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 ^{1}H-NMR studies, to be:

GalNAcβ(1→4)
$\searrow$
      Galβ(1→3)
$\nearrow$ $\searrow$
NeuAcα(2→3)   GalNAc-ol
$\nearrow$
NeuAcα(2→6)
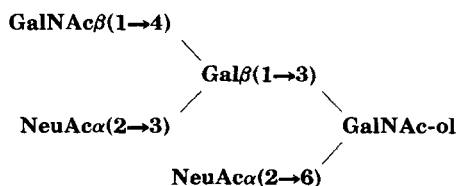
This novel oligosaccharide inhibited strongly the hemagglutination of Cad erythrocytes by the Dolichos biflorus lectin. It shares with the blood group Sd^a determinant a terminal GalNAcβ(1→4)Galβ(1→·) sequence.

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 GalNAc^1 (2, 3), a sugar also involved in blood groups A, Tn, Sd^a, P, and Forssman specificities (4–7). Following the observation that all Cad samples reacted strongly with anti-Sd^a 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^a. In order to prove this assumption, the chemical structure of both the Cad and the Sd^a 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-MS^2 as well as independently on 500-MHz ^{1}H-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→·) with blood group Sd^a 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 sialoglycoproteins obtained after fractionation of 60 ml of packed red cells as described previously (9). 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 KBH$_4$, 0.1 M KOH, and 1.5 to 3 mCi of NaB[^{3}H]$_4$ (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

eliminated as methyl derivatives by concentration *in vacuo* after addition of methanol. The resulting products of the β-elimination procedure were separated on a Bio-Gel P-6 column (1.5 × 50 cm) equilibrated with 1% acetic acid. TLC was performed on 20 × 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 10 min (13). The carbohydrate composition of purified glycophorin A and its alkaline degradation products was determined by GLC after methanolysis (0.5 M HCl/methanol, 24 h, 80 °C) and pertrifluoroacetylation (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 ¹H-NMR spectral analysis, 200 μg of underivatized Cad fraction II were repeatedly exchanged in D₂O (99.96 atom % D; Aldrich) with intermediate lyophilization. The pD of the solution was adjusted to 7. ¹H-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 a neutral solution at 300 K, relative to internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate, but were actually measured by reference to internal acetone: $\delta = 2.225$ ppm, with an accuracy of 0.002 ppm.

Cad blood group activity of β-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 A₁ red cells by *D. biflorus* lectin (0.002 μg of substance inhibited four hemagglutinating doses of lectin) and exhibited a 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. However, the mobility of the control and Cad fraction II oligosaccharides was clearly distinct (namely, $R_{Gal} = 0.60$ 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 A₁ 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 β-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

TABLE I

Molar carbohydrate composition of glycophorin A and of its β-elimination products, prepared from Cad and control erythrocyte membranes

Material	Molar ratio of carbohydrates[a]					
	Gal	Man	Gal-NAc	Glc-NAc	Neu-Ac	Gal-NAc-ol
Cad erythrocytes						
Glycophorin A	1	0.16	1.72	0.33	1.95	0
β-Elimination products[b]						
Fraction II	1	0	1.20	0	2.20	1.01
Fraction III	1	0	0.81	0	2.14	1.19
Control erythrocytes						
Glycophorin A	1	0.18	0.76	0.26	1.70	0
β-Elimination products[b]						
Fraction III	1	0	0	0	1.80	0.94

[a] Sugar analysis was performed as described by Zanetta *et al.* (14) using a Varian 1400 gas chromatograph equipped with flame ionization detector. Trifluoroacetyl derivatives of methylglycosides were separated on a glass column (300 × 0.6 cm) filled with OV-210 5% silicon on chromosorb W (HP) DMCS, 100–200 mesh. Nitrogen flux was 15 ml/min, and the column temperature was raised at 2 °C/min, from 100 to 210 °C. Only traces of L-Fuc and Glc were detected.

