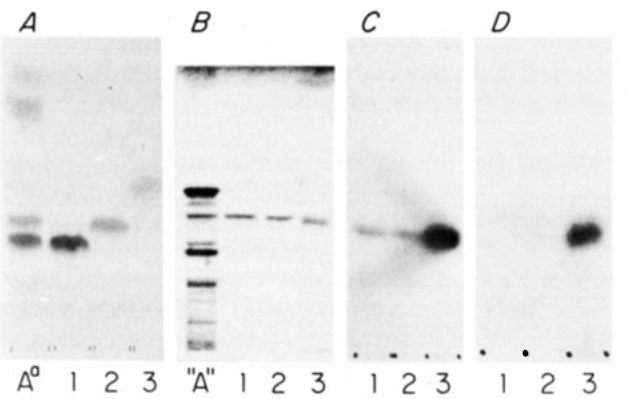


FIGURE 2: Purification of A^a type 1 chain; HPTLC pattern of A^a fraction and its components after purification as acetates. (Panel A) HPTLC of the acetylated A^a fraction. (A^a) Crude A^a fraction; (1–3) purified A-active components separated as acetates on HPTLC. The solvent system was dichloroethane–acetone–water (50:50:1). (Panels B–D) HPTLC of the native components 1–3 (deacetylated A^a fraction). The solvent system was chloroform–methanol–water (50:40:10). The chromatograms in panels A and B were developed with orcinol–H_2SO_4; those in panels C and D were immunostained with AH16 and AH21, respectively. Note that only the lane 3 component showed reactivity with AH21 antibody.

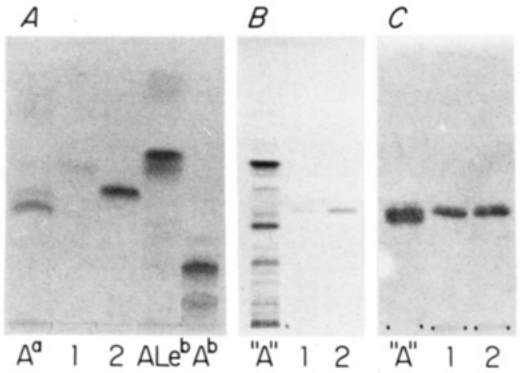


FIGURE 3: Purification of ALe^b; HPTLC pattern of purified ALe^b-active glycolipid fraction as acetates and as deacetylated native compounds. (Panel A) HPTLC of acetylated ALe^b-active components 1 and 2. Reference glycolipids were A^a (crude A^a fraction), ALe^b [ALe^b purified from human intestine (McKibbin et al., 1982)], and A^b (crude A^b fraction). The solvent system was dichloroethane–acetone–water (50:50:0.1). (Panels B and C) HPTLC of the native components 1 and 2; the solvent system was the same as in Figure 2, panels B–D. The chromatograms in panels A and B were visualized with orcinol–H_2SO_4; that in panel C was immunostained with monoclonal antibody HH3.

noclonal antibodies AH16, AH21 (Abe et al., 1984), and HH3, respectively, were further purified by HPLC on Iatrobeads 6RS-8010 (Watanabe & Arao, 1981) and were finally purified after acetylation by preparative HPTLC (see Figures 2 and 3).

Characterization of Glycolipids. The isolated glycolipids were characterized by ^1H NMR according to conditions previously described (Dabrowski et al., 1980a,b, 1981, 1982) and by methylation analysis [Hakomori, 1964; Stellner et al., 1973; Björndal et al., 1967; for a review, see Lindberg & Lönngren (1978)] as adapted for analysis of 10–20 nmol of glycolipid using chemical-ionization mass spectrometry of partially O-methylated hexitol and hexosaminitol acetates separated on a DB-5 capillary column (J & W Scientific, Rancho-Cordova, CA). Details of the procedure will be published elsewhere (S. B. Levery and S. Hakomori, unpublished results).

Monoclonal Antibodies. The monoclonal antibody AH21 defining monofucosyl type 1 chain A was prepared as previously described (Abe et al., 1984), and another monoclonal antibody, HH3, was prepared by immunization of mice with a purified difucosylated type 1 chain A isolated from human intestinal mucosa. The hybridoma producing Ig2a antibody specifically directed toward this structure was selected by positive reactivity with the glycolipid having difucosyl type 1 chain A and negative reactivity with monofucosyl type 1 chain A and difucosyl type 2 chain A. The procedure for establishing this monoclonal antibody and its properties will be described elsewhere (H. Clausen, J. M. McKibbin, and S. Hakomori, unpublished results).

The distribution of glycolipids in various blood group Lewis types was studied by extraction of glycolipids from individual samples of thoroughly washed fresh blood of each Lewis blood type and determination of the presence of Le^a and Le^b glycolipids by HPTLC–immunostaining with monoclonal antibodies directed to Le^a and Le^b antigens. Anti-Le^a antibody CF4C4 (Young et al., 1984) was donated by Dr. William W. Young, Jr. (Department of Pathology, University of Virginia Medical School, Charlottesville, VA), and anti-Le^b antibody was donated by Dr. Donald A. Baker (Chembiomed Ltd., University of Alberta, Edmonton, Alberta, Canada). Samples of fresh blood group A blood with different blood group Lewis status were donated by Marianne Osaki and Dr. Eloise Giblett

