Primary Structure of the Oligosaccharide Determinant of Blood Group Cad Specificity*

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Glycophorin A and B from Cad erythrocyte membranes are the carriers of the blood group Cad determinants. They are characterized by a significant increase in molecular mass, as compared to the corresponding glycophorins from control erythrocytes (Cartron, J.-P., and Blanchard, D. (1982) Biochem. J. 207, 497–504). Lipid-free glycophorin A, purified from Cad red cells, showed an increased GalNAc content in comparison to blood group B, Cad-negative, control cells. Alkaline-borohydride treatment of this Cad glycophorin A released as a predominant species a pentasaccharide; its structure was determined, by methylation analysis and by 500-MHz 1H-NMR studies, to be:

GalNAcβ(1→4)Galβ(1→3)NeuAc(2→3)GalNAc-ol

This novel oligosaccharide inhibited strongly the hemagglutination of Cad erythrocytes by the *Dolichos biflorus* lectin. It shares with the blood group Sd* determinant a terminal GalNAcβ(1→4)Galβ(1→4) sequence.

Cad is a rare human red cell antigen inherited as an autosomal dominant character (1). Cells with such an antigenic activity were first recognized as blood group O or B erythrocytes exhibiting an unexpectedly strong reactivity with the *Dolichos biflorus* lectin. Later, hemagglutination-inhibition of Cad red cells by *D. biflorus* lectin has shown that Cad specificity requires the presence of GalNAcβ(1→4)Galβ(1→4) (2). A sugar also involved in blood groups A, Tn, Sd*, and Forssman specificities (4–7). Following the observation that all Cad samples reacted strongly with anti-Sd* antibodies, which define an antigen of varying strength present on more than 90% of Caucasian red cells, Sanger et al. (3) suggested that Cad was in fact a very strong form of Sd*. In order to prove this assumption, the chemical structure of both the Cad and the Sd* determinants has to be established.

Preliminary investigations have shown that Cad determinants are carried by the main red cell membrane sialoglycoproteins (glycophorin A and B). This was deduced from sodium dodecyl sulfate-polyacrylamide gel electrophoresis and affinity binding on immobilized *D. biflorus* lectin (8, 9). The carbohydrate composition of highly purified lipid-free glycophorin A molecules prepared from Cad erythrocytes indicated an increased GalNAc content and suggested that these residues form part of alkali-labile oligosaccharide chains. In order to clarify the chemical structure of the Cad determinant, the predominant oligosaccharide chains obtained by alkaline-borohydride treatment of purified glycophorin A molecules were isolated and analyzed.

Based on methylation analysis followed by GLC-MS2 as well as independently on 500-MHz 1H-NMR spectral studies, the complete structure of a pentasaccharide bearing the Cad-specific determinant was identified. The novel structure shares the terminal, nonreducing sequence GalNAcβ(1→4)Galβ(1→4) with blood group Sd* determinants (10).

**EXPERIMENTAL PROCEDURES**

The red cells from the original Cad individual (group B) were kindly provided by Monique Monis, Centre de Transfusion Sanguine de Montpellier, France (1). Control red cells were collected from blood donors of the Centre National de Transfusion Sanguine, Paris, France and were typed as group B, Cad negative. The major red cell membrane sialoglycoprotein (glycophorin A) was purified from lipid-free sialoglycoprotein chains obtained after fractionation of 60 ml of packed red cells as described previously (2). Alkaline-borohydride treatment was performed on lipid-free glycophorin A essentially as described by Aminoff et al. (11). Briefly, 5 to 10 mg of purified material was incubated in a medium containing 1 M KBH4, 0.1 M KOH, and 1.5 to 3 mCi of NaB[3H]4 (7 Ci/mmol, New England Nuclear) for 20 h at 45 °C. Samples were neutralized by addition of Dowex 50 × 8 (H+ form) and filtered through glass wool. Borate salts were partially precipitated after 30 min at pH 5.5 and the reaction was stopped by addition of 5% iron (III) chloride.

