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CHARACTERIZATION OF AN EPITOPE (DETERMINANT) STRUCTURE IN A DEVELOPMENTALLY REGULATED GLYCOLIPID ANTIGEN DEFINED BY A COLD AGGLUTININ Fl, RECOGNITION OF α-SIALOSYL AND α-L-FUCOSYL GROUPS IN A BRANCHED STRUCTURE*,†

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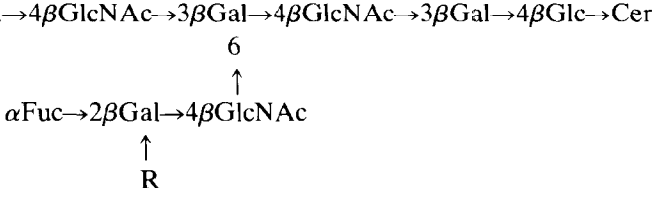
ABSTRACT

The antibody Fl shows preferential reactivity with adult erythrocytes over newborn erythrocytes, and its reactivity is abolished by sialidase treatment of the erythrocyte. The antibody was found to recognize binary determinants linked to the branched *lacto-N*-isooctaosylceramide**



R = OH, or αGalNAc→3, or αGal→3 residue

The presence of an *N*-acetylneuraminyl group at one end and L-fucosyl group at the other end is essential for the reactivity of the antibody. A substitution at the penultimate D-galactosyl residue of one of the chains with an α-D-(1→3)-linked 2-acetamido-2-deoxygalactosyl or galactosyl group did not inhibit the reactivity of the antibody. The new blood group A- and B-active, branched gangliosides are also isolated and characterized.

*Dedicated to Professor Elvin A. Kabat.

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**All sugars in abbreviated form are assumed to have the D configuration (except fucose which has the L configuration), to be in the pyranose form, and to be linked at O-1 (except sialic acid which is linked at O-2) in oligosaccharides.

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INTRODUCTION

Anti-carbohydrate antibodies with well-defined specificities are expedient probes for the study of cell-surface carbohydrates and invaluable reagents in determination of specific carbohydrate structures. Monoclonal anti-carbohydrate antibodies produced by hybridoma techniques as well as paraproteins produced by naturally occurring myeloma and immunoblastoma have greatly enriched our knowledge of the application of anti-carbohydrate antibodies in cell biology and carbohydrate chemistry[1]. A cold agglutinin Fl that reacted with an erythrocyte antigen was found in serum from a patient with immunoblastoma[2]. This agglutinin resembled anti-I antibodies in reacting more strongly with adult erythrocytes than with newborn erythrocytes. However, the activity was destroyed by sialidase treatment, whereas I activity is enhanced by the same treatment. Properties of Fl antigen are also distinct from Pr (ref. 3), Gd (ref. 4), and Sa (ref. 5) antigens, which are all sialidase sensitive but are equally expressed on fetal and adult erythrocytes. This paper describes the characterization of the epitope (determinant) structure recognized by the Fl cold agglutinin.

EXPERIMENTAL

Materials. — The antiserum was the same as previously described[2], and was purified by binding with intact erythrocytes at 0° and warm elution at 37°, after absorption with sialidase-treated erythrocytes at 0°. The antibody had a titer of 1:128 with adult O erythrocytes, and a titer of 1:4 with newborn O erythrocytes. The activity was destroyed by treatment of erythrocytes with *Vibrio cholerae* sialidase. Anti-I antibody (Ma) was a gift from Dr. E. R. Giblett, Puget Sound Blood Bank, Seattle, WA. The reactivity of the antibody with various glycolipid fractions was determined by solid-phase radioimmunoassay on vinyl strip, as previously described[6]. Briefly, each glycolipid (1–20 μg) was mixed with egg-yolk lecithin (5 μg) and cholesterol (3 μg) in ethanol (1 mL). A sample (10 μL) of this solution was placed on a vinyl strip and allowed to evaporate at room temperature. The glycolipid–phospholipid–cholesterol film formed on the vinyl strip was treated with antibody diluted 1:100 (Fl) or 1:1000 (anti-I), washed, treated with the 1:1000 dilution of a second antibody (anti-human IgM rabbit antibody), and finally with ^{125}I-protein A. The radioactivity on the vinyl strip was measured in a gamma scintillation counter.

Methods. — Glycolipids were extracted and isolated as previously described[7] and partitioned according to Folch's method. Glycolipids in the aqueous layer of the partition were further separated into a neutral glycolipid fraction and a ganglioside fraction by DEAE–Sephadex column chromatography[8]. The gangliosides were separated into individual components by 0.3–0.6 MPa liquid chromatography on an Iatrobeads RS8010 column (1.0 × 50 cm, Iatron, Tokyo, Japan), eluted with a gradient of 2-propanol–hexane–water, according to a mod-

