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# Glycolipids of Fetal, Newborn, and Adult Erythrocytes: Glycolipid Pattern and Structural Study of $H_3$-Glycolipid from Newborn Erythrocytes†

Michiko N. Fukuda* and Steven B. Levery

ABSTRACT: The glycolipids of blood group type O adult, newborn, and fetal erythrocytes were compared. The total amount of glycolipids was indistinguishable between adult and newborn erythrocytes. However, glycolipids with long and neutral carbohydrates and the H determinant were greatly reduced in newborn cells. On the other hand, the amount of sialylated glycolipids (gangliosides) was significantly higher in newborn cells, suggesting that during erythropoiesis sialyltransferases are more active in fetuses than in adults. The amount of each core structure, lacto-*N*-tetraosyl, linear lacto-*N*-hexaosyl, and branched lacto-*N*-octaosyl, was compared between adult and newborn erythrocytes. It was found that branched lacto-series glycolipids were reduced in newborn cells compared with adult cells. Thus, development from fetal to adult human erythrocytes is associated with an increase of branching and a decrease of sialylation of *N*-acetyllactosaminyl carbohydrate chains. The study indicates that glycolipids are quantitatively different between adult and newborn or fetus.

Blood group ABH determinants of human erythrocyte membranes are carried by the branched and unbranched species of glycosphingolipid. (Hakomori et al., 1972; Watanabe et al., 1975). In the previous studies the branched and the unbranched species of glycosphingolipid of fetal, newborn, and adult erythrocytes were compared, and the results indicated that adult erythrocytes contained a much higher quantity of branched species ($A^c$ or $H_3$) as compared to the glycolipids of newborn or fetal erythrocytes; the branching process is essentially related to the process of ontogenesis (Watanabe & Hakomori, 1976). The conversion of i to I antigen in erythrocytes during the first year after birth has been classically shown (Marsh, 1961), and the structural basis of Ii antigens was recently established, i.e., a repeating *N*-acetyllactosamine (lacto-*N*-norhexaosyl structure) for i and a branched repeating *N*-acetyllactosamine (lacto-*N*-isooctaosyl structure) for I (Nieman et al., 1978; Watanabe et al., 1979a; Feizi et al., 1979). This led to the development of the concept that a linear polylactosamine structure is converted to a branched polylactosamine structure during erythrocyte development. This concept was verified and further extended to include glycoproteins by methylation analysis and endo-$\beta$-galactosidase digestion of band 3 glycoprotein (Fukuda et al., 1979a). In this study, the band 3 and band 4.5 glycoproteins were found to be the major carriers of polylactosamine as well as Ii antigens (Fukuda et al., 1979b, 1980; Childs et al., 1979). Independent studies on Ii antigens of the polyglycosylceramide of adult and newborn erythrocytes agreed with this view (Koscielak et al., 1979).

Recently, a number of lacto-series gangliosides have been isolated and characterized from human erythrocytes; these indicated that gangliosides also have branched and unbranched species (Watanabe et al., 1978, 1979b; Kundu et al., 1981). However, studies comparing the lacto-series glycolipids of newborn and adult erythrocytes have been limited to neutral glycolipids. This paper describes (1) further studies on the comparison of total glycosphingolipids of fetal, newborn, and adult erythrocytes, focusing in particular on the quantities of these glycolipids, and (2) the structure of the long carbohydrate chain glycolipid of newborn erythrocytes.

## Materials and Methods

*Cells.* Adult and newborn (cord) type O erythrocytes were obtained from the Puget Sound Blood Bank, Seattle, WA, through the courtesy of Dr. E. Giblett. Erythrocytes from umbilical cord blood vessels were obtained from Group Health Hospital and Swedish Hospital, Seattle, WA, and from Scripps Memorial Hospital, San Diego, CA. Each cord blood was

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typed with anti-A and anti-B sera (Ortho Diagnostic), and cells that did not agglutinate with either sera were pooled as type O cord blood. Fetal erythrocytes from fetuses were donated by Dr. T. H. Shepard, School of Medicine, University of Washington.

*Endo-β-galactosidase.* Endo-β-galactosidase was purified from a culture filtrate of *Escherichia freundii*, as described previously (Fukuda & Matsumura, 1976; Fukuda, 1981). The endo-β-galactosidase preparation has a specific activity of 62.8 units/mg of protein and is free from exoglycosidases, sulfatase, and proteases. One unit of the enzyme activity is defined as the activity that hydrolyzes bovine corneal keratan sulfate to release 1 μmol of reducing terminal galactose per min.

*Glycolipids.* Standard glycolipids were prepared from human type O adult erythrocyte membranes and were kindly provided by Dr. S. Hakomori and his associates.

*Analysis of Glycolipids of Adult and Newborn Erythrocytes.* The erythrocytes from adult and cord blood (100 mL each as packed cells) were washed with 0.02 M sodium phosphate buffer, pH 7.4 containing 0.15 M NaCl, and the membranes were prepared as described (Furthmayr & Marchesi, 1976). Glycolipids were extracted from the membranes with 500 mL of hot ethanol and were fractionated according to the method described previouisly (Hakomori & Watanabe, 1976). Briefly, the extracted glycolipids were separated into two fractions by Folch partition with 6 volumes of chloroform/methanol (2:1 v/v) and 1 volume of water. Glycolipids with short carbohydrate chains (in lower organic phase) were further purified on a Florisil column after acetylation. Acetylated Folch lower phase glycolipids were deacetylated and were analyzed by thin-layer chromatography in a solvent system of chloroform/methanol/water (65:30:8 v/v). Neutral glycolipids with longer carbohydrate chains and sialylated glycolipids (gangliosides) were recovered from the upper water phase and were separated from each other by DEAE-Sephadex column chromatography (Momoi et al., 1976). These fractions were analyzed by thin-layer chromatography in solvent systems of chloroform/methanol/water (56:38:10 v/v) for the neutral glycolipids and chloroform/methanol/3.5 M $NH_4OH$ (60:40:9 v/v) for gangliosides. The amount of hexose in each glycolipid fraction was measured by the Anthrone method (Trevelyan & Harrison, 1952).

