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ARTICLE *in* BIOCHEMISTRY · JULY 1988

Impact Factor: 3.02 · DOI: 10.1021/bi00413a003 · Source: PubMed

Articles

Structure of a Ganglioside with Cad Blood Group Antigen Activity†

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Received July 7, 1987; Revised Manuscript Received January 20, 1988

ABSTRACT: The Cad antigen is a rare erythrocyte blood group antigen expressed on both sialoglycoprotein and ganglioside structures. It is related both serologically and biochemically to the Sd^a blood group antigen expressed on over 90% of Caucasian erythrocytes. We reported previously that Cad erythrocytes contain a novel ganglioside that binds *Helix pomatia* lectin and inhibits human anti-Sd^a antibody. We have now purified the Cad ganglioside and determined its structure. The ganglioside contained Glc–Gal–GlcNAc–GalNAc–NeuAc in a molar ratio of 1.00:1.94:0.95:0.93:1.05. Its chromatographic mobility was between that of G_{M1} and G_{D3}. After treatment with β-hexosaminidase (human placenta Hex A), the product migrated with 2–3-sialosylparagloboside ($IV^3NeuAcnLc_4OseCer$), it no longer bound *H. pomatia* lectin, and it acquired the ability to bind an antibody to sialosylparagloboside. Treatment of this material with neuraminidase (*Vibrio cholerae*) yielded a product with the mobility of paragloboside ($nLc_4OseCer$) that bound monoclonal antibody 1B2, which is specific for terminal *N*-acetyllactosaminyl structures. Treatment of the Cad ganglioside with *Arthrobacter ureafaciens* neuraminidase yielded a product reactive with monoclonal antibody 2D4, which is specific for terminal GalNAcβ(1–4)Gal structures. These data provide strong evidence that the Cad ganglioside structure is GalNAcβ(1–4)[NeuAcα(2–3)]Galβ(1–4)GlcNAcβ(1–3)Galβ(1–4)GlcCer. ^1H NMR analysis also supports the conclusion that the terminal GalNAc is linked β(1–4) to Gal. High-performance thin-layer chromatographic ganglioside patterns from three blood group Cad individuals showed a direct correlation between the quantity of Cad ganglioside and the strength of Cad antigen expression on the erythrocytes, as measured by hemagglutination. In addition to the major Cad ganglioside, a minor, slower moving component reactive with *H. pomatia* lectin was detected in all three Cad samples. No *H. pomatia* reactive bands were detected in gangliosides isolated from Sd(a+) cells, and the red cell component carrying the Sd^a antigen remains to be identified.

The blood group Cad antigen is a carbohydrate determinant carried by both sialoglycoproteins and gangliosides of human red cells (Cartron & Blanchard, 1982; Blanchard et al., 1985b). It was first identified by the strong reactivity of group O or B erythrocytes with the GalNAc-specific lectin *Dolichos biflorus*. The major O-linked glycan isolated from Cad red cell glycophorin A is a pentasaccharide, GalNAcβ(1–4)-[NeuAcα(2–3)]Galβ(1–3)[NeuAcα(2–6)]GalNAc (Blanchard et al., 1983), which binds to *D. biflorus* lectin. The major O-linked glycan of normal red cell glycophorin A is a sialotetrasaccharide, NeuAcα(2–3)Galβ(1–3)[NeuAcα(2–6)]-GalNAc (Thomas and Winzler, 1969), which does not bind to *D. biflorus* lectin. More recently, we reported that Cad antigen activity is also present in the ganglioside fraction of Cad red blood cells and the active compound appeared to be derived from the major erythrocyte ganglioside, sialosylparagloboside ($IV^3NeuAcnLc_4OseCer$),[1] by addition of a terminal *N*-acetylgalactosamine residue (Blanchard et al., 1985b).

The Cad antigen is serologically and biochemically related to the Sd^a blood group antigen. Cad red cells, also known as strong Sd^a reactors Sd(a++), are agglutinated more strongly by human anti-Sd^a serum than are Sd(a+) cells (Sanger et al., 1971). Both the glycoproteins and gangliosides from Cad red cells inhibit this antiserum (Blanchard et al., 1985b; Herkt et al., 1985). The terminal nonreducing trisaccharide GalNAcβ(1–4)[NeuAcα(2–3)]Gal of Cad red cell glycoproteins is also present on the Sd^a-active Tamm-Horsfall glycoprotein from human urine (Donald et al., 1983). This trisaccharide is absent from glycophorin A of Sd(a+) red cells

† This work was supported by NIH Grant AI 17712, the Gulf Coast Blood Center, the Institute National de la Sante et de la Recherche Medicale, the Netherlands Foundation for Chemical Research (SON), and the Netherlands Organization for the Advancement of Pure Research (ZWO).