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1 All sugars are of the β-configuration, unless otherwise indicated.

2 The abbreviations used are: GLC-MS, gas-liquid chromatography combined with mass spectrometry; GLC, gas-liquid chromatography; TLC, thin layer chromatography; NMR, nuclear magnetic resonance; Ac, acetyl; Me, methyl.
eliminated as methyl derivatives by concentration in toluene after addition of methanol. The resulting products of the $\beta$-elimination procedure were separated on a Bio-Gel P-6 column (1.5 x 50 cm) equilibrated with 1% acetic acid. TLC was performed on 20 x 20 cm Kieselgel plates (Merck, Darmstadt) in ethanol:water:butanol:pyridine:acetic acid (100:30:10:10:3, v/v) for 6 h at room temperature (12). Carbohydrates were stained with orcinol/sulfuric acid reagent at 105 °C for 30 min (13). The carbohydrate composition of purified glycophorin A and its alkaline degradation products was determined by GLC after methanolsis (0.5 M HCl/methanol, 24 h, 80 °C) and pertr fluoroumacetylization (14). 200 μg of pure oligosaccharide from Cad sialoglycoprotein (Cad fraction II) were methylated according to Finne et al. (15). The permethylated oligosaccharide was methanolyzed and the products were identified by GLC-MS after peracetylation (16).

Prior to $^{1}H$-NMR spectral analysis, 200 μg of underivatized Cad fraction II were repeatedly exchanged in D$_2$O (99.96 atom % D; Aldrich) with intermediate lyophilization. The pH of the solution was adjusted to 7. $^{1}H$-NMR spectroscopic analysis was performed on a Bruker WM-500 spectrometer (Netherlands Foundation for Chemical Research NMR facility, Department of Biophysics, Nijmegen University, The Netherlands) operating at 500 MHz in the Fourier transform mode at probe temperatures of 285 and 300 K (17). Chemical shifts are given for neutral solution at 300 K, relative to internal sodium 4,4-dimethyl-4silapentane-1-sulfonate, but were actually measured by reference to internal acetone: $\delta = 2.252$ ppm, with an accuracy of 0.002 ppm.

Cad blood group activity of $\beta$-eliminated oligosaccharides has been checked by agglutination-inhibition tests carried out as described (9) using the D. biflorus lectin (Serva Laboratory).

RESULTS AND DISCUSSION

Lipid-free glycophorin A purified from Cad red cells (9) inhibited strongly the agglutination of A$_{r}$ red cells by D. biflorus lectin (0.002 μg of substance inhibited four hemagglutinating doses of lectin) and exhibited high GalNAc content as compared to glycophorin A from control cells (Table I).

Purified glycophorin A preparations from Cad and control red cells were submitted to alkaline-borohydride treatment. The reduced oligosaccharides obtained were fractionated on a Bio-Gel P-6 column equilibrated with 1% acetic acid (Fig. 1). The six fractions eluted from this column were lyophilized, redissolved in water, and analyzed by TLC. Fraction I, which eluted in the void volume of the Bio-Gel P-6 column, contained the residual glycoprotein which does not migrate on TLC. Fraction II gave a single spot on TLC, both when the reduced sugars from control and Cad glycoproteins were examined separately, the mobility of the control and Cad fraction II oligosaccharides being clearly distinct (namely, $R_{gal}=0.40$ and 0.45, for control and Cad, respectively), indicating a difference in structure. Fraction III contained, as a predominant species, the same oligosaccharide which is present in fraction II ($R_{gal}=0.60$ and 0.45, for control and Cad, respectively) together with minor components of larger mobility on TLC. Interestingly, three of these minor saccharides had a similar mobility ($R_{gal}=0.74, 0.92, and 1.02$) in material derived from control and Cad cells, suggesting a possible identity of structure. In addition, another oligosaccharide ($R_{gal}=0.58$) with a mobility virtually identical with that of the pure oligosaccharide found in fraction II from control cells ($R_{gal}=0.60$) was also identified in the Cad sample. Fractions IV, V, and VI contained salts, but no detectable sugar.

