

STATUS OF BLOOD GROUP CARBOHYDRATE CHAINS IN ONTOGENESIS AND IN ONCOGENESIS*

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Blocked synthesis of A and B determinants in human epithelial and endodermal tumors have been described based on immunochemical (1, 2), immunohistological (3, 4), and enzymatic (5) studies. The change of Lewis blood group hapten and accumulation of Le^x-like antigen in some adenocarcinoma were described (6). Immunohistological studies indicated that blood group determinants appeared and disappeared in a certain order during ontogenetic development (7), that the H determinant was demonstrated to be a marker of cellular differentiation (8), and that the development of i to I antigen was shown to be associated with postnatal change of erythrocytes (9). The association of I and i antigen with tumor tissue (10) and with carcinoembryonic antigen (11) 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 ontogenesis, and that the program of synthesis is blocked or modified in the process of oncogenesis. This paper is to provide 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 H₃ variants, (b) blocking of the elongation and arborization of a carbohydrate chain occurs during oncogenesis as a result of a blocked ontogenic program.

Materials and Methods

A^a, A^b, A^c, A^d, H₁, H₂, H₃, and H₄ glycolipids were prepared from human erythrocytes, according to the method previously described (12, 13). The structures of each glycolipid were determined, as seen in Table I, by enzymatic degradation, methylation analysis, and mass spectrometry, as previously described. A^d and H₄ glycolipids were still impure and their structures have not been determined. A^c and H₃ glycolipids were identified as ceramide dodeca and decasaccharides, respectively. They have two "type 2" chains which are branched at β -galactosyl residue of "paragloboside" by β (1→3) and β (1→6) linkages (13). Paragloboside and globoside were prepared from human erythrocytes and from a major ganglioside of erythrocytes (14).

Since blood group glycolipids were minor membrane components and only a small amount of fetal and newborn erythrocytes and tissues were available, the change of blood group glycolipids in fetal and newborn erythrocytes were studied by the following two methods: (a) A glycolipids

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TABLE I
Structures of Blood Group H and A Glycolipid Variants

H glycolipid

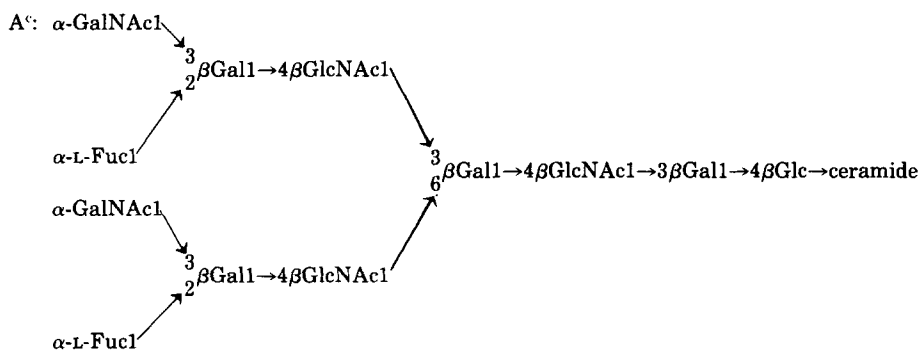
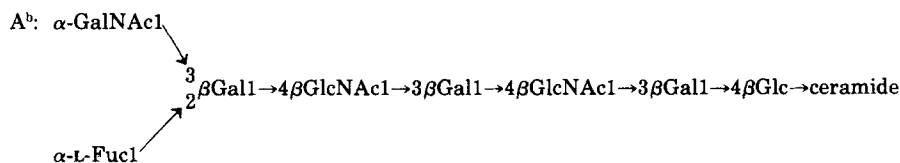
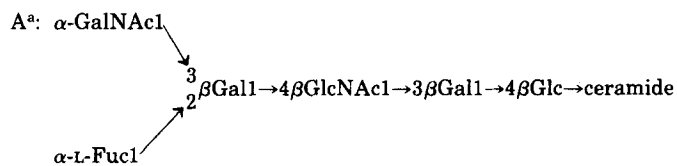
H₁: L- α -Fuc1 \rightarrow 2 β Gal1 \rightarrow 4 β GlcNAc1 \rightarrow 3 β Gal1 \rightarrow 4 β Glc \rightarrow ceramide

H₂: L- α -Fuc1 \rightarrow 2 β Gal1 \rightarrow 4 β GlcNAc1 \rightarrow 3 β Gal1 \rightarrow 4GlcNAc1 \rightarrow 3 β Gal1 \rightarrow 4Glc \rightarrow ceramide

H₃: See Fig. 1.