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[1] Abbreviations: GLC, gas–liquid chromatography; GSL, glycosphingolipid; HPLC, high-pressure liquid chromatography; HPTLC, high-performance thin-layer chromatography; LBSA, lipid-bound sialic acid; MS, mass spectrometry; NMR, nuclear magnetic resonance. Glycosphingolipid structures are abbreviated according to the IUPAC–IUB recommendations (IUPAC–IUB Commission on Biochemical Nomenclature, 1977), except that ganglio series gangliosides are abbreviated according to Svennerholm (1964) and the suffix OseCer is omitted. SPG is sialosylparagloboside, $IV^3NeuAcnLc_4OseCer$; Fors is Forssman glycolipid, $IV^3\alpha GalNAcGb_4OseCer$.

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(Blanchard et al., 1985a). The red cell components carrying the Sd^a antigen have not yet been identified.

In the present study, we have isolated the novel erythrocyte ganglioside which carries the blood group Cad antigen and determined its structure. We have also determined the content of this ganglioside in red blood cells from three different Cad individuals, as well as in cells typed Sd(a+) and Sd(a−). We found a direct correlation between the quantity of Cad ganglioside in erythrocytes and their agglutination by *D. biflorus* lectin.

Materials and Methods

Red blood cells from the original Cad individual (Cad, group B) (Cazal et al., 1968) were kindly provided by M. Monis, Centre de Transfusion Sanguine de Montpellier, France. Other Cad-positive samples (Bui, group B; Des, group O) were obtained through the courtesy of Dr. Guimbretiere and Dr. Assan (Centre de Transfusion Sanguine de Nantes et de Cannes, France, respectively). Sd(a+) and Sd(a−) red cells were collected from blood donors at the Centre National de Transfusion Sanguine, Paris. Control O, A, and B red cells were obtained from the Gulf Coast Blood Center, Houston, TX. The human anti-Sd^a serum was a gift from L. Messeter (Lund, Sweden).

Glycosphingolipids were isolated as described previously (Ledeen & Yu, 1982; Kundu et al., 1985), with modifications for use of HPLC columns. Briefly, lipids were extracted from erythrocyte stroma (Steck & Kant, 1974) with chloroform–methanol–water, 50:50:10, desalted by dialysis, and deproteinized on a small silica column (Svennerholm & Fredman, 1980). Neutral and acidic GSLs were separated by ion-exchange chromatography on a BioSil TSK 540 DEAE column (Bio-Rad). Neutral glycolipids were purified by the acetylation method of Saito and Hakomori (1971. Gangliosides were purified from the acidic glycolipid fraction by base treatment with 0.3 N KOH (Byrne et al., 1985) to remove phospholipids and by chromatography on Iatrobeads (Iatron Laboratory, Tokyo, Japan) to remove sulfatides. The Cad ganglioside was isolated by HPLC on a PLC-Si column (Supelco), with a 2-propanol–hexane–water gradient (Watanabe & Arao, 1981; Kannagi et al., 1982b). Samples were analyzed on precoated silica gel 60 HPTLC glass or aluminum plates from EM Science. Densitometric scanning of the thin-layer chromatograms and autoradiograms was performed with a Quik Scan FlurVis Densitometer (Helena Laboratories, Beaumont, TX).

Sugar composition of the Cad ganglioside was obtained by gas–liquid chromatography of alditol acetate derivatives (Watanabe et al., 1979) on a 3-ft column of Supelco GP 3% SP2340 on 100/120 Supelcoport. Molar ratios were calculated from peak areas, and they were corrected for the response of standard glycolipids. Sialic acid content was determined by the modified resorcinol assay (Ledeen & Yu, 1982).

Enzyme Hydrolysis. Reaction mixtures contained 5–6 nmol of Cad ganglioside or 5–12 nmol of control glycolipids. For experiment 1, reported in Figure 3, β-hexosaminidase treatment was performed with 200 milliunits of human placenta Hex A (Sigma) in 70 μL of 10 mM sodium acetate, pH 4.6, with 1 mg/mL taurodeoxycholate for 48 h at 30 °C. Control G_{M2} was hydrolyzed approximately 75% under these conditions. Neuraminidase treatment was performed with 30 milliunits of enzyme (*Vibrio cholerae*, Calbiochem) in 150 μL of 50 mM sodium acetate, pH 5.4, 150 mM NaCl, 9 mM $CaCl_2$, and 0.5 mg/mL taurodeoxycholate for 7 h at 37 °C. Control SPG and G_{M3} were completely hydrolyzed under these conditions, while G_{M2} was not hydrolyzed. For experiment 2, reported in Figure 4, neuraminidase treatment of Cad ganglioside was performed with 10 milliunits *Arthrobacter ureafaciens* neuraminidase (Calbiochem) in 50 mM sodium citrate buffer, pH 4.4, with 1 mg/mL taurodeoxycholate for 22 h at 37 °C. Control G_{M2} was almost completely hydrolyzed to asialo-G_{M2} (Gg_3) under these conditions. Treatment of the resultant asialo-Cad with α- and β-hexosaminidases was done by addition of either 50 milliunits of β-hexosaminidase from Jack bean (GENZYME) or 10 milliunits of α-galactosaminidase from chicken liver (GENZYME). These enzymes were shown to be specific for GalNAcβ and GalNAcα linkages, respectively, by their action on globoside and Forssman glycolipid. Neither of these hexosaminidases acted on G_{M2} without prior desialylation by neuraminidase. Reactions were stopped by addition of chloroform–methanol, 1:1. Reaction mixtures were desalted on C_{18}-Bond Elut (Analytichem) (Williams & McCluer, 1980), and reaction products were analyzed by HPTLC with colorimetric and antibody overlay (immunostaining) detection systems.

