Miltenberger Class I and II erythrocytes carry a variant of glycophorin A

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The membranes from Miltenberger Class I (Mi I) and II (Mi II) erythrocytes, two rare variants at the blood group MNSs locus, exhibited an abnormal glycoprotein of 32 kDa apparent molecular mass sharply stained by the periodic acid/Schiff procedure and a decreased content of glycoprotein α (synonym glycophorin A, glycoprotein MN) as seen on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Purified ^{125}I -labelled *Vicia graminea* lectin binds to the unusual 32 kDa glycoprotein separated from Mi I and Mi II erythrocyte membrane of blood group NN or MN, but no significant labelling of this band was observed with Mi samples typed MM. On the basis of such lectin-labelling experiments we have described two heterozygous MN, Mi I individuals that carry one copy of an *M* gene producing a normal α -glycoprotein with M-specificity and one copy of a *Mi*^I gene producing a 32 kDa glycoprotein with N-specificity. Further investigations have shown that the 32 kDa glycoprotein was immunoprecipitated by two mouse monoclonal antibodies (R18 and R10) reacting specifically with the external domain of glycoprotein α . These results demonstrate that Mi I and Mi II erythrocytes carry an unusual variant of glycoprotein α .

The Miltenberger subsystem was first recognized by Cleghorn (1966) as defining five classes of human erythrocytes called Mi I, II, III, IV and V on the basis of a phenotypic relationship between red cells carrying rare antigens (Mi^a, V^w, Mur, Hil and Hut) closely linked to the blood-group-MNSs locus (Race & Sanger, 1975). After the discovery of new antisera, the system was extended to seven (Giles *et al.*, 1977) and more recently to eight (Dybckjaer *et al.*, 1981) classes.

As noticed previously, haplotypes carrying Mi III, IV and V specificities display abnormal reactivities within MN and/or Ss antigens (Cleghorn, 1966; Race & Sanger, 1975; Metaxas & Metaxas-Buhler, 1978) as well as an altered sialic acid content as compared with control erythrocytes (Dahr *et al.*, 1978; Anstee *et al.*, 1979; Anstee, 1980). These findings have stimulated biochemical studies of these rare variants and the first approach was an analysis of the membrane glycoproteins solubilized in SDS and separated by polyacrylamide-gel electrophor-

esis. After periodic acid/Schiff staining of the separated components in the discontinuous system of Laemmli (1970), four main monomeric glycoprotein species have been detected in control erythrocytes, namely GP α (synonym glycophorin A monomer, PAS-2, MN glycoprotein), GP β (synonym glycophorin C), GP γ (synonym component E) and GP δ (synonym glycophorin B monomer, PAS-3, Ss glycoprotein), in increased order of mobility (nomenclature from Anstee *et al.*, 1979). Homodimers α_2 (synonym PAS-2) and δ_2 as well as heterodimers $\alpha\delta$ (synonym PAS-4) were also detectable on the gels.

After similar studies performed on native and/or cell-surface-labelled erythrocytes of the Miltenberger complex, it was assumed that Mi I and Mi II red cells carry an unusual variant of GP β with a strong periodic acid/Schiff intensity (Anstee, 1980), whereas Mi III, IV and VI carry an abnormal δ -glycoprotein (Anstee & Tanner, 1978; Anstee *et al.*, 1979; Dahr *et al.*, 1978). The class Mi V is peculiar, since these erythrocytes carry a hybrid glycoprotein molecule made of the N-terminus of GP α and the C-terminus of GP δ (Dahr *et al.*, 1978; Anstee *et al.*, 1979; Vengelen-Tyler *et al.*, 1981). No detectable abnormality of the periodic acid/Schiff-

Abbreviations used: GP α , GP β and GP δ , glycoproteins α , β and δ ; SDS, sodium dodecyl sulphate; Mi, Miltenberger antigen; Vg lectin, *Vicia graminea* lectin; IgG, immunoglobulin G.

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staining profile has been found with class Mi VII (Anstee, 1980) or Mi VIII red cells [from D. J. Anstee; cited by Giles (1982)].