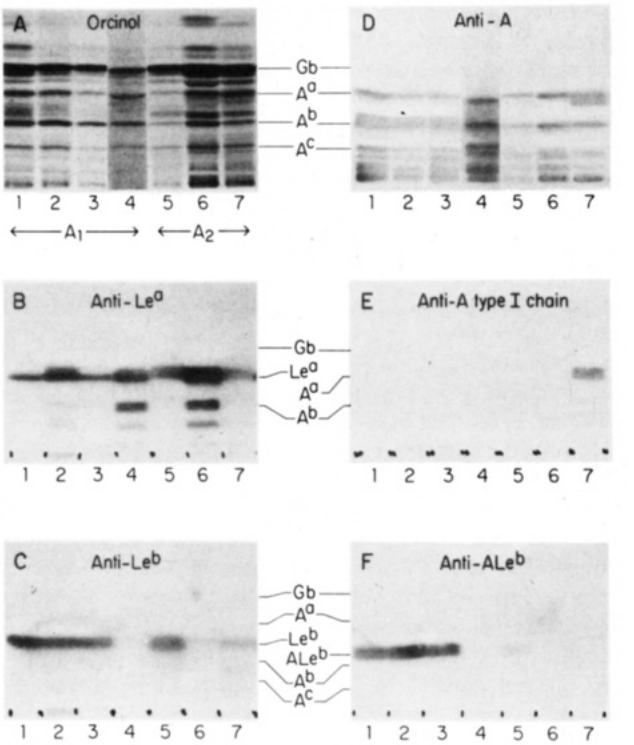


FIGURE 4: TLC pattern of glycolipids isolated from seven individuals with different Lewis blood group status. The upper neutral glycolipid fraction was analyzed on Baker HPTLC plate developed with chloroform–methanol–water (60:35:8). Bands were revealed by orcinol–sulfuric acid in panel A. The HPTLC plates were prepared under the same condition and were immunostained with antibodies to Le^a (in panel B), Le^b (in panel C), type 1 chain A (AH21) (in plate E), and ALe^b (HH3) (in plate F) and by nonrestricted antibody (AH16) in panel D. Samples 1, 3, and 5 were blood group Le^{a-b+}, and samples 4 and 6 were blood group Le^{a+b-}; sample 2 was blood group Le^{a+b+}, and sample 7 was blood group Le^{a-b-}. Samples 1–4 were blood group A_1, and samples 5–7 were blood group A_2.

(Puget Sound Blood Center, Seattle, WA).

RESULTS

Distribution of Monofucosyl Type 1 Chain A and Difucosyl Type 1 Chain A (ALe^b) in Erythrocytes with Various Blood Group Lewis Status. The results of TLC immunostaining of the glycolipids extracted from three cases of Le^{a-b+} (cases 1, 3, and 5), two cases of Le^{a+b-} (cases 4 and 6), one case of Le^{a+b+} (case 2), and one case of Le^{a-b-} (case 7) with five monoclonal antibodies, anti-Le^a, anti-Le^b, AH21, AH16, and HH3, are shown in Figure 4. One major and two minor bands were stained with anti-Le^a antibody in the glycolipids from Le^{a+b-} (lanes 4 and 6, panel B, Figure 4) and Le^{a+b+} (lane 2, panel B, Figure 4) samples. One band was stained with anti-Le^b antibody in the glycolipids from Le^{a-b+} samples (lanes 1, 3, and 5, panel C, Figure 4) and Le^{a+b+} samples (lane 2, panel C, Figure 4). A faint staining was observed with both anti-Le^a and anti-Le^b antibodies in Le^{a-b-} samples (lane 7, panels B and C, Figure 4). A weak but clear staining was also observed with anti-Le^a antibody in Le^{a-b+} samples (lanes 1, 3, and 5, panel B, Figure 4), although those samples were not agglutinated by anti-Le^a anti-sera.[2] Irrespective of the Lewis blood group status, there was no difference in the staining pattern with monoclonal antibody AH16 of the A determinants

[2] The reason the reactive band in the pentasaccharide region was detected by anti-Le^a in all samples, including Le^{a-b-}, is not clear. The antibody may cross-react with unknown structures present in Le^{a-b+} and Le^{a-b-} erythrocytes, or Le^{a-b+} and Le^{a-b-} erythrocytes may contain a trace quantity of the Le^a antigen.

Table I: Glycosyl H-1 Chemical Shifts (ppm from Tetramethylsilane) and $^3J_{1,2}$ Coupling Constants (Hz) of Glycolipids in Dimethyl-d_6 Sulfoxide at 303 ± 2 K

type 1 chain	GalNAcα1→3	Fucα1→2	Galβ1→3	Fucα1→4	GlcNAcβ1→3	Galβ1→4	Glcβ1→1Cer
H_1		4.994 (3.7)	4.450 (7.3)		4.551 (7.9)	4.263 (7.3)	4.176 (7.9)
A^a	4.929 (3.9)	5.070 (4.3)	4.507 (7.9)		4.543 (7.9)	4.267 (7.3)	4.216, 4.205, 4.186 (7.3)
Le^b		4.891 (3.7)	4.531 (7.3)	4.757 (3.7)	4.598 (8.9)	4.274 (6.7)	4.203 (8.3)
ALe^b	5.068 (3.7)	5.033 (4.3)	4.596 (7.9)	4.757 (3.7)	4.596 (7.9)	4.270 (6.7)	4.216 (7.3)

type 2 chain	GalNAcα1→3	Galβ1→4	GlcNAcβ1→3	Galβ1→4	Glcβ1→1Cer	Fucα1→2	Fucα1→3
H_1		5.039 (3.0)	4.335 (7.3)		4.629 (7.9)	4.258 (7.3)	4.168 (7.3)
A^a	4.943 (4.3)	5.157 (3.7)	4.396 (7.9)		4.600 (7.3)	4.259 (7.9)	4.183 (7.9)
Le^y		4.962 (3.7)	4.398 (6.8)	4.852 (3.7)	4.680 (7.3)	4.261 (7.3)	4.218 (8.3)
ALe^y	5.078 (4.3)	5.105 (4.3)	4.441 (7.3)	4.856 (3.7)	4.673 (7.3)	4.254 (7.3)	4.216 (7.3)

carried by various type 2 chain structures, i.e., A^a, A^b, and A^c, and other slower migrating components (panel D, Figure 4), although the Le^{a-b-} sample (case 7) showed a doublet in the A^a region (A^a type 1 chain) and the Le^{a-b+} samples showed a doublet in the A^b region (ALe^b). However, only glycolipids from Le^{a-b-} erythrocytes were stained with AH21, which defines monofucosyl type 1 chain A (lane 7, panel E, Figure 4), and only glycolipids from Le^{a-b+} and Le^{a+b+} erythrocytes were stained with HH3 antibody, which defines difucosyl type 1 chain A (ALe^b) (lanes 1–3 and 5, panel E, Figure 4).