H₄: Similar to H₃ but additional branching, structure not yet determined

A glycolipid



A^d: Similar to A^c but additional branching, the structure not yet determined.

were studied by cell surface labeling with galactose oxidase and tritiated borohydride (15), followed by preparation of membranes (15), extraction, and preparation of long-chain neutral glycolipids (see below). (b) H glycolipids and their degradation products were studied through reactions of erythrocytes and glycolipids fraction of tissues with antibodies directed against the H₃ glycolipid and its degradation products (structures 2, 3, and 4, see Fig. 1).

Cell surface labeling was carried out with a higher concentration of galactose oxidase and tritiated borohydride than that previously described, i.e., 30 U of galactose oxidase (Kabi, Lindhagensgaten 133, Stockholm, Sweden) which was added to 0.5 ml of packed erythrocytes and three times addition of each 5 mCi of tritiated borohydride. The long-chain neutral glycolipid fraction, containing blood group glycolipids, was prepared from the "upper layer" of Folch's extracts of the surface-labeled erythrocytes through DEAE-Sephadex chromatography (16). The fraction was

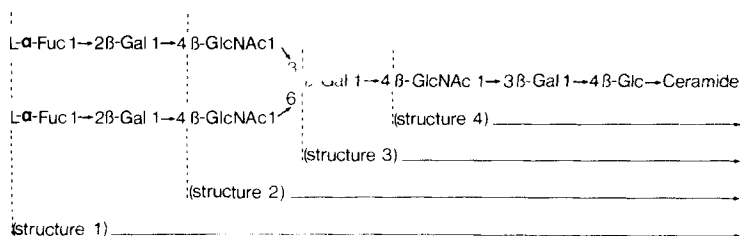


FIG. 1. Structure of H₃ glycolipid and its degradation products to which antibodies were directed.

analyzed through thin-layer chromatography on Silica gel G plate after the addition of standard A variants (A^a, A^b, A^c, A^d), and the radioactivity of each band was counted. The H₃ glycolipid was degraded step-by-step with purified α-L-fucosidase (12, 13), β-galactosidase, and β-N-acetylhexosaminidase (17). Paragloboside was prepared from sialylparagloboside of human erythrocytes (14) and was further degraded into β-N-acetylglucosaminyl(1→3)β-galactosyl(1→4)-glucosylceramide (structure 4, Fig. 1) by β-galactosidase. These glycolipids were purified through thin-layer chromatography. The purified glycolipids were complexed with bovine serum albumin (BSA)¹ and immunized rabbits with a complete Freund's adjuvant (18). Antisera were purified by BSA-Sepharose column to eliminate anti-BSA. Details of the procedure and the properties of antibodies will be described elsewhere. Glycolipids of human gastric and colon cancer, and those of normal mucosa tissue, were extracted directly by chloroform-methanol (2:1) and purified through acetylation procedure (19). In some cases, tissues were first homogenized with water in an "Omnimixer" (Ivan Sorval Inc., Norwalk, Conn.) followed by addition of an equal vol of 2 M perchloric acid, stirred for 30 min. The mixture was centrifuged to separate perchloric acid soluble fraction and insoluble cell residue.² The residue was extracted with chloroform-methanol (2:1), and the neutral glycolipid fraction was prepared by acetylation followed by analysis of glycolipids through thin-layer chromatography (19). The reactivity of individual glycolipids and glycoproteins to anti-glycolipid antisera was determined by complement fixation performed on a microtiter plate (20) and on an Ochtelr long double diffusion agarose plate. The reactivity of an adult, newborn fetal erythrocytes with respective antiserum was determined by hemagglutination on a microtiter plate with 1% erythrocytes and by the capability of erythrocytes absorbing anti-glycolipid antibodies. The absorbing capability of erythrocytes was determined by 10⁶ erythrocytes incubated with 50 μl of an anti-glycolipid antibody which has a titer of 1:128. The erythrocyte suspension, which was incubated with antibody, was centrifuged, and the activity of the supernate was titrated on a microtiter plate.

