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GLYCOSPHINGOLIPIDS WITH BLOOD GROUP A, H, AND I ACTIVITY AND THEIR CHANGES ASSOCIATED WITH ONTOGENESIS AND ONCOGENESIS*

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ABSTRACT

Four sets of glycolipid variants of human A and O erythrocytes were isolated and their structures were determined. The glycolipids carrying A-determinants were termed A^a, A^b, A^c and A^d according to the complexity of the carrier carbohydrate chain in that order. Glycolipids with H-activity were termed H_1, H_2, H_3 and H_4 according to the order of their complexity of the carrier carbohydrate chain. It is suggested that the conversion of complex H variants (H_3/or H_4) to complex A variants (A^c/or A^d) was significantly lower in A_2 erythrocytes. In fetal/or new-born erythrocytes the highly complex variants (A^c, A^d, H_3 and H_4) were significantly lower than adult erythrocytes. These and other studies suggest that step-by-step elongation and arborization of complex glycolipids such as A^c and H_3 chains may take place during the process of development. A remarkable difference between the reaction of glycolipids in human intestinal tumors and the reaction of normal intestinal mucosa was demonstrated by the antibody which was directed against core structure of carrier carbohydrate chain, that is GlcNAcβ1→3Galβ1→4Glc→cer. This structure must be present in greater quantity in tumor tissue than in normal mucosa.

INTRODUCTION

Although already early in the century, blood group activities were found to be associated with the lipid extract of erythrocytes (for a review, see Ref. 1), the idea of blood group glycolipid antigens waited over fifty years for a few investigators who found the presence of a lipid-bound carbohydrate containing neutral and aminosugars with blood group activity (Ref. 2,3,4,5). Yamakawa *et al* (Ref. 6) described that a globoside fraction of human erythrocytes was blood group active, but subsequent studies indicated that the activities were associated with an unidentified glycolipid, which was separated from the major globoside fraction through chromatography (Ref. 7,8,9). It was noteworthy that a small, but measurable amount of fucose, was detected in this blood group active glycolipid fraction (Yamakawa and Irie, Ref. 7). Blood group A- and B-active glycolipids with a similar chemical composition were isolated subsequently by Koscielak (Ref. 10) and by Handa (Ref. 11). These preparations must have been grossly contaminated by gangliosides and other unidentified glycolipids in view of our present day criteria.

The presence of fucose as an essential component for a blood group active glycolipid was revealed later on through a different avenue of approach. The author found a unique fucosyl-glycosphingolipid present in a relatively large quantity in some human adenocardinomas of the stomach and colon (Ref. 12). The glycolipid was H-active as well as Le^a-active (Hakomori *et al* Ref. 13). The presence of a fucolipid and the demonstration of H- and Le^a-activity in the fucose-glycolipid, prompted us to search further into fucose glycolipids as a possible blood group antigen of erythrocytes, because at that time the specific structural relationship between blood group A, B, and H/or Lewis activities was already established by studies of Watkins and Morgan (Ref. 14) and by Cepellini (Ref. 15). This approach was further encouraged by an observation made by Schiffman *et al* (Ref. 16) and Lloyd and Kabat (Ref. 17) *i.e.*: fucose containing oligosaccharide as being the best hapten showing a high activity of blood group A and B, and that the presence of fucose was considered to be essential for the blood group hapten activity. Consequently, a thorough fractionation and purification of blood group ABH glycolipids were carried out when the author was at Brandeis University (Hakomori and Strycharz, Ref. 18). Only a very small quantity of seemingly pure glycolipids with a high A and B activity was isolated through successive chromatographies. These highly purified

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antigens were characterized by the presence of a high content of fucose and demonstrated a noticeable polymorphism (Ref. 18). Three active components were isolated from A_1-erythrocytes, one of which was lacking in A_2-erythrocytes. The simplest component was assumed to be a ceramide hexasaccharide having a similar back-bone structure to a ceramide pentasaccharide which was isolated from H-active or Lewis active glycolipids of tumor tissue (Hakomori et al, Ref. 13). Consequently, a series of structural studies have been undertaken since 1969 at the University of Washington in collaboration with Drs. H.J. Yang, Bader Siddiqui, Klaus Stellner, Kiyohiro Watanabe and Roger A. Laine. These studies have been supported by the generous gift of out-dated human erythrocytes through courtesy of Dr. Eloise R. Giblett, Director of the Puget Sound Central Blood Bank. This venture was only possible through improved isolation methods and through new methods of structural studies as shown in Table 1.

TABLE 1. Methods for Structural Studies of Glycosphingolipids

1. Sugar, fatty acids and sphingosine composition by gas chromatography (Yang and Hakomori Ref. 24).
2. Sequential enzymatic hydrolysis and examination of enzyme-degraded product by TLC (Hakomori et al Ref. 25).
3. Methylation analysis by GC-MS (Hakomori Ref. 26; Bjorndäl et al Ref. 27).
4. Total mass spectrometry of permethylated and reduced glycolipid (Karlsson et al Ref. 28).
5. Combination of enzymatic hydrolysis and total mass spectrometry (Watanabe, Laine and Hakomori Ref. 23).

ISOLATION AND CHARACTERIZATION OF GLYCOSPHINGOLIPIDS HAVING BLOOD GROUP A-, H-, AND I-DETERMINANTS FROM HUMAN ERYTHROCYTES

The major glycolipid of human erythrocyte membranes is globoside and its precursors: ceramide trihexoside, lactosylceramide, and glucosylceramide. The structures of these glycolipids have been well established as seen in Table 2. All the blood group ABH glycolipids found in erythrocyte membranes have been characterized by having the common carbohydrate residue, β-galactosyl(1→4)β-N-acetylglucosaminyl(1→3)β-galactosyl(1→4)β-glucosylceramide ("paragloboside"). A series of glycolipids with blood group A and H activities as seen in Table 3 and 4, were isolated and characterized which can be regarded as the derivative of "paragloboside". In contrast, Le^a- and Le^b-glycolipids are considered to be a derivative of lacto-N-tetraosylceramide rather than a derivative of lacto-N-neotetraosylceramide. This may be related to the fact that Lewis-active glycolipids of erythrocytes were acquired exogenously but were not synthesized in erythorcytes (Marcus and Cass, Ref. 19).