Immunostaining of glycosphingolipids on HPTLC plates was done essentially as described by Magnani et al. (1982) except that the first antibody or lectin was incubated with the HPTLC strip for 18 h at 4 °C. *Helix pomatia* lectin (EY Laboratories) binds to terminal GalNAc (Smith, 1983; Baker et al., 1983). The lectin was radiolabeled with ^{125}I by the Iodogen method (Pierce Chemical Co.) in the presence of 10 mM *N*-acetyl-D-galactosamine and had a specific activity of 1 μCi/μg of protein. Fol human antisera (IgM) is specific for terminal NeuAcα(2–3)Galβ(1–4)GlcNAc– (Schwarting et al., 1977), mouse monoclonal antibody 1B2 (IgM) is specific for terminal lactosamine structure Galβ(1–4)GlcNAcβ(1–3)– (Young et al., 1981), and mouse monoclonal antibody 2D4 (IgM) is specific for GalNAcβ(1–4)Galβ(1–4)Glcβ1–R (Young et al., 1979).

500-MHz ^1H NMR Spectroscopy. Glycolipids were repeatedly exchanged in 1:1 $(C^2H_3)_2SO$ (99.9 atom % ^2H, Janssen Pharmaceutica, Beerse, Belgium): ^2H_2O (99.96 atom % ^2H, Aldrich) with intermediate lyophilization. ^1H NMR spectra were recorded in $(C^2H_3)_2SO$:^2H_2O = 98:2, on a Bruker WM-500 spectrometer (SON hf-NMR facility, Department of Biophysical Chemistry, University of Nijmegen, The Netherlands) operating at 500 MHz in the Fourier-transform mode at a probe temperature of 27 °C. Chemical shifts (δ) were expressed in ppm downfield from internal tetramethylsilane.

Fatty acid analysis of the Cad ganglioside was performed by treatment of the purified material with 0.8 N anhydrous methanolic hydrochloric acid at 80 °C for 18 h. Fatty acid methyl esters were extracted in hexane and analyzed either directly or after trimethylsilylation by gas–liquid chromatography on a fused silica capillary column (0.2 mm × 12 m) wall-coated with SP2100 (Hewlett-Packard, Palo Alto, CA). The carrier gas was helium at 1 mL/min. The oven temperature was programmed between 150 and 270 °C at 5 °C/min.

Quantitative hemagglutination was carried out by measurment of the agglutination percentage of Cad red cells by the GalNAc-specific lectin from *D. biflorus* seeds (affinity purified reagent from E. Y. Laboratories) with a Coulter counter (Model B) as described previously (Lopez et al., 1975). Blood group A erythrocytes were used as the control for complete agglutination.

Results

The ganglioside composition of Cad erythrocytes was abnormal (Figure 1A). The most striking change was seen in

