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Association of human erythrocyte membrane glycoproteins with blood-group Cad specificity

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Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of erythrocyte membranes from a blood-group-B individual with the rare Cad phenotype indicates a lower-than-normal mobility of the main sialoglycoproteins, suggesting an increase in apparent molecular mass of 3 kDa and 2 kDa respectively for glycoprotein α (synonym glycophorin A) and glycoprotein δ (synonym glycophorin B). Since the chief structural determinant of Cad specificity is *N*-acetylgalactosamine, the membrane receptors have been isolated by affinity binding on immobilized *Dolichos biflorus* (horse gram) lectin. The predominant species eluted from the gel was the abnormal glycoprotein α, whereas in control experiments no material could be recovered from the adsorbant incubated with group-B Cad-negative erythrocyte membranes. After partition of the membranes with organic solvents, the blood-group-Cad activity was found in aqueous phases containing the sialoglycoproteins, but not in the organic phases containing simple or complex glycolipids, which, however, retained the blood-group-B activity. The carbohydrate composition of highly purified lipid-free glycoprotein α molecules prepared from Cad and control erythrocytes was determined. Interestingly the molar ratio of *N*-acetylneuraminic acid to *N*-acetylgalactosamine was equal to 2 : 1 in the case of controls and equal to 1 : 1 in the case of Cad erythrocytes. Taken together these results suggest that Cad specificity is defined by *N*-acetylgalactosamine residues carried by the alkali-labile oligosaccharide chains attached to the erythrocyte membrane sialoglycoproteins.

The Cad antigen was described in 1968 by Cazal *et al.* as a dominant character found in three members of a Mauritian family. The cells from these individuals, grouped as O or B, were, however, strongly reactive with the lectin of *Dolichos biflorus* (horse gram) seeds (DBA) and polyagglutinable by all ABO-compatible sera except their own. It was later shown that these Cad erythrocytes were also agglutinated by *Helix pomatia* (Roman or vineyard snail), *Helix aspersa* (garden snail) and *Salvia horminum* (salvia) lectins (Bird & Wingham, 1971, 1974; Uhlenbruck *et al.*, 1971; Sanger *et al.*, 1971) as well as by a naturally occurring antibody found in chicken serum (Bizot & Cayla, 1972). In France the frequency of Cad antigen is 0.071 (Gerbal *et al.*, 1976*a*).

The strength of the Cad antigen estimated by agglutination with DBA varies from one sample to another, and a subdivision into Cad_1, Cad_2 and Cad_3 categories has been proposed in the descending order of reactivity (Cazal *et al.*, 1971).

Hemagglutination inhibition of Cad red cells by DBA has also shown that the chief structural determinant of Cad specificity is *N*-acetylgalactosamine (Bird & Wingham, 1971; Sanger *et al.*, 1971). However, the red-cell membrane components carrying Cad activity have not yet been isolated.

In 1967 the antigen Sd^a (Sid blood group) was independently discovered (MacVie *et al.*, 1967; Renton *et al.*, 1967) on more than 90% of Caucasian red cells, and numerous sources of Sd^a substances have been identified in body fluids and tissues. For instance, urine samples from 96% of human individuals contain large amounts of Sd^a

Abbreviations used: lectins: DBA, *Dolichos biflorus* (horse gram) agglutinin; HPA, *Helix pomatia* (Roman or vineyard snail) agglutinin; WGA, wheat-germ (*Triticum vulgaris*) agglutinin; MPA, *Maclura pomifera* (osage orange seed) agglutinin; PHA-E, *Phaseolus vulgaris* (red kidney bean) agglutinin. SDS, sodium dodecyl sulphate; GPα, GPδ, GPβ, and GPγ, glycoproteins α, δ, β and γ; THGP, Tamm–Horsfall glycoprotein.

