Abnormal Blood-Group-Ss-Active Sialoglycoproteins in the Membrane of Miltenberger Class III, IV and V Human Erythrocytes

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1. We have studied the inherited changes occurring in the sialoglycoproteins of membranes from erythrocytes of type Miltenberger Class III (Mi.III), Miltenberger Class IV (Mi.IV) and Miltenberger Class V (Mi.V) by using sodium dodecyl sulphate/polyacrylamide gel electrophoresis and lactoperoxidase radioiodination. 2. Mi.III erythrocytes lack the normal blood-group-Ss-active sialoglycoprotein but contain an unusual s-active sialoglycoprotein of higher apparent molecular weight. A similar abnormal S-active sialoglycoprotein appears to occur in Mi.IV erythrocytes. 3. The Mi.V condition is associated with the hemizygous absence of both the normal blood-group-MN-active sialoglycoprotein and the normal Ss-active sialoglycoprotein. However, a new sialoglycoprotein component is present in these cells that has properties characteristic of both the MN-active and Ss-active sialoglycoproteins. 4. Our results suggest that the new sialoglycoprotein present in Mi.V erythrocytes is a hybrid of the normal MN sialoglycoprotein and an s-active sialoglycoprotein that has properties similar to the s-active sialoglycoprotein found in Mi.III erythrocytes. We suggest that the unusual Mi.V sialoglycoprotein is derived from chromosomal misalignment with unequal crossing-over between the genes for the MN- and Ss-active sialoglycoproteins in a manner similar to that which gives rise to haemoglobin Lepore. 5. Further studies of S-s-erythrocytes confirm that these cells lack normal Ss-active sialoglycoprotein, but contain an unusual component that shows some of the properties of the normal Ss-active sialoglycoprotein. 6. Analysis of erythrocytes of type M^k/Mi.III confirms that, in addition to the known hemizygous lack of the MN-active sialoglycoprotein, the M^k condition is also associated with a loss of the Ss-active sialoglycoprotein. 7. In order to facilitate discussion of the complex changes that occur in these variant erythrocytes, a new unified nomenclature is used for the erythrocyte sialoglycoproteins.

The human erythrocyte membrane contains at least four different periodate-staining, sialic acid-rich glycoproteins. The major blood-group MN-active sialoglycoprotein (also known as glycophorin A; Tomita & Marchesi, 1975), migrates on SDS/polyacrylamide-gel electrophoresis in both dimeric and monomeric forms as detected by periodic acid-Schiff's-base staining of the gels (PAS-1 and PAS-2 bands; Tuech & Morrison, 1974). One of the minor components, PAS-3 (Hamaguchi & Cleve, 1972; Anstee & Tanner, 1975), carries Ss-blood-group activity, whereas the other minor components (PAS-2'; Mueller & Morrison, 1974; Tanner & Anstee, 1976b) and component E (Dahr *et al.*, 1975a) are not known to be associated with any blood-group determinant.

Abbreviations used: SDS, sodium dodecyl sulphate; Mi.III, Miltenberger Class III; Mi.IV, Miltenberger Class IV; Mi.V, Miltenberger Class V.

Several human erythrocytes carrying genetic variants of these sialoglycoproteins have been described. The major MN-active sialoglycoprotein has been shown to be deficient in the membranes of cells with the En(a-), M^k, and Mi.V phenotypes (Tanner & Anstee, 1976b; Gahmberg *et al.*, 1976; Dahr *et al.*, 1977; Furthmayr, 1978; Anstee & Tanner, 1978), and this protein is altered in cells of the M^s phenotype (Anstee & Tanner, 1978). Erythrocytes that lack S- and s-blood-group antigens have an abnormality of the Ss-blood-group-active glycoprotein (Dahr *et al.*, 1975b; Tanner *et al.*, 1977). No variants of the components PAS-2' and E have yet been described.

The Miltenberger group of erythrocytes (originally defined by their reactivity with the serum Miltenberger) has been subdivided into five classes on the basis of their reactions with four human antisera (Cleghorn, 1966; Crossland *et al.*, 1970). Mi.III erythrocytes react with the antisera Miltenberger,

Murrell and Hill, whereas Mi.IV cells react with the antisera Miltenberger and Murrell only. The Mi.V cells are distinguished from Mi.III and Mi.IV cells by their failure to react with the sera Miltenberger and Murrell, although they do react with antiserum Hill. Mi.III cells have been further subdivided into Mi.III and Mi.VI on the basis of their reactions with antisera Anek and Raddon. Giles *et al.* (1977) summarized the serological evidence for the classification of the Miltenberger classes. All of the Miltenberger group of antigens are very rare in Caucasians. However, the Mi.III condition is found in approx. 10% of the Thai population (Chandanayingyong & Pejrachandra, 1975), and approx. 5% of the American Chinese population [Madden *et al.* (1964) and Sturgeon (1973), both cited by Race & Sanger, 1975].