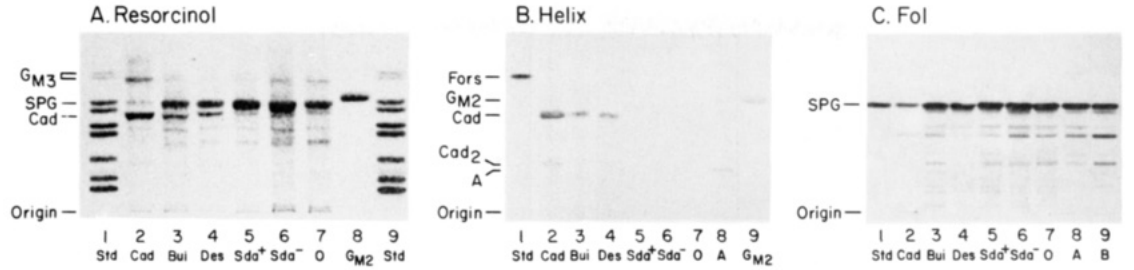

FIGURE 1: Cad and control erythrocyte gangliosides. Samples were developed by HPTLC in chloroform–methanol–0.25% $CaCl_2 \cdot 2H_2O$ (aq), 50:40:10. (A) Detection with resorcinol/HCl reagent. Lanes 2–7 contain erythrocyte gangliosides isolated from donors of the indicated blood types, 7 nmol of lipid-bound sialic acid (LBSA) per lane. Lane 8: G_{M2}, 2 nmol. Lanes 1 and 9: ganglioside standards mixture containing in order of decreasing mobility G_{M3}, SPG, G_{M1}, G_{D3}, G_{D1a}, G_{D1b}, G_{T1b}, and G_{Q1b}, 1 μg each. (B) Immunostaining with ^{125}I-labeled *H. pomatia* lectin. Lanes 2–8 contain gangliosides from the indicated donors, 4 nmol of LBSA per lane. Lane 1: mixture of standard neutral glycolipids, containing GluCer, LcCer, Gb_3OseCer, Gb_4OseCer, nLc_4OseCer, Forssman, and Gg_4OseCer, 0.2 μg each. Lane 9: G_{M2}, 2 nmol. (C) Immunostaining with Fol antiserum followed by ^{125}I-labeled goat anti-human IgM. Lanes 2–9: gangliosides from the indicated donors, spotted at 1 nmol of LBSA per lane. Lane 1: ganglioside standards mixture as in (A), 0.1 μg each.

the sample from donor Cad (Figure 1A, lane 2) in which the major ganglioside of normal erythrocytes, 2–3-sialosylparagloboside, was markedly decreased, and there was a large amount of a new ganglioside, designated Cad ganglioside, with mobility between those of standard G_{M1} and G_{D3}. Samples from Cad donors Bui and Des also contained Cad ganglioside (Figure 1A, lanes 3 and 4), although in lesser amounts than donor Cad. Gangliosides from Sd(a+), Sd(a−), and control O, A, and B cells (Figure 1A, lanes 5–7, and data not shown) all contain faint bands with similar mobility, and no differences between Sd(a+) and Sd(a−) cells were detected.

Immunostaining with *H. pomatia* lectin confirmed a previous report (Blanchard et al., 1985b) that this lectin binds Cad ganglioside (Figure 1B, lanes 2–4). In addition to the major Cad ganglioside band, a minor band reactive with *H. pomatia* lectin was also detected in all three Cad samples (designated Cad_2). This minor Cad ganglioside had slightly greater mobility than the blood group A ganglioside (Figure 1B, lane 8). In contrast to the Cad and blood group A samples, no reactivity with *H. pomatia* lectin was detected in Sd(a+), Sd(a−), and O or B blood group samples (Figure 1B, lanes 5–7, and data not shown). *H. pomatia* lectin also bound strongly to Forssman glycolipid (Figure 1B, lane 1) and weakly to G_{M2} (Figure 1B, lane 9).

The presence of 2–3-sialosylparagloboside in the Cad erythrocyte ganglioside mixtures was demonstrated by immunostaining with human antisera Fol (Figure 1C). This serum binds to structures that contain the terminal trisaccharide NeuAcα(2–3)Galβ(1–4)GlcNAcβ1 (Schwarting et al., 1977). In comparison to normal erythrocytes, Cad erythrocytes contained less 2–3-sialosylparagloboside and less long-chain lacto-series gangliosides that bear this epitope (Figure 1A,C, lanes 2–4).

The relative amounts of Cad ganglioside and 2–3-sialosylparagloboside in the Cad and control samples were determined and compared to the agglutination strength of the donor erythrocytes (Table I). The strength of Cad antigen activity, and the proportion of erythrocytes agglutinable by *D. biflorus* lectin, differs among individual Cad donors. It was reported previously (Blanchard et al., 1985a,b), and confirmed in this study, that a higher proportion of Cad cells were agglutinated by *D. biflorus* lectin than those of donors Bui and Des (Table I, column 3). Percentage of cells agglutinated was directly proportional to Cad ganglioside content (Table I, column 4). Cad and sialosylparagloboside content varied inversely, so that the sum of these gangliosides was approximately constant (Table I, last column). Total ganglioside yield from Cad erythrocytes was not different from that of control erythrocytes: 4.2 ± 1.2 nmol of LBSA/mg of stroma protein (mean ± SEM).