We had recently the opportunity to investigate MM, NN and MN erythrocytes, which express the Mi I and Mi II antigens. In the present paper we report the results obtained by SDS/polyacrylamide-gel electrophoresis, binding of ^{125}I -labelled *Vicia graminea* lectin and immune precipitation with two mouse monoclonal antibodies, showing strong evidence that Mi I and Mi II erythrocytes do indeed carry an unusual variant of GP α rather than an altered GP β component. Before submitting this paper we discovered that our results were in agreement with a preliminary note from Dahr *et al.* (1981), who reached a similar conclusion.

Material and methods

Na^{125}I (16.9 mCi/ μg of iodine) was purchased from The Radiochemical Centre (Amersham, Bucks., U.K.). Lactoperoxidase from bovine milk (60 units/mg) was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). The chemicals used for polyacrylamide-gel electrophoresis were acrylamide, bisacrylamide and *NNN'*-tetramethylethylenediamine from Bio-Rad Laboratories (Touzart et Matignon, Vitry-sur-Seine, France). Bovine serum albumin was obtained from Poviet Laboratories (Organon Teknika, Serfontaine, France) and Protein A-Sepharose CL-4B from Pharmacia (Uppsala, Sweden). All other reagents were of chemical grade. Blood-group-MM, -MN and -NN red cells from control and Miltenberger sources were obtained from Professor C. Salmon, Centre National de Référence sur les Groupes Sanguins, Paris, France, and kept frozen in liquid N_2 until used. Mi I samples from donors Leb. and Aub. were respectively typed MM and NN, whereas Ada. and Blo. were MN. Mi II samples from donors Haf. and Sto. were respectively MM and NN. All these samples were heterozygous for the Miltenberger condition. Different concentrations of *Vicia graminea* lectin were required to agglutinate native M and N red cells (Prigent *et al.*, 1983). Similarly, Miltenberger I and II erythrocytes reacted with *Vicia graminea* according to their MN phenotype. Radioiodination of intact red cells by the lactoperoxidase procedure, preparation of erythrocyte membranes and SDS/polyacrylamide-gel electrophoresis in 10% (w/v) acrylamide were performed as described previously (Blanchard *et al.*, 1982).

Vicia graminea (Vg) lectin was purified from seeds cultivated under artificial conditions (Phytotron du CNRS, Gif-sur-Yvette, France) as previously described (Prigent & Bourillon, 1976) and was radioiodinated by the chloramine-T procedure (Bocci, 1964). Binding of ^{125}I -labelled lectin to

membrane glycoproteins separated by SDS/polyacrylamide-gel electrophoresis was performed in 20 cm-diameter Petri dishes essentially as described by Rostas *et al.* (1977). The gels, fixed and washed as described in the legends to the Figures, were overlaid for 4 h at room temperature with 80 ml of 0.2 M-phosphate buffer, pH 7.4, containing 0.5% (w/v) bovine serum albumin and 500 μg of ^{125}I -labelled lectin (specific radioactivity 0.04–0.06 $\mu\text{Ci}/\mu\text{g}$ of protein). After several further washings in the same buffer devoid of lectin to release unbound radioactive material, the gels were dried and submitted to autoradiography on Kodak X-Omat MA film for 5–10 days. The ^{125}I -labelled lectin can be stored at 4°C in the above buffer for several weeks and re-used in further experiments.

Immune precipitations of ^{125}I -labelled erythrocyte membrane glycoproteins were carried out as described by Mawby *et al.* (1981), with two mouse monoclonal antibodies LICR/LON R18 and R10 kindly provided by Dr. P. Edwards (Ludwig Institute for Cancer Research, Sutton, Surrey, U.K.) and Dr. D. J. Anstee (South West Regional Transfusion Centre, Bristol BS10 5ND, U.K.) respectively. Protein A-Sepharose was used instead of a suspension of fixed *Staphylococcus aureus* (Cowan strain) and the radioactive material eluted was subsequently identified by SDS/polyacrylamide-gel electrophoresis and autoradiography.

Results

SDS/polyacrylamide-gel electrophoresis

Fig. 1(a) shows a typical periodic acid/Schiff-staining profile of membrane glycoproteins obtained after SDS/polyacrylamide-gel electrophoresis of the control erythrocytes. The four monomeric species GP α , GP β , GP γ and GP δ (apparent molecular masses 36, 32, 24 and 20 kDa) were seen, as well as the homodimers (α_2 and δ_2 bands) and heterodimers ($\alpha\delta$) of high molecular mass (86, 42 and 61 kDa respectively).

