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# Two individuals with elliptocytic red cells apparently lack three minor erythrocyte membrane sialoglycoproteins

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1. We have studied the erythrocytes of two individuals (P. L. and K. W.) who lack the Gerbich (Ge) blood-group antigen. 2. The erythrocytes of P. L. and K. W. were not reactive with two monoclonal antibodies (NBTS/BRIC 4 and NBTS/BRIC 10) which reacted with normal erythrocytes. 3. The membranes of P. L. and K. W. erythrocytes appeared to lack three minor sialoglycoproteins ($\beta$, $\beta_1$ and $\gamma$). 4. These three minor sialoglycoproteins were found to be associated with the cytoskeletons of normal erythrocytes. 5. Approx. 10% of the erythrocytes of P. L. and K. W. were frankly elliptocytic. 6. We suggest that one or more of the minor sialoglycoproteins may play a part in maintaining the discoid shape of the human erythrocyte.

The human erythrocyte membrane contains four predominant sialic acid-rich polypeptides (denoted α, β, γ, δ; Anstee *et al.*, 1979). The most abundant of these, α and δ (synonyms glycophorins A and B) have been extensively studied. Individuals whose erythrocytes totally lack either or both of these sialoglycoproteins are known. However, there is, as yet, no evidence that these proteins are essential for normal erythrocyte function and viability (Anstee *et al.*, 1982). The minor glycoproteins β (synonyms glycophorin C, glycoconnectin) and γ are relatively poorly characterized. The *N*-terminal sequence of β has been determined (Dahr *et al.*, 1982), and it has also been suggested that this protein is a membrane attachment site for the erythrocyte cytoskeleton (Mueller & Morrison, 1981). In the present paper we describe two individuals who have elliptocytic red cells lacking detectable β or γ and also appear to lack an additional component ($\beta_1$) that has not previously been described. These results suggest that one or more of the components β, $\beta_1$ and γ have a role in maintaining the shape of the erythrocyte.

Abbreviations used: Ge, Gerbich antigen; NBTS/BRIC, National Blood Transfusion Service/Bristol Immunochemistry; phosphate-buffered saline, iso-osmotic sodium phosphate buffer, pH7.4; SDS, sodium dodecyl sulphate; PAS stain, periodic acid/Schiff's-base stain.

## Materials and methods

Patient P. L. underwent cardiac surgery in January 1982 and is currently receiving warfarin, digoxin and frusemide (furosemide). Patient K. W. has oat-cell carcinoma and at the time of ascertainment was receiving morphine and prednisolone. Neither patient has children or living parents and no relationship has been established between them.

Membranes from washed erythrocytes were prepared by using 5mM-sodium phosphate buffer, pH8.0. Samples of membranes were taken up in 20mM-Tris (pH8.0)/5mM-EDTA/5% SDS/10% glycerol/Bromophenol Blue (0.1mg/ml)/2% β-mercaptoethanol, boiled for 2min and electrophoresed, on a SDS/10%-polyacrylamide slab gel with a 3% overlay, using the buffer system of Laemmli (1970). Intact erythrocytes were labelled by the periodate/$NaB^3H_4$ method by incubating with 1mM-sodium metaperiodate in phosphate-buffered saline, pH7.4, for 10min in the dark on ice, washed twice with phosphate-buffered saline and incubated for 30min at room temperature with 100μCi of $NaB^3H_4$ (Gahmberg & Andersson, 1977). The labelled cells were washed with phosphate-buffered saline and immunoprecipitation with monoclonal antibodies NBTS/BRIC 4 and NBTS/BRIC 10, SDS/polyacrylamide-gel electrophoresis and autoradiography performed as de-