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antigen (Morton *et al.*, 1970). A great variation in the reactivity of the red-cell Sd^a antigen from one individual to another was noticed in the earliest reports (MacVie *et al.*, 1967; Renton *et al.*, 1967) and Sanger *et al.* (1971) made the observation that all Cad samples under their study reacted strongly with anti-Sd^a antibodies. They also noticed that red cells previously known to have a highly reactive form of Sd^a were agglutinated by DBA, therefore suggesting that Cad was a highly reactive form of Sd^a termed 'super Sid' or Sd(a++) cells. There is, however, some controversy about the identity between Cad and Sd^a (Bizot, 1972; Gerbal *et al.*, 1976*b*), which could be resolved only by purification and analysis of the respective structures.

The carbohydrate nature of Sd^a antigen was demonstrated by the partially purified material isolated from human urine being shown to be resistant to proteolytic cleavage (trypsin, pepsin, papain) and to be destroyed by periodate oxidation (Morton & Terry, 1970). More recently investigations carried out on urinary substances have suggested that at least two molecular species, namely the Tamm–Horsfall glycoprotein (THGP) and a mucin, are associated with Sd^a specificity.

(i) THGP is an 82000-M_r glycoprotein occurring in all normal urine samples as a secretory product of the renal tubules (Tamm & Horsfall, 1950; Cornelius *et al.*, 1965; Sikri *et al.*, 1979). THGP isolated from Sd(a+) urine samples is a strong inhibitor of anti-Sd^a antibodies and contains 1–2% of *N*-acetylgalactosamine, whereas THGP isolated from Sd(a–) urine samples does not inhibit anti-Sd^a antibodies and contains less than 0.1% *N*-acetylgalactosamine (Soh *et al.*, 1980).

(ii) A mucin carrying Sd^a specificity has also been isolated recently from pooled blood-group-O urines by affinity chromatography on HPA–sepharose column (Cartron *et al.*, 1982). The purified material has apparent M_r 340000 and a high content of *N*-acetylgalactosamine, but analysis of its amino acid and sugar composition indicates that it is clearly distinct from THGP.

These observations established that *N*-acetylgalactosamine is involved both in the Cad and Sd^a determinants. In the present study we have shown that Cad determinants are carried by several red-cell membrane glycoproteins and provide evidence that, on GPα (synonym glycophorin A), these antigens are located on alkali-labile saccharide chains substituted with new *N*-acetylgalactosamine residues.

Materials and methods

Reagent-grade chemicals were used throughout. Lactoperoxidase from bovine milk (60 units/mg) was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Neuramidase from *Vibrio cholerae* (1 unit/ml) was purchased from Behringwerke AG (Marburg-lahn, Germany). DBA was obtained from Serva (Le Perray en Yveline, France). Agarose derivatives of the DBA, PHA-E and MPA were obtained from E.Y. Laboratories (Sochibo, Boulogne, France). HPA–Ultrogel was from IBF-Pharmindustrie (Villeneuve la Garenne, France) and WGA–Sepharose from Pharmacia (Bois d'Arcy, France). The capacity of the lectin gels was between 2 and 6 mg of purified lectin per ml of packed gel.

The chemicals used for polyacrylamide-gel electrophoresis were acrylamide, bisacrylamide and *NNN'N'*-tetramethylethylenediamine from Bio-Rad Laboratories (Touzart et Matignon, Vitry sur Seine, France) and SDS from Prolabo (Paris, France).

$Na^{125}I$ (16.9 mCi/μg of iodine) was bought from The Radiochemical Centre (Amersham, Bucks., U.K.). Ammonyx-LO was purchased from Franconyx (Lyon, France) and sodium deoxycholate from Koch–Light Laboratories (Sochibo, Boulogne, France).

The red cells from the original Cad individual (group B) were kindly provided by Mrs. Monis, Centre de Transfusion Sanguine de Montpellier, Montpellier, France (Cazal *et al.*, 1968). 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.