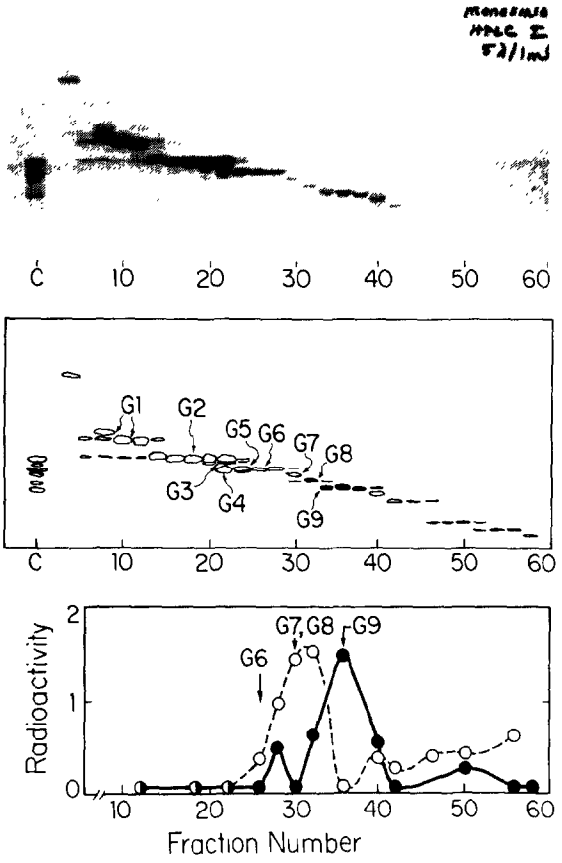


Fig. 1. T.l.c. pattern of series of monosialogangliosides from human blood-group type O erythrocytes separated on 0.3–0.6 MPa l.c., and the reactivity of each fraction to anti-FI and anti-I (Ma) antibody. Top and middle panels: t.l.c. of fractionated gangliosides. Monosialogangliosides prepared from type O erythrocytes were fractionated by l.c. on an Iatrobeads RS8010 column, being eluted with a gradient [55:37:8 to 55:33:12 (v/v)] of 2-propanol–hexane–water for 200 min, at the flow rate of 2.0 mL/min. Fractions (5 mL) were collected each 2.5 min. Aliquots (5 μL) were applied to t.l.c. developed with 60:35:8 (v/v) chloroform–methanol–water. The spots were detected with the orcinol–sulfuric acid reagent. Bottom panel: reactivity of fractionated gangliosides with anti-FI (●—●) or anti-I (Ma) antibody (○--○). Aliquots (0.5 μL) of each fraction were mixed with phosphatidylcholine (0.5 μg) and cholesterol (0.25 μg) in ethanol fraction, and assayed by solid-phase radioimmunoassay (see Experimental section). Abscissa: radioactivity ($\times 10^4$ for anti-FI, $\times 0.5 \times 10^4$ for anti-I). Designation of gangliosides G1 to G9 is the same as previously reported[10].

ified method[7] of Watanabe and Arao[9]. An Iatrobeads RS8060 column (1.0 × 100 cm) was used for the preliminary fractionation of neutral glycolipids. Details of fractionation and solid-phase radioimmunoassay of each fraction are described in the legend to Fig. 1.

^{1}H-N.m.r. spectra for solution of deuterium-exchanged, purified glycolipids (250 μg), dissolved in ($^2H_6$)dimethyl sulfoxide (0.5 mL) containing 2% deuterium

TABLE I

REACTIVITY OF VARIOUS BRANCHED GLYCOLIPIDS TOWARDS ANTI-FI AND ANTI-I (Ma) ANTIBODY

Glycolipid	*Amount (ng)*	*Terminal group of chain linked to Gal*		*Antibody*	
		β-(1→6)	*β-(1→3)*	*Anti-Fl*	*Anti-I (Ma)*
G9-0 (**1**)	1000	αFuc→2	αNeuAc→3	10 471	0
	63			6 940	0
G9-A (**2**)	1000	αFuc→2, αGalNAc→3	αNeuAc→3	7 048	0
G9-B (**3**)	1000	αFuc→2 αGal→3	αNeuAc→3	6 718	883
I-Active bovine ganglioside (**4**)	250	αGal→3	αNeuAc→3	0	17 695
I-Active bovine ganglioside, degalactosylated (**5**)[a]					
(Lot 1)	250		αNeuAc→3	0	11 117
(Lot 2)	250		αNeuAc→3	0	10 905
Bovine disialoganglioside (**6**)[b]	250	αNeuAc→3	αNeuAc→3	212	13 779
H_3-Glycolipid (**7**)	250	αFuc→2	αFuc→2	0	0
Defucosylated H_3-glycolipid (**8**)[c]	250			0	16 826
Desialosylated G9-0 (**1**)[d]	250	αFuc→2		0	3 739

[a]Removal of D-galactose from I-active bovine ganglioside[11] (**4**) was performed with fig α-D-galactosidase, followed by l.c. purification. Hydrolysis of the glycolipid by the enzyme was complete, and the final, degalactosylated ganglioside (both Lot 1 and Lot 2) were essentially 100% pure. The product has the same structure as G8 ganglioside[27]. [b]The structure of this ganglioside[12] is the same as that of the disialoganglioside of human erythrocytes[13]. [c]Removal of L-fucose from **7** (H_3) to give **8** was performed by hydrolysis with 0.1M trichloroacetic acid for 1 h at 100°. The hydrolyzate was purified by acetylation and preparative t.l.c. No contamination of uncleaved **7** was detected. [d]Removal of sialic acid from **1** (G9-0)[10] was performed by hydrolysis with 1.0% acetic acid for 1 h at 100°, followed by l.c. purification. The final preparation was 70% pure, no contamination of uncleaved **1** was observed. However, it contained ~30% of defucosylated material (**8**, having the same structure as defucosylated H_3, **7**), which could account for the observed low activity with anti-I antibody.

oxide and 1% tetramethylsilane, were recorded with a 500-MHz n.m.r. spectrometer (Model WM-500, Bruker, F.R.G.) in the Fourier-transform mode using quadrature detection and an excitation-pulse angle of 90°; 16 k data points for a 5-kHz spectral-width were collected.