[b] The β-elimination products obtained after treatment of the glycophorin A preparation were fractionated on a Bio-Gel P-6 column as described under "Experimental Procedures" and as illustrated in Fig. 1. Only fractions II and III were analyzed quantitatively.
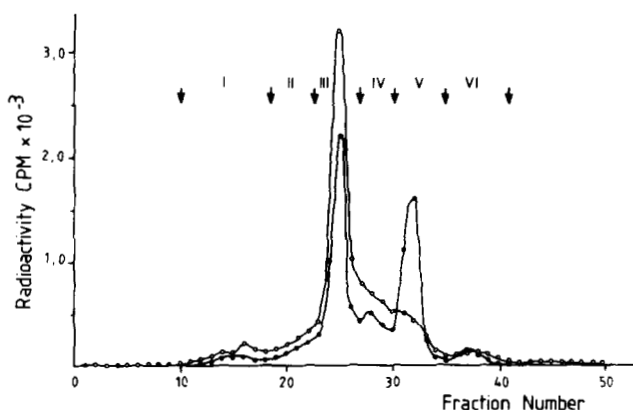

FIG. 1. **Elution pattern of glycophorin A oligosaccharide-alditols from Bio-Gel P-6 column.** The borate-free products obtained after alkaline-borohydride treatment of glycophorin A from control (●) and Cad (○) erythrocyte membranes were solubilized in 1 ml of 1% acetic acid and applied to Bio-Gel P-6 column (1.5 × 50 cm) equilibrated in 1% acetic acid. Fractions of 1 ml were collected and monitored for radioactivity. *I* to *VI*, the six fractions which were pooled, concentrated, and further processed for analysis.

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 ¹H-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-GalNAcNMe, 2,6-di-OMe-Gal, and 1,4,5-tri-OMe-
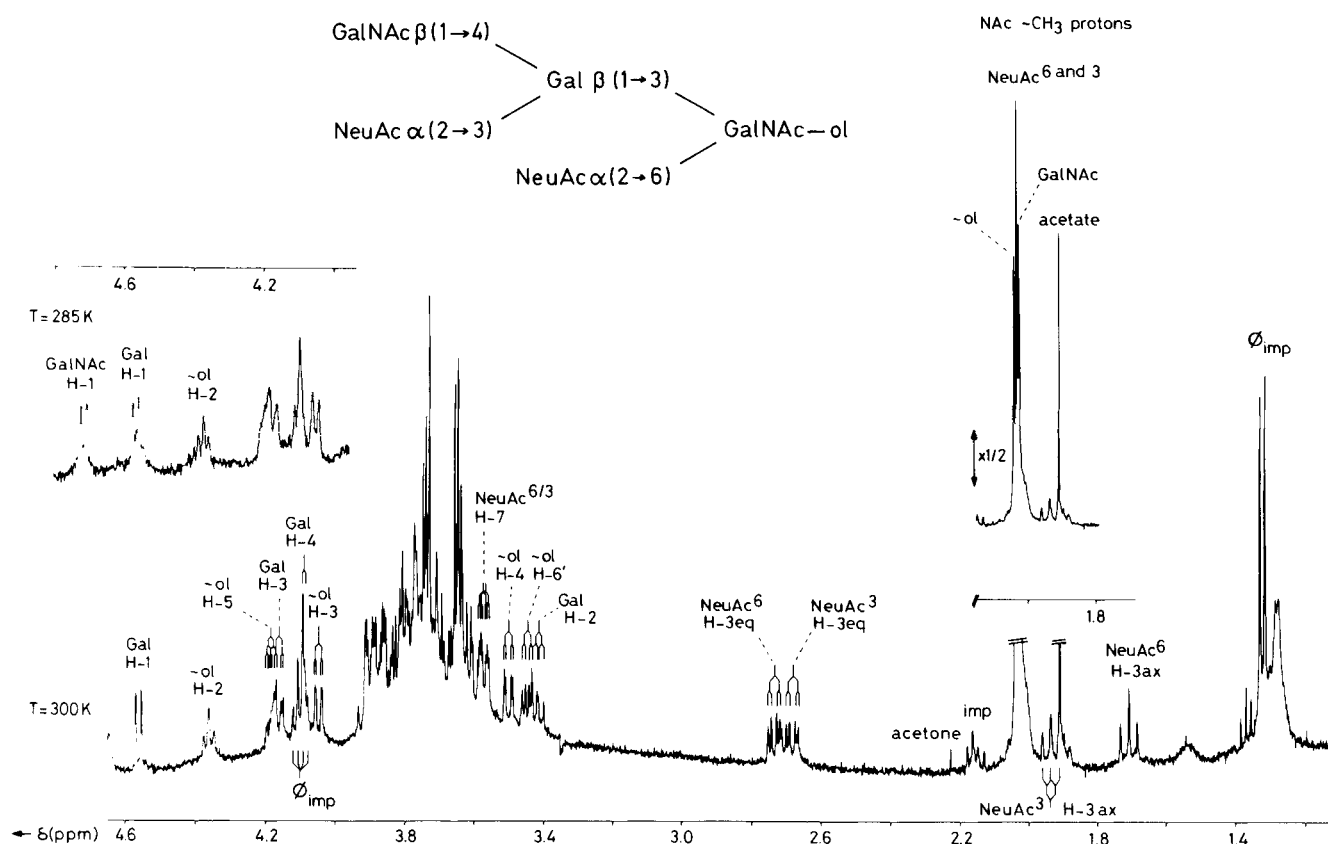
FIG. 2. **The 500-MHz ^{1}H-NMR spectrum of pentasaccharide-alditol fraction II, originating from glycophorin A of erythrocytes with blood group Cad specificity.** The spectrum was recorded in neutral D_2O solution at 300 K. The relative intensity scale of the N-acetyl proton region (see *inset, right*) differs from that of the remaining parts of the spectrum, as indicated. The *inset* at the *left-hand side* shows the $4.0 < \delta < 4.8$ ppm spectral region at 285 K, revealing, in addition, the H-1 doublet of GalNAc, which is hidden under the HOD signal at 300 K. The quartet at $\delta = 4.10$ ppm and the doublet at $\delta = 1.32$ ppm (both marked by Φ) belong to a frequently occurring, nonprotein noncarbohydrate contaminant. Other impurities are indicated by *imp.*

