A Carbohydrate-Deficient Membrane Glycoprotein in Human Erythrocytes of Phenotype S-s-

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1. We investigated the membranes of human erythrocytes which completely lack the blood-group antigens S and s (denoted as S-s-) as part of a study of the structure and function of the surface glycoproteins of the human erythrocyte. 2. The S-s-erythrocyte-membrane glycoprotein PAS-3 band was much less intensely stained in comparison with that of the glycoprotein from normal erythrocyte membranes. The S-s-membrane glycoprotein PAS-4 band also showed decreased staining. 3. Examination with the lectins from *Maclura aurantiaca* (Osage orange) and *Arachis hypogaea* (groundnut) showed that the PAS-3 glycoprotein of S-s-erythrocyte membranes lacked the receptors for these lectins that are present on glycoprotein PAS-3 from normal erythrocytes. 4. Radioiodination with lactoperoxidase showed the presence of the polypeptide of glycoprotein PAS-3 in S-s- cells, although it was more weakly labelled than the protein in the normal erythrocyte. 5. Our results show that the PAS-3 glycoprotein of S-s- erythrocytes is deficient in some of the carbohydrates present in the protein from normal erythrocytes. Glycoprotein PAS-4 of normal erythrocytes is shown to be a complex containing both glycoproteins PAS-1 and PAS-3.

Although the glycoproteins at the surface of the human erythrocyte membrane have been the subject of a number of studies (Steck, 1974; Marchesi *et al.*, 1976), the function of the carbohydrate bound to them remains unknown. In an attempt to gain an insight into the function of these glycoproteins we have studied some human erythrocyte variants. Erythrocytes of type En(a-) completely lack the major membrane sialoglycoprotein, even though individuals with this cell type show no clinical abnormalities (Tanner & Anstee, 1976b). In an extension of this approach we have examined cells of phenotype S-s- (Race & Sanger, 1975). These cells lack the Ss blood-group antigens. There is evidence that in normal individuals the Ss antigens are located on a minor erythrocyte glycoprotein, PAS-3 (Hamaguchi & Cleve, 1972; Anstee & Tanner, 1975). It was therefore decided to determine whether these cells contain a defect in glycoprotein PAS-3 analogous to the total absence of the major sialoglycoprotein found in En(a-) erythrocytes. The S-s- phenotype has not yet been found in Caucasians, has only been reported in one family of Indians (Moore, 1972) and occurs with frequencies ranging from 1 to 35% in different Negroid populations (Lowe & Moore, 1972).

In the present paper we describe the results of a study of erythrocyte membranes from donors of the S-s- phenotype. Our results suggest that the carbo-

hydrate moiety of the glycoprotein PAS-3 is defective in these individuals.

Materials and Methods

Blood samples from three homozygous S-s- donors and one heterozygote, all unrelated, were studied. Blood from donor A. H. (S-s-/S-s-) was provided by Dr. P. Tippett, M.R.C. Blood Group Unit, University College London, London NW1 2HE, U.K. That from donor J. N. (Hoekstra *et al.*, 1975) and donor N. Z. (both S-s-U-/S-s-U-) were provided by Mr. G. Newell, Eastern Province Blood Transfusion Service, Port Elizabeth, South Africa, and Miss P. Moores, Natal Blood Transfusion Service, Durban, South Africa, respectively, and G. E. (S-s+/S-s-) was provided by Dr. R. Lowe, Salisbury and District Blood Transfusion Service, Salisbury, Rhodesia. The U types (Race & Sanger, 1975) of A. H. and G. E. are not known.

The methods for the preparation of erythrocyte 'ghosts', radioiodination of erythrocytes with lactoperoxidase, neuraminidase treatment of erythrocytes and location of lectin-binding components have been described previously (Boxer *et al.*, 1974; Tanner & Anstee, 1976a). The lectin from *Arachis hypogaea* (groundnut) was purified by elution from formaldehyde- and neuraminidase-treated human erythrocytes and iodinated with ¹²⁵I for use in lectin-binding