*Densitometric Determination of Glycolipid Compositions.* The glycolipids were spotted as 10-mm streaks on high-performance thin-layer chromatography plates (10 × 20 cm, silica gel 60 hptlc, E. Merck), and chromatography was developed in the solvent systems described above. The glycolipids on thin-layer chromatograms were visualized with orcinol reagent and were directly scanned with a Gelman automatic computing densitometer, Model ACD-18 (Gelman Science, Ann Arbor, MI). The absorbance at 530 nm was measured in the transmittance mode with a slit of 0.1 × 5.0 mm. Peak areas were measured with an integrator. The areas such as peak shoulder, which the integrator did not detect in the automatic mode, were measured by the same apparatus in the manual scan mode. A linear relationship between the detector response and the amount of standard globoside was found in the range of 0.5–10 μg. The lower limit of detection was roughly 0.1 μg. Thus, the amount of each glycolipid was calculated from the peak areas and expressed as relative amounts of hexose, without correction for the response factor of each glycolipid component.

*Cell-Surface Labeling and Analysis of $^3H$-Labeled Glycolipids.* The galactose and *N*-acetylgalactosamine residues of the erythrocyte surface were labeled by the galactose oxidase and $NaB^3H_4$ method (Gahmberg & Hakomori, 1973). Sialic acid residues were labeled by the periodate and $NaB^3H_4$ method (Gahmberg & Andersson, 1977). $NaB^3H_4$ (9.5 Ci/mol) was purchased from Amersham. Packed erythrocytes (0.5 mL) from adults, newborns, and fetuses were surface labeled, and erythrocyte membranes were prepared with 5 mM sodium phosphate buffer, pH 8.0. Glycolipids were extracted from the membranes and fractionated by Folch's partition. The neutral glycolipids from the Folch's upper phase were prepared from galactose oxidase/$NaB^3H_4$-labeled cells. Gangliosides were prepared from periodate/$NaB^3H_4$-labeled cells. Each of the $^3H$-labeled neutral glycolipids and gangliosides, which amount to $(3\sim6) \times 10^4$ cpm, was analyzed by thin-layer chromatography as described above. One chromatogram was sprayed with 2,5-diphenyloxazole/2-methylnaphthalene (Aldrich) (Bonner & Stedman, 1978), and fluorography was performed by directly exposing Kodak X-ray film (XAR-5) at −80 °C for 7–14 days. The other plate on which standard samples were included was chromatographed at the same time and was visualized with orcinol reagent.

*Isolation of $H_3$-Glycolipid from Umbilical Cord Blood.* Folch upper phase neutral glycolipids from umbilical cord erythrocytes were fractionated by a high-performance liquid chromatography apparatus (Varian Model 5000) using a column (0.5 × 30 cm) of Iatrobeads (6RS-810 (10-μm particle, Iatron Laboratory, Tokyo) in a solvent system described (Watanabe & Arao, 1981). The column was equilibrated with 2-propanol/hexane/water (55:40:5 v/v) and programmed to 2-propanol/hexane/water (55:30:15) during 60 min, followed by a gradient programmed to 2-propanol/hexane/water (55:25:20) during an additional 20 min. Flow rate was constant at 0.5 mL/min, and one fraction was collected per min. The glycolipids in each fraction were examined by thin-layer chromatography on silica gel G (250 μm, prescored, 20 × 20 cm, Analtech) in a solvent system of chloroform/methanol/water (56:38:10). $H_3$-Glycolipid (Watanabe et al., 1975) was also prepared from adult erythrocyte membranes by the same procedure. The blood group H activity of each pooled component was examined by the hemagglutination inhibition assay (Watanabe & Hakomori, 1976) with anti-H hemagglutinin from *Ulex europeus.*

*Endo-β-galactosidase Digestion of $H_3$-Glycolipid.* The $H_3$-glycolipid (10–30 μg) from adult and cord erythrocytes was dissolved in 10 μL of 0.2 M sodium acetate buffer, pH 5.8, containing 20 μg of sodium deoxytaurocholate, and 10 μL of *E. freundii* endo-β-galactosidase (25 milliunits) was added [condition 2, see Fukuda et al. (1978)]. After incubation at 37 °C for 18 h, 80 μL of water and 600 μL of chloroform/methanol (2:1 v/v) were added. The upper aqueous phase was analyzed for the released oligosaccharides by thin-layer chromatography in a solvent of 1-butanol/acetic acid/water (3:3:2 v/v). The glycolipids in the lower phase were analyzed by thin-layer chromatography in a solvent of chloroform/methanol/water (65:30:8). Glycolipids and oligosaccharides were detected with orcinol reagent.

