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Comparative study of glycophorin A derived *O*-glycans from human Cad, Sd(a+) and Sd(a−) erythrocytes

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Glycophorin A was purified from the erythrocyte membranes of blood group Cad, Sd(a+) and Sd(a−) donors and the oligosaccharide alditols, obtained after alkaline borohydride degradation, separated by h.p.l.c. on an alkylamine silica gel column, were characterized by sugar analysis. Structure determination of the major acid components by methylation analysis, g.l.c.–m.s. and ^{1}H-n.m.r. indicated that the three blood group Cad red cells under study (samples Cad., Bui. and Des.) carry the same pentasaccharide GalNAc(β1-4)[NeuAc(α2-3)]Gal(β1-3)[NeuAc(α2-6)]GalNAc-ol (Cad determinant) but in different amounts. This pentasaccharide, however, was absent from glycophorin A of Sd(a+) and Sd(a−) donors, suggesting that the Sd^a determinant is not associated with glycophorins. It was calculated that glycophorin A from the original Cad donor (Cad.) carries about 12 *O*-glycosidically linked pentasaccharide chains per molecule whereas only 2–3 of these chains were present in the samples from the two other unrelated Cad individuals (Bui. and Des.) It is well known from quantitative agglutination studies that the proportion of red cells which can be agglutinated by the *Dolichos biflorus* lectin varies from one Cad blood sample to another. Some are completely agglutinated (Cad. donor) whereas others are only partially agglutinated (Bui. and Des. donors) suggesting that some red cells might not carry the Cad determinants. From the results presented above and sodium dodecyl sulphate/polyacrylamide-gel electrophoresis studies it is suggested that Cad red cells from Bui. and Des. do not carry a mixture of glycophorin A molecules with or without the Cad pentasaccharides but a spectrum of glycoprotein molecules with varying amounts of Cad determinants.

INTRODUCTION

The blood group Cad antigen is a carbohydrate determinant carried both by sialoglycoproteins and gangliosides of the human red cell membranes (Cartron & Blanchard, 1982; Blanchard *et al.*, 1985). The major *O*-glycan isolated from Cad glycoproteins is a pentasaccharide of structure GalNAc(β1-4)[NeuAc(α2-3)]Gal(β1-3)[NeuAc(α2-6)]GalNAc (Blanchard *et al.*, 1983) whereas glycophorin A from normal red cells carries predominantly a sialotetrasaccharide NeuAc(α2-3)Gal(β1-3)[NeuAc(α2-6)]GalNAc (Thomas & Winzler, 1969). A novel ganglioside probably derived from sialosylparagloboside by addition of a βGalNAc residue was also recently identified on red cells from the original Cad donor as well as from two other unrelated Cad individuals (Blanchard *et al.*, 1985).

The Cad antigen is serologically and biochemically related to the Sd^a blood group antigen. Indeed (i) Cad red cells are strongly agglutinated by human anti-Sd^a serum (Sanger *et al.*, 1971) and both the glycoproteins and gangliosides isolated from Cad red cells inhibited this antiserum (Blanchard *et al.*, 1985; Herkt *et al.*, 1985), and (ii) the same terminal non-reducing trisaccharide, GalNAc(β1-4)[NeuAc(α2-3)]Gal, has been characterized from Cad red cells and the Sd^a-active glycoprotein (Tamm–Horsfall glycoprotein) from human urine (Donald *et al.*, 1983). However, the red cell component(s) carrying the Sd^a antigen has not yet been identified and the exact relationship between Cad and Sd^a specificities is still obscure.

In this report we have analysed the alkali-labile oligosaccharides of glycophorin A purified from the red cells of two unrelated Cad donors and from individuals of Sd(a+) and Sd(a−) phenotype. We have also conducted some experiments in order to determine whether the mixed-field agglutination pattern observed after interaction between the *Dolichos biflorus* lectin and most of the Cad samples (Cazal *et al.*, 1971; Lopez *et al.*, 1975) represents a true mixture of antigen 'positive' and 'negative' red cells.