Isolation of Monofucosyl Type 1 Chain A (Type 1 Chain A^a) and Difucosyl Type 1 Chain A (ALe^b) from Human Erythrocytes. The HPLC-purified A^a fraction and ALe^b fraction were purified as acetates by HPTLC as shown in Figures 2 and 3. Seven components were separated from the A^a fraction (Figure 2A), three of which were A active (Figure 2C, lanes 1–3). Only component 3 (Figure 2D) was positive with the type 1 chain A specific antibody, AH21. The remaining components 4–7 reacted with antibodies to Le^a and globo-H (data not shown). Two bands (Figure 3A, lanes 1 and 2) separated from the ALe^b fraction reacted with the antibody HH3 defining ALe^b structure (Figure 3C). Also separated from this fraction were H_2 type 2 chain, H_2 type 3 chain (Clausen et al., 1985), lactonorhexaosylceramide, and Le^b-reactive components (data not shown). The crude ALe^b fraction showed very weak or no reactivity with the antibody HH2 defining ALe^Y (H. Clausen, J. M. McKibbin, and S. Hakomori, unpublished results). After extraction and deacetylation, the bands were characterized by ^1H NMR and methylation analysis as described below.

The yield of type 1 chain A^a glycolipid (ALe^d) from $Le^{a-b-c-d+}$ erythrocytes is approximately 30–40% of the yield of type 2 chain A^a glycolipid, the latter being independent of Lewis and secretor blood group status. The yield of type 2 chain A^a from 250 mg of the upper neutral glycolipid fraction, prepared from 1 kg of packed blood cell membranes, is approximately 2 mg. Type 1 chain A^a (ALe^d) and difucosyl type 1 chain A (ALe^b) glycolipids were prepared, however, from pooled erythrocytes, irrespective of the Lewis blood group status. Therefore, the quantitative estimation of the yield of these glycolipids can vary depending on the population of $Le^{a-b+c-d-}$ and $Le^{a-b-c-d+}$ erythrocytes. Approximately 10–15 μg of the total upper neutral glycolipids prepared from such pooled blood cells will give clearly detectable quantities (1–3 μg per band) of type 2 chain A^a, A^b, A^c, and A^d components as detected by orcinol–sulfuric acid reaction. No bands corresponding to type 1 chain A^a (ALe^d) or difucosyl type 1 chain A (ALe^b) were detectable by orcinol–sulfuric acid reaction when the same quantity of upper neutral glycolipids was separated on TLC. However, the same amount of the upper neutral glycolipid fraction gives clearly detectable bands on TLC immunostaining with monoclonal antibodies AH21 and HH3. The orcinol-detectable level is approximately 50–100 ng on HPTLC. However, the ratio of type 2 chain A^a to monofucosyl type 1 chain A^a (ALe^d) in average pooled blood was estimated to be 45:1; and that of type 2 chain A^a and difucosyl type 1 chain A^a (ALe^b) was estimated to be 10:1.

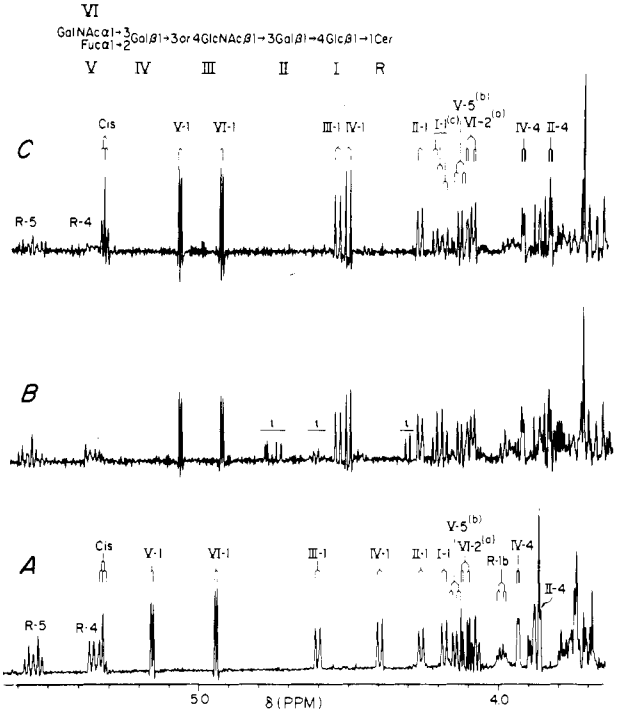


FIGURE 5: Downfield region of resolution-enhanced ^1H NMR spectra of A^a glycolipids. (A) Type 2 chain A^a from A erythrocytes (fraction 2); (B) type 1 chain A^a from A erythrocytes (fraction 3); (C) type 1 chain A^a from MKN45 cells (fraction 3). Arabic numbers refer to protons of residues indicated by roman numerals in the structure drawn at the top of the figure. Resonances marked by "R-" are from sphingosine backbone, while triplets marked "cis" are from *cis*-vinyl protons of unsaturated fatty acids. Resonances marked "i" correspond to anomeric and α-Fuc H-5 protons of Le^a impurity. (a) Assigned by continuous irradiation decoupling from α-GalNAc H-1 (V-1, $^3J_{1,2}$ = 4.3 Hz); (b) assigned by decoupling from α-Fuc methyl doublet ($^3J_{5,6}$ = 6.7 Hz) at 1.087 ppm for type 2 chain and at 1.048 ppm for type 1 chain A^a; (c) assigned by decoupling from Glc H-2 (I-2) at 3.029 ppm ($^3J_{1,2}$ = 7.3 Hz).