In the present paper we report that Mi.III and Mi.IV erythrocytes contain similar abnormal Ss-active glycoproteins with a substantially decreased mobility on SDS/polyacrylamide-gel electrophoresis, and describe the further characterization of the changes in the blood-group-Ss-active sialoglycoprotein that occur in Mi.V, M^k and S-s-erythrocytes. When the periodic acid/Schiff's-base-staining components of human erythrocytes are separated by SDS/polyacrylamide-gel electrophoresis with the system of Fairbanks *et al.* (1971), four or five components can be detected in addition to the erythrocyte anion-transport protein (band 3). These have been denoted PAS-1, -2, -2', -3 and -4 (Steck, 1974; Mueller & Morrison, 1974). The discontinuous electrophoresis system of Laemmli (1970) clearly separates all of these components (Fig. 1*d*) and resolves at least two additional components (Dahr *et al.*, 1975*a*; Mueller *et al.*, 1976). It has become apparent that the complex pattern of bands results from the formation of homo and hetero complexes between two of the four different polypeptides that are present. The existing nomenclatures for these periodate-staining bands (Steck, 1974; Dahr *et al.*, 1975*a*; Mueller *et al.*, 1976) do not reflect the polypeptide composition or aggregation state of the individual bands. Difficulties in utilizing the existing nomenclatures became apparent to us when attempting to describe the changes observed in the gel-electrophoretic patterns of erythrocytes with variant sialoglycoproteins. In many cases, shifts in electrophoretic mobility occur both in individual components and in the complexes that they form, and consequently the description of these changes becomes very cumbersome. Since the polypeptide content of these sialoglycoprotein bands has been established, we suggest that the nomenclature should reflect this knowledge and that the bands be described in terms of their polypeptide composition rather than electrophoretic mobility. A new nomenclature for the sialic acid-rich glycoproteins of the human erythrocyte is proposed.

Materials and Methods

Whole blood from six donors heterozygous for the Mi.III condition (Mr. A, phenotype MSs; Mr. B, phenotype Ms; Mr. Sa, phenotype Ms; Mr. Su, phenotype Ms; Mr. V, phenotype Ms and Mr. M, phenotype Ms) and one donor (Mrs. C. H.) genotype Ms^{Mi.III}/Ms^{Mi.III} (donor I-2 in family C. H. of Chandanayingyong *et al.*, 1977) were provided by Dr. D. Chandanayingyong, Siraraj Hospital, Bangkok, Thailand. Whole blood from donors of genotype Ms^{Mi.III}/M^k (donor E. Q.), Ms^{Mi.III}/M^k (donor H. Q.) and Ns/M^k (donor J. L.) from the family described by Sturgeon *et al.* (1970) were provided by Dr. P. Sturgeon, Cedars-Sinai Medical Center, Los Angeles, CA, U.S.A. Dr. C. M. Giles, Blood Group Reference Laboratory, London SW1W 8QS, U.K., provided cells of genotype M^{Vs}/Ns^{Mi.V} [donor L. R. (Crossland *et al.*, 1970)], Ns/Ns^{Mi.V} (donor H. S.), Ms/Ms^{Mi.V} (donor M. S.) and Ms/NS^{Mi.V} (donor R. H.). S-s-U-cells (donor N. Z.) were provided by Miss P. Moores, Durban, South Africa.

The methods for the preparation of erythrocyte 'ghosts' and radioiodination of intact erythrocytes with lactoperoxidase were described previously (Boxer *et al.*, 1974). SDS/polyacrylamide-gel electrophoresis was by the method of Laemmli (1970); a separating gel containing 10% (w/v) acrylamide was used with either a 3% (tube gels) or 5% (slab gels) stacking gel.