MATERIALS AND METHODS

Neuraminidases from *Vibrio cholerae* (1 unit/ml) and from *Arthrobacter ureafaciens* (0.02 units/mg) were purchased from Behringwerke AG (Marburg/Lahn, Germany). *Dolichos biflorus* lectin was obtained from E.Y. Laboratories (Sochibo, Boulogne, France). NaB^3H_4 (16 Ci/mmol) was from CEA (Gif-sur-Yvette, France) and En^{3}Hance from New England Nuclear (Boston, MA, U.S.A.). X-ray films (RX) were from Fuji. The amino-AS-5A (5 μm; 0.46 cm × 25 cm) h.p.l.c. column

Abbreviations used: SDS, sodium dodecyl sulphate; sialosylparagloboside, NeuAc(α2-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc-ceramide; GalNAc-ol, *N*-acetylgalactosaminitol.

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Table 1. Carbohydrate composition of glycophorin A purified from Cad, Sd(a+) and Sd(a−) erythrocytes and its β-elimination products

Sugar analysis was performed as described by Zanetta *et al.* (1972) using a Varian 1400 gas chromatograph equipped with a flame ionization detector. Trifluoroacetyl derivatives of methylglycosides were separated on a glass column (300 cm × 0.3 cm) filled with OV-210 5% silicon on chromosorb W(HP) DMCS, 100–200 mesh. Nitrogen flow was 7.5 ml/min; the column temperature was raised from 100 °C to 210 °C at 2 °C/min.

Fraction	Molar ratio of carbohydrates					
	Gal	Man	GalNAc	GlcNAc	NeuAc	GalNAc-ol
Glycophorin A from donor:						
Cad.	1	0.20	1.58	0.29	1.58	—
Bui.	1	0.19	0.85	0.39	1.63	—
Sd(a+) or Sd(a−)	1	0.24	0.82	0.31	1.64	—
β-Elimination products of glycophorin A from donor:						
Cad.	0.98	0	1.18	0	2.15	1
Bui.	1.28	0	0.24	0	2.48	1
Sd(a+)	0.78	0	0	0	1.81	1
Sd(a−)	0.68	0	0	0	1.90	1

was purchased from Touzart et Matignon (Vitry-sur-Seine, France). ^2H_2O (99.96 atom% ^2H) was from Aldrich (Milwaukee, WI, U.S.A.). Sd(a−) and Sd(a+) red cells were collected from blood donors at the Centre National de Transfusion Sanguine (Paris, France). Red cells from the original Cad individual (Cad., group B) (Cazal *et al.*, 1968) were kindly provided by Mme Monis, Centre de Transfusion Sanguine de Montpellier, France. Two other Cad positive samples (Bui., group B; Des., group O) were obtained through the courtesy of Dr. Guimbretière and Dr. Assan (Centres de Transfusion Sanguine de Nantes and Cannes, France, respectively).

Lipid-free glycophorin A from Sd(a−), Sd(a+) and Cad red cells was obtained by following a published procedure (Cartron & Blanchard, 1982). *O*-Glycosidically linked oligosaccharides were released by alkaline borohydride treatment (Blanchard *et al.*, 1983). Oligosaccharide alditols were desalted on a Bio-Gel P-6 column (1.5 cm × 90 cm) equilibrated in 0.5% acetic acid and further fractionated by h.p.l.c. on an Amino-AS-5A column according to Herkt *et al.* (1985). Characterization of the purified oligosaccharide alditols was achieved by t.l.c., analysis of carbohydrate composition and g.l.c.–m.s. as described (Herkt *et al.*, 1985). ^1H-n.m.r. spectroscopic analysis was performed after repeated exchange in ^2H_2O using a Brucker spectrometer operating at 400 MHz in the Fourier transform mode at probe temperature of 285 and 300 K.