^1H NMR Spectroscopic Analysis of Type 1 Chain A and ALe^b Glycolipids from Human Erythrocytes. (A) Monofucosyl Type 1 Chain A. The downfield regions of the ^1H NMR spectra of monofucosyl type 1 chain A glycolipids (type 1 chain A^a) are shown in Figure 5. The anomeric protons (see also Table I) can be readily assigned by comparison with

Table II: Fucose H-5 and CH_3 Chemical Shifts (ppm from Tetramethylsilane) for Glycolipids in Dimethyl-d_6 Sulfoxide at 303 ± 2 K

	Fucα1→2		Fucα1→3/4	
	H-5	CH_3	H-5	CH_3
H_1 type 1	4.072	1.051		
H_1 type 2	4.007	1.068		
A^a type 1	4.135	1.048		
A^a type 2	4.145	1.087		
Le^b	4.188	1.079	4.626	1.065
Y	4.020	1.091	4.668	1.051
ALe^b	4.270	1.100	4.572	1.114
AY	4.180	1.123	4.659	1.097

data published by Dabrowski and co-workers for human type 1 and type 2 chain H_1 (Dabrowski et al., 1981) and for human B-II and B-III (Hanfland et al., 1983). Moreover, the α-GalNAc H-1,H-2, α-Fuc H-5,H-6 (CH_3), and β-Glc H-1,H-2 pairs are unambiguously established by simple decoupling experiments in addition to their occurrence in predictable locations (barring unexpected conformational effects) (Dabrowski et al., 1980b, 1981, 1982; Hanfland et al., 1983). The β-Gal H-4 resonances are also readily recognizable and predictable (Dabrowski et al., 1981, 1982), as are the ceramide resonances shown (Dabrowski et al., 1980a,b; Yamada et al., 1980; Koerner et al., 1983).

The anomeric resonances of type 1 chain A^a, as compared with those of type 2 chain, show trends similar to those reported for the H_1 glycolipids. The "anomalous" shifts obtained for type 1 chain H_1 or A can be rationalized by the stereochemical crowding inherent in the Fucα1→2Galβ1→*3*GlcNAc as opposed to the Fucα1→2Galβ1→*4*GlcNAc structure, which may result in a relative reorientation of anisotropic functional groups (such as the GlcNAc 2-acetamido group) or cause changes in the local magnetic environment by way of "nonadditive electronegativity through bond effects" (possible distortion of the dihedral angles Ψ and φ between sugar rings; Dabrowski et al., 1981). Thus, the type 1 β-GlcNAc H-1 is found considerably upfield, the subterminal β-Gal H-1 considerably downfield, and the α-Fuc H-1 downfield of the corresponding resonances in type 2 chain. The internal β-Gal-II H-1 in type 1 chain is also found slightly downfield of that in type 2 chain (Table I).

Glycosylation-induced shift changes readily apparent on addition of the α-GalNAc residue to the subterminal β-Gal of either type 1 or type 2 H_1 are a downfield shift of the β-Gal-IV H-1 by approximately 0.06 ppm and a downfield shift of the β-Gal-IV H-4 by approximately 0.3 ppm, to 3.933 ppm for type 2 and 3.926 ppm for type 1. Other changes for protons of this residue are expected, as for the B-active termini (Hanfland et al., 1983), but were not determined. Significant downfield shifts were apparent for the α-Fuc H-1 and H-5 and smaller changes for H-6 (CH_3) (Table II). In each of these cases, the shift change is somewhat smaller for type 1 chain, which may be reasonably expected for a structure already under more severe steric restraints. For type 2 chain structures, the shift changes noted are quite similar to those found for the analogous addition of Galα1→3 to convert H to B terminal (Hanfland et al., 1983), indicating that the α-GalNAc *N*-acetyl group makes little contribution to these particular effects.

It is obvious from the ^1H NMR spectra presented in Figure 5 that A^a fraction 3 (Figure 5B) is identical in carbohydrate structure with the type 1 chain glycolipid isolated from MKN45 cells (Figure 5C) and previously characterized (Abe et al., 1984), in agreement with the methylation analysis. On the other hand, the ^1H NMR spectra show definite differences