Intact red cells were radioiodinated by the lactoperoxidase procedure derived from that described by Shin & Carraway (1974). Red-cell ghosts were prepared by the method of Steck & Kant (1974) and submitted to SDS/10% (w/v)-polyacrylamide-gel electrophoresis performed in the discontinuous buffer system of Laemmli (1970) as previously described (Blanchard *et al.*, 1982). Components were identified after periodic acid/Schiff or Coomassie Blue R250 staining (Fairbanks *et al.*, 1971) and autoradiography of the dried gels.

Ghosts prepared from ^{125}I-labelled erythrocytes (1 vol.) were solubilized for 30 min at 0°C in 1 vol. of 100 mM-Tris/HCl buffer (pH 8.0)/0.3 M-NaCl (buffer A) containing 2% (w/v) sodium deoxycholate. Portions of the deoxycholate lysates adjusted to 0.5% (w/v) detergent were incubated with 50 μl of immobilized lectin (DBA–agarose, HPA–Ultrogel, WGA–Sepharose, MPA–agarose and PHA-E–agarose) for 30 min at room temperature. In some experiments, neuraminidase-treated membranes were used and were obtained from ^{125}I-radiolabelled red cells incubated for 30 min at 37°C with neuraminidase (0.1 unit/ml of packed control red cells or 0.5 unit/ml of packed Cad red cells). Control experiments were carried out in the presence of the appropriate inhibitory sugar at 0.3 M concentration (*N*-acetylgalactosamine for DBA, HPA, PHA-E; *N*-acetylglucosamine for WGA, and galactose for MPA). Unbound material was removed from

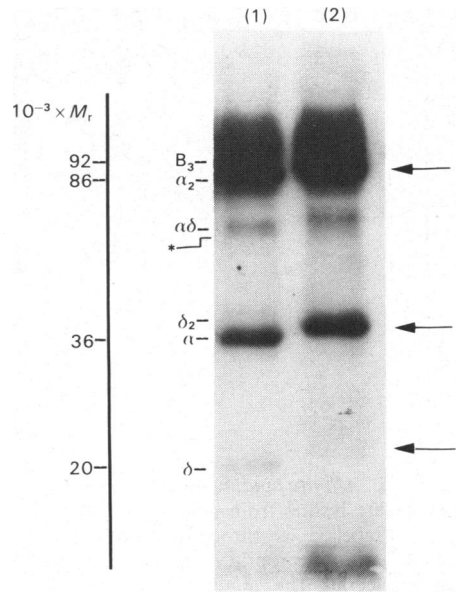


Fig. 1. *SDS/polyacrylamide-gel electrophoresis of radioiodinated red-cell membranes from Cad and control erythrocytes*

Packed red cells (0.5 ml) were ^{125}I-labelled with 0.5 mCi of Na^{125}I and 100 μg of lactoperoxidase. Iodination was initiated by 25 successive additions of 0.02 ml of 2.5 mM-H_2O_2 at 20 s intervals. About 150 μg of membrane protein solubilized in SDS was loaded on the gel. Electrophoresis was performed on a 1.5 mm-thick gel slab containing 10% (w/v) acrylamide, 0.27% (w/v) bisacrylamide, with a stacking gel of 3% (w/v) acrylamide in the discontinuous buffer of Laemmli (1970). After staining by the periodic acid/Schiff procedure the gel was dried on a filter paper and exposed to Kodak X-Omat MA film for 2 days. (1) Radiolabelled ghosts from control erythrocytes; (2) radiolabelled ghosts from Cad erythrocytes. Arrows indicate glycoproteins with abnormal mobility. The band denoted * might be either a minor component or a degradation product of band 3. The nomenclature of the glycoproteins is that of Anstee *et al.* (1979).

the gel by six washings in buffer A containing 0.5% (v/v) Triton X-100. The material remaining firmly bound was eluted from the gel in 100 μl of 10 mM-Tris/HCl (pH 6.8)/1 mM-EDTA/2% (w/v) SDS, heated for 3 min at 100°C and analysed by SDS/polyacrylamide-gel electrophoresis.