*Methylation Analysis.* The adult $H_3$-glycolipids and cord $H_3$-glycolipids were permethylated in dimethyl sulfoxide with methyl iodide and sodium methanesulfonyl carbanion as catalyst (Hakomori, 1964; Stellner et al., 1973a,b). Permethylated materials were hydrolyzed in 90% acetic acid with 0.5 N $H_2SO_4$ at 80 °C for 6 h, and partially methylated sugars were analyzed as partially methylated alditol acetate derivatives by gas–liquid chromatography–mass spectrometry (Stellner et al., 1973a,b). The sample was analyzed on columns packed with 3% OV-225 on supelcoport (80–100 mesh)

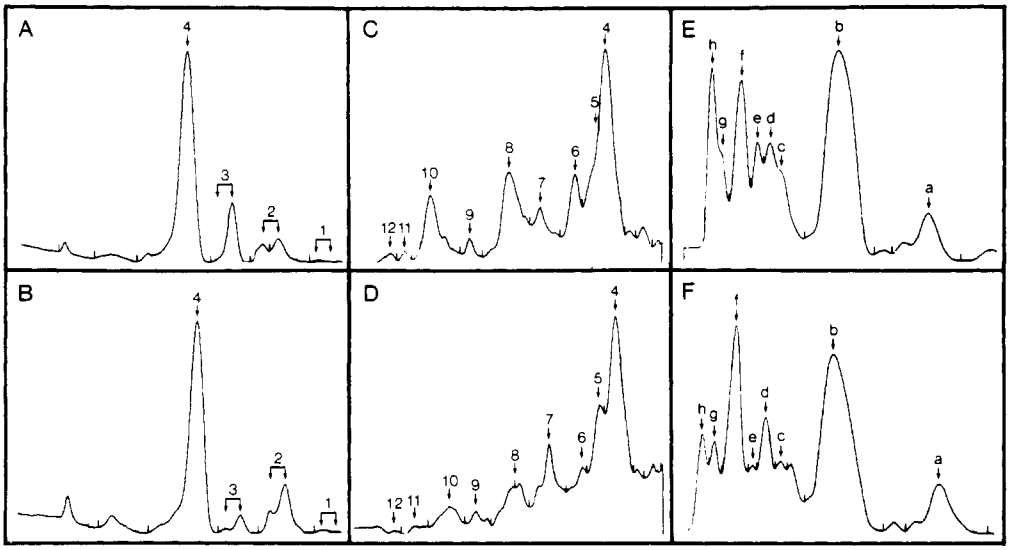



FIGURE 1: Densitometric pattern of glycolipids from adult and newborn erythrocytes. Glycolipids from adult and cord erythrocytes were fractionated into short carbohydrate chain glycolipids (Folch organic phase), neutral glycolipids with long carbohydrate chains (Folch water phase, DEAE-Sephadex unbound), and gangliosides (Folch water phase, DEAE-Sephadex bound). Glycolipids in each fraction were separated by high-performance thin-layer chromatography and were visualized with orcinol reagent. Chromatograms were monitored densitometrically. (A) Adult and (B) cord short carbohydrate chain glycolipids; (C) adult and (D) cord long carbohydrate chain neutral glycolipids; (E) adult and (F) cord gangliosides. In both adult and cord, the chromatograms obtained by using pooled erythrocytes are presented in this figure. The arrows indicate the position of the following glycolipids: (1) CMH; (2) CDH; (3) CTH; (4) globoside; (5) paragloboside; (6) $H_1$-glycolipid plus $\beta$-galactosylparagloboside; (7), $X_2$-glycolipid; (8) $H_2$-glycolipid; (9) $Y_2$-glycolipid; (10) $H_3$-glycolipid; (11) Z-glycolipids; (12) $H_4$-glycolipid; (a) $G_1$; (b) $G_2$; (c) $G_3$; (d) $G_4$; (e) $G_5$; (f) $G_6$; (g) $G_7$; (h) $G_8$ plus $G_9$.

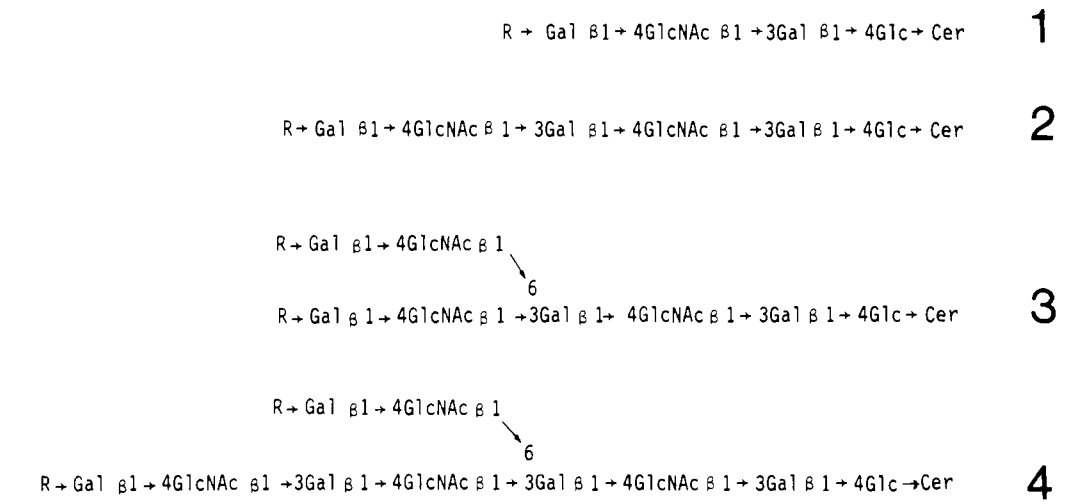



FIGURE 2: Structure of blood group ABH-active glycolipids of human erythrocytes: (1) $A^a$- or $H_1$-glycolipid; (2) $A^b$- or $H_2$-glycolipid; (3) $A^c$- or $H_3$-glycolipid; (4) $A^d$- or $H_4$-glycolipid. (R) Fuc$\alpha$1→2 in H determinant and GalNAc$\alpha$1→3(Fuc$\alpha$1→2) in A determinant (Fukuda & Hakomori, 1982).