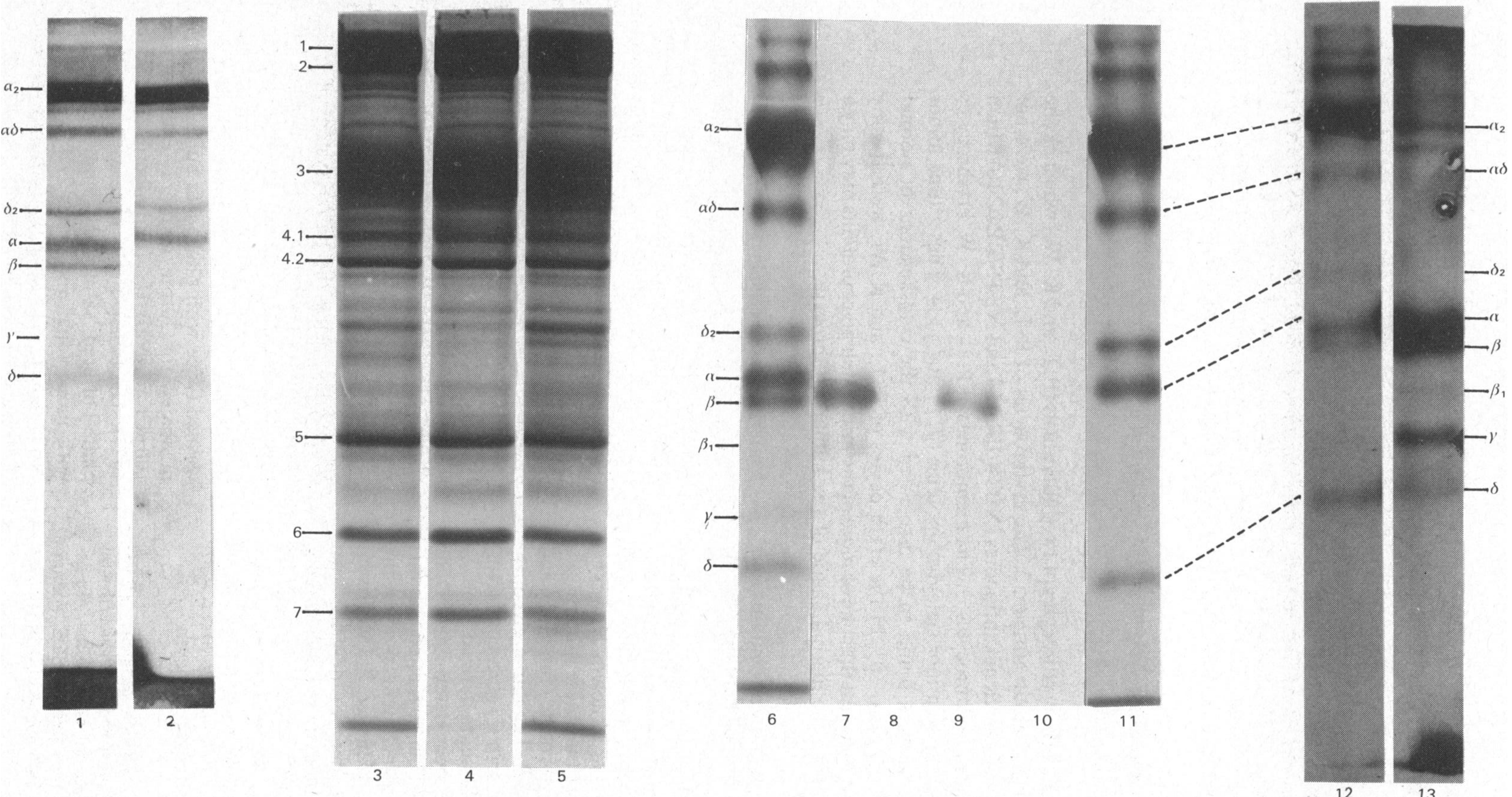


Fig. 1. *Solubilized erythrocyte membranes stained for sialoglycoproteins and proteins, and immunoprecipitation of sialoglycoproteins from $^3$H-labelled intact erythrocytes*
Tracks 1—5: 1, control erythrocyte membranes stained with PAS stain; 2, P. L. erythrocyte membranes stained with PAS stain; 3, control erythrocyte membranes stained with Coomassie Blue; 4, P. L. erythrocyte membranes stained with Coomassie Blue; 5, K. W. erythrocyte membranes stained with Coomassie Blue. Tracks 6–11, autoradiographs of: 6, membranes from $^3$H-labelled intact control erythrocytes before immunoprecipitation (3 weeks exposure); 7, components immunoprecipitated from $^3$H-labelled control erythrocytes by antibody BRIC 4; 8, components immunoprecipitated from $^3$H-labelled P. L. erythrocytes by BRIC 4; 9, components immunoprecipitated from $^3$H-labelled control erythrocytes by BRIC 10 (inspection of the autoradiograph showed that band $\beta_1$ was also immunoprecipitated by BRIC 10, although this is not apparent in the illustration); 10, components immunoprecipitated from $^3$H-labelled P. L. erythrocytes by BRIC 10; 11, membranes from $^3$H-labelled intact P. L. erythrocytes before immunoprecipitation (3 weeks exposure). Tracks 12–13: Triton-insoluble cytoskeletons from $^3$H-labelled normal erythrocytes; 12, autoradiograph of membranes from $^3$H-labelled intact control erythrocytes; 13, autoradiograph of cytoskeletons derived from the control membranes shown in track 12.

scribed by Ridgwell *et al.* (1983). The erythrocyte cytoskeleton preparations were performed as described by Mueller & Morrison (1981).