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 oligosaccharide from Cad fraction II contains 1 GalNAc residue and 2 NeuAc residues in terminal, nonreducing positions. The identification of the methyl 2,6-di-OMe-3,4-di-OAc-galactoside indicates that the Gal residue is substituted at positions C-3 and C-4.

In order to elucidate the complete primary structure of the pentasaccharide-alditol, the Cad fraction II sample was analyzed by 500-MHz ^{1}H-NMR spectroscopy. The overall spectrum, recorded at 300 K, is presented in Fig. 2. Relevant ^{1}H chemical shift data at this probe temperature are listed in Table II. The signals in the spectrum were assigned by using the ^{1}H-NMR data, acquired at 500 MHz, of the reference substances GgOse$_3$,[3] that is, GalNAcβ(1→4)Galβ(1→4)Glc, and II^{3}NeuAc-GgOse$_3$, 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)Galβ(1→3)[NeuAcα(2→6)]GalNAc-ol (17, 20) (see Table II).

The chemical shift of H-2 ($\delta = 4.360$ 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 ($\delta = 4.181$ ppm) and H-6' ($\delta = 3.445$ 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-3_{ax} = 1.707 ppm; δH-3_{eq} = 2.733 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 $\delta = 1.933$ ppm (H-3_{ax}) and $\delta = 2.681$ (H-3_{eq}). It should be noted that the H-3_{ax} triplet is partly obscured by the singlet at $\delta = 1.908$ 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 ($\delta = 2.032$ ppm). From comparison with the data for the ganglioside oligosaccharide II^{3}NeuAc-GgOse$_3$ (see Table II), it can be inferred that this very typical set of H-3 chemical shifts, in particular, δH-3_{ax} $\simeq$ 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 $\delta \simeq 4.16$ 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 $\delta = 2.025$ ppm (see Fig. 2). However, at 300 K, only one anomeric signal could be observed (at $\delta = 4.561$ ppm) instead of the two expected; thus, no H-1 doublet for the GalNAc residue was observable. In order to attempt visual-

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[3] Nomenclature for liquids follows the recommendation of the IUPAC-IUB Commission on Biochemical Nomenclature.