experiments as described by Anstee *et al.* (1977). SDS*/polyacrylamide-gel electrophoresis was done in three systems, those described by Fairbanks *et al.* (1971), by Laemmli (1970) modified as described by Dahr *et al.* (1975b), and in a system which used the buffer of Fairbanks *et al.* (1971) in a separating gel containing 10% (w/v) acrylamide with a gel overlay containing 3% (w/v) acrylamide. Polyacrylamide gels were scanned at 560nm for periodic acid/Schiff's-base-stained gels and at 500nm for radioautographs in a Hilger-Gilford scanner. To measure radioactivity quantitatively, radioactive bands were excised from dried polyacrylamide gels by using the radioautographs as a guide. The gel slices were incubated for 2 days with 0.5ml of 1% SDS, and then 2ml of scintillation fluid [containing 4g of 2,5-diphenyloxazole, 75mg of 1,4-bis-(5-phenyloxazol-2-yl)benzene and 120g of naphthalene in 1 litre of 1,4-dioxan] was added. After a further 48h the material was counted for radioactivity in a liquid-scintillation counter.

Results and Discussion

Membranes from the three donors homozygous for the S-s- conditions (A. H., J. N., N. Z.) and one donor heterozygous (S-s+) for the condition (G. E.) were compared with those from normal control cells by SDS/polyacrylamide-gel electrophoresis. No difference was observed in the banding patterns of the protein-stained gels. However, gels stained with the periodic acid/Schiff's-base stain revealed that membranes from homozygous S-s- cells gave bands with considerably decreased staining when compared with those from normal erythrocytes, in the regions corresponding to glycoproteins PAS-3 and PAS-4. Fig. 1 shows the results for one of these donors (A. H.). On occasions the PAS-3 band was resolved into two components (denoted PAS-3 and PAS-3'), as shown in Fig. 1. It is not clear whether this resolution was an artifact of the electrophoretic system or a real separation of two components. Both of these components showed decreased staining in gels of membrane glycoproteins from the homozygous S-s- cells. The results of several experiments on the membranes from homozygous S-s- cells showed that an appreciable decreased staining intensity of bands PAS-3 and PAS-4 (to 14–27% of normal and 24–38% of normal respectively) was always obtained (Table 1). The wide range of values obtained for this decrease probably reflects the difficulty in accurately determining bands which stain as weakly as PAS-3 and PAS-4. The heterozygous S-s+ cells showed a decrease in periodic acid/Schiff's-base staining of bands PAS-3 and PAS-4 intermediate between that of normal and homozygous S-s- cells (Table 1).

* Abbreviation: SDS, sodium dodecyl sulphate.

Fig. 1. SDS/polyacrylamide-gel electrophoresis of 'ghosts' from normal and S-s- erythrocytes

Samples of 'ghosts' from control (—) and S-s- (·····) erythrocytes (A. H.) were used. Electrophoresis was carried out with gels containing 8% (w/v) acrylamide and with the buffer system described by Fairbanks *et al.* (1971). The gels were stained with the periodic acid/Schiff's-base stain and scanned at 560nm.

Table 1. Decreased staining of bands PAS-3 and PAS-4 in erythrocytes lacking Ss antigens

Parallel gels containing known amounts of sample were stained for protein and carbohydrate and scanned at 550 and 560nm respectively. Protein was determined from the area of band 4.2 (Steck, 1974). This value was used to normalize the area of the PAS-3 and PAS-4 peaks to the same unit concentration of band 4.2. Control normal erythrocyte membranes were separated at the same time as the S-s- erythrocyte membranes. Data are expressed as the means $\pm$ S.D., for the numbers of determinations in parentheses.

Donor	Ss genotype	Percentage staining relative to normal controls	
		Band PAS-3	Band PAS-4
A. H.	S-s-/S-s-	27 $\pm$ 10 (3)	38 $\pm$ 5 (2)
J. N.	S-s-/S-s-	22 $\pm$ 11 (3)	24 $\pm$ 4 (2)
N. Z.	S-s-/S-s-	14 $\pm$ 3 (3)	26 $\pm$ 5 (2)
G. E.	S-s+/S-s-	47 $\pm$ 20 (3)	47 $\pm$ 20 (3)

The PAS-1 and part of the PAS-2 band have been shown to represent interconvertible forms of the major membrane sialoglycoprotein (Tuech & Morrison, 1974). This glycoprotein is also a component of the PAS-4 band, since the PAS-4 glycoprotein was absent from cells lacking the major sialoglycoprotein (Tanner & Anstee, 1976b; Anstee *et al.*, 1977). The results reported here demonstrate that glycoprotein