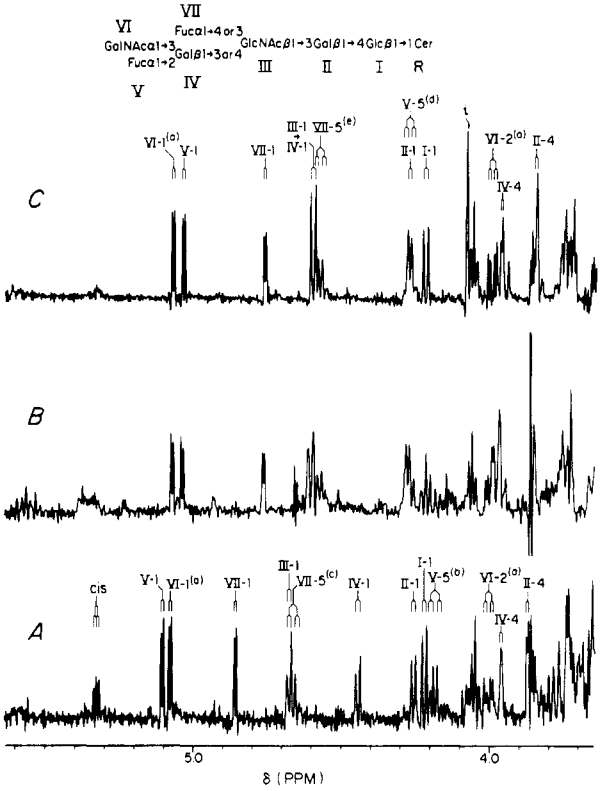


FIGURE 6: Downfield region of resolution-enhanced ^1H NMR spectra of difucosyl A^a glycolipids. (A) Type 2 chain (ALe^y) from dog intestine; (B) type 1 chain (ALe^b) from A erythrocytes; (C) type 1 chain (ALe^b) from human intestine. (a) α-GalNAc H-1 and H-2 resonances assigned by continuous irradiation decoupling experiments ($^3J_{1,2}$ = 4.3 Hz); (b–e) α-Fuc H-5 quartets assigned by continuous irradiation decoupling from methyl doublets ($^3J_{5,6}$ = 6.7 Hz) at (b) 1.123 and (c) 1.097 ppm (for ALe^y) and at (d) 1.100 and (e) 1.114 ppm (for ALe^b), respectively.

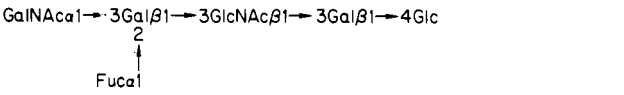
in ceramide structure among the three compounds shown. Thus, the type 2 chain A^a glycolipids (Figure 5A; the NMR of fraction 1 was essentially identical) show a "typical" erythrocyte pattern, with mostly unsaturated sphingosine (R-4, R-5 trans vinylic protons) and some (~20–30%) unsaturated fatty acids. The type 1 chain A^a from MKN45 cells (Figure 5C) shows an elevation in saturation of sphingosine (as evidenced by the reduced intensity of R-1b, R-4, and R-5 signals) and an increase of unsaturated fatty acids ("cis"). These changes in the ceramide are also indicated by multiple signals for β-Glc-I H-1. Finally, the type 1 chain A^a from erythrocytes (Figure 5B) shows both an increase in saturated sphingosines and a virtual disappearance of unsaturated fatty acids. These differences, in the case of the erythrocyte glycolipids, may reflect either a differential processing of ceramide types (Kannagi et al., 1983a) or a nonerythrocyte origin for the type 1 chain species.

(B) Difucosyl Type 1 Chain A (ALe^b). If one views the formation of difucosyl A^a structures as the result of the GalNAcα1→3 glycosylation of respective type 1 chain (Le^b) and type 2 chain (Y) precursors, the interpretation of the ^1H NMR spectra (Figure 6) is fairly straightforward. As for the monofucosyl A^a results, the obvious changes are in the terminal residues, with a downfield shift of the β-Gal-IV H-1 ($\Delta\delta \simeq$ 0.04–0.07 ppm) and H-4 ($\Delta\delta \simeq$ 0.3 ppm) and the terminal α-Fuc-V H-1 ($\Delta\delta \simeq$ 0.04 ppm), H-5 and H-6 (CH_3) (Tables I and II). Significantly, the internal residues are little affected, leaving the complex of resonances diagnostic for 3α- or 4α-fucosylation of GlcNAcβ1→4/3 [β-GlcNAc-III H-1, α-Fuc-VII H-1, H-5, and H-6 (CH_3)] virtually unchanged from

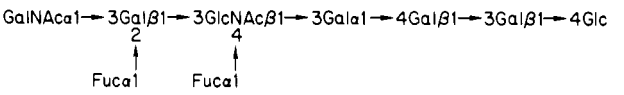
Table III: Blood Group A Determinant Carried by Four Types of Carbohydrate Chains in Human Erythrocytes

type 1 chain

$A^{a\,a}$

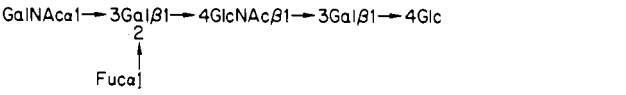


$ALe^{b\,a}$

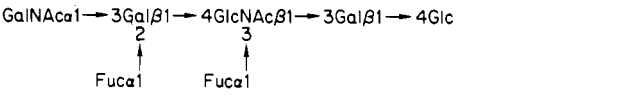


type 2 chain

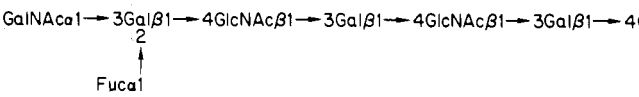
$A^{a\,b}$



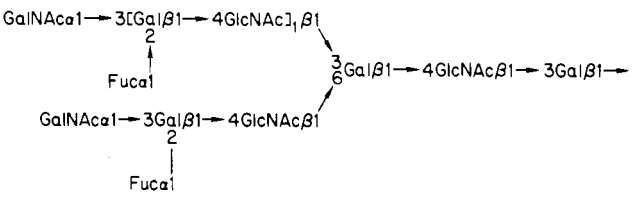
$ALe^{y\,c}$



$A^{b\,b}$



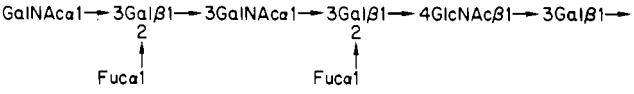
$A^{c\,b}$



$A^{d\,d}$ same as A^c, but the unit Galβ1→4GlcNAc in β1-3 side chain is repeated twice, i.e., the number of the unit in brackets is two

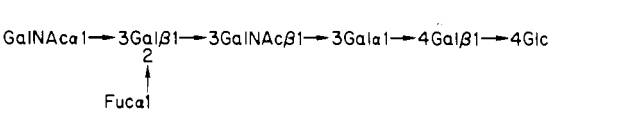
type 3 chain $A^{b\,e}$

repetitive A



type 4 chain (A^x)

globo-A^f



[a] This paper. [b] Hakomori (1981). [c] H. Clausen, J. M. McKibbin, and S. Hakomori, unpublished observation. [d] Fukuda & Hakomori (1982). [e] Clausen et al. (1985). [f] Clausen et al. (1984).

their positions in Y (Abe et al., 1983) vs. Le^b (Dabrowski et al., 1981; Abe et al., 1983) glycolipids.