and was monitored by electron-impact mass spectrometry in a Finnigan 3300 quadropole mass spectrometer combined with a Finnigan 6110 data system. The gas chromatogram was programmed from 160 to 230 °C with 1 °C increment/min (Watanabe et al., 1978). The mass spectrum of each peak was compared to the standard mass spectrum previously reported (Bjorndal et al., 1970; Stellner et al., 1973a,b).

## Results

*Quantities of Glycolipids of Adult and Newborn Erythrocytes.* The glycolipids were extracted from adult and cord (newborn) erythrocytes and were fractionated into three components (Table I). There was no significant difference between the total amount of glycolipid in adult and newborn erythrocytes. A difference was observed in long-chain neutral glycolipids and gangliosides. Newborn erythrocytes have more gangliosides and less long-chain neutral glycolipids compared with adult erythrocytes.

Each glycolipid fraction was analyzed by thin-layer chromatography followed by densitometric scanning (Figure 1). In short-chain glycolipid fractions (panels A and B for adult and cord, respectively), the adult erythrocytes have approximately equal amounts of CDH[1] and CTH, whereas cord cells have three times more CDH than CTH.

Neutral glycolipids with long carbohydrate chains (panels C and D for adult and cord, respectively) showed a significant decrease of H-antigenic glycolipids (8 and 10 in Figure 1) in cord cells. A relatively higher level of X- and Y-glycolipids[2] were shown, although absolute amounts of these glycolipids were not higher than those in adult cells (see below). Gang-

[1] Abbreviations: CMH, ceramide monohexoside; CDH, ceramide dihexoside; CTH, ceramide trihexoside or ceramide trisaccharide.

[2] The glycolipids between $H_1$ and $H_2$ were termed X-glycolipids, and those between $H_2$ and $H_3$ were termed as Y-glycolipids (Kannagi et al., 1982a,b).

Table I: Quantities of Glycolipids in Adult and Newborn Erythrocyte Membranes

| | adult ($n$ = 3) | newborn ($n$ = 3) |
|---|---|---|
| total glycolipids ($\mu$mol of hexose/100 mL packed erythrocytes) | 13.4 ± 2.1[a] | 13.2 ± 1.9 |
| short-chain glycolipids[b] (% hexose) | 91.2 ± 2.2 | 93.2 ± 1.9 |
| long-chain neutral glycolipids[c] (% hexose) | 7.6 ± 0.6 | 2.5 ± 0.2 |
| gangliosides[c] (% hexose) | 1.2 ± 0.1 | 4.3 ± 0.2 |

[a] Values represent means ± standard deviation. Analyses were done on three glycolipids preparations each from adult and cord erythrocyte membranes. Galactose was used as standard. [b] Lower phase of Folch's partition. [c] Upper phase of Folch's partition followed by DEAE-Sephadex column chromatography to separate neutral and acidic glycolipids.

Table II: Quantities [Weight as % of Total Hexose (as Galactose) in Glycolipids] of Each Glycolipid and of Each Core Structure in Adult and Newborn Erythrocytes

| glycolipids | adult ($n$ = 4)[a] | newborn ($n$ = 3) | glycolipids | adult ($n$ = 4)[a] | newborn ($n$ = 3) |
|---|---|---|---|---|---|
| CMH | 0.07[b] | 0.09 | $G_1$ | 0.09 | 0.39 |
| CDH | 8.34 | 14.64 | $G_2$ | 0.48 | 1.63 |
| CTH | 9.74 | 4.18 | $G_3$ | 0.07 | 0.20 |
| Glo | 59.73 | 60.05 | $G_4$ | 0.09 | 0.26 |
| PG | 0.61 | 0.39 | $G_5$ | 0.09 | 0.17 |
| $H_1$ + β-GalPG[c] | 0.79 | 0.11 | $G_6$ | 0.18 | 0.84 |
| X[d] | 0.45 | 0.22 | $G_7$ | 0.08 | 0.29 |
| $H_2$ | 1.44 | 0.13 | $G_8$ + $G_9$ | 0.09 | 0.21 |
| Y[e] | 0.14 | 0.06 | | | |
| $H_3$ | 0.88 | 0.06 | | | |
| Z | 0.05 | 0.01 | | | |
| $H_4$ + others | 0.08 | <0.01 | | | |

| core structure | adult ($n$ = 4) | newborn ($n$ = 3) |
|---|---|---|
| lacto-*N*-tetraosylceramide[f] | 2.49 | 2.81 |
| linear lacto-*N*-hexaosylceramide[g] | 1.84 | 1.32 |
| branched lacto-*N*-octaosylceramide[h] | 0.97 | 0.27 |