1) A-glycolipids

Four types of blood group A-active glycolipids, A^a, A^b, A^c and A^d have been separated through chromatography with either DEAE-cellulose or Anasil S (a mixture of magnesium oxide and silicic acid, Analab Co., Conn.). These A-variants differ in their carrier carbohydrate structure, but the structure of A-determinants is identical among themselves (Hakomori et al, Ref. 21).

(a) A^a-glycolipids: This component was isolated from other glycolipids such as H_1-, Le^a-glycolipids and "paragloboside" through thin-layer chromatography as acetylated compounds and was identified as ceramide hexasaccharide. The compound was hydrolyzed by α-N-acetylgalactosaminidase of Chalonia lampus and was converted to H_1-glycolipid. On the other hand, H_1-glycolipid can be converted to A^a-glycolipid through serum N-acetylgalactosaminyltransferase and UDP-GalNAc. The structure was further assessed by methylation studies (Watanabe, Stellner and Hakomori, unpublished observation). Through systematic degradation and methylation analysis, the structure of this glycolipid was determined as seen in Table 3.

(b) A^b-glycolipids: This component was eluted from a DEAE-cellulose column using solvent numbers 7 and 8 (see Note a), and was further purified through thin-layer chromatography or through a chromatography on a long column of silicic acid ("Biosil A") with a chloroform-methanol-water system. The glycolipid was finally purified as acetylated compound through thin-layer chromatography. A^b-glycolipids were converted to H_2-glycolipid through Chalonia lampus α-N-acetylgalactosaminidase and H_2-glycolipid were converted to A^b-glycolipids by incubation with UDP-GalNAc and serum α-N-acetylgalactosaminyltransferase, and the product was chromatographically indistinguishable from A^b-glycolipids, on chromatography on silicic acid thin-layer and on silica gel-impregnated paper (Watanabe, Stellner and Hakomori, unpublished observation). These results indicate that A^b-glycolipids must be a ceramide octasaccharide with a structure as shown in Table 3.

TABLE 2. Classes of glycosphingolipids found in human erythrocytes

Globoside Series	
Glcβ1→1ceramide	CMH
Galβ1→4Glcβ1→1ceramide	CDH
Galα1→4Galβ1→4Glcβ1→1ceramide	CTH
GalNAcβ1→3Galα1→4Galβ1→4Glcβ1→1ceramide	globoside
Lacto-N-tetraoside Series	
(Galβ1→3GlcNAcβ1→3Galβ1→4Glcβ1→1ceramide) 4 ↑ 1 Fuc	Le^a
(Galβ1→3GlcNAcβ1→3Galβ1→4Glcβ1→1ceramide) 2 ↑ 1 Fuc; 4 ↑ 1 Fuc	Le^b
Lacto-N-neotetraosyl Series	
Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→1ceramide	paragloboside (reactive to anti-type XIV pneumococcal polysaccharide)
H_1, H_2, H_3 glycolipids	
A^a, A^b, A^c glycolipids see Table 3.	
BI, BII glycolipids	
Ganglioside Series (sialosyl glycolipids)	
NANAα2→3Galβ1→4Glcβ1→1ceramide	
NANAα2→3Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→1ceramide	
NANAα2→3Galβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→1ceramide	

(c) A^c-glycolipids were eluted from a DEAE-cellulose column using solvent numbers 9 and 10 and partially solvent number 11 (see Note a). The glycolipid was further purified on a long column of silica gel ("Biosil A:) with chloroform-methanol-water system and finally purified as acetylated compound through thin-layer chromatography on silica gel H. This compound was converted through α-N-acetylhexosaminidase of Chalonia lampus (see Note b) to a substance which was indistinguishable from H_3-glycolipids. Methylation studies indicated that the branching structure of A^c-glycolipids could be analogous to the one as was established in H_3-glycolipid. There are two possibilities for the branching structure, namely, a) two identical A-determinants (GalNAcl→3[L-Fucl→2]Gall→R) are linked to the type 2 chains which are further linked to paragloboside, b) the second possibility is that one of the type 2 chain structures may be incompleted and devoid of A-determinant, but H-determinant or its precursor form is present at the terminal of one of the branches. These two possibilities were studied by mass spectrometry of permethylated A^c compound. The mass #228 (260-32) is the major and the absence of the mass numbers 393 or 219 could exclude the second possibility as described above. Their terminal non-reducing structures, therefore, must be both substituted with α-N-acetylhexosamine residue, since no terminal galactose was detected through mass spectrometry of fully methylated A^c-glycolipids.

The presence of the fourth component of A-active glycolipids (A^d-glycolipid) has been noticed although the fraction was extremely difficult to further purify by the present technique and the chemical structure of this glycolipid has not been elucidated. The fraction is still heterogenous and further purification has to be made. This fraction contained branching structures as evidenced through methylation studies.

TABLE 3. Structures of blood group A-active glycolipids isolated from human erythrocyte membranes

A^d Similar to A^c, but with an additional branching structure, exact structure undetermined.

2) H-glycolipids

Four types of H-active glycolipids have been separated, and designated as H_1, H_2, H_3-, and H_4-glycolipids (Stellner et al Ref. 20; Watanabe et al Ref. 23).

(a) H_1-glycolipid was eluted from an Anasil S column with solvent numbers 5-7, and with solvent number 6 from a DEAE-cellulose column (see Note a). H_1 component was co-eluted with many other glycolipids such as paraglobosides, Le^a-, Le^b-glycolipids, and β-galactosyl-paraglobosides (Stellner and Hakomori, Ref. 29). These components were separated from one another on a long column of silica gel using the chloroform-methanol-water system or thin-layer chromatography as acetylated compound. Based on enzymatic degradation, partial acid hydrolysis, and methylation studies, the structure of H_1-glycolipid was established as L-fucosyl-α1→2paragloboside as shown in Table 5. The major fatty acid was identified as C20 and the long-chain base exclusively as C18 (octadecasphingenine).