If difucosyl A^a structures are viewed as Fucα1→3 or -4 (to GlcNAc) derivatives of respective type 1 or type 2 A^a precursors, the effects seen are the expected downfield shifts of β-GlcNAc-III and β-Gal-IV H-1 resonances and an upfield shift for the terminal α-Fuc-V H-1 (these have been demonstrated for the conversion of type 1 H_1 to Le^b; Dabrowski et al., 1981); in addition, a significant downfield shift is seen for the α-GalNAc-VI H-1 resonance regardless of chain type.

It is apparent even from the poorer quality NMR of the difucosyl A^a from A erythrocytes (Figure 6B) that its structure must be the same as that from human (Figure 6C) rather than dog (Figure 6A) intestine. That it is of type 1 chain (ALe^b) is information that cannot be inferred from a single methylation analysis by GC–MS, since both ALe^b and ALe^y yield the same set of partially methylated alditol acetates. Normally this information would have to be derived from the results of degradation studies with additional GC–MS analysis, requiring much more material than was used here.

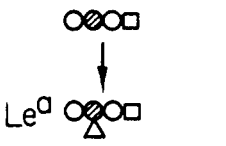
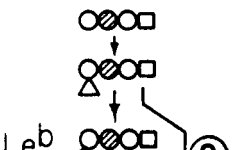
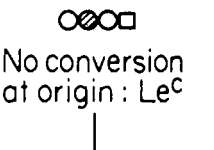
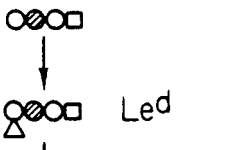
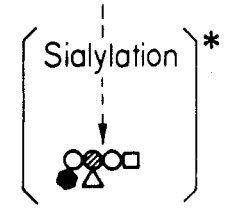
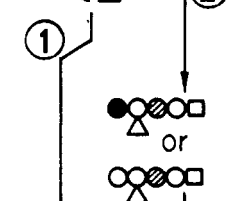
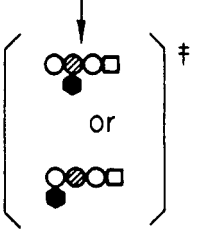
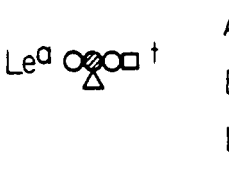
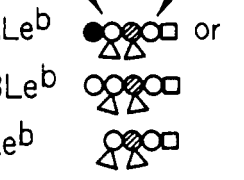
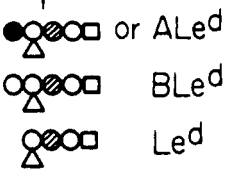
From the NMR it is apparent that the ceramide from A erythrocyte difucosyl type 1 chain A^a (Figure 6B) is high in saturated sphingosine and contains virtually no unsaturated fatty acids. This similarity to monofucosyl type 1 chain A^a (Figure 5B) suggests a common origin.

Methylation Analysis of Type 1 Chain A^a and ALe^b Glycolipids. The GC–MS patterns of partially O-methylated hexitol and hexosaminitol acetates from the hydrolysates of permethylated monofucosyl type 1 chain A^a and difucosyl type 1 chain A (ALe^b) are shown in panels A and B of Figure 7, respectively. The presence of 4,6-di-*O*-Me-GlcNAcMe and the absence of 3,6-di-*O*-Me-GlcNAcMe and 6-mono-*O*-Me-GlcNAcMe are characteristic of monofucosyl type 1 chain A^a, while the presence of a prominent peak for 6-mono-*O*-Me-GlcNAcMe and the absence of 4,6-di-*O*-Me-GlcNAcMe are characteristic for difucosyl type 1 chain A (ALe^b). A small peak corresponding to 3,6-di-*O*-Me-GlcNAcMe detected in the hybrid ALe^b must be a contamination by an unsubstituted type 2 chain, such as lactonorhexaosylceramide (X_4a glycolipid; Okada et al., 1984).

Discussion

Blood group ABH determinants in human erythrocytes have been characterized as being carried mainly by type 2 chain with different chain lengths and degrees of branching. Thus, there are multiple forms of A, B, and H glycolipids, designated respectively as A^a, A^b, A^c, and A^d (Hakomori et al., 1972; Hakomori, 1981), B_I (Koscielak et al., 1973) and B_{II} (Hanfland, 1975), and H_1, H_2, H_3, and H_4 [Hakomori et al., 1972; Watanabe et al., 1975; see, for review, Hakomori (1981)]. More recently, A and H determinants carried by globo-series structure (Kannagi et al., 1983b; Kannagi et al., 1984; Clausen et al., 1984; Breimer & Jowall, 1984) and a novel repetitive A structure (Clausen et al., 1985) have been isolated and

Table IV: Allogeneic Serocellular Expression of Type 1 Chain in Man[a]

Phenotype	Le^a ($Le^{a+b-c-d-}$)	Le^b ($Le^{a-b+c-d-}$)	Le^c ($Le^{a-b-c+d-}$)	Le^d ($Le^{a-b-c-d+}$)
Genotype	Le, se	Le, Se	le, se	le, Se
Main conversion at unknown synthetic site(s) and main product released in sera				
Subsequent substitutions at unknown site				
Type 1 chain transferred to and found in erythrocytes				