These values are in good agreement with those found by others (Dahr *et al.* 1976a) and, as far as GP α monomer is concerned, it is very close to the expected value (31 kDa) deduced from chemical composition (Tomita & Marchesi, 1976).

The periodic acid/Schiff-staining pattern of Mi I erythrocyte membrane resembles that of the control, except that a band of apparent molecular mass 32 kDa, migrating in the same position as GP β , exhibited a very strong staining intensity (Fig. 1b). A similar pattern was obtained with Mi II erythrocytes (not shown), as previously reported by Anstee (1980). However, careful examination of the glycoprotein profile performed under identical assay conditions (protein concentration, solubilization and loading on the acrylamide gel) showed that the

Fig. 1. SDS/polyacrylamide-gel electrophoresis of red-cell membranes from Mi I and normal erythrocytes. Electrophoresis of ghosts was performed on a 1.5 mm-thick slab gel containing 10% (w/v) acrylamide, 0.27% (w/v) bisacrylamide with a stacking gel of 3% (w/v) acrylamide in the discontinuous buffer system of Laemmli (1970) in a Bio-Rad 220D electrophoresis system. A 150 μ g sample of protein was loaded and after electrophoresis (3 h at 30 mA/gel), the gel was stained with the periodic acid/Schiff reagent and scanned on a Vernon spectrophotometer equipped with a green filter (no. 57A). (a) Normal erythrocytes; (b) Mi I erythrocytes. Nomenclature of glycoproteins as given by Anstee *et al.* (1979). The arrow indicates position of the Bromophenol Blue tracking dye (TD).

staining intensity of band GP α was decreased in Mi I (and Mi II) as compared with the control, suggesting a decreased content of the main red-cell membrane glycoprotein. Similar results were recently reported by Dahr *et al.* (1981). The band GP α_2 is

also slightly decreased, but the peak was broader in Mi I than in the control. In contrast, the GP δ content appeared unchanged. However, control red cells were not typed for Ss blood groups, and it is known that the staining intensity of GP δ is 1.5 times higher in SS than in ss membranes (Dahr *et al.*, 1976b).

Like Anstee (1980) we have observed that the abnormal Mi I (and Mi II) band was strongly radioiodinated from intact cells (see below and Fig. 3), whereas GP β was not, or only to a very limited extent.

Fig. 2. Binding of the radioiodinated lectin from *Vicia graminea* to human erythrocyte components with Miltenberger I and II specificity

Ghosts from control and Miltenberger I and II erythrocytes were solubilized with SDS and separated by SDS/polyacrylamide-gel electrophoresis as described in Fig. 1. After electrophoresis the gel was fixed overnight with 25% (v/v) propan-2-ol in 10% (w/v) acetic acid. A washing procedure on a rocked platform with 200 ml of each solution was applied as follows: 2 $\times$ 1 h in 10% acetic acid, 3 $\times$ 30 min in distilled water, 2 $\times$ 30 min in 1 M-phosphate buffer, pH 7.4 (buffer A), 24 h in 0.2 M-potassium phosphate, pH 7.4, containing 0.5% (w/v) bovine serum albumin (buffer B, three changes). The gel was then overlaid for 4 h at room temperature with 80 ml of buffer B containing 500 μ g of radioiodinated Vg lectin. The gel was then washed in buffer B until no significant radioactivity was eluted, dried and submitted to autoradiography under exposure to Kodak X-Omat MA film for 5–10 days. The nomenclature of glycoproteins is as in Fig. 1. AG 1 and AG 2 stand respectively for aggregates of GP α and GP δ . Lanes 1 and 7, controls MM and MN respectively; lane 2, NN, Mi I (Aub.) lane 3, MM, Mi II (Haf.); lane 4, NN, Mi II (Sto.); lane 5, MN, Mi I (Ada.); lane 6, MN, Mi I (Blo.).