Results

The ratios of the surface-labeled activities, newborn to adult erythrocytes of each A variant (A^a, A^b, A^c, and A^d), are shown in Table II. Activities of each A variant of newborn erythrocytes are expressed as percent 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.³ Alternatively,

¹ Abbreviation used in this paper: BSA, bovine serum albumin.

² The insoluble residue contained essentially all neutral glycolipids except those containing a long-carbohydrate chain; the perchloric acid soluble fraction contained glycoprotein, gangliosides, and some long-chain glycolipids (Watanabe and Hakomori; unpublished observation). The procedure was used for simultaneous extraction and fractionation of glycoprotein and glycolipid from a small amount of surgical specimen.

³ The subterminal galactosyl residue of α-L-Fuc1→2βGal can be oxidized by galactose oxidase and hence can be labeled by the procedure as described. The reaction was, however, not stoichiometric (Matsubara and Hakomori; unpublished observation).

TABLE II
*Surface-Labeled Activities of Blood Group A Glycolipids Variant:
 Percent of Activity of Newborn Erythrocytes to that of Adult
 Erythrocytes*

	Percent activity of newborn 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

A erythrocytes of adult and newborn were surface labeled by galactose oxidase and tritiated borohydride (15), membranes were isolated, glycolipid fractions were prepared by DEAE-Sephadex chromatography, and the nonradioactive standard A variants glycolipids were added, and A^a, A^b, A^c, and A^d fractions were separated on thin-layer chromatography (12, 13). Activities of each variant obtained from newborn erythrocytes were compared with those of adult erythrocytes. Values were expressed as percent of adult erythrocytes.

TABLE III
Inhibition of Anti-H₃-Dependent Hemagglutination by H₁, H₂, and H₃ 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 three hemagglutination doses of anti-H₃ glycolipid rabbit antisera (purified by BSA-column) and in the presence of two times weight of cholesterol and lecithin.

the reactivities of erythrocytes to various antibodies, which are directed against H₃ and its degradation products, were compared.

The antibody that was directed against whole H₃ glycolipid (structure 1) did not strongly cross-react to H₁ and H₂ structures (see Table III). This indicates that the antibody recognizes a whole branched structure. The antibody that was directed against H₃ strongly reacted to adult human erythrocytes, weakly reacted to cord erythrocytes, and did not react at all to fetal erythrocytes (Fig. 2A). The higher reactivity of adult erythrocytes to anti-H₃ glycolipid has been further confirmed through an absorption experiment, i.e., adult erythrocytes absorb about eight times the quantity of anti-H₃ antibody than newborn erythrocytes absorb. 10⁶ adult erythrocytes absorb almost all of the anti-H₃ activity which was present in 50 μl of antisera (titer 1:128), whereas the supernate of the same amount of antiserum, which was incubated with the same number of newborn erythrocytes, contained the anti-H₃ activity with a titer of 1:32 to 1:64. No diminished activity in the supernate was observed when fetal erythrocytes were incubated with the anti-H₃ antiserum.

The difference in reaction to an anti-H₃ antibody between adult and fetal erythrocytes became greater after trypsin treatment of erythrocytes, i.e., enhanced reaction of adult erythrocytes through trypsin treatment was observed, no increase in reaction of fetal erythrocytes after trypsin treatment, and only a slight increase of cord erythrocytes (see Fig. 2A). The agglutinability of both