EXPLANATION OF PLATE 1

SDS/polyacrylamide-gel electrophoresis of 'ghosts' from lactoperoxidase-radioiodinated control and $S^{-}s^{-}$ erythrocytes
 Radioautographs of dried-down gels are shown. Experimental details are described in the legend to Fig. 2. (a) Donor N. Z. ($S^{-}s^{-}$); (b) donor J. N. ($S^{-}s^{-}$); (c) donor A (normal); (d) donor B (normal).

PAS-4 is a complex that contains glycoprotein PAS-3 as well as the major sialoglycoprotein.

Dahr *et al.* (1975c) reported that the PAS-3 and PAS-4 glycoproteins were completely absent from cells homozygous for the S-s- phenotype. However, in our hands, low extents of periodic acid/Schiff's-base staining in the PAS-3 and PAS-4 regions were consistently observed in cells homozygous for the S-s- phenotype. The parallel decrease in staining of the PAS-3 and PAS-4 regions for S-s- cells suggested that the residual staining in the PAS-3 region was not due to the presence of a further component in the PAS-3 band.

The lectins from *Maclura aurantiaca* (synonym *M. pomifera*; Osage orange) and *A. hypogaea* bind to sugar residues present on glycoprotein PAS-3 (Tanner & Anstee, 1967a; Anstee *et al.*, 1977). Unlike the periodic acid/Schiff's stain, which is mainly dependent on the presence of sialic acid, these lectins have a primary specificity towards N-acetylgalactosamine and galactose residues respectively (Dahr *et al.*, 1975a; Terao *et al.*, 1975; Lotan *et al.*, 1975). The binding of these lectins was used to give information on the other sugar residues present in the PAS-3 glycoprotein.

The pattern of binding of radioiodinated *M. aurantiaca* lectin to the components of homozygous S-s- erythrocytes is shown in Fig. 2. The absence of

binding of this lectin to glycoprotein PAS-3 in the homozygous S-s- erythrocyte membrane showed that these receptors were not present in glycoprotein PAS-3 of S-s- cells. The binding of the *M. aurantiaca* lectin to glycoprotein PAS-4 of S-s- cells was also substantially decreased. The results of Furthmayr *et al.* (1975) suggest that glycoprotein PAS-3 constitutes 5–10% of the periodic acid/Schiff's-reactive sialoglycoprotein present in the normal erythrocyte membrane, and the relative intensity of periodic acid/Schiff's-base staining of band PAS-3 was consistent with this value. However, when *M. aurantiaca* lectin was used to detect these components in normal erythrocytes in experiments similar to that shown in Fig. 2, glycoprotein PAS-3 took up approximately one-half [$53 \pm 5\%$ (s.d.; $n = 4$)] of the radioactively labelled lectin bound by glycoprotein PAS-1. The apparent difference in the decrease in glycoprotein PAS-4 in S-s- cells detected by the *M. aurantiaca* lectin and the periodic acid/Schiff's stain (cf. Figs. 1 and 2) may therefore reflect the different sensitivities of the two methods in detecting the PAS-3 band as compared with the PAS-1 band.

The lectin from *A. hypogaea* binds more strongly to the membrane components of neuraminidase-treated erythrocytes than to those of untreated erythrocytes (Bird, 1964; Anstee *et al.*, 1977). Fig. 3 shows the binding of radioiodinated *A. hypogaea* lectin to the components of neuraminidase-treated homozygous S-s- erythrocytes. There was no binding of this lectin to the band that probably represents the desialylated product of glycoprotein PAS-3 in these cells. It was not possible from this experiment to determine whether the desialylated product of glycoprotein PAS-4 from S-s- cells bound the lectin.

All the above data suggested that a deficiency occurs in the carbohydrate moiety of glycoprotein PAS-3 in homozygous S-s- erythrocytes.