Partially purified fig α-D-galactosidase and beet kidney α-L-fucosidase (Boehringer Manheim, F.R.G.) were used for enzymic degradation of various glycolipids. Methods for the chemical defucosylation and desialylation are described in the footnote to Table I. Derivatives of glycolipids were further purified by l.c. (Iatrobeads RS8010, 0.5 × 30 cm column) with a gradient of 2-propanol–hexane–water 55:37:8 to 55:33:12 (v/v) for 200 min, under a pressure of 0.3–0.6 MPa.

RESULTS

Glycolipid antigen, reactive with anti-Fl antibody, in type O human erythro-

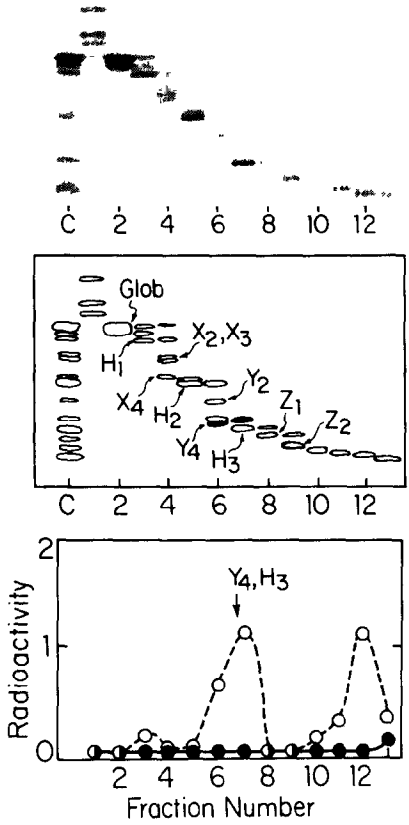


Fig. 2. T.l.c. pattern of neutral glycolipids from human blood-group type O erythrocytes separated on 0.3–0.6 MPa l.c., and the reactivity of each fraction to anti-Fl and anti-I (Ma) antibody. Top and middle panels: t.l.c. of fractionated neutral glycolipids. Neutral glycolipids prepared from type O erythrocytes were fractionated by l.c. on a Iatrobeads RS8060 column, being eluted with a gradient 11:8:1 to 11:6:3 (v/v) 2-propanol–hexane–water for 400 min, followed by elution with 11:5:4 (v/v) for an additional 100 min, at a flow rate of 2.0 mL/min. Fractions (5 mL) were collected each 2.5 min, and fractions showing similar glycolipid composition were pooled. Aliquots (5 μL) of pooled fractions 1–13 were examined by t.l.c. in 60:35:8 (v/v) chloroform–methanol–water. The spots were detected with the orcinol–sulfuric acid reagent. Bottom panel: reactivity of fractionated, neutral glycolipids with anti-Fl (●—●) or anti-I (Ma) antibody (○--○). Aliquots (0.5 μL) of each fraction were mixed with phosphatidylcholine (0.5 μg) and cholesterol (0.25 μg) in ethanol fraction, and assayed by solid-phase radioimmunoassay (see Experimental section). Abscissa, radioactivity ($\times 10^4$ for anti-Fl, $\times 0.5 \times 10^4$ for anti-I). For designation of H_1, H_2, and H_3, see references 15 and 16; designation for X and Z series, see references 7 and 14.

cytes. — The reactivities of various neutral glycolipids and gangliosides to F1 antibody are shown in Figs. 1 and 2. When various gangliosides from type O erythrocytes were tested with anti-Fl antibody after elution from the l.c. column, reactivity was clearly observed for fractions near 36, where ganglioside G9 was eluted (Fig. 1). An additional small peak of reactivity was observed for Fraction 28, where ganglioside G6 was eluted. The reactivity with anti-I (Ma) was found in Fractions

TABLE II

REACTIVITY OF TYPE O, A, B, AND AB ERYTHROCYTES WITH ANTI-Fl ANTIBODY

Dilution of antibody	*Erythrocytes*			
	O	A_1	*B*	A_1B
1:1	+++	+++	+++	+++
1:2	+++	+++	+++	+++
1:4	+++	++	+++	+++
1:8	++	+	++	++
1:16	+	±	±	+
1:32	-			