(b) H_2-glycolipids: This component was eluted using solvent number 9 from "Anasil S" column, and solvent number 7 and 8 from a DEAE-cellulose column (see Note a). Sequential hydrolysis using various glycosyl hydrolases, as well as methylation analysis established the structure of H_2-glycolipids as seen in Table 3. The H_2-glycolipids were not hydrolyzed through endo-β-N-acetylglucosaminidase (Tarentino et al Ref. 30), excluding the possibility of such a linkage as a di-N-acetylchitobiosyl structure within the H_2-carbohydrate chain.

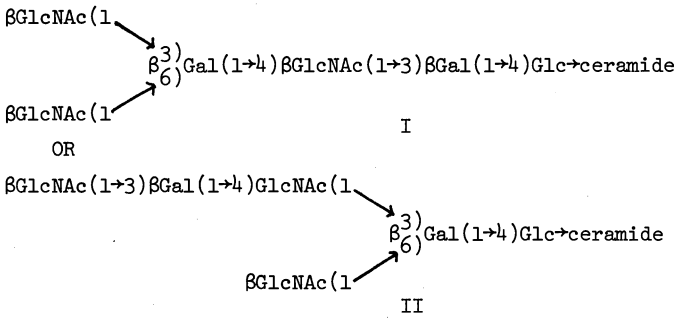
(c) H_3-glycolipids: Incubating H_3-glycolipids with α-L-fucosidase of Chalonia lampus turned the chromatography migration rate of H_3-glycolipids to a substance with a slightly higher chromatography migration rate than that of the original H_3-glycolipids. Neither β-galactosiadase, α-galactosiadase, nor β-N-acetylglucosaminidase altered the properties of the H_3-glycolipid. The hydrolysis product of H_3-glycolipid was then hydrolyzed with Jack bean β-galactosidase to a substance which had a slightly higher mobility than H_2-glycolipid. The compound was further degraded using β-hexosaminidase, resulting in a degradation product identical to paraglobosides in chromatography and in its immunological property. The paraglobosides-like material was further identified through successive hydrolysis with β-galactosiadase and β-N-acetylhexosaminidase. H_3-glycolipid was not susceptible to endo-β-N-acetylhexosaminidase as has been described by Tarentino et al Ref. 30). The result of the successive degradation through various hydrolases as described above, and through carbohydrate analysis of intact H_3-glycolipids, clearly indicated the presence of 2 moles of fucose per glucosylceramide. Both fucosyl residues as well as two of the additional four galactosyl residues, were easily destroyed by periodate oxydation under controlled oxidation conditions (Hakomori et al Ref. 25), suggesting the presence of two H-active carbohydrate chains linked to glycosylceramide by branching.

The concept of a branched structure was further supported by extensive methylation studies and through a characterization of partially methylated hexosaminitol and a partially

O-methylated 2-deoxy-2-N-methyl-acetamido-hexitol compound. Mass spectrometry of an enzyme degraded permethylated product, as has already been described, was used to determine the branching point (Watanabe et al Ref. 23). Enzymatic degradation and methylation study do not identify the branching point.

This distinction can be carried out by enzymatic hydrolysis combined with total mass spectrometry of the enzyme-degraded compound, after methylation and reduction (Karlsson, Ref. 28). However, the original method was modified in the purification of permethylated compound and in the use of sodium bis(2-methoxyethoxy)aluminum hydride for reduction. The glycolipids were permethylated (Ref. 26); permethylated glycolipid was isolated by chromatography on LH-20 column followed by thin-layer chromatography on silica gel G, developed with benzene-acetone (1:1 v/v). The permethylated glycolipid, eluted from the silica gel with acetone and evaporated under nitrogen in a Teflon-lined screw-capped Pyrex glass tube, was dissolved in 1 ml of benzene, and to it 0.5 ml of sodium bis(2-methyoxyethoxy)aluminum hydride ("Vitride", 70% in benzene, Eastman Kodak) was added. It was then heated at 80° for 2 hr. After cooling, 1 ml of water and 0.1 N sodium hydroxide was added, shaken well, and centrifuged. The upper benzene layer was separated, and the lower layer was partitioned twice with benzene. The first benzene layer and the benzene extract from the lower phase were combined and evaporated to dryness. The dried residue was dissolved in 1 ml of chloroform and shaken with 1 ml of water. The chloroform layer was evaporated to dryness in a glass "direct-probe tube" first under nitrogen stream and then in vacuo over phosphorus pentoxide. The residue was subjected to direct probe mass spectrometry in the Finnigan quadrupole instrument.

By successive treatment of H_3-glycolipid with α-L-fucosidase and with β-galactosidase, H_3-glycolipid was converted to a glycolipid with two terminal N-acetylglucosamines, which were characterized by having a strong precipitin reaction with wheat germ lectin and by giving 3,4,6-tri-O-methyl-2-deoxy-2-N-methylglucitol after methylation analysis. This compound could have either of the following structures, I or II.



Identification of either structure I or II was of critical importance in determining the branching point of the parent glycolipid. It was expected that the mass spectrum of the degraded glycolipid, structure I, would show a difference from that of structure II, although mass spectra of the parent compound may be difficult to distinguish. The method of Karlsson (Ref. 28) using the volatile permethylated and reduced derivatives of glycosphingolipids was employed. This gave 2-deoxy-2-N-methylethyl amino sugars which displayed an ion at *m/e* 246 when terminally located. An N-acetylhexosaminosylhexosyl residue at the terminal of the carbohydrate chain could be identified by the presence of *m/e* 460 and 246, as was exemplified in the mass spectra of permethylated and reduced GlcNAcβ(1→3)Galβ(1→4)Glc→ceramide (see Fig. 1A) and globoside (see Fig. 1B). The *m/e* 466 is the ion with oxygen. A similar mass spectra should be given by compounds with structure II but not by those with structure I.