[a] Analysis includes three samples derived from three different individuals and one prepared from pooled adult blood. [b] Numbers represent mean values. Standard deviations were within 12%. [c] The structures of H-active glycolipids in human erythrocytes are given in Figure 2. [d] The major component of X-fraction component is $X_2$-glycolipid, the structure of which was determined as GalNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4GlcCer (Kannagi et al., 1982a). [e] The major component of Y-fraction component is $Y_2$-glycolipids and one of the Z-fraction components is $Z_1$-glycolipid. The structures of these were shown as Galβ1→4(Fucα1→3)-GlcNAcβ1→3(Galβ1→4GlcNAcβ1→3)$_{1\sim2}$Galβ1→4GlcCer (Kannagi et al., 1982b). [f] Subtotals of PG, $H_1$, β-GalPG, $G_2$, $G_3$, $G_4$, and X. [g] Subtotals of $H_2$, Y, $G_6$, and $G_7$. [h] Subtotals of $H_3$, $G_8$, and $G_9$.

liosides from cord erythrocytes showed a high level of $G_6$ component[3] (linear lacto-*N*-hexaosyl structure) (f in panel F) and a low level of $G_8$ and $G_9$ components (branched lacto-*N*-octaosyl structure) (h in panel F) (see Figure 2).

[3] The trivial name and structure of gangliosides $G_1$–$G_9$ are as follows: $G_1$, $GM_3$ or hematoside; $G_2$, sialosyl-α2→3-paragloboside ($IV^3$NeuAcLcnOse$_4$Cer); $G_3$, sialosyl-α2→3GalNAcβ1→3Galβ1→-4GlcNAcβ1→3Galβ1→4Glc→Cer (NeuAc2→3GalNAcLcnOse$_4$Cer); $G_4$, sialosyl-α2→6-paragloboside ($IV^6$NeuAcLcnOse$_4$Cer); $G_5$, sialosyl-α2→3-ganglio-*N*-tetraosylceramide ($IV^3$NeuAcGgOse$_4$Cer); $G_6$, sialosyl-α2→3-lacto-*N*-norhexaosylceramide ($IV^3$NeuAcLcnOse$_6$Cer); $G_7$, sialosyl-α2→6-lacto-*N*-norhexaosylceramide ($VI^6$NeuAcLcnOse$_6$Cer); $G_8$, sialosyl-α2→3-lacto-*N*-isooctaosylceramide; $G_9$, fucosylganglioside. For details, see Watanabe et al. (1978), Watanabe et al. (1979a,b), and Watanabe & Hakomori (1979).

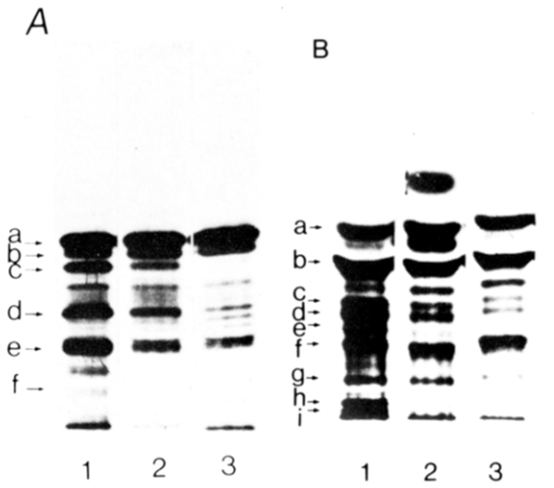



FIGURE 3: Fluorographs of thin-layer chromatograms of neutral glycolipids (A) and gangliosides (B). (A) Folch upper phase neutral glycolipids prepared from galactose oxidase/$NaB^3H_4$-labeled cells: (a) globoside; (b) paragloboside; (c) $H_1$-glycolipid; (d) $H_2$-glycolipid; (e) $H_3$-glycolipid; (f) $H_4$-glycolipid. (B) Ganglioside fractions prepared from periodate/$NaB^3H_4$-labeled cells: (a) $G_1$; (b) $G_2$; (c) $G_3$; (d) $G_4$; (e) $G_5$; (f) $G_6$; (g) $G_7$; (h) $G_8$; (i) $G_9$. For trivial names and the structures for $G_1$–$G_9$, see abbreviation footnote. (A and B) (Lane 1) Adult erythrocytes; (lane 2) cord (newborn) erythrocytes; (lane 3) fetal erythrocytes. A spot above (a) and the other spot between (a) and (b) in lane 2 of (B) are presumed to be lactones of gangliosides derived from (a) and (b), respectively, since these spots appeared during storage of glycolipid samples at a slightly acidic pH.

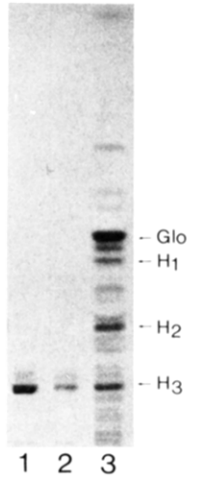



FIGURE 4: Thin-layer chromatogram of $H_3$-glycolipids prepared from adult and cord erythrocytes. (Lane 1) $H_3$-glycolipid from adult; (lane 2) $H_3$-glycolipid from cord; (lane 3) Folch upper phase neutral glycolipids fraction from adult type 0 erythrocytes. Abbreviations: Glo, globoside; $H_1$, $H_1$-glycolipid; $H_2$, $H_2$-glycolipid; $H_3$, $H_3$-glycolipid. Glycolipids were detected by orcinol/$H_2SO_4$ spray.