In hemagglutination-inhibition assays using group A$_{r}$ red cells and the D. biflorus lectin, 1.0 μg of fraction II or III from Cad was sufficient to inhibit agglutination, whereas the fractions from control cells were inactive.

The sugar composition of the products obtained after $\beta$-elimination is given in Table I. The predominant oligosaccharide in fraction III from control ($R_{gal}=0.60$) has a carbohydrate composition close to that found for the sialic acid-rich tetrasaccharide structure isolated by Thomas and Winzler (18) from the main human red cell membrane glycoprotein (see Table I). Fraction III isolated from Cad cells contains nonreduced GalNAc in addition to the sugars of control fraction III. The carbohydrate composition of Cad fraction III (Table I) indicates that the main species of this fraction might represent a pentasaccharide structure. Fraction II from Cad cells has a carbohydrate composition essentially identical with that of fraction III. Since Cad fraction II contained a single oligosaccharide species ($R_{gal}=0.45$), this fraction was further studied by methylation analysis and $^{1}H$-NMR spectroscopy.

The molar ratios of the monosaccharide methyl ethers obtained by methanolysis and acetylation of the permethylated pentasaccharide-alditol from Cad fraction II were determined by GLC-MS (16). 4,7,8,9-Tetra-OMe-NeuAcMe, 3,4,6-tri-OMe-GalNAcMe, 2,6-di-OMe-Gal, and 1,4,5-tri-OMe-
GalNAcNMe-ol were found in the molar ratios of 2.1:1.2:0.9:1. From the nature of the latter derivative it can be concluded that GalNAc-ol bears substituents at C-3 and C-6. Moreover, the pentasaccharide-alditol, the Cad fraction I1 sample was analyzed by 500-MHz 'H-NMR spectroscopy. The overall spectrum, recorded at 300 K, is presented in Fig. 2. Relevant 'H chemical shift data at this probe temperature are listed in Table II. The signals in the spectrum were assigned by using the chemical shift data at this probe temperature are listed in Table II. The signals in the spectrum were assigned by using the 'H-NMR data, acquired at 500 MHz, of the reference substances GgOse₅, that is, GalNAcβ(1→4)Galβ(1→4)Glc, and II₃NeuAc-GgOse₅, that is, GalNAcβ(1→4)[NeuAcα(2→3)]Galβ(1→4)Glc, both derived from gangliosides (19), and those of the so-called classical tetrasaccharide-alditol of the mucin type, NeuAcα(2→3)[NeuAcα(2→6)]Galβ(1→3)[NeuAcα(2→6)]GalNAc-ol (17, 20) (see Table II).

The chemical shift of H-2 (δ = 4.360 ppm) of GalNAc-ol in the Cad II pentasaccharide-alditol indicates that this residue bears a Gal residue in β(1→3)-linkage (17, 20–23). The set of chemical shifts of GalNAc-ol H-5 (δ = 4.181 ppm) and H-6' (δ = 3.445 ppm) points to the presence of a sialic acid residue α(2→6)-linked to GalNAc-ol (17, 20, 23). This NeuAc residue is further characterized by its set of H-3 chemical shifts (δH-3ax = 1.707 ppm; δH-3eq = 2.373 ppm), which were previously found to be unique for the NeuAcα(2→6)[Galβ(1→3)]GalNAc-ol sequence (17, 20, 24) (compare with the disialo tetrasaccharide-alditol in Table II).