Lipid-free sialoglycoproteins were obtained from 20–25 ml of unlabelled packed red cells as described by Dejter-Juszynski *et al.* (1978). Freeze-dried membranes from Cad and blood-group-B control erythrocytes were extracted with 9 vol. of chloroform/methanol (2:1, v/v). The organic phase was dried *in vacuo* and resuspended in 2 ml of water. The aqueous phase was concentrated to 50 ml and dialysed against water adjusted to pH 8 with KOH and then extracted twice with 1 vol. of butanol. The upper butanol phases obtained after centrifugation (15000 *g*, 15 min) were pooled, dried and solubilized in 2 ml of water. The aqueous butanol phase was freeze-dried, resolubilized in 1.5 ml of 50 mM-Tris/HCl/0.1 mM-EDTA, pH 8.0, containing 1% (v/v) Triton X-100, and submitted to ion-exchange chromatography on a 1 cm × 15 cm column of DEAE-cellulose. The unbound poly(glycosyl)-ceramide fraction was dialysed against distilled water and freeze-dried. The detergent was removed by extraction with ethanol before resuspension in 350 μl of water. The sialoglycoproteins were subsequently eluted with 0.5 M-NaCl in the above buffer, extensively dialysed against water and freeze-dried.

Cad as well as B-blood-group activities of each fraction were investigated by agglutination-inhibition tests carried out in conventional assays (Mollison, 1974).

The GPα component (in the nomenclature of Anstee *et al.*, 1979) was further purified from the sialoglycoproteins with a Bio-Rad A 1.5 column (1.5 cm × 30 cm) equilibrated in 5 mM-phosphate buffer (pH 8.0)/25 mMNaCl containing 0.1% (v/v) Ammonyx-LO, as described by Furthmayr *et al.* (1975).

The sugars were estimated by g.l.c. in a Varian Aerograph model 1400 apparatus as described by Zanetta *et al.* (1972) after methanolysis of the sialoglycoprotein and GPα components and trifluoroacetylation (internal standard: *meso*-inositol).

Sialic acids were also determined after hydrolysis *in situ* by *Vibrio cholerae* neuraminidase. Packed red cells (1 ml) were incubated in 1 ml of 50 mM-acetate buffer (pH 5.5)/9 mM-$CaCl_2$/150 mM-NaCl containing 0.01–1 unit of neuraminidase. After 30 min at 37°C the supernatant obtained by centrifugation (2500 *g*; 15 min) was passed through a Millipore membrane (40 μm pore size) and submitted to a Warren (1959) assay.

Results

SDS/polyacrylamide-gel electrophoresis

The human red-cell membrane polypeptides radioiodinated by the lactoperoxidase procedure were first separated by SDS/polyacrylamide-gel electrophoresis as shown in Fig. 1. Band-3 glycoprotein as well as the main glycoprotein species, GPα and GPδ (in the nomenclature of Anstee *et al.*, 1979), were heavily labelled *in situ*. Homodimers and heterodimers of these glycoproteins (GPα_2, GPδ_2 and GP$\alpha\delta$) were also detected.

As compared with control erythrocytes, it is clearly shown that both the monomeric form of GPα

and GPδ from Cad red cells have a lower-than-normal electrophoretic mobility, suggesting an increase in their apparent molecular masses of 3 kDa and 2 kDa respectively.

The periodic acid/Schiff staining of the Cad membranes, although displaying a normal pattern, also indicated an abnormal mobility of GPα and GPδ (results not shown). In addition, the mobility of GPγ and two GPβ minor red-cell membrane glycoproteins hardly labelled by the radioiodination process seems to be affected, but further investigations are needed to firmly establish this point.

On a quantitative basis the distribution of the glycoproteins from Cad erythrocytes was similar to that of the control (Dahr *et al.*, 1976).

Affinity binding of red cell components to immobilized lectins

Since Cad erythrocytes have receptors for DBA, this property was used to identify the molecular species carrying the specific determinants.