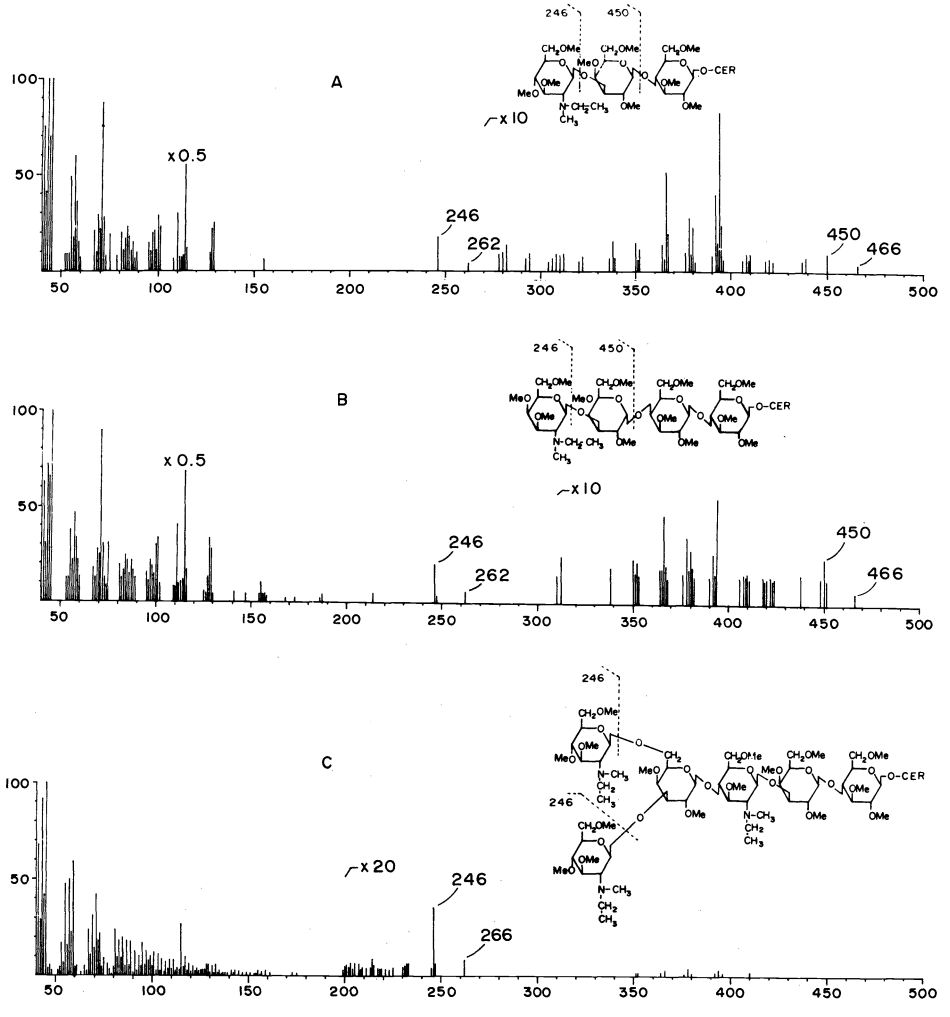
Note a. Chloroform-methanol-water mixtures of the following proportions by volume: Solvent number 1, 9:1:0.02; number 2, 8.75:1.25:0.02; number 3, 8.5:1.5:0.02; number 4, 8:2:0.02; number 5, 7.75:2.25:0.03; number 6, 7.5:2.5:0.03; number 7, 7:3:0.03; number 8, 6.75:3.25:0.04; number 9, 6.5:3.5:0.04; number 10, 6:4:0.04; number 11, 5.75:4.25:0.05; number 12, 5.5:4.5:0.05. Elution schedules were indicated in a few papers already published. (Ref. 22, 23).

Note b. Chalonia lampus enzyme cocktail obtained through a courtesy of Dr. Takashi Okuyama (Director of the Research Institute, Seikagaku Kogyo Co., Ltd.) contained a powerful α-N-acetylgalactosaminidase and A-structure was readily converted to H-structure. Fractionation of Chalonia lampus enzyme was published (Iijima, Muramatsu and Egami, Ref. 47; Fukuda and Egami, Ref. 48).

As shown in Fig. 1C, the enzyme-degraded compound derived from H_3-glycolipid gave only intensive mass with *m/e* 246 (and 266), but none at 450. This clearly indicates that the compound should have both GlcNAc terminals branched at the penultimate hexose; namely, the compound should have structure I rather than structure II. The mass spectra of an intact permethylated and reduced H_3-glycolipid were characterized by the presence of *m/e* 189 and 393, which is derived from the terminal fucosyl and fucosylgalactosyl residue, respectively (See Fig. 1D). The structure of H_3-glycolipids was thus determined as having two identical H-active type-2 chains attached to a paragloboside by β1→3 and β1→6 linkages.

H_3-glycolipid showed I-antigen activity both in inhibition of I-hemagglutination and in precipitin reaction. This is in striking contrast to H_1- and H_2-glycolipids which had no I-activity at all (Watanabe et al, Ref. 23). Glycolipids with blood group H- and B-activities of erythrocytes were studied by Koscielak et al (Ref. 31), that of gastrointestinal tract have been studied by McKibbin and his associates (Ref. 32,33) and by Slomiany et al (Ref. 34, 35). Structures of H-glycolipids proposed by Koscielak et al (Ref. 31) was identical to that of H_1 and H_2, and that of A-glycolipid of dog intestine by Smith et al, (Ref.32) was identical to that of A^a-glycolipid. A- and H-glycolipids of dog gastric mucosa described by Slomiany and Horowitz (Ref.34) and Slomiany et al, (Ref. 35) were similar to that of A^a- and H_1-glycolipid but had one more mole of galactose at the inside of the carrier carbohydrate chain.