The second NeuAc residue present shows its H-3 signals at δ = 1.933 ppm (H-3ax) and δ = 2.681 (H-3eq). It should be noted that the H-3eq triplet is partly obscured by the singlet at δ = 1.908 ppm stemming from contaminating acetate (see Fig. 2). The N-acetyl singlet of this NeuAc residue coincides with that of the aforementioned α(2→6)-linked residue (δ = 2.032 ppm). From comparison with the data for the ganglioside oligosaccharide II₃NeuAc-GgOse₅ (see Table II), it can be inferred that this very typical set of H-3 chemical shifts, in particular, δH-3ax = 1.93 ppm, points to the occurrence of a so-called internal sialic acid residue (19, 25). That means NeuAc is α(2→3)-linked to a Gal residue that bears also a β-linked substituent at C-4. The attachment of NeuAc at C-3 of Gal is corroborated by the appearance of the Gal H-3 signal at δ = 4.16 ppm, clearly separated from those of the remaining sugar skeleton protons (compare Refs. 17 and 20).

According to sugar and methylation analysis, a GalNAc residue should be the substituent at C-4 of Gal. This is supported by the presence of an N-acetyl signal in the NMR spectrum at δ = 2.025 ppm (see Fig. 2). However, at 300 K, only one anemic signal could be observed at δ = 4.561 ppm instead of the two expected; thus, no H-1 doublet for the GalNAc residue was observable. In order to attempt visual-
**Table II**

$^1$H chemical shifts of structural reporter groups of constituent monosaccharides for the Cad-specific pentasaccharide-alditol, together with those for some reference compounds

| Residue     | Reporter group | Chemical shift$^a$ in | (GlcOse) | Anomer of oligosaccharide | (1PNeuAc-GlcOse) | (Classical tetrasaccharide) | (Cad pentasaccharide) |
|-------------|----------------|-----------------------|----------|----------------------------|------------------|-----------------------------|-----------------------|
| Glc         | H-1            | 5.219                 | $\alpha$ | 5.218                      |                  | 4.378                       | 4.360                 |
|             | 4.665          | $\beta$               |          | 4.665                      |                  | 4.067                       | 4.047                 |
|             | 3.272          | $\beta$               |          | 3.272                      |                  | 3.524                       | 3.500                 |
| GalNAc-ol   | H-2            | 4.441                 | $\alpha$ | 4.530                      |                  | 4.091                       | 4.162                 |
|             | 4.439          | $\beta$               |          | 4.528                      |                  | 4.117                       | 4.162                 |
|             | ND$^b$         |                       |          |                             |                  | 4.147                       | 4.091                 |
| Gal         | H-1            | 4.096                 | $\alpha$ | 4.116                      |                  | 4.714$^c$                   | 4.714$^c$             |
|             | 4.638          | $\alpha$              |          | 4.744                      |                  | 3.927                       | 4.714$^c$             |
|             | 4.633          | $\beta$               |          | 4.741                      |                  | 3.927                       | 4.714$^c$             |
| GalNAc      | H-2            | 3.419                 | $\alpha$ | 3.370                      |                  | 3.920                       | 3.416                 |
|             | 3.410          | $\beta$               |          | 3.361                      |                  | 3.920                       | 3.416                 |
|             | 3.912          | $\alpha, \beta$       |          | 3.920                      |                  | 3.920                       | 3.920                 |
|             | NAc            | 2.057                 | $\alpha$ | 2.018                      |                  | 2.025                       | 2.025                 |
|             | 2.054          | $\beta$               |          | 2.015                      |                  | 2.025                       | 2.025                 |
| NeuAc$^{cd}$| H-3ax          | 1.922                 | $\alpha, \beta$ | 1.922 | 1.800 | 1.933 |
|             | H-3eq          | $\alpha, \beta$       |          | 2.664                      | 2.774            | 2.681                       | 2.681                 |
|             | NAc            | $\alpha, \beta$       |          | 2.032                      | 2.032            | 2.032                       | 2.032                 |
| NeuAc$^{cd}$| H-3ax          | 1.922                 | $\alpha, \beta$ | 1.922 | 1.922 | 1.922 |
|             | H-3eq          | $\alpha, \beta$       |          | 2.733                      | 2.733            | 2.733                       | 2.733                 |
|             | NAc            | $\alpha, \beta$       |          | 2.032                      | 2.032            | 2.032                       | 2.032                 |