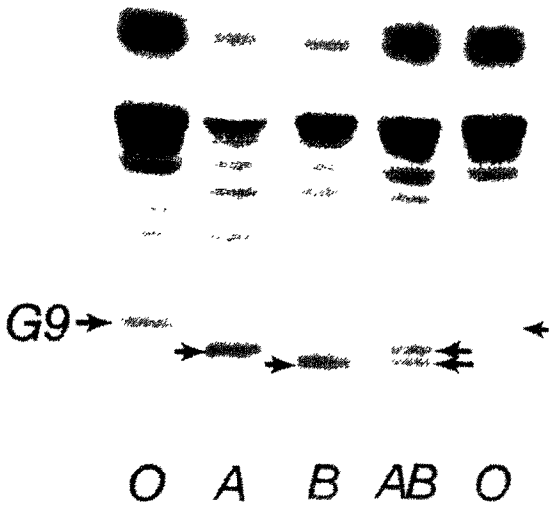


Fig. 3. Thin-layer chromatographic pattern of monosialoganglioside and "G9" glycolipids prepared from blood-group type O, A, B, and AB erythrocytes. Type O and AB, total monosialoganglioside fraction; type A and B, the latter half of the monosialoganglioside fraction eluted from a DEAE-column chromatography [most of G1 (GM_3) and G2 (sialosylparagloboside) glycolipids were removed]. Gangliosides that correspond to G9 glycolipids in each blood type are marked by an arrow. The G9 glycolipid of type A erythrocytes (G9-A) moved slower than the G9 glycolipid of type O erythrocytes (G9-O, 1), and G9-B (3) was even slower than G9-A (2) glycolipid. For type AB erythrocytes, two spots corresponding to G9-A (2) and G9-B (3) were clearly observed. The solvent system was 60:35:8 (v/v) chloroform–methanol–water. The spots were detected with the orcinol–sulfuric acid reagent.

30–32, which contain ganglioside G8[27] (5). Neutral glycolipid fractions, including H_1, H_2, and H_3 (refs. 15, 16) (for H_3, see 7), showed no detectable reaction with anti-Fl antibody, whereas reactivity with anti-I (Ma) was noted in Fractions 6 and 7 (Fig. 2). These activity profiles with anti-I were clearly different from those with anti-Fl antibody; thus, it is obvious that the specificity of anti-Fl antibody is distinct

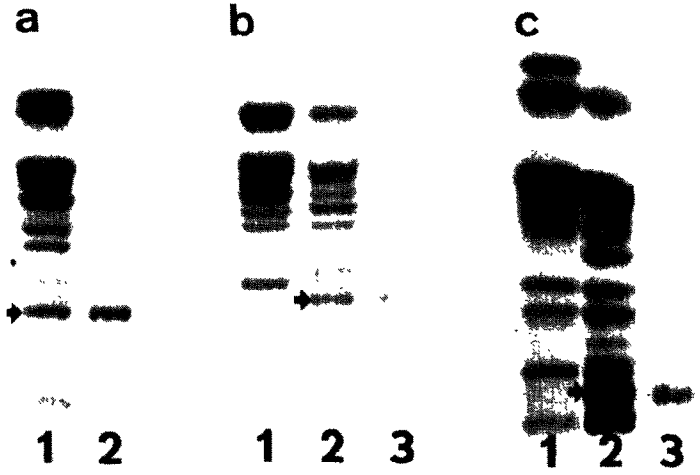


Fig. 4. T.l.c. of purified G9 glycolipids in 60:35:8 (v/v) chloroform–methanol–water. The spots were detected with the orcinol–sulfuric acid reagent: (a) Lane 1, monosialosyl gangliosides of type O erythrocytes; lane 2, purified G9-0 (**1**). (b) Lane 1, monosialosyl gangliosides of type O erythrocytes; Lane 2, monosialosyl gangliosides of type A erythrocytes; and Lane 3, purified G9-A (**2**). (c) Lane 1, monosialosyl gangliosides of type O erythrocytes; Lane 2, monosialosyl gangliosides of type B erythrocytes; and Lane 3, purified G9-B (**3**).

from typical, cold agglutinins with anti-I or -i specificity, and also from Sa-type or Gd-type antigens[3,4], which react well with sialosylparagloboside (G2) and sialosyl-neo*lacto*norhexaosylceramide (G6). The antigenic glycolipid for the anti-Fl antibody was found exclusively in the ganglioside fraction, and the major fraction, from blood-group O erythrocytes, that showed a strong reactivity was G9 ganglioside[10], a branched, H-active fucoganglioside (G9-O, **1**).

TABLE III

REACTIVITY OF ANALOGS OF *lacto-N*-ISO-OCTAOSYLCERAMIDE WITH ANTI-I (MA) AND ANTI-FL ANTIBODIES[a]

Compound	*Antibody*	
	Anti-I (MA)	*Anti-Fl*
1 (G9-0)	–	+
2 (G9-A)	–	+
3 (G9-B)	–	+
4	+	–
5 (G8)	+	–
6	+	–
7 (H_3)	–	–
8	+	–

[a]Detected by solid-phase radioimmunoassay on a vinyl strip (see Experimental section).