Fig. 1



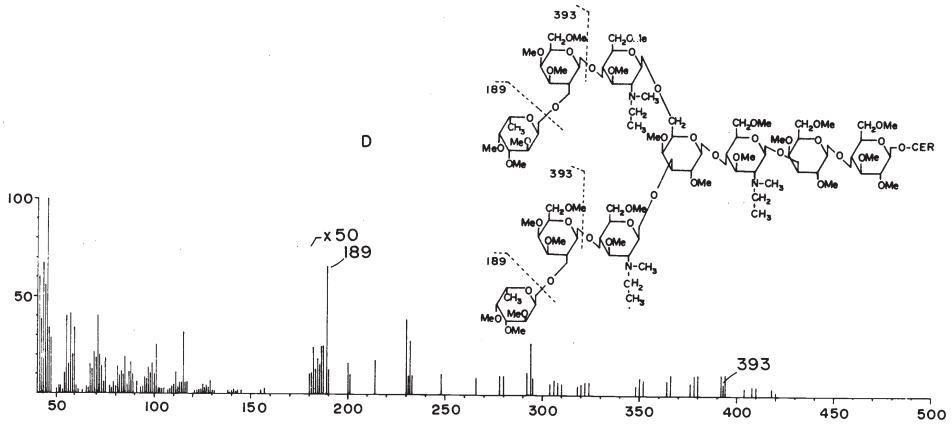


Fig. 1 Mass spectra of permethylated and reduced product of some glycosphingolipids. Permethylation was according to Hakomori's method (Ref. 26) and reduction with sodium bis(2-ethoxymethoxy) aluminum hydride, as modified from Karlsson (Ref. 28b,28c: (A) GlcNAcβ(1→3)Galβ(1→4)Glc→ceramide, degradation product from "paragloboside"; (B) globoside of human erythrocyte membranes; (C) degradation product from H_3-glycolipid by α-L-fucosidase; and by β-galactosidase (D) intact H_3-glycolipid. The condition for mass spectrometry is as follows: ion source pressure, 2 X 10^{-5} Torr; ionization current, 0.50 mA; electric energy, 70 eV, ion energy, 6.2 eV: sensitivity. 10^{-7} A/V: mass range. 0-500 amu. The mass peak 214 (246 minus 32 for methanol) was clearly seen in Figures 1B and 1C, but was barely detectable with the sensitivity recorded for Figure 2A. At higher sensitivity this peak was detected clearly. The intensive peaks between 350 and 400 due to fragments derived from ceramides (Karlsson et al, Ref. 28).

TABLE 4. Structures of blood group H-active glycolipids isolated from human erythrocyte membranes

H_1 L-Fucα1→2Galβ1→4GlcNAcβ1→3Galβ1→4Glc→Ceramide

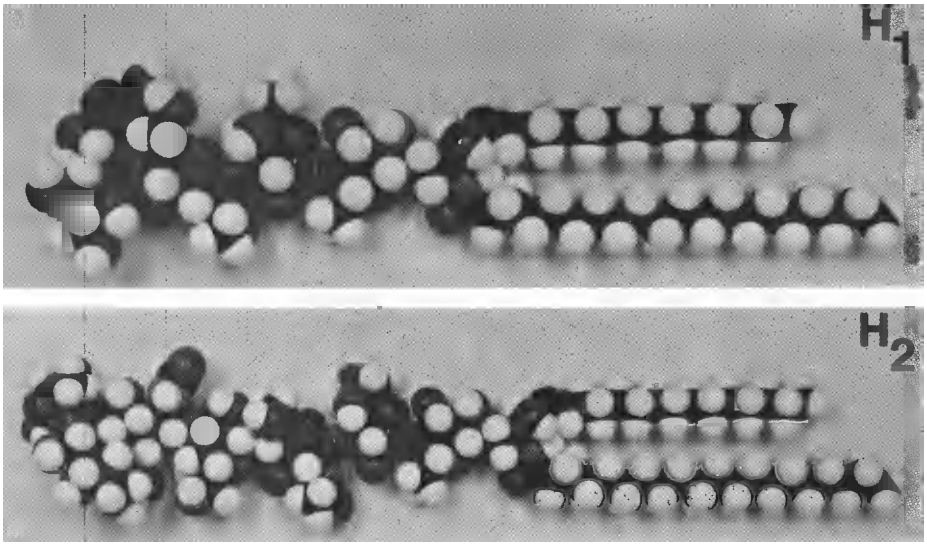
H_2 L-Fucα1→2Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glc→Ceramide

H_3 L-Fucα1→2Galβ1→4GlcNAcβ1 → 3 / 6 Galβ1→4GlcNAcβ1→3Gall→4Glc→Ceramide

L-Fucα1→2Galβ1→4GlcNAcβ1

H_4 Similar to H_3, but with an additional branching structure, exact structure undetermined.

Fig. 2
The molecular models of these glycolipids are shown in Fig. 2.