Table I: Cad Ganglioside Content of Cad and Sd^a Erythrocytes[a]

erythrocyte phenotype	donor	percentage agglutinated[b]	% Cad	% SPG	% (Cad + SPG)
Cad	Cad	100	58	12	70
Cad	Bui	50	24	43	67
Cad	Des	50	28	45	73
$Sd(a^+)$		0	<5[c]	65	70
Sd(a−)		0	<4	61	65
O		0	<2	49	51

[a] Cad and 2–3-sialosylparagloboside ganglioside content was calculated as a percentage of the total peak area of densitometric scans of thin-layer chromatograms, developed with resorcinol/HCl. [b] Percentage of the red cell population agglutinated by *D. biflorus* lectin. [c] Cad ganglioside was not detectable by *H. pomatia* staining in the Sd(a+), Sd(a−) and O samples. The noted "Cad peak areas" correspond to resorcinol-positive material with the mobility of Cad ganglioside.

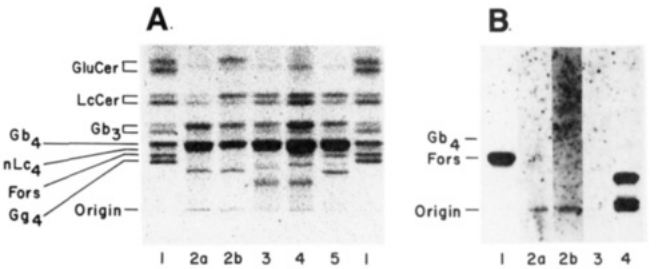

FIGURE 2: Cad and control erythrocyte neutral glycosphingolipids. Samples were developed by HPTLC in chloroform–methanol–0.1% KCl (aq), 50:40:10, and stained with (A) α-naphthol/H_2SO_4 or (B) ^{125}I-labeled *H. pomatia* lectin. Lanes 1: neutral glycolipids standard mixture, same as Figure 1B, lane 1. Lanes 2a and 2b: two different preparations from Cad erythrocytes. Lanes 3–5: glycolipids from type B, A, and O erythrocytes, respectively.

The neutral glycosphingolipid composition of Cad erythrocytes did not appear to be significantly different from that of normal erythrocytes (Figure 2). Globoside (Gb_4) was the major neutral glycolipid component in all cases (Figure 2A). Immunostaining with *H. pomatia* lectin (Figure 2B) gave strongly positive bands with standard Forssman glycolipid (Figure 2B, lane 1) and with neutral glycolipids from blood group A erythrocytes (Figure 2B, lane 4) but only weak nonspecific staining of Cad or control group B erythrocyte neutral glycolipids (Figure 2B, lanes 2a, 2b, and 3). These data are consistent with the absence of Cad antigenic activity in the neutral glycosphingolipid fraction (Blanchard et al., 1985b).

The Cad ganglioside was purified from the ganglioside mixture by HPLC. The purified material migrated as a single band in two solvent systems on HPTLC. In chloroform–

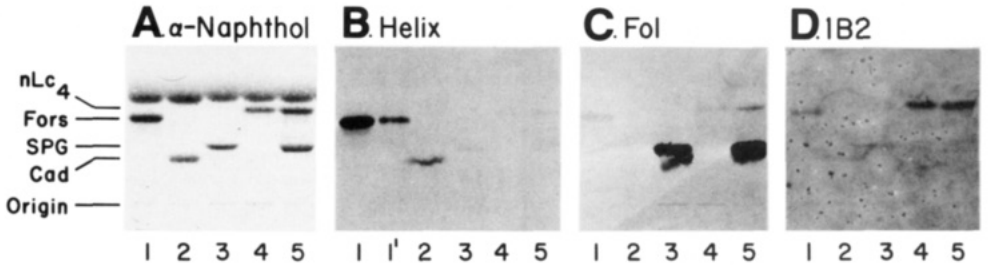

FIGURE 3: Enzymatic hydrolysis of purified Cad ganglioside, experiment 1. Cad ganglioside was incubated in buffer alone (lanes 2), with Hex A β-hexosaminidase (lanes 3), or with Hex A β-hexosaminidase followed by *V. Cholerae* neuraminidase (lanes 4). The reaction products were desalted; one-third of each sample was spotted on plates A and B and one-sixth on plates C and D. Plates were stained with (A) α-naphthol/H_2SO_4, (B) ^{125}I-labeled *H. pomatia* lectin, (C) Fol antiserum, and (D) mAb 1B2. Standards for mobility and immunostaining were incubated in buffer alone. Lanes 1 and 1′: Forssman glycolipid, 4 and 0.4 nmol per lane, respectively. Lanes 5: nLc_4 and $IV^3NeuAcnLc_4$, 2 nmol per lane. The upper band in plate A is taurodeoxycholate.

methanol–0.25% $CaCl_2 \cdot 2H_2O$ (aq), 50:40:10, it migrated between standard G_{M1} and G_{D3}, as in Figure 1A, lane 2. In chloroform–methanol–2.5 M NH_4OH (aq), 50:40:10, it migrated between G_{M1} and G_{D1a}, in contrast to the increased relative mobility of G_{D3} which migrated above G_{M1} in this basic solvent (data not shown). The sugar composition of the purified Cad ganglioside was 1.00:1.94:0.95:0.93:1.05 Glc:Gal:GlcNAc:GalNAc:NeuAc.