Elution and re-electrophoresis of ¹²⁵I-labelled bands was performed as follows. Radioiodinated samples were subjected to electrophoresis on slab gels as described above and the radioactive bands detected by radioautography of the wet gel for approx. 3h. The appropriate labelled bands were then excised, homogenized and eluted into 0.1% SDS/5mm-NH₄HCO₃/0.1mm-EDTA/1mm phenylmethanesulphonyl fluoride containing 100μg of bovine γ-globulin/ml for 2–3 days. The resulting supernatants were recovered, freeze-dried and subjected to re-electrophoresis on slab gels as described above. Quantitative assessment of the relative radioactivity of individual bands was carried out as follows: labelled bands were excised from dried gels, immersed in 0.5 ml of 0.5% SDS and shaken for 2 days at 37°C. A 10 ml portion of scintillation fluid {containing 4 g of PPO (2,5-diphenyloxazole), 75 mg of POPOP [1,4-bis-(5-phenyloxazol-2-yl)benzene] and 120 g of naphthalene in 1 litre of 1,4-dioxan} was added, and after 48 h the material was counted for radioactivity in a liquid-scintillation counter.

The method for locating lectin-binding components was described previously (Tanner & Anstee 1976*a*). Wheat (*Triticum vulgaris*)-germ lectin (3.25 mg; Calbiochem, Bishops Cleeve, Herts, U.K.) was dissolved in iso-osmotic sodium phosphate buffer, pH 7.4 (2 ml), containing 0.3 M-N-acetylglucos-

amine (Sigma) and radioiodinated with 0.5 mCi of ^{125}I with 100 mg of chloramine-T (BDH, Poole, Dorset, U.K.). After 1 min at room temperature, 150 μg of sodium metabisulphite was added, followed 15 min later by 30 mg of bovine haemoglobin (Sigma). After exhaustive dialysis against phosphate-buffered saline (1 vol. of iso-osmotic sodium phosphate buffer, pH 7.4, diluted with 9 vol. of 0.15 M-NaCl), pH 7.4, the labelled protein (agglutination titre > 1:2000 against Pronase-treated human erythrocytes; 1.9×10^7 c.p.m. of ^{125}I /ml) was used directly in lectin-binding experiments.

Sialic acid contents of intact erythrocytes were assessed by assay of the sialic acid released from a known number of erythrocytes after incubation with neuraminidase (Aminoff *et al.*, 1976). Packed erythrocytes (50 μl ; range 2×10^8 – 4×10^8 cells in different experiments) in 0.15 M-NaCl, pH 6.0, containing 1 mM- CaCl_2 (total vol. 300 μl) were incubated at 37°C for 30 min with neuraminidase (20 μl ; 500 units/ml; from *Vibrio cholerae*; Hoechst Pharmaceuticals, Brentford, Middx., U.K.) and 100 μl of the resulting supernatant material assayed by the thiobarbituric acid method (Aminoff, 1961). Quadruplicate assays were performed and the results were compared with the values obtained by using at least three different samples of normal control cells.

Results and Discussion

Nomenclature and composition of the periodate staining sialoglycoproteins of the normal erythrocyte

We propose that the four different sialoglycoprotein polypeptides of the erythrocyte membrane be denoted α , β , γ and δ in order of increasing electrophoretic mobility (Fig. 1). These components have also been reproducibly detected in two other laboratories (Dahr *et al.*, 1975a; Mueller *et al.*, 1976). It is likely that other more weakly periodate-staining components also occur, and indeed additional bands can be found in occasional electrophoresis runs. Component α is the major blood-group-MN-active sialoglycoprotein (glycophorin A; Tomita & Marchesi, 1975). Component β is PAS-2' (Mueller & Morrison, 1974; Tanner & Anstee, 1976b). Component γ is the minor PAS-staining polypeptide identical with component E (Dahr *et al.*, 1975a) and the 27000-mol.wt. component (Mueller *et al.*, 1976). Component δ is the blood-group-Ss-active glycoprotein (glycophorin B; Furthmayr *et al.*, 1975). PAS-1, the dimeric form of the major MN-active sialoglycoprotein (Marton & Garvin, 1973; Tuech & Morrison, 1974; Furthmayr & Marchesi, 1976), is denoted α_2 . PAS-4 ($\alpha\delta$) is a hybrid dimer of the α and δ components (Dahr *et al.*, 1976a; Tanner *et al.*, 1977),

Fig. 1. SDS/polyacrylamide-gel electrophoresis of membranes from Mi.V, Mi.III, S-s- and normal erythrocytes. Electrophoresis was in a slab containing 10% (w/v) acrylamide with an overlay of 5% acrylamide in the buffer system of Laemmli (1970). The gel was stained with the periodic acid/Schiff-base stain. (a) Heterozygous Mi.V (donor H. S.); (b) homozygous Mi.III/Mi.III (donor C. H.); (c) S-s-U- (donor N. Z.); (d) Normal control. Abbreviation used: T.D., tracking dye.