Purified glycophorin A (50 μg) was incubated overnight with 0.01 unit of *Vibrio cholerae* neuraminidase in 50 μl of 0.05 M-acetate buffer, pH 5.5, containing 0.009 M-$CaCl_2$ (buffer A) at 37 °C. The glycoproteins were then treated for 15 min at 37 °C with 0.01 M-sodium metaperiodate, reduced with 150 μCi of NaB^3H_4 for 30 min at room temperature and desalted by passage over a column of Sephadex G-50 (1.5 cm × 15 cm) equilibrated and developed in 0.5% acetic acid. Fractions (0.5 ml) were monitored by counting an aliquot in a liquid-scintillation counter and analysed by SDS/polyacrylamide-gel electrophoresis as described below.

Intact red cells were treated with increasing amounts (0–1 unit/ml) of neuraminidase either from *Vibrio cholerae* or from *Arthrobacter ureafaciens*. Following 30 min incubation at 37 °C the supernatant obtained by centrifugation (1500 *g*, 10 min) was filtered through a Millipore filter (0.45 μm) and submitted to sialic acid analysis (Warren, 1959).

Untreated or neuraminidase-treated cells (*Vibrio cholerae*; 0.1 unit/ml) were labelled with NaB^3H_4 after periodate oxidation according to Gahmberg & Anderson (1977).

Membranes were obtained by hypo-osmotic lysis (Steck & Kant, 1974), solubilized in SDS and submitted to SDS/polyacrylamide-gel electrophoresis in a 10% polyacrylamide gel (Laemmli, 1970). Sialoglycoproteins were revealed by periodate/Schiff staining (Fairbanks *et al.*, 1971) or by fluorography performed by incubating the gel in En^{3}Hance scintillation cocktail and exposure to X-ray film at −80 °C. Inhibition–hemagglutination tests with human anti-Sd^a serum (Dr. Messeter, Lund, Sweden) were performed in microtitre plates as described before (Cartron & Blanchard, 1982).

RESULTS

Biological activity of glycophorin A from Cad, Sd(a+) and Sd(a−) red cells

Preliminary experiments using agglutination–inhibition tests indicated that the glycophorin A purified from the original Cad donor (Cad.) inhibited strongly the agglutination of Cad erythrocytes (donor Des.) by the human anti-Sd^a antibody (0.12 μg dry wt./40 μl total volume). About 25 times more glycophorin (3.10 μg) from another Cad individual (donor Bui.) was required for complete inhibition of this antibody. The preparations from Sd(a+) and Sd(a−) red cells were inactive at the concentration used (> 50 μg/40 μl), suggesting that the Sd^a determinant is not present on the glycophorin A molecules from these donors.

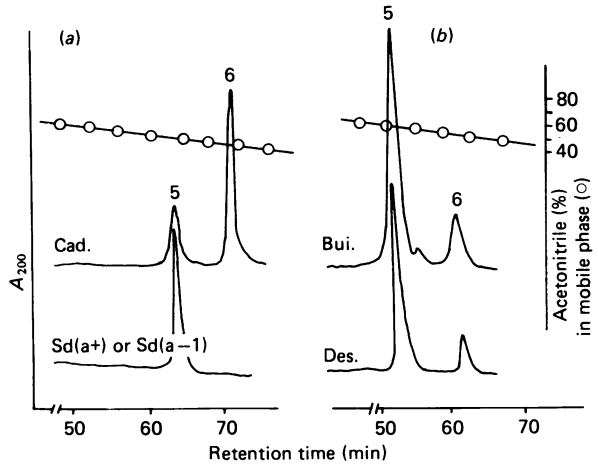

Fig. 1. Separation by h.p.l.c. of the oligosaccharide alditols obtained after alkaline borohydride treatment of glycophorin A from Cad (Cad., Bui., Des.), Sd(a+) and Sd(a−) donors

β-Elimination products, desalted on a Bio-Gel P-6 column (90 cm × 15 cm) equilibrated in 0.5% acetic acid, were injected on an Amino AS 5A (0.4 cm × 25 cm, 5 μm) h.p.l.c. column. Separation occurred at 1 ml/min by isocratic elution for 25 min in acetonitrile/15 mM-phosphate buffer, pH 5.2 (4:1, v/v) followed by a linear gradient in which the ionic strength of the buffer increased at the rate of 0.6% (v/v) per minute. Sugars were detected by u.v. absorbance at 200 nm. Only the relevant part of the elutions patterns (retention time 50–80 min) showing the separation of oligosaccharide 5 {NeuAc(α2-3)Gal(β1-3)[NeuAc(α2-6)]GalNAc-ol} from oligosaccharide 6 {GalNAc(β1-4)[NeuAc(α2-3)]Gal(β1-3)[NeuAc(α2-6)]GalNAc-ol} are presented.