The sugar sequence of the Cad ganglioside was determined by sequential reactions with β-hexosaminidase and neuraminidase (Figure 3). The native Cad ganglioside bound *H. pomatia* lectin (Figure 3B, lane 2) but not Fol serum (Figure 3C, lane 2) nor antibody 1B2 (Figure 3D, lane 2). The reaction product produced by treatment of Cad ganglioside with Hex A β-hexosaminidase (Figure 3, lanes 3) comigrated with standard erythrocyte 2–3-sialosylparagloboside (lower band, lane 5); it no longer bound *H. pomatia* lectin (Figure 3B, lane 3) but now stained strongly with Fol antiserum (Figure 3C, lane 3). Heterogeneity in the ceramide portion of both the Cad ganglioside reaction product and standard 2–3-sialosylparagloboside was detected by immunostaining with Fol (Figure 3C, lanes 3 and 5) but not by the less sensitive α-naphthol colorimetric reaction (Figure 3A, lanes 3 and 5). The reaction product produced by sequential incubation of the Cad ganglioside with β-hexosaminidase followed by *V. cholerae* neuraminidase (Figure 3, lanes 4) comigrates with standard paragloboside (Figure 3A, lanes 4 and 5) and also bound antibody 1B2 (Figure 3D, lanes 4 and 5). These results indicate that the Cad ganglioside structure consists of a terminal GalNAc group linked to 2–3-sialosylparagloboside. The susceptibility to hydrolysis by Hex A β-hexosaminidase indicates that the anomeric linkage is β, but the enzyme preparation did contain a small amount of contaminating α-hexosaminidase activity.

Further evidence for a β anomeric configuration for the terminal GalNAc linkage was obtained by treating the Cad ganglioside with *A. ureafaciens* neuraminidase to obtain asialo-Cad (Figure 4, lane 2) and testing the susceptibility of asialo-Cad to α- and β-hexosaminidase preparations which did not contain any detectable anomeric cross reactivity. Asialo-Cad was hydrolyzed by β-hexosaminidase (Figure 4, lane 3) but not by α-galactosaminidase (Figure 4, lane 4) to a product with the mobility of paragloboside. The positional configuration of the terminal GalNAc was tested by immunostaining asialo-Cad with monoclonal antibody 2D4 (Figure 4B, lanes 2–4). This antibody is specific for the terminal GalNAcβ(1–4)Gal– of asialo-G_{M2} (Gg3) (Young et al., 1979). It does not bind globoside, Forssman glycolipid, G_{M2}, or asialo-G_{M1} (Gg_4) (Young et al., 1979; data not shown). The strong binding of asialo-Cad to antibody 2D4 indicates that the terminal GalNAc is linked β(1–4) to Gal.

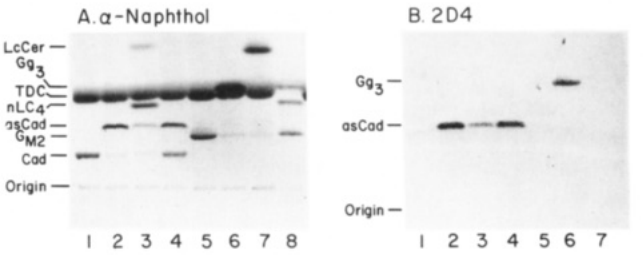

FIGURE 4: Enzymatic hydrolysis of Cad ganglioside, experiment 2. Cad ganglioside (lanes 1–4) or control G_{M2} (lanes 5–7) was incubated in buffer alone (lanes 1 and 5), with *A. ureafaciens* neuraminidase (lanes 2 and 6), or with neuraminidase and either Jack bean β-hexosaminidase (lanes 3 and 7) or chicken liver α-galactosaminidase (lanes 4). The reaction products were desalted; three-fourths of each sample was spotted on plate A and one-fourth on plate B. Plates were stained with (A) α-naphthol/H_2SO_4 or (B) monoclonal antibody 2D4. Standards in lane 8 are Gg_3, nLc_4, and G_{M2}. Band TDC is taurodeoxycholate.