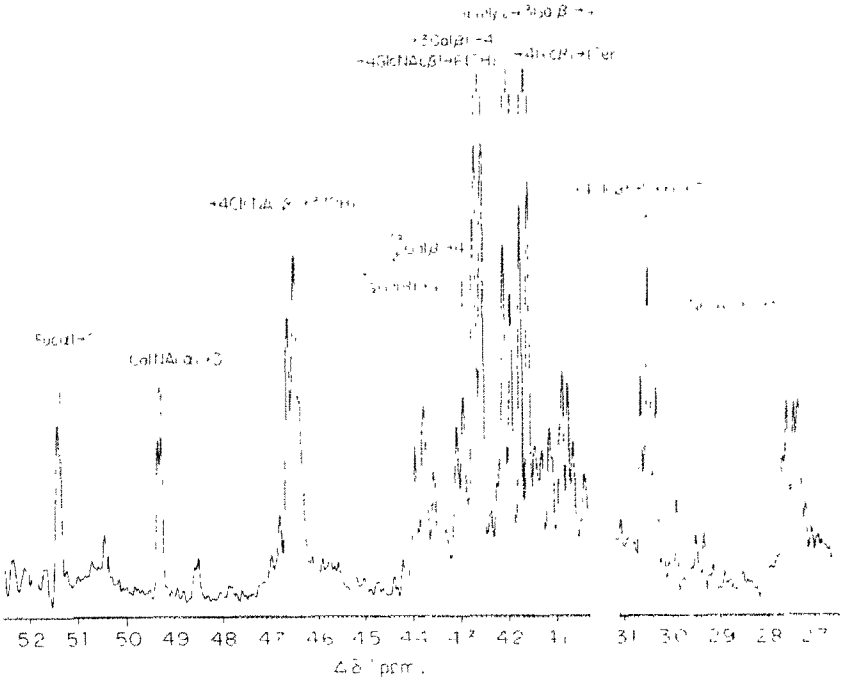


Fig. 5. Partial ^{1}H-n.m.r. spectrum of G9-A ganglioside (**2**) (~250 μg) for a solution in ($^2H_6$)dimethyl sulfoxide (0.5 mL) (1200 pulse at 35°), a resolution enhancement was applied before transformation of FID.

Fl-Antigens in type A, B, and AB erythrocytes. — As shown in Table II, anti-Fl antibody reacts not only with type O erythrocytes, but also with type A, B, and AB erythrocytes. Since the results described earlier showed that the ganglioside antigen in type O erythrocytes carries the H-active terminus[10], it was concluded that the antigen in blood types A and B has the A- and B-determinant structures, respectively. Fig. 3 shows the monosialoganglioside pattern of blood-type O, A, B, and AB erythrocytes. Clearly, the G9 ganglioside present in type O erythrocytes is absent in type A, B, and AB erythrocytes, and gangliosides having t.l.c. mobilities significantly slower than that of G9 ganglioside are present in type A, B, and AB erythrocytes (Fig. 3). These gangliosides were tentatively named G9-O (**1**), G9-A (**2**), and G9-B (**3**), and purified by l.c. to homogeneity on t.l.c. (see Fig. 4 for t.l.c. of purified G9s).

The reactivity of G9-A (**2**) and G9-B (**3**) with the anti-Fl antibody was ascertained by solid-phase radioimmunoassay. As shown in Table III, all G9 glycolipids (**1**–**3**) reacted well with the antibody.

Structure of Fl antigens in type A and B erythrocytes. — G9-A (**2**) was reactive with anti-A antibody, and G9-B (**3**) was reactive with anti-B antibody; neither was reactive to anti-H antibody, to which G9-O (**1**) was reactive. In analogy with the

structure of G9-0 (**1**) elucidated previously[10], these findings suggest that the structure of G9-A (**2**) and G9-B (**3**) is a branched ganglioside having a sialosyl group in one chain, and an A- or B-active determinant group at the other chain terminal. This tentative structure was further supported by the ^{1}H-n.m.r. data for the G9-A ganglioside (**2**), as shown in Fig. 5. H-1 resonances for H-1 of a terminal α-L-linked fucosyl group at δ 5.14 (J 3.0 Hz) and of an α-L-(1→3)-linked GalNAc group 4.93 (J 3.7 Hz), possibly accompanied by small resonances[17] at δ 5.04 and 4.85, were clearly observed; the resonance for H-3*eq* of the α-(2→3)-linked NeuAc group was present at δ 2.75 (J 5.12 Hz). The resonance usually observed for H-1 of the internal, branched β-D-(1→4)-linked D-Gal residue, substituted at O-3 and -6, was observed[18] at δ 4.30 (J 7.4 Hz)*. The H-1 resonance for the penultimate β-D-Gal residue linked to the A-antigen terminus was present at δ 4.39 and 4.37 (J 8.0 Hz) and the H-1 resonance for the residue linked to the sialosyl residue was present at δ 4.20 (J 7.9 Hz), within 0.01 p.p.m. of its observed position in both α-(2→3)-linked sialosylparagloboside (Type II) and sialosylneo*lacto*norhexosylceramide[19]. Resonances from the internal β-D-(1→3)-linked, 4-*O*-substituted GlcNAc residue were observed at δ ~4.65–4.67, and that from the β-D-(1→6)-linked 4-*O*-substituted GlcNAc residue was tentatively assigned to the same position as that of the 3-*O*-substituted, β-D-(1→4)-linked Gal residue at δ 4.27. Further information on the structural analysis of these A- and B-active gangliosides will be described elsewhere.