The amount of each glycolipid is given in Table II. From these data, the ratios of lacto-*N*-tetraosyl, linear lacto-*N*-hexaosyl, and branched lacto-*N*-octaosyl structures can be calculated to be 1.00:0.73:0.39 in adult and 1.00:0.47:0.09 in newborn erythrocytes (Table II). By use of the methods of analysis described, the relative amount of each glycolipid component was indistinguishable whether the material was prepared from blood samples of individual adults or from pooled samples.

*Surface-Labeled Glycolipid Pattern of Adult, Cord, and Fetus Erythrocytes.* Figure 3 shows the fluorography pattern of the thin-layer chromatogram of neutral glycolipids and gangliosides prepared from surface-labeled cells. Erythrocytes from umbilical cord and fetus have the components corre-

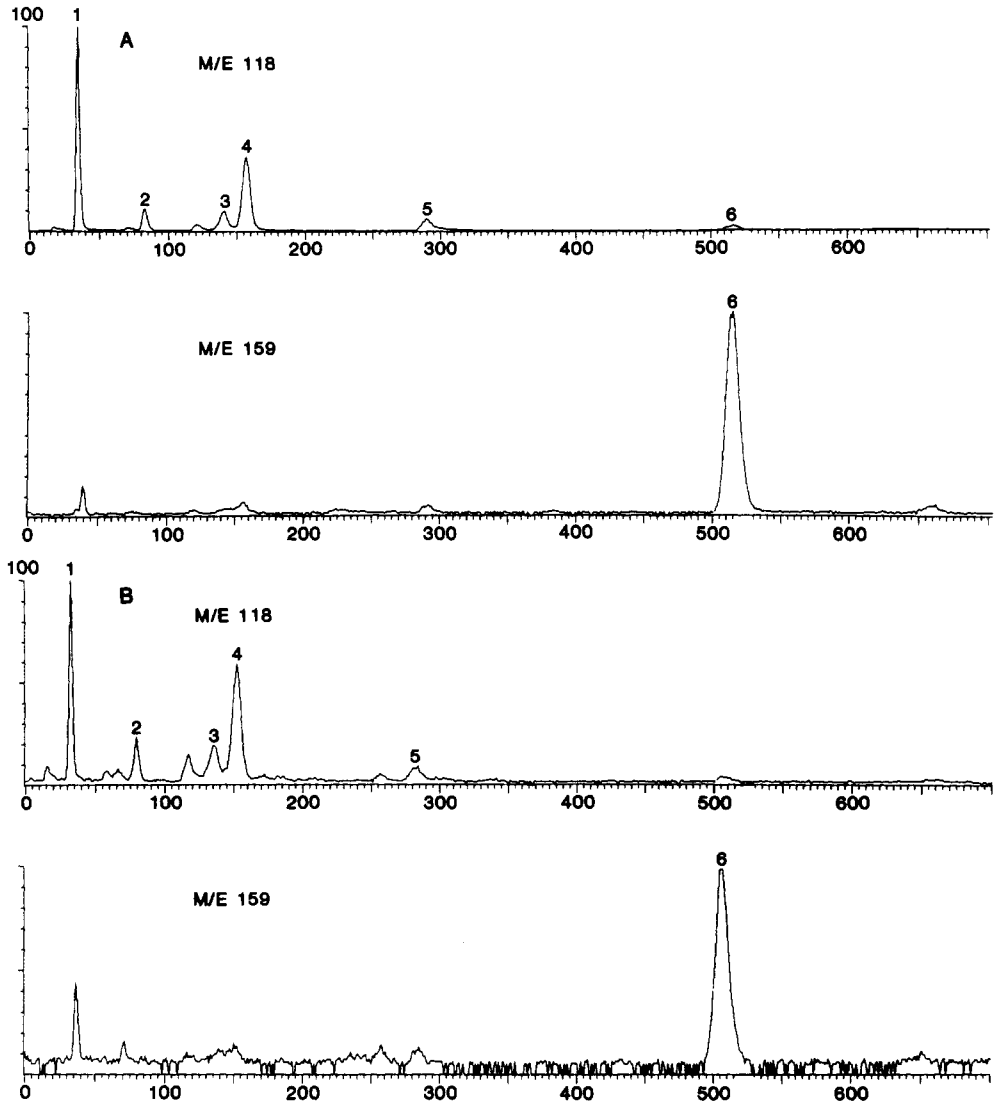



FIGURE 5: Gas chromatogram of partially O-methylated sugars obtained from adult $H_3$-glycolipid (A) and cord $H_3$-glycolipid (B). The chromatograms were shown by monitoring the ions *m/e* 118 and 159, respectively. The following peaks were identified as alditol acetates of (1) 2,3,4-tri-*O*-methylfucose, (2) 2,3,4,6-tetra-*O*-methylgalactose, (3) 2,4,6-tri-*O*-methylgalactose, (4) 3,4,6-tri-*O*-methylgalactose and 2,3,6-tri-*O*-methylglucose, (5) 2,4-di-*O*-methylgalactose, and (6) 3,6-di-*O*-methyl-2-(*N*-methylacetamido)-2-deoxyglucose. Each peak was identified on the basis of its mass spectrum. (Ordinate) Relative intensity (%); (abscissa) retention time (expressed in about 3 s).

sponding to $H_1$-, $H_2$-, and $H_3$-glycolipid, but all appeared to be present in lower amounts than those from adult erythrocytes (Figure 3A).