The red-cell membrane components from Cad and control individuals which bind to DBA–agarose in the presence of deoxycholate are shown in Fig. 2. The predominant species from Cad erythrocytes which adhere to the column are represented by the monomeric and dimeric forms of the main red-cell membrane glycoprotein (GPα and GPα_2 in Fig. 2, lane 5). This binding was completely inhibited by 0.3 M-*N*-acetylgalactosamine (Fig. 2, lane 9) and there was no material eluted from DBA–agarose incubated with deoxycholate lysates obtained from A_1 or B control erythrocytes (Fig. 2, lanes 3 and 4). GPδ also carries some Cad determinants, but is much less ^{125}I-labelled and is not clearly distinguished on the autoradiograph.

The interaction between Cad determinants and DBA is enhanced after partial removal of sialic acids (Fig. 2, lane 8), and the binding in these conditions is also completely inhibited by 0.3 M-*N*-acetylgalactosamine (Fig. 2, lane 10).

However, the DBA–agarose adsorbant does not retain labelled material from control group A_1 or B neuraminidase-treated red cells.

It is noteworthy that the electrophoretic mobility of GPα is slightly decreased in neuraminidase-treated red cells as compared with native Cad red cells (Fig. 2, lanes 11 and 12). This is reminiscent of a recent finding from Gahmberg & Andersson (1982) that, after partial neuraminidase treatment, the GPα from control red cells exhibits a decrease in mobility on SDS/polyacrylamide-gel electrophoresis. In our conditions, where *N*-acetylneuraminate residues are totally removed, this effect is not detectable (Fig. 2, lanes 1 and 2 show at variance the expected increase in mobility of GPα after complete removal of the sialic acids), but it occurs with Cad red-cell membranes, which appear to be

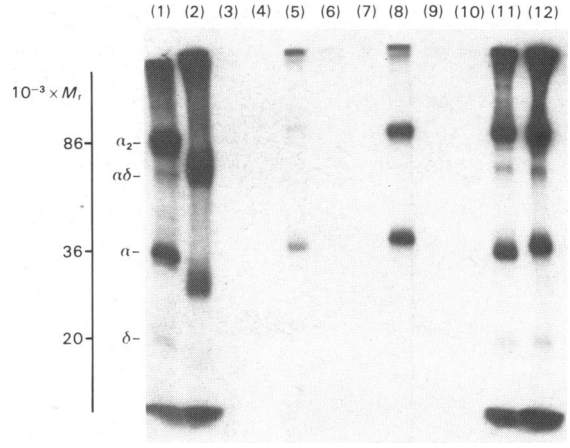


Fig. 2. *Affinity binding on DBA–agarose*
Deoxycholate lysates from blood group-A_1, -B and -Cad ^{125}I-labelled erythrocyte membranes were incubated with appropriate amounts of the immobilized lectin. The material bound was eluted from the gel and subjected to SDS/polyacrylamide-gel electrophoresis as described in Fig. 1. The radioactive bands were detected by autoradiography of the dried gels after exposure to Kodirex film for 15 days. Radiolabelled ghosts: (1) native or (2) neuraminidase-treated A_1 erythrocytes; (11) native or (12) neuraminidase-treated Cad erythrocytes. Eluates from DBA–agarose: (3) untreated or (6) neuraminidase treated A_1 membranes; (4) untreated or (7) neuraminidase-treated blood-group-B membranes; (5) untreated or (8) neuraminidase-treated Cad membranes. Eluates from control experiments carried out in the presence of 0.3 M-*N*-acetylgalactosamine: (9) untreated or (10) neuraminidase-treated Cad membranes.

partly resistant to neuraminidase treatment (Fig. 2, lanes 11 and 12). Although 0.1 unit of neuraminidase liberates more than 90% of all the *N*-acetylneuraminate residues from 1 ml of control packed red cells, 0.5 unit cleave only about 80% of *N*-acetylneuraminate residues from Cad red cells.