Carbohydrate composition of glycophorin A prepared from Cad, Sd(a+) and Sd(a−) red cells

We have shown earlier that the GalNAc content was much higher in glycophorin A isolated from the donor Cad. than from Cad-negative red cells (Cartron & Blanchard, 1982). The ratio NeuAc:GalNAc was 2.0 for Cad-negative cells but only 1.0 for Cad-positive cells. However, the carbohydrate analysis of a glycophorin A preparation from the Cad-positive donor Bui. did not show a significant increase in GalNAc content as compared with glycophorin samples from Sd(a+) and Sd(a−) donors (Table 1). Since the content of other sugars is similar in these samples it is suggested that only a few numbers of Cad pentasaccharides are present on Bui. erythrocytes.

Analysis of oligosaccharide alditols obtained by alkaline borohydride cleavage of glycophorin A

The oligosaccharide alditols released by alkaline borohydride degradation of glycophorin A preparation from Cad, Sd(a+) and Sd(a−) red cells were submitted to sugar analysis.

As shown in Table 1, the sugar-alditols isolated from Cad donors Bui. and Cad. after β-elimination contain GalNAc in addition to GalNAc-ol. The relative amount of GalNAc was 5 times higher in the Cad. sample as compared to the Bui. sample. A low amount of GalNAc was also present in the β-elimination products from Des., another Cad individual (results not shown). On the other hand, unreduced GalNAc was not detectable in the oligosaccharide alditols isolated from Sd(a+) and Sd(a−) red cells.

Analysis of the oligosaccharide alditols from Cad. glycophorin A by h.p.l.c. on an alkylamine silica gel column resulted in the separation of six major acidic oligosaccharides (Herkt *et al.*, 1985). The disialylated components were identified as the sialotetrasaccharide and the Cad-specific pentasaccharide (Blanchard *et al.*, 1983) (oligosaccharides 5 and 6, respectively, Fig. 1*a*). When β-elimination products of glycophorin A from Sd(a−) or Sd(a+) erythrocytes were submitted to this separation procedure only the tetrasaccharide (oligosaccharide 5) was identified (Fig. 1*a*). We were unable to detect any oligosaccharide with a retention time corresponding to that of the Cad-specific pentasaccharide (oligosaccharide 6) even using the highest u.v. absorbance

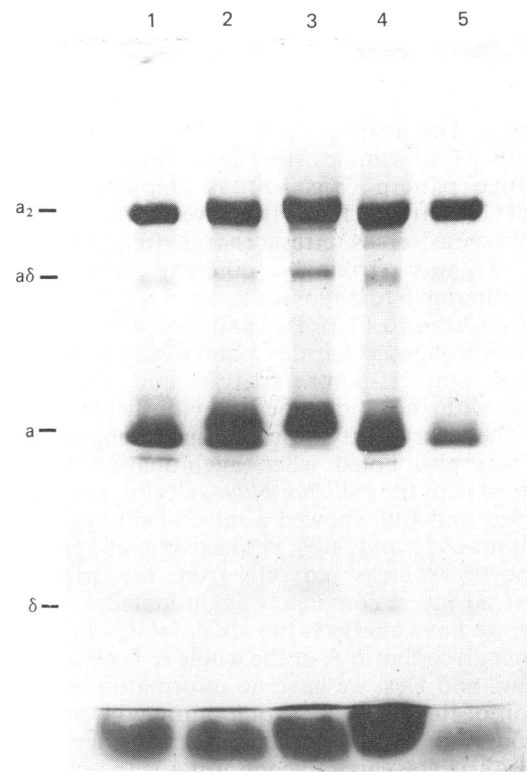

Fig. 2. Polyacrylamide-gel electrophoresis of red cell membranes from control and Cad erythrocytes