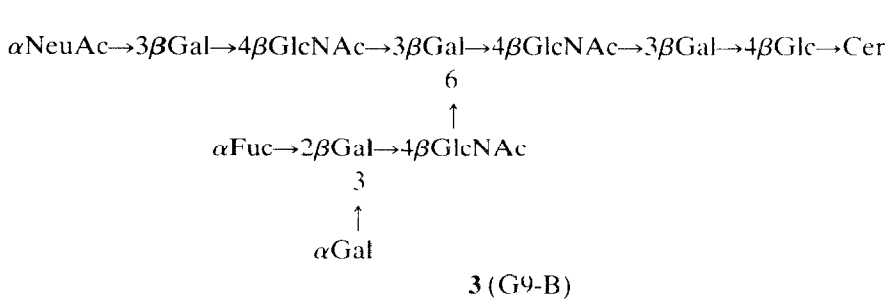
αNeuAc→3βGal→4βGlcNAc→3βGal→4βGlcNAc→3βGal→4βGlc→Cer
6
↑
αFuc→2βGal→4βGlcNAc

1 (G9-0)

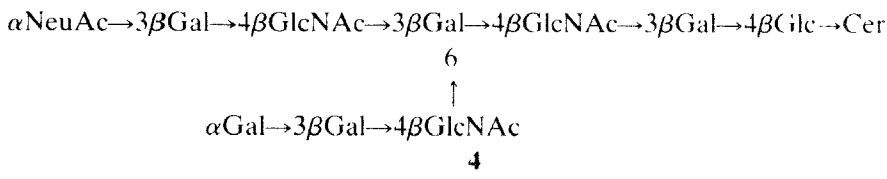
αNeuAc→3βGal→4βGlcNAc→3βGal→4βGlcNAc→3βGal→4βGlc→Cer
6
↑
αFuc→2βGal→4βGlcNAc
3
↑
αGalNAc

2 (G9-A)

*Hanfland *et al.*[18] observed a 3-proton resonance for H-1 of the galactosyl residue of the β-D-Gal-(1→4)-D-GlcNAc structure at δ 4.30 in the ^{1}H-n.m.r. spectrum of a weak-B-active branched neutral glycolipid, and unambiguously assigned it in part to the 3,6-di-*O*-substituted, β-D-(1→4)-linked galactosyl residue. In line with this observation, we have observed[19] the same resonance in the spectra of other branched glycolipids, including H_3, G9-0 (**1**), G10, and defucosylated H_3.



3 (G9-B)



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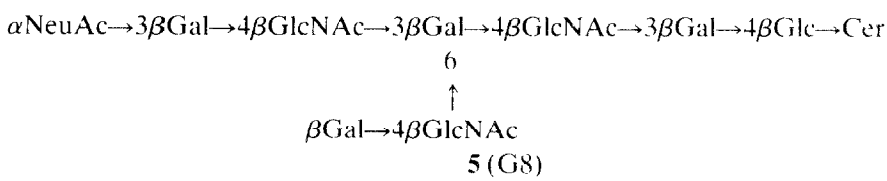
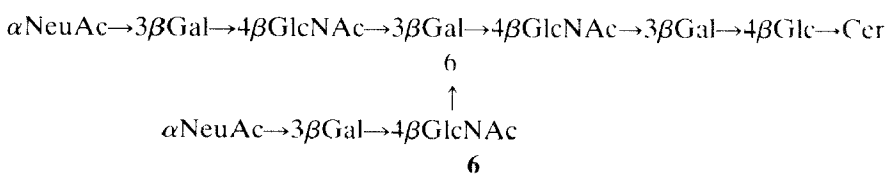


5 (G8)



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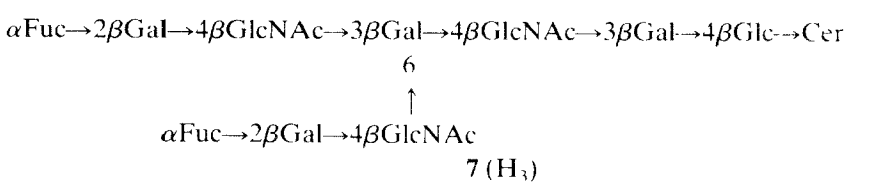
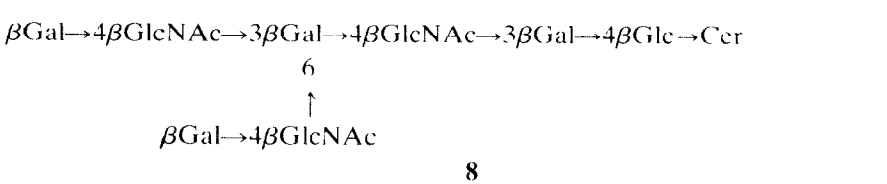


7 (H_3)