Two monoclonal antibodies (BRIC 4 and BRIC 10) were produced in mice in response to intact human erythrocytes by the method of Parsons *et al.* (1982). Serological methods were as described in Parsons *et al.* (1982). Estimation of the number of binding sites of monoclonal antibodies was as described by Merry *et al.* (1984).

## Results and discussion

Two individuals (P. L. and K. W.) who lack the common erythrocyte antigen Gerbich (Ge; Race & Sanger, 1975) were found to be unreactive with two monoclonal antibodies (BRIC 4 and BRIC 10) that reacted with normal human erythrocytes.

SDS/polyacrylamide-gel electrophoresis of erythrocyte membrane proteins from P. L. stained with PAS stain showed that bands $\beta$ and $\gamma$ were absent (Fig. 1, track 2). Membranes from K. W. gave an identical pattern (results not shown). When gels of P. L. and K. W. membranes were stained with Coomassie Blue, differences were observed in staining intensities of several bands in the region between bands 4.2 and 5 (Fig. 1, tracks 3–5). Since the blood samples were subject to different conditions before the preparation of membranes, we cannot be certain whether these changes are significant. No differences were evident in the region of band 4.1 in either P. L. or K. W. membranes.

The sialic acid-containing membrane glycoproteins of normal erythrocytres were labelled by using the periodate/$NaB^3H_4$ method and the labelled components separated by SDS/polyacrylamide-gel electrophoresis. After prolonged fluorography, additional labelled bands were revealed on the gels which were not apparent when the PAS stain was used. Apart from bands of $M_r$ higher than that of $\alpha_2$ (which are assumed to be oligomeric complexes of $\alpha$ and/or $\delta$), two faintly labelled bands of similar mobility were seen in the region between $\beta$ and $\gamma$ (Fig. 2). For convenience, we have denoted these bands $\beta_1$ and $\varepsilon$. The apparent $M_r$ values for $\beta_1$ and $\varepsilon$ were $34000 \pm 1000$ (five determinations) and $32000 \pm 1100$ (five determinations) respectively. When membranes from P. L. and K. W. were labelled in a similar manner, one of the bands ($\beta_1$) was absent, as well as bands $\beta$ and $\gamma$ (Fig. 2 shows data for P. L.). Immunoprecipita-

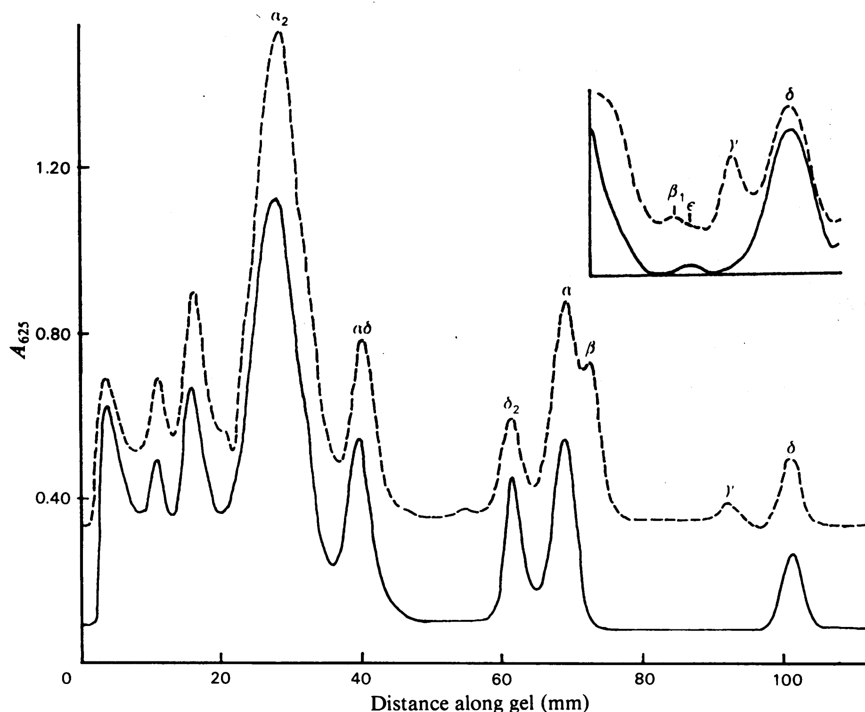


Fig. 2. *Scans of autoradiographs of normal and P. L. membranes*

Scans of autoradiographs shown in Fig. 1, tracks 6 and 11; ----, control erythrocyte membranes; ——, P. L. erythrocyte membranes; the inset shows a scan of the $\beta_1$ region after 8 weeks exposure.