About 150 μg of membrane protein solubilized in 10 mM-Tris/HCl, pH 6.8, containing 1 mM-EDTA and 5% SDS was loaded on the gel. Electrophoresis was performed at 40 mA constant current on a 1.5 mm thick gel slab containing 10% (w/v) acrylamide and 0.27% (w/v) bisacrylamide with a stacking gel of 3% (w/v) in the discontinuous buffer of Laemmli (1970). The gel was stained by the periodate/Schiff procedure (Fairbanks *et al.* 1971). Membranes are: 1, control; 2, mixture of control (75 μg) and Cad. (75 μg); 3, Cad.; 4, Bui.; 5, Des. Nomenclature of sialoglycoproteins according to Anstee *et al.* (1979); α_2 and α, respectively the dimer and monomer of glycophorin A; δ, monomer of glycophorin B; αδ, heterodimer of glycophorins A and B.

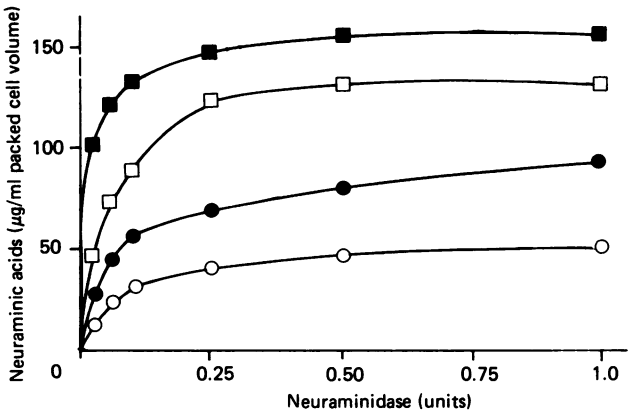

Fig. 3. Enzymic removal of sialic acids from control and Cad. red cells

Cad. (○, ●) and control (□, ■) red cells were submitted to neuraminidase from *Vibrio cholerae* (■,●) and *Arthrobacter ureafaciens* (□, ○) as described in the Materials and methods section. Sialic acid liberated in the supernatants was determined by the Warren (1959) procedure.

sensitivity. The analysis of the oligosaccharide alditols from other Cad samples (Bui. and Des.) gave a similar qualitative pattern (Fig. 1*b*) as compared with the original Cad pattern (Fig. 1*a*). However, the quantitative ratio of penta- versus tetrasaccharide (oligosaccharide 6 versus 5) was strikingly different. The ratio of oligosaccharide 6 to oligosaccharide 5 was 3.40 for Cad. and only 0.20 and 0.17 for Bui. and Des., respectively. The structure of oligosaccharides 5 and 6 were confirmed by g.l.c.-m.s. and ^{1}H-n.m.r. analysis and shown to be identical with those identified previously (Herkt *et al.*, 1985). The heterogenity observed on the oligosaccharide ratios was also noted when agglutination tests were performed with the *Dolichos biflorus* lectin, since red cells from Des. and Bui. showed a mixed-field agglutination with about 60% and 50%, respectively, of agglutinated erythrocytes whereas red cells from the original Cad donor (Cad.) were completely agglutinated.

Since we have analysed the alkali-labile oligosaccharides from glycophorin A of the whole red cell population from Bui. and Des. we have no information on the distribution of the pentasaccharides among glycophorin A molecules. The mixed-field agglutination suggested that some erythrocytes carry glycophorin A with tetrasaccharides (Cad-negative cells) whereas other cells carry mainly alkali-labile pentasaccharides (Cad-positive cells). In order to test this hypothesis we investigated the periodate/Schiff profile of membrane preparations from several Cad individuals after SDS/polyacrylamide-gel electrophoresis.