[a] Four allogeneic types can be distinguished in man according to their type 1 chain structures distributed in serum, erythrocytes, and endothelial and epithelial cells. The origin of such a genetic distinction is unknown, perhaps endothelial cells/or gastrointestinal cells, but the major loci expressing such an allogeneic difference are sera and secretions, and the major products are Lewis antigens [see, for review, Watkins (1980)]. Each antigen can be modified subsequently at unknown sites as shown. Symbols for structures are as follows: (○) Gal; (hatched circle) GlcNAc; (□) Glc; (△)Fuc; (●) GalNAc; (◆) sialic acid. The sialylation of Le^a (*) and that of Le^c (§) are highly limited to certain glandular tissue; Le^a antigen in erythrocytes can occur as a hybrid of type 2 chain; i.e, long-chain Le^a can be present, which is carried by type 2 chain (R. Kannagi, S. B. Levery, and S. Hakomori, unpublished observation). Le^c antigen in serum and in erythrocytes is not fully identified; i.e., polyclonal antibodies to Le^c agglutinated Le^c erythrocytes (Pendu et al., 1982), but type 1 chain paragloboside (Lc_4) did not inhibit Le^c hemagglutination (Hanfland et al., 1982). ALe^b or BLe^b can be synthesized by either route 1 or route 2.

characterized. The repetitive A and globo-A are both found in A_1 erythrocytes but are absent or present in trace quantities in A_2 erythrocytes and are classified as type 3 chain and type 4 chain A, respectively (see Table III) (Clausen et al., 1985).

The blood group Lewis antigens have been characterized as fucosylated type 1 chain [see review by Watkins (1980)], and they are essentially glycolipids synthesized at an unknown site and present in serum carried by lipoproteins transferred onto erythrocytes (Marcus & Cass, 1969). Several serological studies indicated a close relationship between the Lewis (Le,le) and secretor/nonsecretor (Se,se) system and the ABO system. A large amount of heterogeneity is known in anti-Le^b antibodies. Most anti-Le^b antibodies react strongly with OLe^b and A_2Le^b and only weakly with A_1Le^b erythrocytes (Andreasen, 1948). Others preferentially react with A_1Le^b (Seaman et al., 1968; Crookston et al., 1970; Jeannett et al., 1972), and a few react preferentially with A_1Le^d and only weakly with A_2Le^d (Andersen, 1958; Iwaki, 1982). The amount of A antigen in serum was found to be dependent on the Le/Se status (Tilley et al., 1975). These findings suggest that transferases specific for A_1, A_2, and B may convert type 1 chain structures (Lewis gene products) as has been suggested [see, for a review, Watkins (1980)], although the chemical properties of type 1 chain products in erythrocytes have been unknown.

Since we have established two monoclonal antibodies, AH21 and HH3, which define mono- and difucosyl type 1 chain A, respectively, we have studied a possible correlation between Lewis antigen expression and two types of type 1 chain A. A clear correlation between Lewis blood group status and the presence of these two forms of type 1 chain A was found; i.e., monofucosyl type 1 chain A^a was only found in Le^{a-b-} erythrocytes and was absent in Le^{a+b-} and Le^{a-b+} erythrocytes and difucosyl type 1 chain A was found only in Le^{a-b+} erythrocytes and was absent in Le^{a+b-} and Le^{a-b-} erythrocytes. The presence of ALe^b glycolipid in Le^{a-b+} erythrocytes can be explained by a sequential conversion of unsubstituted lactotetraosylceramide by the H-fucosyltransferase, the A enzyme, and the Lewis fucosyltransferase, all acting at the site of synthesis of the Lewis structures, since the A enzyme (of human milk) cannot act on the Le^b structure (route 2 in Table IV) (Kobata & Ginsburg, 1970). However, an alternative pathway (route 1 in Table IV) could be possible, i.e., conversion of Le^b structure to ALe^b or BLe^b. The apparent weaker expression of ALe^b in the A_2Le^{a-b+} sample (Figure 4, case 5) as compared to the A_1Le^{a-b+} samples (Figure 4, cases 1–3) would be consistent with less efficient A_2 enzyme competing with the Lewis enzyme for the H type 1 chain substrate.

In contrast to the presence of a series of type 2 chain A determinants (Hakomori, 1981) and type 3 chain A determinants (repetitive A) (Clausen et al., 1985), only one band of monofucosyl type 1 chain A defined by AH21 and one band of difucosyl type 1 chain A defined by HH3 were detected. It was clearly demonstrated that the type 1 chain A determinants were correlated with Lewis and secretor status and not with A_1 and A_2 phenotypes. Although a structural basis for a qualitative distinction between A_1 and A_2 phenotypes, as has been previously proposed (Economidou et al., 1967; Lloyd & Kabat, 1968; Moreno et al., 1979; Kisailus & Kabat,