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Cer = ceramide

Scheme 1. Structures of analogs of *lacto-N-iso*-octaosylceramide

Comparison of specificity of anti-Fl and anti-I (Ma) towards various branched glycolipids. — To further establish the specificity of the antibody, various branched glycolipids of *lacto-N*-isooctaosylceramide analogues (see Scheme 1) were prepared either enzymically or chemically, and tested for the reaction with the Fl-antibody by solid-phase radioimmunoassay (Table III). The terminal α-NeuAc group of the (1→3)-linked branch was found to be essential to antibody activity; no desialylated materials showed reactivity. In addition, the α-L-Fuc group at the terminus of the other (1→6)-linked branch was also found to be essential for the reactivity on the basis of the following findings: (a) the reactivity with anti-Fl antibody was decreased when G9-0 (**1**) was enzymically defucosylated (data not shown); (b) no reactivity was observed with bovine I-active gangliosides that have the same structure as G9-B (**3**), minus the α-L-Fuc group (**4**); (c) a branched ganglioside with the same structure as G8 (**5**) (G9-0, **1**, minus the α-L-Fuc group), which was prepared from I-active ganglioside of bovine erythrocytes by treatment with ficin α-D-galactosidase, did not react with the anti-Fl antibody; and (d) a branched disialosylganglioside (**6**) from bovine erythrocytes that has sialosyl groups at both termini did not react with the anti-Fl antibody. From these results, it is concluded that the

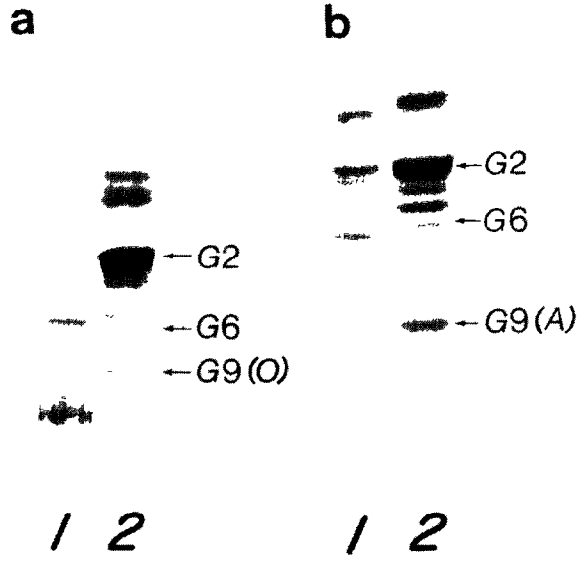


Fig. 6. Absence of G9 ganglioside in cord erythrocytes as shown by t.l.c in 60:35:8 (v/v) chloroform–methanol–water and detection of the spots by the orcinol–sulfuric acid reagent: (a) Lane *1*, gangliosides of type O cord erythrocytes; and Lane *2*, gangliosides of type O adult erythrocytes. (b) Lane *1*, gangliosides of type A cord erythrocytes; and Lane *2*, gangliosides of type A adult erythrocytes. Almost no G9 gangliosides are present in cord erythrocytes of either type O or A. Another characteristic of the ganglioside composition of cord erythrocytes is the relatively low proportion of sialosylparagloboside (G2), whereas the amount of G6 glycolipid (sialosylnorhexaosylceramide, i-antigen) is comparable to that in adult erythrocytes. The quantity of gangliosides is compared with the same quantity of membrane protein (1–1.5 mg dry protein basis).

anti-Fl antibody reacts with binary determinants (NeuAc and Fuc) linked to a branched *lacto-N*-isooctaosylceramide, and the major natural antigens present in human type O, A, and B erythrocytes are G9-0 (**1**), G9-A (**2**), and G9-B (**3**), respectively.

Fl antigens in cord erythrocytes. — Fig. 6 shows the ganglioside pattern of type O and type A cord erythrocytes as compared to that of the corresponding adult erythrocytes. Cord erythrocytes contain mainly gangliosides with shorter carbohydrate chains, and the amount of G9 gangliosides is almost negligible. This explains the low activity of cord erythrocytes with anti-Fl antibody. Another characteristic of the cord ganglioside pattern is the smaller amount of sialosylparagloboside as compared to that of adult erythrocytes, although the amount of sialosylneolacto—→norhexaosylceramide (G6, i-active) does not differ greatly from that in adult erythrocytes.

DISCUSSION

Cold agglutinins are classified into two major categories: one group consists of antibodies having Ii specificities, and the other group is directed to sialidase-sensitive structures. Pr, Gd, and Sa antibodies are good examples of the latter category. The antigens of the former group of antibodies show a remarkable developmental dependency; they are well-developed in adult erythrocytes, but are not fully expressed in cord erythrocytes. The antigenic conversion from i to I, associated with development from fetal and newborn to adult erythrocytes, has been shown to be correlated with a shift from an unbranched to a branched poly(lactosamine) structure based on the structural assignment of I and i antigens by inhibition of Ii activities by various glycolipid structures[11,20,21]. A similar conclusion was also drawn from methylation analysis of i-active and I-active poly(glycosyl)ceramide[22] and of poly(lactosamine) associated with the purified Band-3 glycoprotein of newborn, adult i, and adult I erythrocytes[23] (see ref. 24 for a review).

In contrast, cold agglutinin directed to sialidase-sensitive structures generally shows no developmental dependency. In this respect, the cold agglutinin Fl shows a very unique property. It is directed to a sialidase-sensitive structure, but, nevertheless, the antigen is minimally expressed in fetal erythrocytes and fully expressed in adult erythrocytes. It is assumed, therefore, that the determinant could be similar to I, but with sialic acid as a part of its epitope structure.