SDS/polyacrylamide-gel electrophoresis of erythrocyte membrane glycophorins

It is known that glycophorins A and B from donor Cad. show a decreased mobility in SDS/polyacrylamide-gel electrophoresis corresponding to an increase in the molecular mass of 3 and 2 kDa respectively (Cartron & Blanchard, 1982; Fig. 2, lane 3). When a mixture of Cad-positive (Cad. donor) and Cad-negative membranes is prepared and analysed by gel electrophoresis, two well-separated bands of glycophorin A (band α) as well as glycophorin B (band δ) monomer are resolved (Fig. 2, lane 2). However, only one band in the regions of glycophorin A and B is detectable with the Bui. and Des. samples (Fig. 2, lanes 4 and 5) suggesting that these red cells do not carry two distinct populations of glycophorins.

Taking advantage of the observation that sialic acids from Cad. donor red cells are partially resistant to neuraminidases from *Vibrio cholerae* and *Arthrobacter ureafaciens* (Fig. 3) we studied the incorporation of ^{3}H (from NaB^3H_4) into preparations of glycophorin A from Sd(a−) and Cad samples after neuraminidase treatment and periodate oxidation. Incorporation of ^{3}H was low in periodate-treated glycophorin A from Sd(a−) cells (400 c.p.m./μg) when compared with the radioactivity incorporated into sialic acid residues still present on sialoglycoproteins from Bui. (1200 c.p.m./μg) or Cad (2700 c.p.m./μg). SDS/polyacrylamide-gel electrophoresis showed that the ^{3}H label incorporated into Sd(a−) cells migrated as broad bands (Fig. 4, lane 1) the mobility of which was clearly distinct from those identified in Cad glycoprotein (Fig. 4, lane 3). In addition, the bands from the Sd(a−) sample were faint whereas those from donor

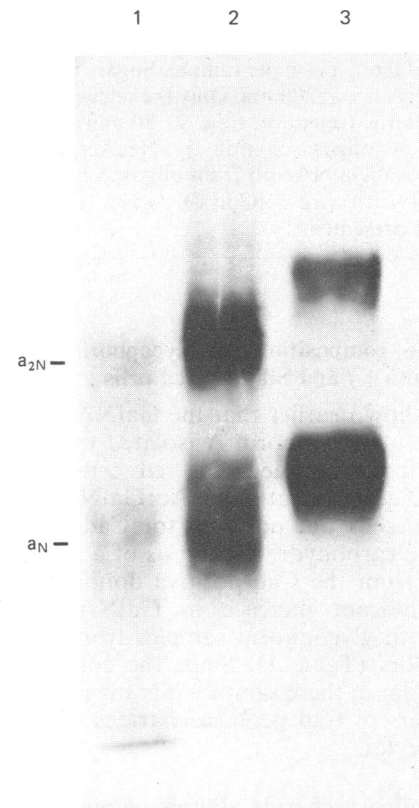

Fig. 4. Polyacrylamide-gel electrophoresis of ^{3}H-labelled glycophorin A preparation from Cad and Sd(a−) individuals

Neuraminidase-treated and ^{3}H-labelled glycophorin A from Sd(a−), Bui. and Cad. donors were loaded on a 10% (w/v) polyacrylamide gel as described in the legend to Fig. 2. The gel was treated for fluorography, dried on a filter paper and exposed to Fuji RX film. Neuraminidase-treated glycophorin A was from: 1, Sd(a−); 2, Bui.; 3, Cad. α_{2N} and α_N stand for desialylated glycophorin A (α) dimeric and monomeric forms respectively.

Cad. were of strong intensity and migrated clearly, as expected for glycophorin A dimer and monomer. Interestingly, the glycophorin A from Bui. is seen as diffuse bands with an intermediate mobility and intensity (Fig. 4, lane 2).

Very similar results were obtained after labelling of neuraminidase-treated red cells, indicating that glycophorin A from Cad. is also strongly labelled and displays an abnormal mobility whereas glycophorin B from Bui. and Des. is less intensely labelled and migrates as a broad band (results not shown).