We used lactoperoxidase-catalysed radioiodination of intact S-s- erythrocytes to detect the presence of the polypeptide core of glycoprotein PAS-3 in these cells. Fig. 4 shows that glycoprotein in a band of the same mobility as band PAS-3 was labelled in homozygous S-s- erythrocytes, but the extent of labelling was much decreased compared with the control. However, the amount of label in glycoprotein PAS-4 did not appear to be substantially different from that of the normal control (Plate 1). Measurement of the radioactivity in the gels from normal erythrocytes showed that glycoprotein PAS-3 took up $3.7 \pm 2.8\%$ (s.d.; $n = 4$) of the radioactivity taken up by glycoprotein PAS-1, but in the samples from S-s- cells glycoprotein PAS-3 took up $0.8 \pm 0.7\%$ (S.D.; $n = 4$) of the radioactivity taken up by glycoprotein PAS-1. The high background radioactivity under the PAS-4 band made accurate quantification of this band difficult. Since glycoprotein PAS-4 contains glycoproteins PAS-1 and PAS-3, the labelling observed in

Fig. 2. Binding of radioiodinated lectin from *M. aurantiaca* to components of 'ghosts' from normal and S-s- erythrocytes. Samples of 'ghosts' from control (—) and S-s- (·····) erythrocytes (J. N.) were separated by electrophoresis in SDS/polyacrylamide gels containing 8% (w/v) acrylamide, and were fixed and stained with ^{125}I -labelled *M. aurantiaca* lectin as described by Tanner & Anstee (1976a); 0.1 ml of lectin was used in each incubation, and this contained 350 000 c.p.m. of ^{125}I and had a haemagglutination titre of 1:160 against normal human group-O erythrocytes. No bands were observed on control gel slices incubated with the lectin in the presence of 0.2 M-D-galactose.

Fig. 3. Binding of radioiodinated *A. hypogaea* lectin to the components of neuraminidase-treated normal and S-s- erythrocytes

A 6% (w/v) suspension of normal (—) and S-s- (····) erythrocytes (J. N.) in 0.15M-NaCl/0.02M-sodium phosphate buffer, pH 6.5, containing 1 mM-CaCl₂ was incubated at 37°C for 30 min with 5 units of neuraminidase/ml (from *Vibrio cholerae*; Hoechst Pharmaceuticals, Brentford, Middx., U.K.). 'Ghosts' prepared from these cells were separated on SDS/polyacrylamide gels containing 8% (w/v) acrylamide, and were fixed and stained with ¹²⁵I-labelled *A. hypogaea* lectin as described by Anstee *et al.* (1977); 0.05 ml of lectin was used in each incubation, and this contained 250 000 c.p.m. of ¹²⁵I and had a haemagglutination titre of 1:640 against neuraminidase-treated human group-O erythrocytes. No bands were observed in control slices incubated with the lectin in the presence of 0.2M-lactose. PAS-1_N, PAS-2_N etc. denote the positions of the desialylated products of PAS-1, PAS-2 etc.

the glycoprotein PAS-4 band in these experiments was predominantly due to the labelling of glycoprotein PAS-1. Therefore the decreased labelling of glycoprotein PAS-3 in the S-s- cells would have a very small effect on the total labelling of the PAS-4 component.

The observation that the labelling of glycoprotein PAS-4 was apparently not significantly changed in S-s- cells suggests that these cells contain amounts of PAS-3 polypeptide which are similar to that found in normal cells. This suggests that the decreased labelling of glycoprotein PAS-3 found in S-s- cells probably results from a change in the labelling properties of the glycoprotein in the intact erythrocyte rather than a decrease in the amount of the polypeptide chains of glycoprotein PAS-3.

These results indicate that, although the polypeptide carrier of glycoprotein PAS-3 is present in homozygous S-s- cells, the protein lacks the receptors for *M. aurantiaca* and *A. hypogaea* lectins. Our results

show that in S-s- cells there is a substantial decrease in the amount of the sugar components of the normal PAS-3 glycoprotein that stain with the periodic acid/Schiff's-base stain (probably predominantly sialic acid). Since the carbohydrate content of normal glycoprotein PAS-3 is not known, we cannot estimate the extent of the decrease in the total carbohydrate in the glycoprotein PAS-3 of S-s- erythrocytes. Marchesi *et al.* (1976) have reported that the carbohydrate composition of glycoprotein PAS-3 is similar to that of the major sialoglycoprotein, but that the polypeptide portion appears to be different. We have suggested (Tanner & Anstee, 1976a; Anstee *et al.*, 1977) that the two lectins used here detect different residues of the same oligosaccharide in glycoprotein PAS-3, and that this oligosaccharide is the same as, or very similar to, the alkali-labile sialotetrasaccharide described by Thomas & Winzler (1969), the predominant oligosaccharide of the major erythrocyte sialoglycoprotein. If this is the case then our data suggest