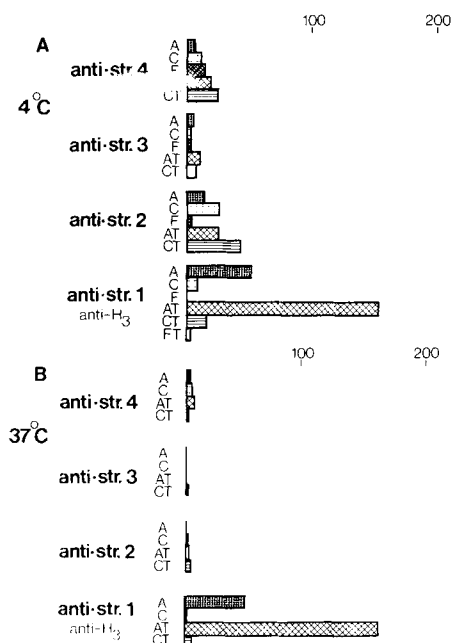


FIG. 2. Agglutinability of adult, cord (newborn), and fetal erythrocytes determined with anti-structure 1 (intact H₃), anti-structure 2, anti-structure 3, and anti-structure 4 antibodies. Agglutinabilities were determined on microtiter plates with 1% erythrocytes and were expressed by reciprocals of the highest dilution of antibody that could cause obvious hemagglutination. The values are the mean of 10 cases. A: adult erythrocytes, C: umbilical cord erythrocytes (newborn erythrocytes), F: fetal erythrocytes obtained from abortion cases. AT: trypsin-treated adult erythrocytes (0.25%, 37°C 15 min). CT: trypsin-treated cord erythrocytes. FT: trypsin-treated fetal erythrocytes. The upper panel (A) is the results determined at 4°C; the lower panel (B) is the results determined at 37°C.

fetal and adult cells through anti-H₃ antibodies was not thermosensitive as compared to the reactivity of erythrocytes as demonstrated by an anti-I antibody.

In striking contrast, antibodies directed against the core structures of H₃, such as structure 2 and 4 (see Fig. 1) reacted more strongly to fetal and/or cord erythrocytes than to adult erythrocytes (Fig. 2 A). The hemagglutination caused by these antibodies was extremely thermosensitive and completely disappeared at 37°C (Fig. 2 B) and intensified at 0–4°C (Fig. 2 A). 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, newborn, 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. 2 A compared to 2 B). Both adult and fetal erythrocytes intensified their reaction through protease treatment.

In using the antibodies that were directed against structures 1, 2, and 4, comparisons were made between the complement fixation reaction of glycolipids that were isolated from human intestinal mucosa and glycolipids from human

TABLE IV
Complement Fixing Reactivities of Glycolipids Extracted from Normal and Colon Mucosa

	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-H ₃)	160	80	160	80	80	80	80	80

Complement fixing reactivities of glycolipids extracted from normal colon mucosa and colon tumors with antibodies that 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 $\mu\text{g}/50 \mu\text{l}$ of glycolipid antigen complexed with two times amount of lecithin and cholesterol.

colon carcinoma. The results of only four cases are shown in Table IV. 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 mucosa tissue and 1:750 for cancer tissue, respectively. While the antibody directed against structure 4 (Fig. 1) 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. Glycolipids of several cases of colon carcinomas, as compared to normal mucosa tissue, were analyzed by thin-layer chromatography. An intensified spot corresponding to $\text{GlcNAc}\beta 1 \rightarrow 3\text{Gal}\beta 1 \rightarrow 4\text{Glc}\beta 1 \rightarrow 1\text{ceramide}$ (structure 4) was observed in glycolipid fraction of colon carcinoma as compared to normal mucosa (see Fig. 3).

Discussion

The results clearly indicate that highly complex variants of blood group glycolipids such as A^c and H₃ glycolipid are present in an appreciable quantity in adult erythrocytes, but low in newborn and not in fetal erythrocyte membranes. The low reactivity of fetal or newborn erythrocytes with anti-H₃ antibody was not enhanced by protease treatment. This indicates that H₃ structure was absent or very low in fetal or newborn erythrocytes rather than that H₃ structure was in cryptic state in fetal or newborn erythrocytes. In fact, the concentration of A^c structure, the branched analog of H₃ in A erythrocytes, was significantly low in newborn erythrocytes as compared to adult erythrocytes through the surface-label procedure.⁴ In striking contrast, the structures corresponding to the precursor of blood group glycolipids were detected by immunological reaction in appreciable quantity in fetal and newborn erythrocytes, whereas such reactions, due to the precursors, were less active in adult erythrocytes. This is particularly remarkable for structure 4, i.e., the first aminosugar-containing precursor for synthesizing a series of compounds which lead to various blood group chains, including the H₃ structure. According to the definition (21) the property of the anti-H₃ glycolipid behaves like that of the anti-I