DISCUSSION

On human red cell glycoproteins the blood group Cad determinant is associated with alkali-labile pentasaccharides of which the structure has been recently elucidated (Blanchard *et al.*, 1983). The presence of a (β1-4)GalNAc residue on these sugar chains results in the resistance of the (α2-3)-linked sialic acids to hydrolysis by neuraminidases from *Vibrio cholerae* and *Arthrobacter ureafaciens*. This phenomenon is also observed with the GM_2 ganglioside which contains a closely related terminal non-reducing sequence (Harris & Thornton, 1978). In addition, the terminal βGalNAc unit provides the resistance of the Cad red cells towards infestation *in vitro* by the malarial parasite *Plasmodium falciparum* (Cartron *et al.*, 1983). The findings described above were obtained using the red cells from the first case of Cad erythrocytes (Cad. donor) described by Cazal *et al.* (1968). In this report, we have provided further studies on two unrelated cases of Cad samples (Des. and Bui.) and both immunological as well as biochemical analysis demonstrate that these erythrocytes carry the same oligosaccharide determinants present on Cad. red cells, but in lower amount. The ratio of pentasaccharide versus sialotetrasaccharide was higher on glycophorin A from the original Cad sample (value 3.40) than on glycophorin A from Bui. and Des. (value 0.20 and 0.17 respectively).

Since glycophorin A carries 15 alkali-labile sialotetrasaccharide chains (Tomita *et al.*, 1978) we calculated that glycophorin A from the original Cad. donor carries about 12 pentasaccharide structures whereas the same calculation gives 2–3 GalNAc-containing oligosaccharide chains per molecule of glycophorin A from Bui. or Des. Analysis of chymotryptic peptides derived from the glycophorin A of Bui. red cells showed that, in contrast to the observation on M_1 reactive red cells where the additional GlcNAc residues are mainly present on sugar chains located in the *N*-terminal region of the molecule (Baumeister *et al.*, 1981), the additional GalNAc residues responsible for blood group Cad specificity are randomly distributed among alkali-labile oligosaccharides (W. Dahr, D. Blanchard & J. P. Cartron, unpublished work). Polyacrylamide-gel electrophoresis suggests that Cad erythrocytes from Bui. or Des., which exhibited a mixed-field agglutination pattern with the *Dolichos biflorus* lectin, carry a spectrum of glycophorin A molecules substituted by variable amounts of GalNAc residues and not a mixture of GalNAc-positive and GalNAc-negative molecules. These results correlate with serological studies showing that there are no distinct subpopulations of Cad erythrocytes, since part of the separated Cad-negative cells are again agglutinated by the *Dolichos biflorus* lectin (D. Blanchard & J. P. Cartron, unpublished work). This finding is clearly different from the true mixed-field seen with Tn red cells, where there are two distinct subpopulations of cells, Tn-positive and Tn-negative, the latter being unagglutinable by Tn-specific reagents (Cartron *et al.*, 1980).

The Sd^a blood group determinant was identified as a carbohydrate structure carried by the urinary Tamm–Horsfall glycoprotein (Donald *et al.*, 1983). It shares with the Cad determinant the GalNAc(β1-4)[NeuAc(α2-3)]-Gal sequence which explains the strong reactivity of Cad cells with the anti-Sd^a antibody (Sanger *et al.*, 1971). The results presented in this study show that glycophorin A from Sd(a+) cells does not carry detectable amounts of the pentasaccharides present on Cad red cells. From recent findings we suggest that the Sd^a determinant of the red cells might be carried by red cell gangliosides rather than glycoproteins (Blanchard *et al.*, 1985). It is therefore expected that the (β1-4)GalNAc transferases involved in biosynthesis of Cad and Sd^a antigens might also be different. (β1-4)GalNAc transferases were recently reported in guinea-pig kidney (Serafini-Cessi & Dall'Olio, 1983) and in a murine cytotoxic T-cell line (Conzelmann & Kornfeld, 1984) and both enzymes were able to transfer [^{14}C]GalNAc from UDP-[^{14}C]GalNAc to human glycophorin as expected for the blood group Cad enzyme. However, experiments from our group (Piller *et al.*, 1985) suggest that the (β1-4)GalNAc from human kidney has a different substrate specificity since it is inactive towards native or partially desialylated and/or resialylated glycophorin A preparations. Further studies are required to identify the biochemical basis of Sd^a specificity.

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