The gangliosides in cord and fetal erythrocytes also showed a decrease in the levels of higher molecular weight glycolipids. Both $G_8$ and $G_9$, which are known to have branched structures (Watanabe et al., 1978, 1979b), are present but in significantly smaller amounts in cord and fetal erythrocytes (Figure 3B). Thus, the results obtained by surface labeling are similar to those obtained by the chemical means described above (Tables I and II and Figure 1). These results showed that both neutral glycolipids and gangliosides with branched lactosamine structure decreased greatly in newborn and fetal erythrocytes.

*Methylation Analysis of Cord $H_3$-Glycolipid.* In order to know the structure of a slow-migrating, H-active glycolipid of umbilical cord erythrocytes, which had the same mobility to $H_3$-glycolipid on thin-layer chromatography, the component was isolated and referred to as "cord $H_3$-glycolipid" (see Materials and Methods). The thin-layer chromatograms of cord $H_3$-glycolipids and adult $H_3$-glycolipids are shown in Figure 4.

The cord $H_3$-glycolipid was subjected to analysis by permethylation, and partially O-methylated sugars were analyzed as alditol acetate derivatives. Adult $H_3$-glycolipid, whose structure has been determined (Watanabe et al., 1975), was permethylated in the same manner and was used as a reference. As shown in Figure 5 and Table III, cord $H_3$-glycolipids and adult $H_3$-glycolipids gave very similar gas chromatograms. The presence of 2,4-di-*O*-methylgalactose derivative clearly

Table III: Relative Proportions of Methylated Sugars from Adult and Cord $H_3$-Glycolipids

| methylated sugars | mol/mol[a] adult $H_3$-glycolipid | mol/mol[a] cord $H_3$-glycolipid |
|---|---|---|
| 2,3,4-tri-*O*-methylfucose | 2.0 | 1.7 |
| 2,3,4,6-tetra-*O*-methylgalactose | 0.5[b] | 0.4 |
| 2,4,6-tri-*O*-methylgalactose | 1.0 | 1.0 |
| 3,4,6-tri-*O*-methylgalactose plus 2,3,6-tri-*O*-methylglucose | 3.0 | 3.3 |
| 2,4-di-*O*-methylgalactose | 1.0 | 0.7 |

[a] Relative amount of each sugar derivative was calculated by using the peak area shown by *m/e* 118 (Figure 3) as adult $H_3$-glycolipid as standard. [b] The same response factor as 2,3,4-tri-*O*-methylfucose was used.

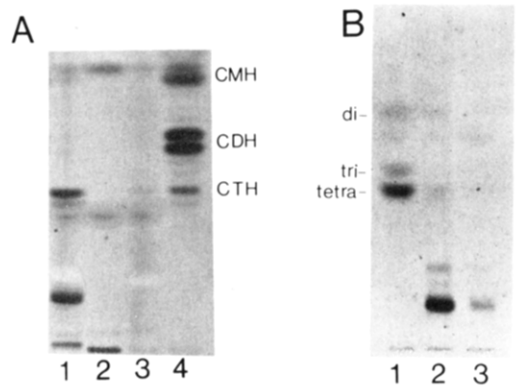
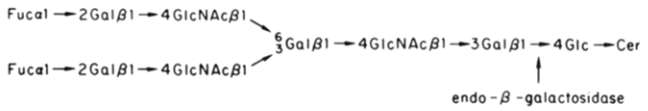



FIGURE 6: Thin-layer chromatogram of hydrolysis products of $H_3$-glycolipids by endo-$\beta$-galactosidase. (A) Short-chain glycolipids released from $H_3$-glycolipids are shown: (lane 1) hydrolysis products of $H_2$-glycolipid; (lane 2) hydrolysis products of adult $H_3$-glycolipid; (lane 3) hydrolysis products of cord $H_3$-glycolipid; (lane 4) standards. Hydrolysis conditions and the fractionation of reaction products were described under Materials and Methods. The spots below CTH were sodium deoxytaurocholate. (B) Oligosaccharides released from $H_3$-glycolipids are shown: (lane 1) standards [(di) GlcNAc$\beta$1→3Gal; (tri) Gal$\beta$1→4GlcNAc$\beta$1→3Gal; (tetra) Fuc$\alpha$1→2Gal$\beta$1→-4GlcNAc$\beta$1→3Gal)]; (lane 2) oligosaccharide from adult $H_3$-glycolipid; (lane 3) oligosaccharide from cord $H_3$-glycolipid.

showed the presence of a branching structure in cord $H_3$-glycolipid.

The digests of cord and adult $H_3$-glycolipids by endo-$\beta$-galactosidase were analyzed (Figure 6). Cord $H_3$-glycolipids gave a large oligosaccharide and CMH as the major hydrolysis products. These results strongly suggest that cord $H_3$-glycolipid has the branched lactosaminyl structure:




As a minor component, a spot corresponding to ceramide trisaccharide was seen as a hydrolysis product of cord $H_3$-glycolipid (Figure 6A, lane 3), although neither small oligosaccharides nor disaccharides were detected in oligosaccharide products (Figure 6B, lane 3). The results indicate the presence of linear lacto-series glycolipids in the cord $H_3$-glycolipid preparation as minor components. These results, together with the methylation analysis (Figure 5), indicate that the major component of cord $H_3$-glycolipid has the same branched *N*-acetyllactosaminyl structure as the adult $H_3$-glycolipid.