⁴ Similar experiments for determining the chemical concentration of A^c and A^d variants in fetal erythrocytes was unsuccessful because the quantity of fetal erythrocytes obtained from abortion cases was extremely limited.

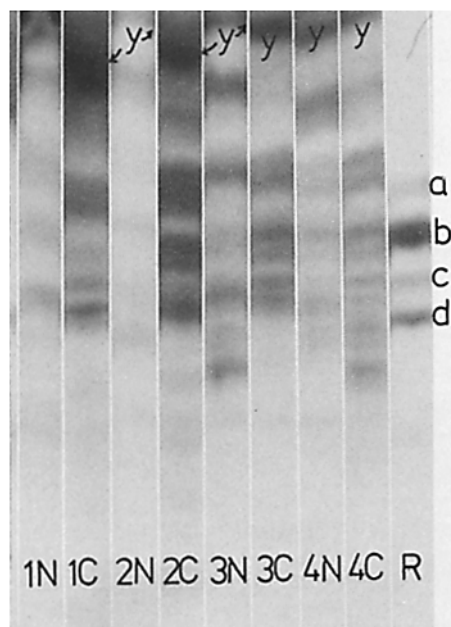


FIG. 3. Neutral glycolipid profile of colon mucosa and colon carcinoma by thin-layer chromatography on Silica gel G with chloroform-methanol-water (60:35:8). Neutral glycolipids were isolated from perchloric acid extract residue of tissue (see Text). N: normal mucosa, C: cancer tissue, 1, 2, 3, and 4 represent case numbers, R: reference glycolipid a, b, c, d are lactosylceramide, structure 4 ($\text{GlcNAc} \rightarrow \text{Gal} \rightarrow \text{Glc} \rightarrow \text{ceramide}$), globoside, and Forssman glycolipid, respectively. Y: yellow colored spot (nonglycolipid).

antibody, although thermosensitivity of hemagglutination caused by anti- H_3 was not as remarkable as the thermosensitivity that was displayed by anti-I.⁵ H_3 glycolipid inhibited I hemagglutination (13) caused by anti-I (Ma) whose specificity was directed towards $\beta\text{-Gal1} \rightarrow 4\beta\text{-GlcNAc1} \rightarrow 6\beta\text{Gal}$ (22), and the same structure was found in H_3 (13). The reaction of the antibodies that are directed against structures 2 and 4, is, by definition, similar to the reactivity of erythrocytes displayed by anti-i, and is 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 -carbohydrate chains, may take place during the development of human erythrocytes, but the exact correlation between the stage of development and the appearance of a definite structure in the red blood cell membranes is not known and further extensive studies are required.

A remarkable difference between the reaction in glycolipids of human intestinal tumors and the reaction of normal intestinal mucosa was demonstrated by the antibody that was directed against structure 4. The difference in immuno-

⁵ This reactivity is, however, probably due to anti-"branched H" rather than due to anti-I because Marcus' anti-H (23) showed a similar preferential agglutination of adult erythrocytes as well (Watanabe and Hakomori, unpublished observation). Furthermore, "anti- H_3 " do not react with "I-glycoprotein without H" (observation by Dr. Ten Feizi, Medical Research Council, Middlesex, England: personal communication).