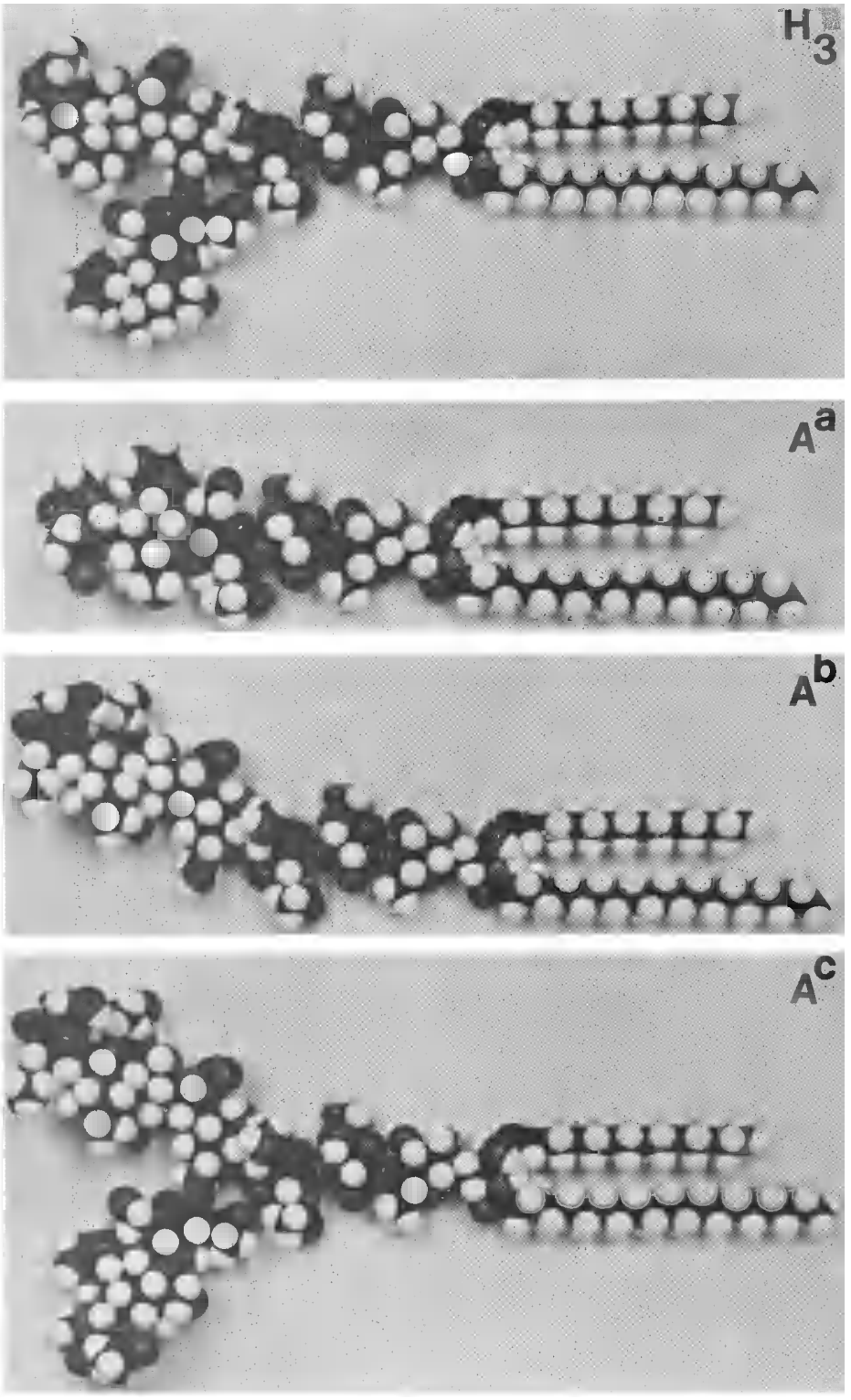


Fig. 2 Molecular models of blood group H and A active glycolipid.
H_1, H_2, H_3, A^a, A^b, A^c glycolipid in that order.
Only H_3-glycolipid was found to be I-active (with "Ma"-serum; Cf Ref. 50.

STATUS OF BLOOD GROUP CARBOHYDRATE CHAINS AND THEIR CHANGES DURING ONTOGENESIS AND ONCOGENESIS.

A blocked synthesis of A- and B-determinants in human epithelial and endodermal tumors have been described, based on immunochemical (Ref. 36, 13), immunohistological (Ref. 37,38), and enzymatic (Ref. 39) studies. The change of Lewis blood group hapten and accumulation of Le^x-like antigen in some adenocarcinoma were described (Ref. 13,24). Immunohistological studies indicated that blood group determinants appeared and disappeared in a certain order during ontogenetic development (Ref. 40,41), that the H-determinant was demonstrated to be a marker of cellular differentiation (Ref. 42), and that the development of i- to I-antigen was shown to be associated with post-natal change of erythrocytes (Ref. 43). The association of I- and i-antigen with tumor tissue (Ref. 44), and with carcinoembryonic antigen (Ref. 45) has been described.

All these findings suggest that a genetic or epigenetic program, for synthesizing blood group determinants and their carrier carbohydrate chains, develops step-by-step during the process of ontogenetic development, and that the program of synthesis is blocked or modified in the process of malignancy. This section is to provide a new experimental evidence to support the following general concepts: a) ontogenesis of a carbohydrate chain occurs as a step-by-step elongation and arborization of a complex carbohydrate chain, as for example that of A^c and H_3 variants, b) blocking of the elongation and arborization of a carbohydrate chain occurs during oncogeny as a result of a blocked ontogenic program.

Since blood group glycolipids were minor membrane components and only a small amount of fetal and new-born erythrocytes were available, the change of blood group glycolipids in fetal and new-born erythrocytes were studied by the following methods: (1) A-glycolipids were studied by cell-surface labeling with galactose oxidase and tritiated borohydride according to the method of Gahmberg and Hakomori (Ref. 46), followed by a preparation of membrane extraction, and by a preparation of long-chain neutral glycolipids. (2) H_2-glycolipids and their degradation products were studied through reactions of erythrocytes and glycolipid fractions of tissues with antibodies directed against H_3-glycolipid and its degradation product (Structures 2, 3, 4 see Fig. 3). The H_3-glycolipid was degraded step-by-step with purified α-L-fucosidase, β-galactosidase, and β-N-acetylhexosaminidase. Paragloboside and its degradation product were also prepared. These degraded glycolipids were purified through thin-layer chromatography. Purified glycolipids were complexed with bovine serum albumin and immunized rabbits with a complete Fruend adjuvant. Anti-sera were purified by BSA-Sepharose column to eliminate anti-BSA.