## Discussion

The present study showed quantitative differences in glycolipids between adult, newborn, and fetal erythrocytes. In both neutral and acidic glycolipids, newborn and fetal erythrocytes contained smaller quantities of long-chain carbohydrate glycolipids. The amount of branched glycolipids in newborn is especially reduced compared to those in adults. These results essentially confirmed the previous study on neutral glycolipids (Watanabe & Hakomori, 1976) and extended the idea of a branching process associated with development to ganglioside species. The relative decrease of branched gangliosides, $G_8$ and $G_9$, in fetal and newborn erythrocytes was obvious (Table II, Figure 1E,F, Figure 3B).

It was suggested that the branched-chain blood group A and H glycolipids are significantly decreased in fetus and cord erythrocytes (Watanabe $\alpha$mp Hakomori, 1976). This is also in agreement with the reported absence of highly branched chain polyglycosylceramide in cord cells (Koscielak et al., 1979). The mass spectrometric analysis of neutral glycolipids from cord erythrocytes could not detect chains longer than eight sugars nor branched-chain structures (Larson & Samuelsson, 1980). From the present study, it is concluded that branched glycolipid is also present in cord cells although the amount is significant reduced. The structure of cord $H_3$-glycolipid showed the branched carbohydrate chain as seen in adult $H_3$-glycolipids. Thus the difference between cord and adult glycolipids is essentially quantitative. This evidence indicates that the conversion of i to I antigen during erythrocyte development is due to the quantitative increase of branched *N*-acetyllactosaminyl structure. In fact, the small degree of branching was observed in cord (i-active) band 3 glycopeptide.[4] It is thus interesting to see whether the glycolipids from adult i individual lack the branched species, since adult i is assumed to be defective in the branching enzyme (Fukuda et al., 1979a,b).

We investigated fetal glycolipids by using surface-labeled erythrocytes, because large amounts of fetal blood is not available for chemical analysis. The terminal sialic acid of gangliosides was labeled by using periodate, and the fluorogram (Figure 3B) might reflect the quantities of sialic acid. However, neutral glycolipids were labeled by using galactose oxidase, so quantitative interpretation of the results solely by surface labeling may not be appropriate by the following reasons. The reactivity of glycolipids toward galactose oxidase is known to be greatly affected by the ceramide moiety when they are present on surface membranes (Young et al., 1981; Urdal & Hakomori, 1980), by the presence of other cell-surface components such as glycoproteins, and by the presence of lectins (Gahmberg & Hakomori, 1973). Significant variation in sensitivity to galactose oxidase among the erythrocytes from different individuals was also noticed (Lis et al., 1982). Thus, the fluorogram (Figure 3A) shows the profiles of neutral glycolipids, but they may not represent the exact amount of each glycolipid variant.

The lower expression of ABH antigens in fetus and newborn has previously been described (Race & Sanger, 1975), and such low reactivity of fetal erythrocytes toward anti-A or -B sera is believed to minimize the reaction of feto–maternal incompatibility, which is caused by transplacental anti-A/B antiodies (Romans et al., 1980). One explanation for the reduced ABH antigenicity of fetal erythrocytes was in the terminal valency of these antigenic determinants, i.e., monovalency in fetuses and di- or multivalency in adults, which corresponds to the linear and branched core carbohydrate structure.

As shown in this study, a total decrease of neutral glycolipids is seen in cord cells. The decrease of H-glycolipids occurs not only in branched species ($H_3$- and $H_4$-glycolipids) but also in short ($H_1$-glycolipids) and linear long-chain species ($H_2$-glycolipids) (see Table II and Figure 2D). The present study also showed that the glycolipids in newborn erythrocytes are more sialylated than those in adult. Such increased sialylation of the polylactosaminyl carbohydrate chain was seen in band 3 carbohydrates from cord erythrocytres (Fukuda et al., 1979a,b). It is thus possible that sialyl transferase is enhanced in cord cells and the substantial sialylation of the precursor glycolipids results in lesser production of neutral glycolipids such as H-active glycolipids. Therefore, the reduced number of H-active determinants, the predominant monovalency of H-antigenic sites, and the increase of surface sialic acids may all explain the lower ABH reactivity of these cells in comparison with adult erythrocytes.

[4] M. Fukuda and M. N. Fukuda, unpublished data.

It is obvious from the present study that there is neither increased accumulation of linear structures nor replacement of the branched chain with linear, long-chain glycolipids. In newborn or fetal erythrocytes in which the branching process is not active as it is in adult cells, a linear elongation reaction may not occur efficiently enough to synthesized such glycolipids. Systematic studies are required of the mechanism controlling chain elongation vs. branching, which is the crucial key step determining the phenotypic expression of cell-surface carbohydrates during the differentiation of erythroid cells (Fukuda et al., 1979b, 1980), early mouse embryo (Kapadia et al., 1981), and teratocarcinoma [Muramatsu et al., 1979; see also a review, Hakomori et al. (1982)].

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**Registry No.** **1**, 56573-54-7; **2**, 86993-34-2; **3**, 86993-35-3; 2,3,4-tri-*O*-methylfucose, 78513-65-2; 2,3,4,6-tetra-*O*-methylgalactose, 4060-05-3; 2,4,6-tri-*O*-methylgalactose, 5856-20-2; 3,4,6-tri-*O*-methylgalactose, 31655-52-4; 2,3,6-tri-*O*-methylglucose, 4234-44-0; 2,4-di-*O*-methylgalactose, 4301-53-5; globoside I, 11034-93-8; β-galactosylparagloboside, 86993-33-1; 3,6-di-*O*-methyl-2-(*N*-methylacetamido)-2-deoxyglucose, 86900-83-6.

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