logical reactivity of glycolipids may not always indicate the difference in chemical quantity of glycolipids, as the immunologic reactivity of glycolipids can be modulated by the quality and the quantity of coexisting lipids and other glycolipids (12, 22). However, the differential reactivity of glycolipids between normal and tumor tissue was only observed through anti-structure 4 but not through other antibodies. This suggested a possibility that structure 4 is present in greater quantity in tumor tissue than in normal mucosal tissue. Qualitative analysis of glycolipids of normal and tumor tissue through thin-layer chromatography supported this possibility, i.e., an enhanced spot was observed corresponding to $\beta\text{GlcNAc}1\rightarrow3\beta\text{Gal}1\rightarrow4\beta\text{Glc}\rightarrow\text{ceramide}$. Since a number of compounds could be in the same position on thin-layer chromatography, further purification and identification of this compound is obviously needed. Furthermore, immunofluorescent staining of frozen sections of various cases also supported this possibility, i.e., some subpopulation of colon tumor cells were stained strongly with anti-"structure 4" by indirect fluorescent method (Wang, S. M., T. Huang, K. Watanabe, and S. Hakomori, unpublished observation). An increase in human erythrocytes of a glycolipid with a structure similar to structure 4 was reported in a certain hereditary disease (24). In some cases, reaction against H_3 was slightly higher in normal tissue than in tumor tissue (see Table IV, last line, case 1 and 2).

It is plausible that an accumulation of structure 4, (i.e., $\beta\text{GlcNAc}(1\rightarrow3)\text{-}\beta\text{Gal}\rightarrow\text{R}$ in tumor tissue) results as a consequence of blocked or inhibited synthesis of blood group chains. An analogous situation was reported in mammary carcinoma glycoprotein in which precursor structures for M and N determinants increased and were immunologically detectable, whereas the M and N haptens were not significantly decreased. The precursor structures for M and N, which were present in human mammary carcinoma, were identified as T and Tn antigen (25). A similar accumulation of precursor glycolipids, in relationship to a blocked synthesis of higher glycolipids, has been exemplified in many cases of transformed cell systems *in vitro* (26, 27). It has been clearly demonstrated in this study, however, that elongation and arborization of carbohydrate chains in human erythrocyte membranes are associated with ontogenetic 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, newborn fetal erythrocytes, colon mucosa, and colon tumors. Further extensive comparison of carbohydrate chains in various tissues and their correlation to ontogenesis is obviously necessary.

The change of a carbohydrate chain length and the state of arborization associated with the process of ontogenesis and oncogenesis may indicate that the structural complexity of a carbohydrate chain will influence membrane fluidity through altered interaction with proteins and other components of membranes.

Summary

Blood group ABH determinants in human erythrocytes are carried by four kinds of glycolipid carbohydrate chains, differing in their structural complexity.

They are A^a, A^b, A^c, and A^d for A variants, and H₁, H₂, H₃, and H₄ for H variants (Table I and Fig. 1). Based on the surface labeling of A variants and on the reactivity of erythrocytes to antibodies directed against H₃ and against its degradation products, it is concluded that complex variants of A or H determinants (A^c and A^d/or H₃ and H₄) are absent or significantly low in fetal erythrocytes (80–150 days after gestation) and in newborn erythrocytes, whereas these complex structures are fully developed in adult erythrocytes. In contrast, A determinants linked to simpler carbohydrate chains (A^a, A^b variants) are fully developed before birth and do not show significant change after birth.

The precursor of blood group carbohydrate chains seems to be abundant in fetal or newborn erythrocytes. This assumption is based on the higher reactivity of fetal or newborn erythrocytes to an antibody, which is directed against the precursor *N*-acetylglucosaminyl β 1 \rightarrow 3galactosyl β 1 \rightarrow 4glucosylceramide than in adult erythrocytes.

Reactions of glycolipids of gastrointestinal mucosa, with antibodies directed against H₃ glycolipid and its degradation products, were compared to that of gastrointestinal tumors. The reaction to β GlcNAc1 \rightarrow 3 β Gal1 \rightarrow 4Glc \rightarrow ceramide (structure 4), which is the precursor of all blood group glycolipids, was consistently high in many cases of tumor glycolipid than that of normal glycolipid. This as well as other evidence supports a general concept that the process of ontogenesis of a blood group carbohydrate chain occurs as step-by-step elongation and arborization, and that blocking of such a development of a carbohydrate chain occurs in the process of oncogenesis.

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