Fig. 2. Re-electrophoresis of radiolabelled bands eluted from electrophoretograms of radioiodinated normal erythrocytes

Packed cells (200 μ l) were radioiodinated by using 0.4 mCi of Na^{125}I and 90 μ g of lactoperoxidase. Membranes from these erythrocytes were separated on a 10% acrylamide gel with a 5% overlay, with the buffer system of Laemmli (1970). The wet gel was radioautographed for up to 3 h and the radioactive bands excised and eluted as described in the Materials and Methods section. (a) Normal erythrocytes; (b) re-electrophoresis of the δ -band from normal erythrocytes; (c) re-electrophoresis of the α -band; (d) re-electrophoresis of the $\alpha\delta$ -band; (e) re-electrophoresis of the α_2 -band. [The labelling of the band marked * was very variable. The band remained substantially undissociated on re-electrophoresis and did not correspond to any of the periodic acid/Schiff's-base-staining bands. The band may represent a poorly sialylated membrane component exposed on the outer surface of the cell or possibly a proteolytic digestion product of band III.]

whereas PAS-2, -2' and -3 are the monomeric forms α , β and γ respectively, δ_2 is only resolved in a discontinuous electrophoresis system and its dimeric nature has been established by Dahr *et al.*, (1976a).

We have confirmed the composition of the component- α - and - δ -containing bands by elution and re-electrophoresis of each of these components in membranes derived from normal erythrocytes that had been radioiodinated with lactoperoxidase. Components β and γ are labelled very poorly by the lactoperoxidase method (Mueller *et al.*, 1976; Fig. 2a). Fig. 2 shows that elution and re-electrophoresis of the α_2 band yielded only components α and α_2 , whereas the $\alpha\delta$ band gave mainly components α and δ with smaller amounts of components α_2 , $\alpha\delta$ and δ_2 . Band α yielded mostly component α with a little component α_2 . Band δ yielded predominantly component δ with traces of component δ_2 .

Membranes from Mi.III erythrocytes

The Mi.III condition is associated with elevated amounts of erythrocyte sialic acid (Luner *et al.*, 1975; Bergen & Morel, 1978). We obtained sialic acid values of $121 \pm 8\%$ (4) for homozygous Mi.III cells and $113 \pm 3\%$ (4) for heterozygous cells compared with normal controls.

SDS/polyacrylamide-gel electrophoresis. Membranes from one donor homozygous for the Mi.III condition and six donors heterozygous for the condition were examined by SDS/polyacrylamide-gel electrophoresis. The protein-staining patterns did not show any difference when compared with normal controls. When gels of membranes from the homozygous Mi.III donor were stained for carbohydrate with the periodic acid/Schiff's-base stain they showed normal amounts of the α -component and α_2 -band, but bands

Fig. 3. SDS/polyacrylamide-gel electrophoresis of 'ghosts' from heterozygous Mi.III, homozygous Mi.III/Mi.III and normal erythrocytes

Separation of 'ghosts' containing 150 μ g of membrane protein from (a) normal, (b) homozygous Mi.III/Mi.III (donor C. H.) and (c) heterozygous Mi.III erythrocytes (donor Mr. B.) was carried out on tube gels containing 10% (w/v) acrylamide with a 3% overlay, with the buffer system of Laemmli (1970). The gels were stained with the periodic acid/Schiff's-base stain and scanned at 560 nm.

corresponding to components δ or δ_2 were absent; however, a new component, which we denote as $\delta^{\text{Mi.III}}$ was apparent (Figs. 1b and 3b). The $\delta^{\text{Mi.III}}$ component of homozygous Mi.III/Mi.III cells stained with the periodic acid/Schiff's-base stain with approximately twice the intensity of the normal monomeric form of the δ polypeptide [1.9 ± 0.1 (2)]. The apparent mol.wt. of component $\delta^{\text{Mi.III}}$ on 10% acrylamide gels was 36000, whereas the normal δ component gave an apparent mol.wt. of 25000 under the same conditions. Mi.III/Mi.III cells also contained periodate/Schiff-staining bands with the expected mobilities of the complexes $\alpha\delta^{\text{Mi.III}}$ and $\delta_2^{\text{Mi.III}}$ (apparent mol.wts. 79000 and 72000 respectively). Membranes from cells heterozygous for the Mi.III condition contained both δ and $\delta^{\text{Mi.III}}$ components. In addition to bands $\alpha\delta^{\text{Mi.III}}$ and $\delta_2^{\text{Mi.III}}$, a further band, corresponding to $\delta\delta^{\text{Mi.III}}$ (apparent mol.wt. 63000), was observed (Fig. 3c). The heterozygous cells contained lower amounts of component δ than normal erythrocytes [$45\% \pm 14$ (3)] and less component $\delta^{\text{Mi.III}}$ [$39\% \pm 8$ (3)] than the homozygous Mi.III/Mi.III cells. Prior treatment of either Mi.III/