In addition to DBA, other lectin receptors have been investigated at the surface of native and neuraminidase-treated Cad erythrocytes. In contrast with its strong agglutinating property, the HPA adsorbant does not bind significant amounts of material from Cad membranes, perhaps because of the loose bond that is established in the presence of deoxycholate (results not shown). The specific interactions between the sialoglycoproteins of Cad red cells and immobilized WGA, MPA or PHA-E are similar to those found previously with control erythrocytes (Tanner *et al.*, 1980) and reflect the presence of oligosaccharide chains attached by alkali-labile and alkali-stable linkages to the protein

backbone of GPα (carrying receptors for WGA, MPA and PHA-E). By contrast, there are receptors for WGA and MPA, but not for the PHA-E lectin on GPδ molecules, which carry only alkali-labile oligosaccharide chains.

Distribution of blood-group-Cad and -B antigens among red-cell membrane components

Agglutination-inhibition tests carried out on the aqueous and organic phases resulting from the extraction procedure of Dejter-Juszynski *et al.* (1978) point to a different distribution of the Cad- and B-blood-group antigens (Table 1). The blood-group-B activity was mainly located in organic phases and the most active fractions are poly-(glycosyl)ceramides (80% of the total blood-group-B activity).

However, the starting material in these experiments came from chloroform/methanol extracts almost completely devoid of membrane components such as band 3 and band 4.5, which were subsequently shown to carry a large proportion of ABH-blood-group determinants (Finne, 1980; Karhi & Gahmberg, 1980).

In contrast, the Cad activity estimated by agglutination-inhibition tests was mainly located in aqueous phases containing the sialoglycoproteins (Table 1). The most active fractions were the lipid-free sialoglycoproteins fractionated on DEAE-cellulose, since as little as 0.002 μg and 0.5 μg respectively of this material inhibited the agglutination of A_1 and Cad erythrocytes by DBA. The sialoglycoprotein fractions from control blood-group-B red cells were not inhibitory in this system. From 25 ml of packed Cad erythrocytes, 5 mg (dry weight) of lipid-free sialoglycoproteins were obtained by the procedure outlined above and were fractionated on Bio-Rad A 1.5 gel to give about

Table 1. *Biological activity of organic and aqueous fractions obtained from Cad and control erythrocyte membranes after the extraction procedure of Dejter-Juszynski et al. (1978)*

Freeze-dried membranes from 25 ml of packed red cells were first extracted with 9 vol. of chloroform/methanol (2:1, w/v). The aqueous phase was concentrated 50 times, dialysed against water adjusted to pH 8 with KOH and extracted twice with 1 vol. of n-butanol. The aqueous butanolic phase was freeze-dried, resolubilized in 50 mM-Tris/HCl/0.1 mM-EDTA, pH 7.5, containing 1% (v/v) Triton X-100 and submitted to DEAE-cellulose chromatography (see the text). The unbound poly(glycosyl)ceramides and the organic phases from the chloroform/methanol and n-butanol extracts were dried *in vacuo* and resuspended in 0.3 ml and 2 ml of water respectively before determination of the biological activity. The sialoglycoproteins contained in the aqueous butanolic phase and those lipid-free eluted from the DEAE-cellulose column with 0.5 M-NaCl (in the above buffer without detergent) were extensively dialysed against water, freeze-dried and resolubilized in water before used. (1) The blood-group activities of the organic phases are given as the titre of hemagglutination-inhibition of the DBA lectin against A_1 and Cad red cells as well as human anti-(blood-group B) against group-B red cells. A 10 μl portion of each phase was used with 10 μl of DBA or human anti-(blood-group B) reagent at the appropriate dilution. (2) The blood-group activities of the aqueous phases are given as the minimum concentration of substance (μg in 40 μl total volume) giving a complete hemagglutination-inhibition of the DBA and human anti-(blood-group B) as described above. Abbreviation used: nt, not tested.