Binding of *Vicia graminea* lectin to erythrocyte membrane components

The binding of ^{125}I -labelled Vg lectin to erythrocyte membrane glycoproteins separated by SDS/polyacrylamide-gel electrophoresis was investigated after protein fixation and removal of the detergent. In agreement with previous findings, Vg lectin binds both to $\text{GP}\alpha$ and $\text{GP}\delta$ molecules from native NN erythrocytes, whereas it binds only to $\text{GP}\delta$ molecules from native MM erythrocytes (Duk *et al.*, 1982; Prigent *et al.*, 1983). Fig. 2 (lane 7) shows that the major membrane components which bind ^{125}I -labelled Vg lectin were bands α_2 , $\alpha\delta$, δ_2 , α and δ from heterozygous MN individuals, and only bands $\alpha\delta$, δ_2 and δ within MM erythrocytes (Fig. 2, lane 1). High-molecular-mass components that bind a small amount of Vg lectin are also present at the top of the gel and might represent aggregates of $\text{GP}\alpha$ and $\text{GP}\delta$ glycoproteins. Indeed the slow-migrating component denoted the 'AG1 band' is not seen with MM erythrocytes (Fig. 2, lanes 1 and 3) and might represent an aggregate of $\text{GP}\alpha$, and/or of the 32 kDa component. In contrast, the fast-migrating component denoted 'AG2' is always present and might represent an aggregate of $\text{GP}\delta$. The $\text{GP}\beta$ band is not, or only to a very small extent, recognized by Vg lectin, but this glycoprotein is present in low concentration in the membrane.

Fig. 2 (lanes 2 and 4) shows the pattern of binding of ^{125}I -labelled Vg lectin to membrane components from two NN individuals typed Mi I and Mi II respectively. These profiles are identical with those obtained with normal NN or MN (lane 7) individuals except that a 32 kDa component migrating as $\text{GP}\beta$ is strongly labelled. As is also shown in Fig. 2 (lane 3), the Vg-lectin-binding components of an MM individual carrying the Mi II antigen are identical with those from control MM individuals (lane 1), both showing a faint labelling in the region of 32 kDa.

The results obtained with the two Mi I individuals heterozygous for MN are of particular interest (Fig. 2, lanes 5 and 6). Careful examination shows that the Vg lectin binds to the 32 kDa component, to $\text{GP}\delta$ and δ_2 , but clearly not to $\text{GP}\alpha$ monomer. Such results suggest that N-specificity from these erythrocytes is related to the presence of the 32 kDa component (and $\text{GP}\delta$), but not to the normal $\text{GP}\alpha$ glycoprotein, which appears to carry the M-specificity with a typical polymorphism at position 1 and 5 of the N-terminal sequence (Wasniowska *et al.*, 1977; Dahr *et al.*, 1977). We assume that the labelled bands seen in the area of α_2 and $\alpha\delta$ (Fig. 2, lanes 5 and 6) represent respectively two components. In the area of $\text{GP}\alpha_2$ we might expect an homodimer of the 32 kDa component and an heterodimer of the 32 kDa component with $\text{GP}\alpha$. In the area of $\alpha\delta$ one can find the normal $\alpha\delta$ together

Fig. 3. Immunoprecipitation of radiolabelled sialoglycoproteins from Mi I and Mi II variant erythrocytes

The intact red cells from control and Miltenberger erythrocytes were radioiodinated as previously described (Blanchard *et al.*, 1982). The labelled ghosts were solubilized (ghosts/buffer, 1:1, v/v) in 100 mM-Tris/HCl, pH 8.0, containing 300 mM-NaCl and 2% (w/v) sodium deoxycholate, and 100 μl of lysate was incubated at 0°C for 1 h with 10 μl of non-immune mouse serum, followed by a 30 min incubation with 20 μl of rabbit anti-mouse IgG (1 mg/ml). After a further incubation for 30 min at 0°C with 50 μl of a 50% suspension of Protein A-Sepharose in 50 mM-Tris/HCl (pH 8.0)/150 mM-NaCl/1% sodium deoxycholate, the gel was removed by centrifugation. The supernatant (150 μl) was immunoprecipitated in the cold with 10 μl of the mouse monoclonal anti-(human erythrocyte) antibody (R18) followed by 20 μl of rabbit anti-mouse IgG and 50 μl of Protein A-Sepharose as described above. The gels were finally washed three times with cold 150 mM-NaCl/5 mM-EDTA/0.5% (v/v) Triton X-100, and the immune complexes were eluted by heating for 3 min at 100°C in 100 μl of Laemmli buffer. SDS/polyacrylamide-gel electrophoresis of the samples was performed in a Bio-Rad Protein electrophoresis system as described in the legend of Fig. 1. The gel was then dried on a filter paper and exposed to Kodak X-Omat MA film for 2 days. Lanes 1 and 2, ^{125}I -labelled ghosts from control and Mi I (Blo.) erythrocytes respectively; lanes 3 to 6, immune precipitations carried out with non-immune mouse serum using MN, NN, Mi I (Blo.) and Mi II (Haf.) ^{125}I -labelled red-cell-membrane lysates respectively; lanes 7 and 8, immune precipitation with mouse monoclonal R18 antibody and Mi I (Blo.) and control ^{125}I -labelled ghosts.

with an heterodimer of GP δ with the 32kDa component.