Mi.III or heterozygous Mi.III erythrocytes with trypsin did not degrade the $\delta^{\text{Mi.III}}$ component.

Lactoperoxidase radioiodination of Mi.III erythrocytes. The patterns of radioiodination of intact homozygous and heterozygous Mi.III erythrocytes and normal control erythrocytes are shown in Figs. 4(a)–4(f). The homozygous Mi.III/Mi.III cell showed strong labelling of the bands containing component $\delta^{\text{Mi.III}}$. No labelling of the components corresponding to the normal δ - and δ_2 -bands was observed. The electrophoresis patterns of lactoperoxidase-radioiodinated heterozygous Mi.III cells showed labelling of normal δ - and δ_2 -bands (but this was weaker than in the normal control) as well as of bands $\delta^{\text{Mi.III}}$ and $\delta_2^{\text{Mi.III}}$. An additional labelled band corresponding to $\delta\delta^{\text{Mi.III}}$ (mol.wt. 63000) was also observed.

Although the homozygous Mi.III/Mi.III erythrocytes lacked the normal δ components, prolonged radioautography revealed the presence of three weakly labelled components migrating in the δ -region that were not detectable in the normal control cells (X, Y and Z; Figs. 4d–4f). None of these components

Fig. 4. Radioautographs of SDS/polyacrylamide gels of membranes from lactoperoxidase-catalysed radioiodinated erythrocytes from Mi.III, Mi.V, S-s- and normal erythrocytes

(a)–(f) Lactoperoxidase-catalysed radioiodination of heterozygous Mi.III and homozygous Mi.III/Mi.III erythrocytes. Packed erythrocytes (0.1 ml) were radioiodinated by using 0.5 mCi of Na^{125}I and 50 μg of lactoperoxidase. Membranes from the radioiodinated cells were separated on a 10% (w/v) acrylamide with a 5% overlay, with the buffer system of Laemmli (1970). Radioautographs of dried electrophoretograms obtained from two different exposures are shown: (a)–(c) 1 h; (d)–(f) 36 h. Samples (a) and (d) are normal erythrocytes, (b) and (e) are homozygous Mi.III/Mi.III erythrocytes (donor C. H.), and (c) and (f) are heterozygous Mi.III erythrocytes (donor Mr. V.). (g)–(h) Lactoperoxidase-catalysed radioiodination of heterozygous Mi.V erythrocytes. Packed erythrocytes (200 μl) were radioiodinated by using 0.4 mCi of Na^{125}I and 90 μg of lactoperoxidase. Membranes were separated on 10% (w/v) acrylamide with a 5% overlay by using the buffer system of Laemmli (1970). Radioautographs: (g) heterozygous Mi.V (donor H. S.); (h) normal erythrocytes. (i)–(l) Lactoperoxidase-catalysed radioiodination of S-s- and normal erythrocytes. Packed cells (300 μl) were radioiodinated by using 0.6 mCi of Na^{125}I and 120 μg of lactoperoxidase. Membranes were separated on a 10% (w/v) acrylamide with a 5% overlay, with the buffer system of Laemmli (1970). Radioautographs of dried electrophoretograms obtained from two different exposures are shown: (i)–(j) 1 day; (k)–(l) 7 days. (i) and (k) are normal erythrocytes, (j) and (l) are S-s- erythrocytes (donor N. Z.). For explanation of the * band, see the parenthetical note in Fig. 2.

corresponds in mobility to the normal δ -component. These components were also found in the heterozygous Mi.III cells together with normal δ -component. It is not clear whether these are pre-existing components that become more susceptible to lactoperoxidase labelling as a consequence of the changes at the cell surface that occur in Mi.III cells or whether they represent the appearance *de novo* of new surface components as a consequence of the genetic events associated with the appearance of the $\delta^{\text{Mi.III}}$ -component.