Membrane fraction	Biological activity (titre)		
Agglutination-inhibition of ...	DBA against:		Human anti-(blood-group B) against group-B red cells
	A_1 red cells	Cad red cells	
Control (group B)			
(1) Organic phases			
Chloroform/methanol	nt	0	8
Butanol	0	0	64
Poly(glycosyl)ceramides	0	0	2048
(2) Aqueous phases			
Sialoglycoproteins (butanol)	>180	>180	>180
Lipid-free sialoglycoproteins (DEAE-cellulose)	>100	>100	>100
Cad erythrocytes (group B)			
(1) Organic phases			
Chloroform/methanol	4	0	16
Butanol	0	0	32
Poly(glycosyl)ceramides	0	0	2048
(2) Aqueous phases			
Sialoglycoproteins (butanol)	0.02	2.5	>160
Lipid-free sialoglycoproteins (DEAE-cellulose)	0.002	0.5	>130

Table 2. *Sugar composition of red-cell membrane glycoproteins prepared from Cad and control erythrocytes*
Sugar analysis were performed as described by Zanetta *et al.* (1972), with a Varian 1400 gas chromatograph equipped with ionization flame detector. Trifluoroacetate derivatives of *O*-methyl glycosides were separated on a glass column filled with OV 210 5% silicone on chromosorb W (HP) DMCS (100/200 mesh). Nitrogen flux was 15 ml/min and the column temperature was raised at 2°C/min between 100 and 210°C. Only traces of fucose and glucose were detected. The sialoglycoprotein fraction obtained after DEAE-cellulose chromatography and the GPα were purified as described in the text and as reported in Table 1. The numbers in parentheses indicate the absolute content of each sugar expressed as nmol per mg.

	Molar ratio of sugars (Gal = 1.0)					Ratio $\frac{\text{NeuAc}}{\text{GalNAc}}$
Fraction	Gal	Man	GalNAc	GalNAc	NeuAc	
Control erythrocytes						
Sialoglycoprotein fraction after DEAE-cellulose	1 (673)	0.18 (117)	0.76 (507)	0.26 (174)	1.70 (1130)	2.2
GPα	1 (660)	0.24 (156)	0.82 (541)	0.31 (206)	1.64 (1080)	2.0
Cad erythrocytes						
Sialoglycoprotein fraction after DEAE-cellulose	1 (512)	0.16 (83)	1.72 (882)	0.33 (169)	1.95 (1000)	1.1
GPα	1 (577)	0.20 (115)	1.58 (911)	0.29 (165)	1.58 (910)	1.0

1.5 mg (dry weight) of purified GPα. The biological activity of this purified glycoprotein was identical with that of total lipid-free sialoglycoprotein fraction (Table 1).

Carbohydrate analysis of Cad-specific components

The sugar composition of the lipid-free sialoglycoproteins and purified GPα was determined by g.l.c. (Table 2).

In controls the results were in agreement with other published results which have established a molar ratio *N*-acetylneuraminate/*N*-acetylgalactosamine equal to 2:1 (Fukuda & Osawa, 1973; Furthmayr *et al.*, 1975; Kahane *et al.*, 1976; Liljas *et al.*, 1976; Ebert *et al.*, 1979).

Similar analysis performed on glycoproteins prepared from Cad erythrocyte membranes indicate an increased content of *N*-acetylgalactosamine residues, so that the molar ratio *N*-acetylneuraminate/*N*-acetylgalactosamine reaches 1:1.

Discussion

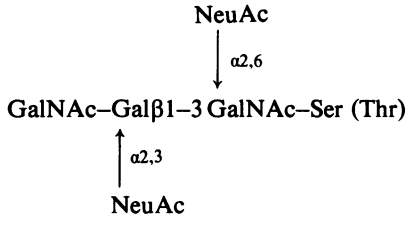
The present results demonstrate that GPα and GPδ, the main red-cell membrane sialoglycoproteins, have a higher apparent molecular mass in Cad than in control erythrocytes. The increase is about 3 kDa for GPα and 2 kDa for GPδ, as revealed by SDS/polyacrylamide-gel electrophoresis. Affinity binding experiments have also shown that GPα (and very likely GPδ) from the Cad red cells under study (a blood-group-B individual) have specific receptors for DBA, which is highly specific for *N*-acetylgalactosamine residues (Hammarström *et al.*, 1977). Taken together these results strongly suggest that GPα and GPδ might carry additional *N*-acetylgalactosamine residues involved in the blood-group-Cad specificity. HPA, another *N*-acetylgalactosamine binding lectin, agglutinates Cad red cells but was ineffective for binding to Cad-reactive structures when used in the immobilized state in the presence of detergent. It is possible that the binding affinity of this lectin as many others is strongly decreased in the presence of deoxycholate (Lotan & Nicolson, 1979).