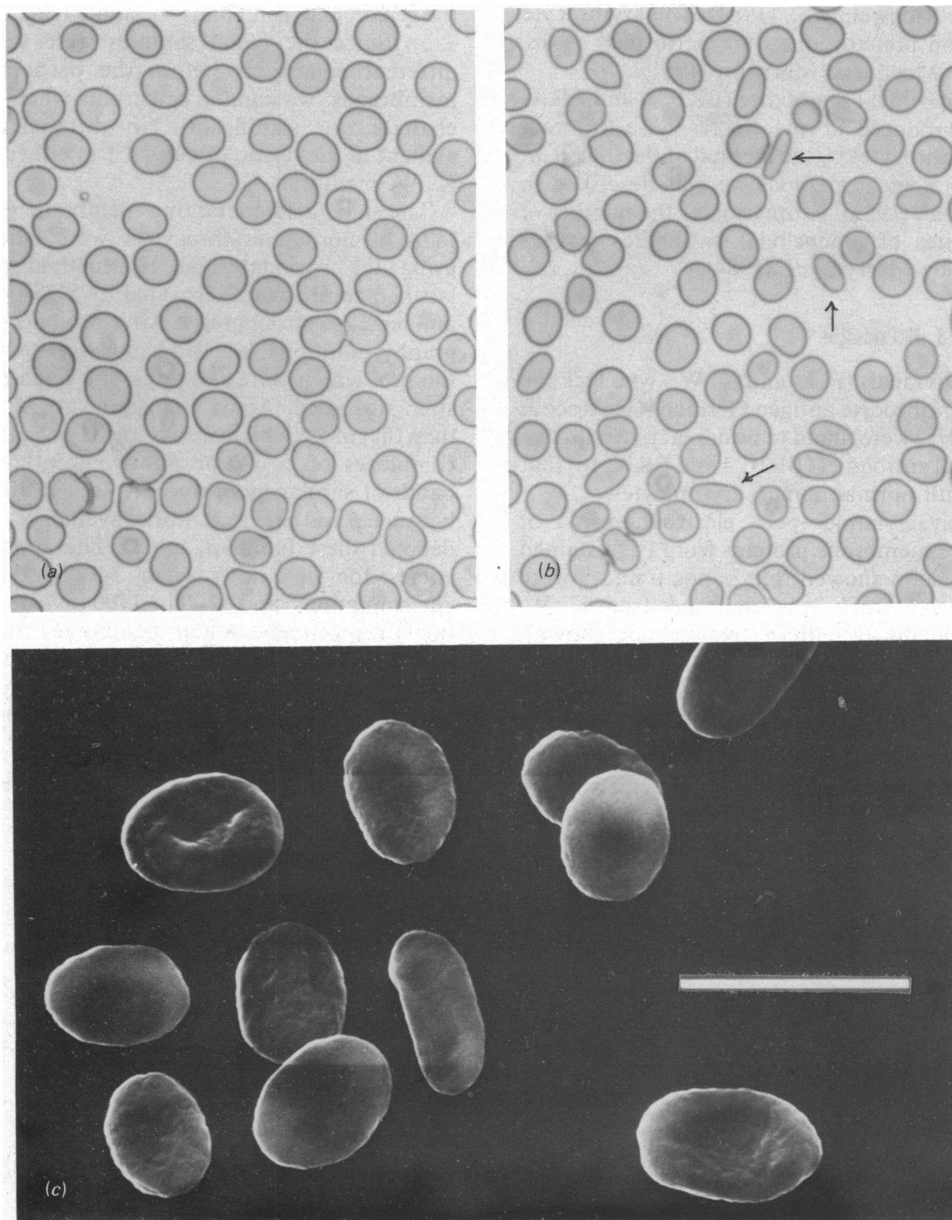


Fig. 3. *Morphology of erythrocytes from a normal donor and P. L.*
(*a*) and (*b*) are photomicrographs of smears of blood taken into EDTA and stained with May–Grunwald–Giemsa stain (Leitz Dialux 20EB microscope; original magnification × ~1300). (*a*), normal donor; (*b*), P. L. (*c*) is a scanning electron micrograph of erythrocytes from P. L. Erythrocytes were fixed in 2.5% glutaraldehyde overnight at room temperature, dehydrated and gold-coated before examination at 25kV in a Philips (Eindhoven, The Netherlands) scanning electron microscope. The white scale bar corresponds to 10 μm.