Table II: ^{1}H Chemical Shifts of Constituent Monosaccharides for PG, SPG, and Cad Ganglioside and Those for Reference Compound G_{M2}

		chemical shift (ppm) in compound[a]			
residue[e]	proton(s)	PG[b]	SPG	G_{M2}[c]	Cad
Glc	H-1	4.166	4.166	4.150	nd[d]
Gal^4	H-1	4.252	4.254	4.274	nd
GlcNAc	H-1	4.649	4.629		nd
	NAc	1.812	1.811		1.811
$Gal^{4,3}$	H-1	4.200	4.188		nd
NeuAc	NAc		1.887	1.875	1.873
GalNAc	H-1			4.794	nd
	NAc			1.777	1.775

[a] Chemical shifts are in ppm downfield from internal TMS in $(C^2H_3)_2SO/^2H_2O$ (98:2) at 27 °C acquired at 500 MHz. [b] Assignments are based on the data reported in Dabrowski et al. (1981). [c] Data reported in Koerner et al. (1983). [d] nd = not determined. [e] A superscript at the name of the sugar residue indicates to which position of the adjacent monosaccharide it is glycosidically linked. A second superscript is used to discriminate between identically bound Gal residues; it indicates the type of linkage of the adjacent monosaccharide.

Independent evidence for the terminal GalNAc linkage and the sugar sequence was obtained by NMR analysis. The 500-MHz ^{1}H NMR spectra were recorded for the Cad ganglioside and for paragloboside and sialosylparagloboside as reference compounds. Relevant ^{1}H NMR data, as well as published data for G_{M2} (Koerner et al., 1983), are presented in Table II. The H-1 and *N*-acetyl signals of the constituent monosaccharides in sialosylparagloboside were assigned by comparison to those found for paragloboside. The *N*-acetyl signal of GlcNAc resonates at essentially the same position in both spectra and is independent of the presence of NeuAc.

Table III: Fatty Acid Composition of the Cad Ganglioside

fatty acid	%	fatty acid	%	fatty acid	%
14:0	4.8	18:1	13.6	24:0	20.3
15:0	1.6	20:0	1.1	24:1	6.5
16:0	19.8	22:0	6.7	25:0	0.7
16:1	4.5	23:0	1.2	26:0	1.7
18:0	17.4				

The quantity of Cad ganglioside was very limited, and from the carbohydrate portion of the molecule only the *N*-acetyl signals could be clearly discerned. Three singlets were observed at 1.775, 1.873, and 1.811 ppm, respectively. Comparison of data from the Cad ganglioside with those of paragloboside and sialosylparagloboside revealed that the signal at 1.811 ppm originated from the *N*-acetyl group of GlcNAc. The remaining two signals demonstrated the presence of the same structural element, GalNAcβ(1–4)[NeuAcα(2–3)]Galβ(1–4), as in G_{M2}. The signal at 1.873 ppm in the spectrum of the Cad ganglioside corresponds to the *N*-acetyl signal of NeuAc (1.875 ppm) in the spectrum of G_{M2}, whereas the signal at 1.775 ppm in the spectrum of the Cad ganglioside corresponds to the *N*-acetyl signal of GalNAc (1.777 ppm) in the spectrum of G_{M2}.

Fatty acid analysis (Table III) did not reveal the presence of α-hydroxylated fatty acids in Cad ganglioside. The major fatty acids contained aliphatic chains of the C_{16}, C_{18}, and C_{24} series.

Discussion

The data presented above provide strong evidence that the Cad ganglioside structure is GalNAcβ(1–4)[NeuAcα(2–3)]Galβ(1–4)GlcNAcβ(1–3)Galβ(1–4)Glcβ1–Cer. The terminal position of the GalNAc is indicated by the binding of Cad ganglioside to *H. pomatia* lectin and by its susceptibility to Hex A β-hexosaminidase. That the core structure of this ganglioside is sialosylparagloboside is indicated by the mobility of the reaction product after Hex A β-hexosaminidase treatment, by its strong binding to Fol antiserum, and by the reaction product's susceptibility to *V. cholerae* neuraminidase. Sequential treatment of Cad ganglioside with Hex A β-hexosaminidase and neuraminidase released paragloboside as indicated by the HPTLC mobility of the reaction product and by its reaction with antibody 1B2. That the terminal GalNAc is linked β(1–4) is further supported by the binding of asialo-Cad to the anti-asialo-G_{M2} monoclonal antibody 2D4 and the resistance of asialo-Cad to hydrolysis by chicken liver α-galactosaminidase.

The ^{1}H NMR data also indicate the presence of GalNAcβ(1–4)[NeuAcα(2–3)]Galβ(1–4)GlcNAcβ(1–3)Gal as a structural element of the Cad ganglioside. First, the NMR spectrum of the *N*-acetyl region of the Cad ganglioside reported here is identical with that previously reported for the Cad determinant on glycophorin A (Blanchard et al., 1983), after correction for solvent effects (DMSO vs D_2O). This structure was identified unequivocally as GalNAcβ(1–4)Gal. Second, near identity of the terminal GalNAc and NeuAc *N*-acetyl signals for Cad ganglioside and G_{M2} (Table II) indicates that these groups have the same anomeric and positional configuration in the two compounds.