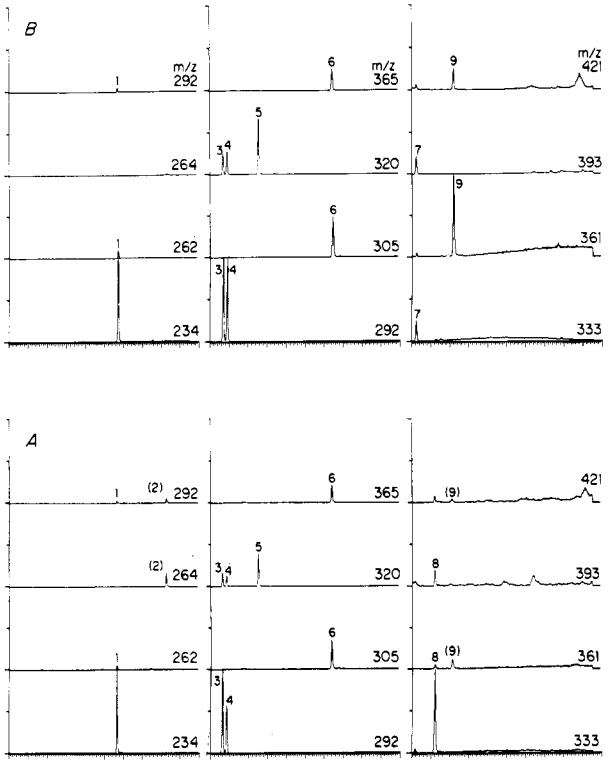


FIGURE 7: Selected mass chromatograms of partially O-methylated alditol and hexosaminitol acetates from hydrolysis of permethylated A^a glycolipids isolated from A erythrocytes. (A) Type 1 chain A^a; (B) type 1 chain difucosyl A^a (ALe^b). (Ordinate) Intensity of ion of mass number indicated; (abscissa) scan numbers: (1) 2,3,4-tri-*O*-MeFuc; (2) 2,3,4,6-tetra-*O*-MeGal; (3) 2,3,6-tri-*O*-MeGlc; (4) 2,4,6-tri-*O*-MeGal; (5) 4,6-di-*O*-MeGal; (6) 3,4,6-tri-*O*-MeGalNAcMe; (7) 3,6-di-*O*-MeGlcNAcMe; (8) 4,6-di-*O*-MeGlcNAcMe; (9) 6-*O*-MeGlcNAcMe. In panel A, the peaks marked 2 and 9 are derived from Le^a impurity in agreement with ^1H NMR (see Figure 5B). In panel B, the peak marked 7 is believed to be derived from an impurity of type 2 chain norhexaosylceramide.

1978), may exist, the data presented here do not support the hypothesis that the structural difference is based on type 1/type 2 chains. Furthermore, it appears that a newly isolated repetitive A epitope, present mainly in A_1 erythrocytes and expressed independently of Lewis and secretor status, may provide the structural basis for the A_1 and A_2 phenotype distinction (Clausen et al., 1985).

Four allogeneic types of type 1 chain in man have been known, which are the interaction products of three glycosyltransferases defined by each specific gene, i.e., $\alpha 1 \rightarrow 4$ fucosyltransferase (defined by gene Le; recessive form le), $\alpha 1 \rightarrow 2$ fucosyltransferase nonspecific for type 1 as well as type 2 (defined by gene H, recessive form h), and $\alpha 1 \rightarrow 2$ fucosyltransferase for type 1 chain (defined by gene Se, recessive form se). The products Le^a, Le^b (interactive product of Le and Se), Le^c, and Le^d [Graham et al., 1977; see, for review, Watkins (1980)] can subsequently be converted as shown in Table IV, released into serum, and subsequently transferred onto erythrocytes. The present concept of allogeneic distinction (serocellular distribution) of type 1 chain is illustrated in Table IV and its legend.

ADDED IN PROOF

Blood group A^a glycolipid with type 1 chain was recently isolated and characterized from plasma of an A_1 Le^{a-b-} secretor, albeit presented only in abstract from (Jowall et al., 1984). It is possible, therefore, that type 1 chain A glycolipid antigens of erythrocytes as described in this paper are acquired from plasma as are Lewis antigens.

Registry No. AH21, 87501-61-9; HH3, 96502-23-7.

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A Small Hydrophobic Domain That Localizes Human Erythrocyte Acetylcholinesterase in Liposomal Membranes Is Cleaved by Papain Digestion†

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ABSTRACT: A small hydrophobic domain in isolated human erythrocyte acetylcholinesterase is responsible for the interaction of this enzyme with detergent micelles and the aggregation of the enzyme on removal of detergent. Papain has been shown to cleave this hydrophobic domain and to generate a fully active hydrophilic enzyme that shows no tendency to interact with detergents or to aggregate [Dutta-Choudhury, T. A., & Rosenberry, T. L. (1984) *J. Biol. Chem. 259*, 5653-5660]. We report here that the intact enzyme could be reconstituted into phospholipid liposomes while the papain-disaggregated enzyme showed no capacity for reconstitution. More than 80% of the enzyme reconstituted into small liposomes could be released by papain digestion as the hydrophilic form. Papain was less effective in releasing the enzyme from large liposomes that were probably multilamellar. In a novel application of affinity chromatography on acridinium resin, enzyme reconstituted into small liposomes in the presence of excess phospholipid was purified to a level of 1 enzyme molecule per 4000 phospholipid molecules, a ratio expected if each enzyme molecule was associated with a small, unilamellar liposome. Subunits in the hydrophilic enzyme form released from reconstituted liposomes by papain digestion showed a mass decrease of about 2 kilodaltons relative to the intact subunits according to acrylamide gel electrophoresis in sodium dodecyl sulfate, a difference similar to that observed previously following papain digestion of the soluble enzyme aggregates. The data were consistent with the hypothesis that the same hydrophobic domain in the enzyme is responsible for the interaction of the enzyme with detergent micelles, the aggregation of the enzyme in the absence of detergent, and the incorporation of the enzyme into reconstituted phospholipid membranes.

Acetylcholinesterase (AChE)[1] (EC 3.1.1.7) forms in vertebrate tissues are classified either as asymmetric if they include a collagen-like tail structure or as globular if they are devoid of such a structure [see Massoulié & Bon (1982) and Rosenberry (1985)]. In addition to soluble and secreted forms, the globular AChEs include a class of integral membrane

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[1] Abbreviations: AChE, acetylcholinesterase; RBC AChE, human erythrocyte acetylcholinesterase; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; DFP, diisopropyl fluorophosphate; [^{125}I]TID, 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; kDa, kilodalton(s).

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