tion from periodate/$NaB^3H_4$-labelled normal erythrocytes using BRIC 4 and BRIC 10 antibodies showed that these antibodies specifically precipitated sialoglycoproteins $\beta$ and $\beta_1$ (Fig. 1, tracks 7 and 9). As expected, no immunoprecipitation of these components was obtained with either antibody from the cells of P. L. which had been similarly labelled (Fig. 1, tracks 8 and 10).

Measurement of the number of binding sites per cell, using radioiodinated monoclonal antibodies BRIC 4 and BRIC 10 on normal erythrocytes, gave values of 49000 ($\pm$ 11600; six determinations) and

49700 (±9400; five determinations) respectively. By comparison, the values obtained for the binding of these antibodies to erythrocytes of P. L. were 4900 and 4300 respectively (assumed to result from non-specific binding).

Examination of the erythrocytes of P. L. and K. W. by optical microscopy of stained blood films showed that approx. 10% of the cells were frankly elliptocytic (Fig. 3*b* shows data for P. L.). Further examination by scanning electron microscopy revealed that, in addition to the pencil-shaped elliptocytic cells, cells with characteristics intermediate between these and discocytes were also present (Fig. 3*c* shows data for P. L.). There was no evidence of erythrocyte fragmentation.

The two available siblings of P. L. were also studied (V. R. and H. L.); no elliptocytes were present in blood samples from either of these donors. The SDS/polyacrylamide-gel electrophoresis patterns of erythrocyte membrane proteins from these siblings were also apparently normal.

Since it has been suggested that sialoglycoprotein $\beta$ (glycoconnectin) is connected to the erythrocyte cytoskeleton via band 4.1 (Mueller & Morrison, 1981), it was important to determine whether $\beta_1$ and $\gamma$ are also associated with the cytoskeleton. The erythrocyte cytoskeleton has been defined as the insoluble residue remaining after extraction of erythrocyte membranes with Triton X-100 (Lux, 1979). When cytoskeletons were prepared from periodate/$NaB^3H_4$-labelled normal erythrocytes, a selective enrichment of sialoglycoproteins $\beta$, $\beta_1$, $\varepsilon$ and $\gamma$ was observed (Fig. 1, tracks 12 and 13). This suggests that all the components that appear to be absent from the erythrocytes of P. L. and K. W. ($\beta$, $\beta_1$ and $\gamma$) are associated with the cytoskeletal complex. A component with similar mobility to that of $\varepsilon$ was found in cytoskeletons prepared from the cells of P. L. (results not shown). Coomassie Blue staining of the cytoskeletons from P. L. and K. W. showed that band 4.1 remained associated with the cytoskeletons as in normal cells. Thus the minor sialoglycoproteins are not required for the retention of band 4.1 either with the cytoskeleton or with the erythrocyte membrane. Mueller & Morrison (1981) have recently described an individual with elliptocytic erythrocytes which lack band 4.1. These authors were unable to show whether sialoglycoproteins $\beta$ or $\gamma$ were absent from intact erythrocyte membranes from this band 4.1-deficient individual, although cytoskeletons isolated from these erythrocyte membranes did lack both $\beta$ and band 4.1. A deficiency in band 4.1 has also been shown to be associated with hereditary elliptocytosis in another individual (Tchernia *et al.*, 1981), but it is not clear in either case whether the shape change results simply from the absence of band 4.1. In the cases described here, band 4.1 is present in both the intact erythrocyte membranes and isolated cytoskeletons. The possible absence of $\beta$, $\beta_1$ and $\gamma$ may be significant in determining the elliptocytic shape of the erythrocytes of both P. L. and K. W.

The estimation of the number of binding sites on normal erythrocytes for the monoclonal antibodies BRIC 4 and 10 suggests that $\beta$ and $\beta_1$ together comprise approx. 50000 sites per cell. Thus the number of membrane attachment sites for the cytoskeleton that involve $\beta$ and $\beta_1$ is comparable in magnitude with the number of sites involved in the well-documented interaction between erythrocyte band 3 and ankyrin (100000; Bennett, 1982). It seems likely that the minor sialoglycoproteins, $\beta$, $\beta_1$ and/or $\gamma$ will be of significance in the association of the erythrocyte cytoskeleton with the membrane.

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