The fatty acid composition of the Cad ganglioside resembles that found for sialosylparagloboside, with predominance of C16, C18, and C24 species (Siddiqui & Hakomori, 1973; Kannagi et al., 1982a). In contrast, the Cad ganglioside does not contain a large amount of myristic acid (C14), and thus, its fatty acid composition differs markedly from that of its structural isomer NeuAcα(2–3)GalNAcβ(1–3)Galβ(1–4)-GlcNAcβ(1–3)Galβ(1–4)GlcCer (G3 ganglioside) previously identified in human erythrocyte membranes (Watanabe & Hakomori, 1979).

The synthesis of the Cad ganglioside appears to follow an ordered pathway via a sialyated precursor substrate. It was previously reported that a β-GalNAc transferase from kidney of Sd(a+) individuals adds GalNAc to sialosylparagloboside (Piller et al., 1986). Further analyses of the reaction product by GLC–MS indicated that the GalNAc residue is transferred to the C-4 position of the IV Gal residue (F. Piller, B. Fournet, and J. P. Cartron, unpublished data). Paragloboside was not an acceptor, indicating the strict requirement of the enzyme for NeuAc-containing substrates (Piller et al., 1986). A synthetic trisaccharide corresponding to the asialo-Cad determinant GalNAcβ(1–4)Galβ(1–3)GalNAc, could not be resialylated in vitro by either a purified α(2–3)-sialyltransferase or by a purified α(2–6)-sialyltransferase (F. Piller, J. Paulson, P. Sinay, and J. P. Cartron, unpublished results). These findings are consistent with the observation that no sialic acid free oligosaccharides containing a terminal GalNAcβ(1–4)Gal disaccharide have been identified so far in Sd^a/Cad tissues (Blanchard et al., 1983; Herkt et al., 1985; Donald et al., 1983). It is of interest to note that the synthetic asialo-Cad trisaccharide does not inhibit human anti-Sd^a serum but strongly inhibits hemagglutination by the *D. biflorus* lectin (D. Blanchard, P. Sinay, and J. P. Cartron, unpublished data).

Amounts of both Cad ganglioside and Cad glycoprotein are highest in red cells of donor Cad and lower in cells of donors Bui and Des. The relative contribution of gangliosides and glycoproteins to Cad erythrocyte antigen activity is not known. In purified form, Cad ganglioside activity is approximately equivalent to that of the Cad determinant on glycophorin A. Cad ganglioside at a concentration of 1 μM NeuAc in liposomes has the same inhibitory activity as 0.5 μM NeuAc on glycophorin A in micellar solution (Blanchard et al., 1985b). The density of Cad ganglioside determinants on red cells is directly correlated with the percentage of cells agglutinated by *D. biflorus* lectin.

Sd(a+) erythrocytes are also agglutinated by anti-Sd^a sera, but the chemical nature of the Sd^a erythrocyte antigen is not yet known. Sd(a+) erythrocytes did not contain Cad glycophorin A (Blanchard et al., 1985a). In this study we did not detect any difference in Cad ganglioside content of Sd(a+) and Sd(a–) erythrocyte samples by densitometry. With the more sensitive radiolabeling method, however, a small amount of Cad ganglioside was detected in Sd(a+) erythrocytes (Blanchard et al., 1985b). Human erythrocytes contain a family of sialylated lactosamine ganglioside structures identified with the serum Fol [Figure 1 and Okada et al. (1984)], which might represent other potential acceptor structures for β-GalNAc transferase enzyme(s). ^{125}I-labeled *H. pomatia* lectin immunostaining of Cad ganglioside samples revealed both the major Cad ganglioside and a minor band, presumably the Cad antigen on a longer lacto-series core structure. More sensitive techniques may detect related structures in Sd^a samples.

Acknowledgments

We thank Mary A. Jones and Susan M. Osovitz for technical assistance and Charlene Shackelford for the preparation of the manuscript. We also thank Dr. S. Hakomori (Seattle, WA) for monoclonal antibodies 1B2 and 2D4, Dr. Pierre Sinay (Orléans, France) for the supply of the synthetic asialo-Cad trisaccharide, and Dr. James Paulson (Los Angeles, CA) for sialylation experiments.

Registry No. 14:0, 544-63-8; 15:0, 1002-84-2; 16:0, 57-10-3; 16:1, 373-49-9; 18:0, 57-11-4; 18:1, 112-80-1; 20:0, 506-30-9; 22:0, 112-85-6;

23:0, 2433-96-7; 24:0, 557-59-5; 24:1, 506-37-6; 25:0, 506-38-7; 26:0, 506-46-7; Cad ganglioside, 97708-84-4.

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