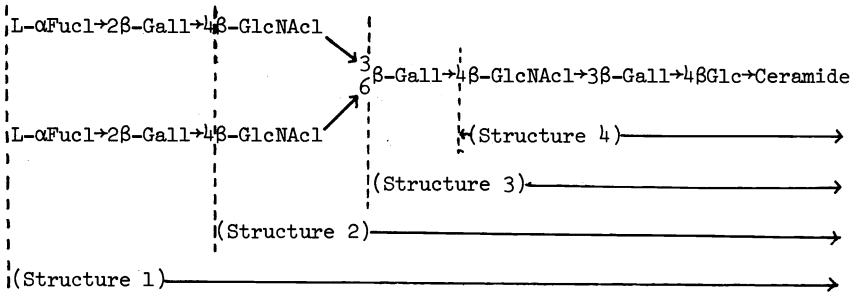


Fig. 3 Structure of H_3-glycolipid and its degradation products to which antibodies were directed.

The ratios of the surface-labeled activities, new-born to adult erythrocytes of each A-variant (A^a, A^b, A^c, and A^d) are shown in Table 5. Activities of each A-variant of new-born erythrocytes are expressed as % of adult erythrocytes. The labels of A^c- and A^d-variants in fetal erythrocytes were significantly lower than those of adult erythrocytes. A similar experiment cannot be carried out for H-determinants, because they were not quantitatively labeled. Alternatively, the reactivities of erythrocytes to various antibodies, which are directed against H_3 and its degradation products, were compared. The antibody which was directed against whole H_3-glycolipids (structure 1), did not strongly cross-react to H_1- and H_2-structures (see Table 6). This indicates that the antibody recognizes a whole branched H-structure. This antibody strongly reacted to adult human erythrocytes, weakly reacted to cord erythrocytes, and did not react at all to fetal erythrocytes (Fig. 4A). The higher reactivity of adult erythrocytes to anti-H_3-glycolipids has been further confirmed through an absorption experiment, i.e. adult erythrocytes absorb about 8 times the quantity of anti-H_3-antibodies than new-born erythrocytes absorb. 10^6 adult erythrocytes absorb almost all of the anti-H_3 activity which was present in 50 µl of antisera (titer 1:128), whereas the supernatant of the same amount of antiserum, which was incubated with the same number of new-born erythrocytes, contained the anti-H_3 antiserum.

TABLE 5. Surface-labeled activities of blood group A-glycolipids variant: % of activity of new-born erythrocytes to that of adult erythrocytes.

A-erythrocytes of adult and new-born were surface-labeled by galactose oxidase and tritiated borohydride (46), membranes were isolated, glycolipid fractions were prepared. Activities of each variant, A^a, A^b, A^c and A^d fractions obtained from new-born erythrocytes were compared with those of adult erythrocytes. Values were expressed as % of adult erythrocytes.

	% activity of new-born erythrocytes to adult erythrocytes			
	A^a	A^b	A^c	A^d
Experiment 1	95	65	24	20
Experiment 2	84	92	45	30
Experiment 3	110	96	46	35

TABLE 6. Inhibition of anti-H_3-dependent hemagglutination by H_1, H_2, and H_3-glycolipid*

H_1-glycolipid	Not inhibited by 25 µg/50 µl, partially inhibited by 50 µg/50 µl.
H_2-glycolipid	Not inhibited by 25 µg/50 µl, partially inhibited by 50 µg/50 µl.
H_3-glycolipid	Inhibited completely by 12 µg/50 µl.
	Inhibited partially by 3 µg/50 µl

*Determined by 3-hemagglutination doses of anti-H_3 glycolipid rabbit antisera (purified by NSA-column) and in the presence of two times weight of cholesterol and lecithin.

The difference in reaction to an anti-H_3 antibody between adult and fetal erythrocytes, became greater after trypsin treatment of erythrocytes, i.e. enhanced reaction of adult erythrocytes after trypsin treatment, and only a slight increase of cord erythrocytes (see Fig. 4A). The agglutinability of both fetal and adult cells through anti-H_3 antibodies are not thermosensitive as compared to the reactivity of erythrocytes as demonstrated by an anti-I antibody.

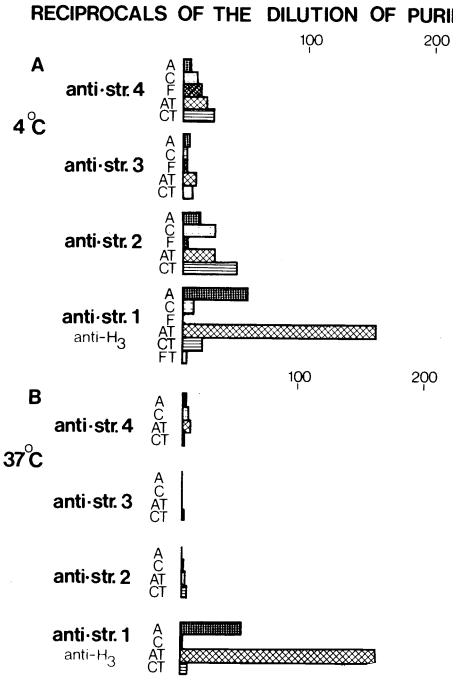


Fig. 4 Agglutinability of adult, cord (new-born) and fetal erythrocytes determined with anti-structure 1 (intact H_3), anti-structure 2, anti-

Legend for Fig. 4 cont'd. structure 3, and anti-structure 4 antibodies. Agglutinabilities were determined on microtiter plates with 1% erythrocytes and were expressed by reciprocals of the highest dilution of anti-body that could cause obvious hemagglutination. The values are the mean of 10 cases. A: adult erythrocytes, C: umbilical cord erythrocytes (new-born erythrocytes), F: fetal erythrocytes obtained from abortion cases. AT: trypsin-treated adult erythrocytes (0.25%, 37° C 15 minutes). CT: trypsin-treated cord erythrocytes. FT: trypsin-treated fetal erythrocytes. The upper panel (A) is the results determined at 37° C; the lower panel (B) is the results determined at 4° C.