Immunoprecipitation with mouse monoclonal antibodies

Immunoprecipitation of membrane glycoproteins solubilized in deoxycholate from intact cells labelled by the lactoperoxidase procedure were performed with two mouse monoclonal antibodies, R18 and R10, which specifically recognized the external domain of GP α between residues 40 and 72 and in the region of residue 26 respectively (Mawby *et al.*, 1981; Anstee & Edwards, 1982). As Fig. 3 shows, the R18 antibody precipitated GP α from control red cells (MM or NN) and also both GP α and the 32kDa component from Mi I (and Mi II) erythrocytes. Similar results were obtained with the R10 antibody (not shown), but faint bands were seen on the gel, presumably because the R10 is of weaker affinity. These results demonstrate a direct structural relationship between GP α and the 32kDa glycoprotein.

Discussion

We have presented here some experimental results that favour the view that Mi I and Mi II erythrocytes carry an unusual 32kDa-apparent-molecular-mass glycoprotein that is related to GP α , the main red-cell membrane glycoprotein. Several lines of evidence strongly support this conclusion: (i) Mi I and II erythrocytes carry a new glycoprotein and a decreased amount of GP α , and (ii) binding of the ¹²⁵I-labelled Vg lectin to membrane components separated by SDS/polyacrylamide-gel electrophoresis shows that the 32kDa glycoprotein of Mi I and II is strongly labelled when originating from NN and MN, but not from MM individuals. It is known from previous studies that this lectin binds to GP α and GP δ from NN and MN individuals, but only to GP δ from group-MM individuals (Prigent *et al.*, 1983). Most noteworthy and highly significant is the result obtained with two samples from MN, Mi I subjects, showing that the 32kDa glycoprotein, but not the GP α molecule, is recognized by Vg lectin. Therefore these two MN, Mi I individuals should carry one copy of a normal *M* gene, producing a GP α molecule (not recognized by Vg lectin) and one copy of a *Mi*^I gene producing the unusual 32kDa glycoprotein with N (and Mi I) specificity (recognized by Vg lectin). As a result a reduced content of normal GP α molecule is expected. Lastly (iii) evidence is provided by the immune precipitation of both GP α and 32kDa component by two mouse monoclonal antibodies which are specific for the external domain of GP α .

That the *Mi*^I and *Mi*^{II} genes might represent alleles of the α gene and produce a glycoprotein structurally related to GP α is also in agreement with the trypsin-sensitivity of Mi I and Mi II antigens (Anstee, 1980; Giles, 1982). The present results also support the preliminary results obtained by Dahr *et al.* (1981) showing by chemical analysis that Mi I and Mi II glycoproteins might be due to Thr→Met and Thr→Lys substitutions respectively at position 28 of GP α . It is not yet known, however, if the anti-Vw and anti-Hut antisera which serologically define the Mi I and II classes are detecting this polymorphism. It was also shown that ¹²⁵I-labelled *Phaseolus vulgaris* lectin does not bind to Mi I, II components (the 32kDa glycoprotein), which signifies that the asparagine residue at position 26 is not glycosylated. This is also expected from the above substitutions, since the Asn-Xaa-Thr (Ser) sequence required for *N*-glycosylation (Pless & Lennarz, 1977) is no longer present. The fact that Mi I and Mi II glycoproteins lack *N*-glycosylation might explain their decreased apparent molecular weight compared with that of the normal GP α molecule.

It is concluded that rare antigens gathered under the Miltenberger complex represent a heterogeneous group in which some entities are variants of GP α (Mi I and Mi II), others are presumed variants of GP δ (Mi III, IV, and VI), and still others are hybrid glycoproteins (Mi V) resulting from unequal crossing-over between α and δ genes. Further investigations should establish whether Mi VII and Mi VIII variants belong to one of this group or introduce new complexities.

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