$^a$ All data were acquired at 500 MHz, for neutral solutions of the compounds in D$_2$O at 300 K.
$^b$ Value could not be determined.
$^c$ Measured at 285 K (signal is hidden under HOD-line at 300 K).
$^d$ A superscript at the name of a sugar residue indicates to which position of the adjacent monosaccharide it is linked.
izing this signal, another spectrum of the Cad II sample was recorded, at a probe temperature of 285 K. This results in a downfield shift of the relatively broad HOD signal (from δ = 4.758 ppm to δ = 4.922 ppm), thereby enabling one to observe the spectral region upfield from δ = 4.85 ppm undisturbed. The relevant part of the spectrum recorded at 285 K has been included in Fig. 2. At this temperature, two doublets are recognizable, at δ = 4.714 ppm (J1,2 = 7.6 Hz) and at δ = 4.564 ppm (J1,2 = 8.3 Hz), respectively. The combination of the chemical shift and the J1,2 coupling constant values for each of these doublets points unambiguously to the β-configuration of the glycosidic linkages in which the sugars are involved. The former signal is attributed to the GalNAc anomeric proton, on the basis of comparison with the ganglioside oligosaccharides (see Table II). In consequence, the latter doublet belongs to Gal H-1.

Thus, the Cad II pentasaccharide-alditol can be conceived as an extension of the distal tetrasaccharide-alditol with a GalNAc residue in β(1→4)-linkage to Gal. Attachment of this GalNAc residue results in a downfield shift for H-1 of the substituted Gal (Δδ = 0.02 ppm). For a similar extension, namely, that from Galα(1→4)Glc (lactose) (26) to GlcOse, hardly any shift effect was observed for H-1 of Gal. The aberrant shift effect observed in this study might be due to the crowdedness of the C-3,4-C4-disubstituted Gal residue in the Cad pentasaccharide.

The results from the present investigation demonstrate clearly that the blood group Cad specificity is associated with a pentasaccharide, the structure of which could be deduced from 500-MHz 1H-NMR analysis in combination with methylation analysis: GalNAcb(1→4)[NeuAcα(2→3)]Galβ(1→3)[NeuAcα(2→6)]GalNAc-ol. This oligosaccharide represents a novel mucin-type structure, originally being attached via an O-glycosidic linkage from GalNAc to Ser and/or Thr residues of glycoporin A. It is not known how many of such pentasaccharide chains are present on a single glycoporin A molecule. However, the 3,000-dalton increase in apparent molecular mass of glycoporin A from Cad erythrocytes in comparison to controls (8, 9) strongly suggests that most of the 15 Ser/Thr-linked O-glycosidic oligosaccharide chains normally present on this glycoprotein (27) might be of that novel type.

Nevertheless, isolation of minor components after alkaline-borohydride treatment indicates that some chains with a lower content in NeuAc or GalNAc, or both, might be present.

Since the pentasaccharide isolated from Cad red cells is a potent inhibitor of D. biflourus lectin, the specificity of this lectin reported to be highly specific for terminal α-linked GalNAc (28) should be reconsidered. Interestingly, the GM3 ganglioside containing the trisaccharide sequence GalNAcb(1→4)[NeuAcα(2→3)]Galβ(1→3) (19, 29) is a strong inhibitor of D. biflourus lectin (0.04 μg inhibits four hemagglutinating doses of lectin).4 In agreement with these observations, Donald et al. (10) have shown recently that the disaccharide GalNAcb(1→4)Gal isolated after hydrazinolysis of the Tamm-Horsfall glycoprotein prepared from Sd(a+) individuals, also inhibited the D. biflourus lectin. Finally, our results lend firm biochemical support to the predicted direct relationship between Cad and Sd determinants (3) since both receptors are characterized by a terminal GalNAcb(1→4)Galβ(1→3) sequence. Further studies should determine to which extent the two determinants are similar and whether the same β-N-acetylglactosaminyltransferase is involved in their biosynthesis.

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