After separation of glycolipid and glycoprotein components from Cad erythrocytes with solvents, we have demonstrated that the Cad antigenic activity is associated with sialoglycoprotein fractions. The final products obtained were lipid-free glycoproteins that exhibited a strong Cad reactivity but were completely devoid of blood-group-B reactivity.

Carbohydrate analysis of GPα molecules purified from Cad red cells reveals an abnormal sugar composition characterized by a high content in *N*-acetylgalactosamine and a molar ratio *N*-acetylneuraminate/*N*-acetylgalactosamine of 1:1 (other sugars were in the usual range). This was at variance with the GPα isolated from control erythrocytes (Cad-negative), in which the molar ratio was 2:1.

GPα has been extensively studied and carries 15 alkali-labile and one alkali-stable oligosaccharide chains (Tomita & Marchesi, 1975). Since glycoproteins generally exhibit abnormal SDS binding, a reliable estimate of their molecular weight using a single acrylamide concentration in SDS-containing gels is rather uncertain. However, the 3 kDa in-

crease in molecular mass of GPα fits with the presence of about 15 extra *N*-acetylgalactosamine residues per molecule of glycoprotein. Considering both the presence of an extra *N*-acetylgalactosamine residue and its increase in molecular mass, it is likely that the GPα from Cad erythrocytes carries new alkali-labile pentasaccharide chains in which the sialic acid-rich tetrasaccharides described by Thomas & Winzler (1969) are substituted by an *N*-acetylgalactosamine residue in a still-unknown position and linkage. A tentative structure of the Cad-red-cell antigen is proposed:



The lower-than-normal mobility of GPδ from Cad erythrocytes correlates with this model, since the substitution with an additional *N*-acetylgalactosamine residue of ten oligosaccharide chains carried by this glycoprotein would give an increase of about 2kDa molecular mass (with the restriction stated above). The minor GPγ and GPβ components have not yet been studied, but might also carry similar determinants.

Preliminary observations have suggested that the sialic acids of such a pentasaccharide structure exhibit a partial resistance to cleavage by the neuraminidase from *Vibrio cholerae*. Interestingly it is well known that the *N*-acetylneuraminate residue from GM_1 and Tay–Sachs gangliosides is resistant to hydrolysis by the neuramidase from *Vibrio cholerae*, since substituents of the 4-axial hydroxy group of the galactose adjacent to the sialic acid residue prevent effective binding of the enzyme (Ledeen & Salsman, 1965).

Further support to the model proposed above requires a detailed carbohydrate analysis of Cad-red-cell glycoproteins as well as the identification of the specific *N*-acetylgalactosaminyltransferase which would be the direct product of the *Cad* gene.

Very recently, Donald *et al.* (1982) have isolated, from the human Sd^a-active urinary THGP, a disaccharide with the structure:

$$GalNAc\beta 1 \rightarrow 4Gal$$

This disaccharide, obtained by fragmentation of THGP by hydrazinolysis and acid hydrolysis, is very likely attached to alkali-stable carbohydrate chains joined to THGP peptide through *N*-acetylglucosamine–asparagine linkage. It is noteworthy that a terminal non-reducing *N*-acetylgalactosamine–galactose disaccharide is also present as part of the Cad receptor. If it is confirmed, a direct relationship between Cad and Sd^a would be established.

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