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TABLE II

¹H 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	Anomer of oligosaccharide	GalNAcβ(1→4)\\Galβ(1→4)Glc (GgOse$_3$)	GalNAcβ(1→4)\\Galβ(1→4)Glc /NeuAcα(2→3) (II^{3}NeuAc-GgOse$_3$)	Galβ(1→3)\\GalNAc-ol /NeuAcα(2→6) NeuAcα(2→3) (Classical tetrasaccharide)	GalNAcβ(1→4)\\Galβ(1→3)\\GalNAc-ol /NeuAcα(2→6) NeuAcα(2→3) (Cad pentasaccharide)
Glc	H-1	α	5.219	5.218		
		β	4.665	4.665		
		β	3.272	3.275		
GalNAc-ol	H-2				4.378	4.360
	H-2				4.067	4.047
	H-3				3.524	3.500
	H-4				4.240	4.181
	H-5				3.475	3.445
	H-6'					
	NAc				2.042	2.039
Gal	H-1	α	4.441	4.530	4.541	4.561
		β	4.439	4.528		
	H-3	α	ND[b]	4.153	4.117	4.162
		β		4.147		
	H-4	α,β	4.096	4.116	3.927	4.091
GalNAc	H-1	α	4.638	4.744		4.714[c]
		β	4.633	4.741		
	H-2	α	3.419	3.370		3.416
		β	3.410	3.361		
	H-4	α,β	3.912	3.920		3.92
	NAc	α	2.057	2.018		2.025
		β	2.054	2.015		
NeuAc^{3}[d]	H-3ax	α,β		1.923	1.800	1.933
	H-3eq	α,β		2.664	2.774	2.681
	NAc	α,β		2.032	2.032	2.032
NeuAc^{6}[d]	H-3ax				1.692	1.707
	H-3eq				2.723	2.733
	NAc				2.032	2.032

[a] All data were acquired at 500 MHz, for neutral solutions of the compounds in D₂O 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 $\delta = 4.758$ ppm to $\delta = 4.922$ ppm), thereby enabling one to observe the spectral region upfield from $\delta \simeq 4.85$ 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 $\delta = 4.714$ ppm ($J_{1,2} = 7.6$ Hz) and at $\delta = 4.564$ ppm ($J_{1,2} = 8.3$ Hz), respectively. The combination of the chemical shift and the $J_{1,2}$ 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 disialo tetrasaccharide-alditol with a GalNAc residue in $\beta(1\rightarrow4)$-linkage to Gal. Attachment of this GalNAc residue results in a downfield shift for H-1 of the substituted Gal ($\Delta\delta = 0.02$ ppm). For a similar extension, namely, that from Gal$\beta(1\rightarrow4)$Glc (lactose) (26) to GgOse$_3$, 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,C-4-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 ^{1}H-NMR analysis in combination with methylation analysis: GalNAc$\beta(1\rightarrow4)$[NeuAc$\alpha(2\rightarrow3)$]Gal$\beta(1\rightarrow3)$[NeuAc$\alpha(2\rightarrow6)$]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 glycophorin A. It is not known how many of such pentasaccharide chains are present on a single glycophorin A molecule. However, the 3,000-dalton increase in apparent molecular mass of glycophorin 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 also 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. biflorus* lectin, the specificity of this lectin reported to be highly specific for terminal α-linked GalNAc (28) should be reconsidered. Interestingly, the GM$_2$ ganglioside containing the trisaccharide sequence GalNAc$\beta(1\rightarrow4)$[NeuAc$\alpha(2\rightarrow3)$]Gal$\beta(1\rightarrow\cdot)$ (19, 29) is a strong inhibitor of *D. biflorus* 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 GalNAc$\beta(1\rightarrow4)$Gal isolated after hydrazinolysis of the Tamm-Horsfall glycoprotein prepared from Sd(a+) individuals, also inhibited the *D. biflorus* lectin. Finally, our results lend firm biochemical support to the predicted direct relationship between Cad and Sd^a determinants (3) since both receptors are characterized by a terminal GalNAc$\beta(1\rightarrow4)$Gal$\beta(1\rightarrow\cdot)$ sequence. Further studies should determine to which extent the two determinants are similar and whether the same β-N-acetylgalactosaminyltransferase is involved in their biosynthesis.

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[4] J.-P. Cartron, D. Blanchard, unpublished results.

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