In striking contrast, antibodies directed against the core structures of H_3, such as structure 2 and 4 (see Fig. 3), reacted more strongly to fetal and/or cord erythrocytes than to adult erythrocytes (Fig. 4A). The hemagglutination caused by these antibodies was extremely thermosensitive and completely disappeared at 37° C (Fig. 4B), and intensified at 0-4° C (Fig. 4A). The agglutinability of erythrocytes by antibodies directed against structure 2 and 4 was completely reversible between high and low temperatures. There was a clear difference of agglutinability between fetal, new-born, and adult erythrocytes caused by anti-structure 4, 3 and 2 antibodies, however, this difference was not as remarkable as the agglutinability caused by an anti-structure 1 antibody (see Fig. 4A compared to 4B). Both adult and fetal erythrocytes intensified their reaction through protease treatment.

In using the antibodies which were directed against structures 1, 2, and 4, comparisons were made between the complement fixation reaction of glycolipids which were isolated from human intestinal mucosa, and glycolipids from human colon carcinoma. The results of only four cases are shown in Table 7. A number of other cases were also determined (in total 16 cases). The average activity with anti-structure 4 antisera was 1:160 for normal tissue and 1:750 for cancer tissue, respectively. While the antibody directed against structure 4 (Fig. 3) showed a remarkable differential reactivity between normal and tumor glycolipids, the antibodies directed against structure 1 and 2 showed a similar reactivity towards normal and tumor glycolipids.

TABLE 7. Complement fixing reactivities of glycolipids extracted from normal colon mucosa and colon tumors with antibodies which are directed against structure 1, 2, and 4 of Fig. 3. Numbers are reciprocals of the dilution of antisera that could fix complement by 1.2 µg/50 µl of glycolipid antigen complexed with 2 times amount of lecithin and cholesterol.

	Case 1		Case 2		Case 3		Case 4	
	Normal	Cancer	Normal	Cancer	Normal	Cancer	Normal	Cancer
Reactivity with anti-structure 4	320	1280	160	640	320	1280	320	1280
Reactivity with anti-structure 2	80	320	160	160	160	160	160	160
Reactivity with anti-structure 1 ($anti\text{-}H_3$)	160	80	160	80	80	80	80	80

DISCUSSION AND CONCLUSION

1) The presence of four sets of glycolipid variants in human A- and O-erythrocytes, respectively have been established. The glycolipids carrying A-determinants were termed A^a, A^b, A^c, and A^d according to the complexity of the carrier carbohydrate chain in that order. Similarly, four sets of glycolipids with H-activity were found in O-erythrocytes, they were termed H_1, H_2, H_3 and H_4 according to the order of their complexity of the carrier carbohydrate chain. Except for A^d- and H_4-glycolipid, their structures were established. These structures are characterized by having exclusively "type 2 chain". The structure of the carrier carbohydrate chains of H_1, H_2, H_3 and H_4 is identical to that of A^a, A^b, A^c and A^d, respectively. Thus, it is assumed that H_1, H_2, H_3 and H_4 are the precursors of A^a, A^b, A^c and A^d. This idea has been assessed by conversion of H_1 to A^a, and H_2 to A^b, by a serum A-enzyme as first described by Kim et al (Ref. 49).

2) A possible molecular development of blood group carrier carbohydrate chains during ontogenesis has been suggested by the change of the proportion of various variants in erythrocytes. The highly complex variants of blood group glycolipids, such as A^c and H_3, were lower in new-born than in adult erythrocytes and virtually absent in fetal erythrocytes. The low reactivity (agglutinability) of fetal/or new-born erythrocytes to anti-H_3

glycolipids was not enhanced by protease treatment. This indicated that an H_3-structure was absent or very low in fetal/or new-born erythrocytes. In fact, the cell surface-labeling experiment indicated that the concentration of an A^c-structure, which is a branched analog of H_3-erythrocytes, was significantly lower in new-born erythrocytes as compared to adult erythrocytes. In striking contrast, the structures corresponding to the precursors of blood group glycolipids were detected by immunological reaction in appreciable quantity in fetal and new-born erythrocytes, whereas, such reactions, due to the precursors, were less active in adult erythrocytes. This is particularly remarkable for the structure 4, the first amino sugar containing precursor for synthesizing a series of compounds, which led to blood group chains including the H_3-structure. According to definition (Ref. 50) the property of the anti-H_3 glycolipid behaved like that of anti-I antibody although thermosensitivity of hemagglutination caused by anti-H_3 was not as remarkable as that displayed by anti-I. Whereas, the reactivity of antibodies which were directed against structures 2, 3, and 4 was by definition similar to the reactivity of erythrocytes displayed by anti-i, and was characterized by thermosensitivity as well.

The result of these studies suggests that step-by-step elongation and arborization of complex glycolipids such as A^c- and H_3-chains may take place during the development and the appearance of a definite structure in the red blood cell membrane is not known and further extensive studies are required.

A remarkable difference between the reaction of glycolipids in human intestinal tumors and the reaction of normal intestinal mucosa was demonstrated by the antibody which was directed against structure 4. This difference was not observed with other antibodies, anti-structures 1, 2 and 3. It may be that structure 4 is present in greater quantity in tumor tissue than in normal mucosa tissue. In some cases, reaction against H_3 was slightly higher in normal tissue than in tumor tissue. It is plausible that an accumulation of structure 4, i.e. β-GlcNAcl→3βGalR in tumor tissue results as a consequence of a blocked or inhibited synthesis of blood group chains. A similar accumulation of precursor glycolipids, in relation to a blocked synthesis of higher glycolipids, has been exemplified in many cases of transformed cell systems in vitro (Ref. 51,52). It has been clearly demonstrated in this study, however, that elongation and arborization of carbohydrate chains in human erythrocyte membranes are associated with ontogenic development. On the other hand, the development of carbohydrate chains in gastrointestinal mucosa is modified or blocked by an accumulation of one of the precursors in intestinal tumors.

The present study is, however, limited to adult, new-born, and fetal erythrocytes, colon mucosa and colon tumors. Further extensive comparison of the carbohydrate chains in various tissues and their correlation to ontogenesis is obviously necessary. The change of a link between carbohydrate chains and the state of arborization associated with the process of ontogeny and oncogeny may indicate that the structural complexity of a carbohydrate chain will influence membrane fluidity through altered interaction with other components of membranes.

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