Fig. 4. Lactoperoxidase labelling of control and S-s- erythrocytes

Samples of control erythrocytes (A) and S-s- erythrocytes (N. Z.) were radioiodinated with lactoperoxidase as described in the Materials and Methods section. 'Ghosts' from these erythrocytes were separated by SDS/polyacrylamide-gel electrophoresis in the buffer system described by Fairbanks *et al.* (1971). The separating gel contained 10% (w/v) acrylamide and was overlaid by a 5 mm-high gel containing 4% (w/v) acrylamide. The gel was dried, stained with the periodic acid/Schiff's-base stain, and the radioactive bands were detected by radioautography. The Figure shows scans of the radioautographs at 500 nm. The complete scan was from a radioautograph which had been exposed for 1 h. The inset shows the PAS-3 peaks from the same gels radiographed for 4 h and scanned at a higher sensitivity.

that the defect in erythrocytes homozygous for the S-s- phenotype causes the loss of *O*-glycosidically linked sialic acid-rich oligosaccharides from glycoprotein PAS-3. Since there is no indication of any abnormality of the similar oligosaccharides in the major sialoglycoprotein of S-s- erythrocytes, the ability of the S-s- erythrocyte to synthesize these oligosaccharides on the appropriate substrate is probably unimpaired. This suggests that the defective glycosylation of the polypeptide chain of glycoprotein PAS-3 in S-s- cells probably results from an alteration in the substrate for this glycosylation process, namely the amino acid sequence of the protein. Such an alteration could also give rise to the observed decrease in the extent of radioiodination by lactoperoxidase of glycoprotein PAS-3 of S-s- cells.

Dahr *et al.* (1975c) have reported that the *Vicia graminea* lectin (which detects galactose residues in the *O*-glycosidically linked oligosaccharides of the major sialoglycoprotein and will agglutinate trypsin-treated normal erythrocytes) does not agglutinate trypsin-treated S-s- erythrocytes. We have confirmed this observation and have also noted that the *M. aurantiaca* lectin shows a substantially diminished ability to agglutinate trypsin-treated S-s- cells (lectin titre 1:32 against trypsin-treated normal erythrocytes and 1:2 against trypsin-treated S-s- erythrocytes). Since trypsin treatment removes the major sialoglycoprotein but not glycoprotein PAS-3, this shows that a further source of lectin receptors, probably the *O*-glycosidically linked oligosaccharides, must be present in normal cells but absent from S-s- cells. This result is consistent with the absence of sialotetrasaccharides from glycoprotein PAS-3 in S-s- erythrocytes. The defect in S-s- cells seems to be of a different type from that found in another erythrocyte variant, the En(a-) cell. The polypeptide of the major sialoglycoprotein is absent in the latter case (Tanner & Anstee, 1976b; Gahmberg *et al.*, 1976). However, as with En(a-) erythrocytes, the resulting carbohydrate deficiency does not give rise to any known clinical abnormalities.

The chemical nature of the determinants specifying the Ss-blood-group antigens is not known, although neuraminidase treatment of intact erythrocytes does not result in the loss of Ss antigenic activity (Bird & Wingham, 1970). Our data indicate the absence of sialotetrasaccharides from glycoprotein PAS-3 and a possible alteration in amino acid sequence of glycoprotein PAS-3 in cells that lack Ss antigens. Although apparently similar sialotetrasaccharides are present in the major membrane sialoglycoprotein, this protein does not have Ss antigenic activity (Hamaguchi & Cleve, 1972; Anstee & Tanner, 1975). Thus it is unlikely that these oligosaccharides alone

constitute the Ss determinants. It is possible that the polypeptide component of glycoprotein PAS-3, perhaps with the additional involvement of sub-terminal residues of the sialotetrasaccharides, specifies the Ss antigenic determinants.

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