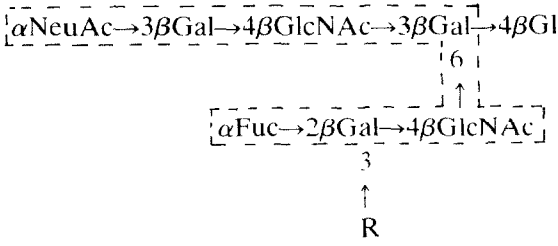
The results obtained were in good agreement with this assumption. A systematic study of various glycolipid fractions isolated from human blood-group O, A, and B erythrocytes indicated clearly that only one type of ganglioside in each blood group had a strong reactivity with Fl. The ganglioside of O erythrocytes was identified as a branched structure with an α-sialosyl group linked to the penultimate D-galactosyl residue of the β-(1→3)-linked branch, and an L-fucosyl group linked to the other penultimate D-galactosyl residue of the β-(1→6)-linked branch of *lacto-N*-isooctaosylceramide structure, and has been previously designated as G9 ganglioside[10]. The active gangliosides isolated from blood-group A and B erythro-

cytes had a branched structure analogous to that of G9 of O erythrocytes, but one penultimate residue was substituted with the A-active or B-active determinant group, instead of the H-active L-fucosyl group as in G9 ganglioside. These A- and B-active gangliosides were equally reactive with the anti-Fl antibody as G9 fucogangliosides of type O erythrocytes.

These A- and B-active, branched gangliosides are structures reported herein for the first time. Interestingly, both A- and B-active gangliosides co-exist in type AB erythrocytes. Since this antigen has a unique structure, the antigenicity of G9-A (**2**) and G9-B (**3**) may be different from that of the simple A- or B-active glycolipids, such as A^a, A^b, BI, and BII. It has been reported that some anti-A antibodies have anti-AI specificities as they react well with adult A erythrocytes, but react to a lesser degree with cord A erythrocytes[25]. The antigen corresponding to such antibodies could, of course, have an A^c structure, but could also have the G9-A structure (**2**). Neither A^c nor G9-A (**2**) structures are present in cord erythrocytes.

Interestingly, the Fl antibody did not react with H_3 glycolipid[16] (**7**), G8 ganglioside[27] (**5**), nor I-active ganglioside[11] nor disialosylganglioside of bovine erythrocytes. It is assumed, therefore, that the Fl antibody recognizes both the α-sialosyl group at one chain-end and the α-L-fucosyl group at the other chain-end of *lacto*-isooctaosylceramide. This is a clear example of one antibody recognizing binary determinants. Previously, it was observed that some of the I antibodies, such as Low and Sch, recognize binary galactosyl groups present at each chain-end of a branched *N*-acetyllactosamine structure[21]. The Fl antibody is an example of another type of binary recognition by a single antibody. It is also possible that the actual antigenic site may be on one branch that is held in an appropriate conformation by interaction with the other branch.

The structure of the Fl antigen is closely related to that of the I antigen; they share a branched poly(lactosaminyl) core structure. Thus, the enzymic processes of branching as well as addition by α-L-fucosyl- and α-sialosyl-transferases are necessary for the synthesis of the Fl antigen. The same branching process plays an essential role in the synthesis of I antigen. It is quite expected that cord erythrocytes, which have a very low quantity of glycolipids with branched structure, have only negligible amounts of both Fl and I antigens. Although the Fl antigen is chemically related to the I antigen, they are entirely different immunologically. As shown in Table III, the major Fl antigens of human erythrocytes have no I-antigenicity, whereas the most I-active structures have no reactivity with the anti-Fl antibody. It may be concluded that the presence of an α-L-fucosyl group at one chain-end of *lacto-N*-isooctaosylceramide is inhibiting the expression of I activity, and that the presence of an α-L-fucosyl group is essential for activity with the Fl antibody. A terminal α-sialosyl group is also essential for the Fl antibody, but it has less effect on the reactivity of the anti-I antibody. The epitope structure for the Fl antibody is shown in Scheme 2. Thus, antigenic epitopes recognized by Fl and I antibodies are entirely different from each other, although they both require common, branched-



1 R = OH
2 R = αGalNAc→3
3 R = αGal→3

Scheme 2. Structure of "G9" gangliosides in blood-group type O, A, B, and AB erythrocytes, and proposed recognition site of anti-Fl antibody (within dashed line). Both G9-A (**2**) and G9-B (**3**) were present in the same fraction prepared from type AB erythrocytes (see Fig. 3)

core structures. This suggests that a great diversity of antigenic structures are carried by binary poly(lactosaminyl) compounds.

One of us has previously observed anti-Pr[3] and anti-Gd[4] antibodies that react with various types of sialosyl residues in glycoproteins as well as glycolipids. These antibodies, however, do not require a branched structure, and depend on any end-chain with an α-NeuAc-(2→3)-D-Gal group linked to an appropriate carbohydrate chain[26]. The reactivity of the anti-I (Ma) and anti-Fl antibodies with various determinant structures (Table III, Scheme 1) suggest their use for analyzing the sialosyl structures of the cell surface that are developmentally regulated and may be altered upon oncogenic transformation.

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