Composition of the sialoglycoprotein complexes in Mi.III cells. The polypeptide composition of the components in each of the unusual complexes found in Mi.III cells was confirmed by elution of the radio-

Fig. 5. Re-electrophoresis of radiolabelled bands eluted from electrophoretograms of radioiodinated Mi.III erythrocytes

Heterozygous Mi.III and homozygous Mi.III/Mi.III erythrocytes were radiolabelled as described in the legend to Fig. 3. The membranes were separated and the eluted bands analysed as described in Fig. 1. (a) Radioautograph of normal whole erythrocyte membranes; (b) re-electrophoresis of the $\delta\delta^{\text{Mi.III}}$ -band (see Fig. 4c) isolated from heterozygous Mi.III cells (donor Mr. V.) [Note: labelling in the α - and α_2 -regions of this radioautograph is due to contamination from the adjacent slot (not shown) of the slab gel]; (c) re-electrophoresis of the $\delta_2^{\text{Mi.III}}$ -band from homozygous Mi.III/Mi.III cells (donor C. H.); (d) re-electrophoresis of the $\delta_2^{\text{Mi.III}}$ -band from heterozygous Mi.III cells (donor Mr. V.); (e) and (f) re-electrophoresis of the $\delta^{\text{Mi.III}}$ -band from homozygous (donor C. H.) and heterozygous (donor Mr. V.) Mi.III cells respectively; (g) whole erythrocyte membranes from homozygous Mi.III/Mi.III cells (donor C. H.). For an explanation of the band marked †, see legend to Fig. 9.

active bands obtained from gels of membranes derived from lactoperoxidase-labelled erythrocytes, and re-electrophoresis of the eluted components (Fig. 5). The $\delta^{\text{Mi.III}}$ -component from homozygous (Fig. 5e) and heterozygous (Fig. 5f) Mi.III cells predominantly yielded the monomeric form (component $\delta^{\text{Mi.III}}$) with traces of dimeric $\delta_2^{\text{Mi.III}}$ band. No trace of bands corresponding to normal δ -containing components was found. Significant amounts of one of the weakly labelled bands, Y (Fig. 4e), with an electrophoretic mobility slightly faster than normal δ -component were found in the $\delta^{\text{Mi.III}}$ band. A dimeric form of band-Y component therefore co-migrates with component $\delta^{\text{Mi.III}}$.

Re-electrophoresis of the $\delta_2^{\text{Mi.III}}$ band (Figs. 5c and 5d) gave $\delta^{\text{Mi.III}}$ and $\delta_2^{\text{Mi.III}}$ bands as expected, but in addition significant amounts of band X (Fig. 4e) as well as normal component- α . Presumably a complex of component- α and band X co-migrates with component $\delta_2^{\text{Mi.III}}$. Re-electrophoresis of the $\delta\delta^{\text{Mi.III}}$ band from heterozygous Mi.III cells (Fig. 5b) gave the δ -, $\delta^{\text{Mi.III}}$ -, $\delta\delta^{\text{Mi.III}}$ - and $\delta_2^{\text{Mi.III}}$ -bands as expected. On re-electrophoresis of the $\delta^{\text{Mi.III}}$ band of heterozygous Mi.III erythrocytes, the $\delta^{\text{Mi.III}}$ - and $\delta_2^{\text{Mi.III}}$ -components contained 5.2 ± 1 (2) times more radioactivity than did the sum of the δ - and δ_2 -components. Since it is likely that the $\delta\delta^{\text{Mi.III}}$ complex contains equimolar amounts of δ - and $\delta^{\text{Mi.III}}$ -monomers, it is possible to estimate the molar ratios of δ - and $\delta^{\text{Mi.III}}$ -components by summing the radioactivity present in the component- δ -containing bands and the component- $\delta^{\text{Mi.III}}$ -containing bands in the original membrane sample from heterozygous Mi.III cells. The value obtained for the molar ratio $\delta^{\text{Mi.III}}/\delta[1.1 \pm 0.1$ (2)] suggests that δ - and $\delta^{\text{Mi.III}}$ -components are present in equimolar amounts in heterozygous Mi.III erythrocytes.

Nature of the abnormal component in Mi.III erythrocytes. Our results suggest that component- $\delta^{\text{Mi.III}}$ replaces normal component- δ in Mi.III erythrocytes. Heterozygous Mi.III erythrocytes contain equimolar amounts of δ - and $\delta^{\text{Mi.III}}$ -components. Component $\delta^{\text{Mi.III}}$ readily forms complexes with itself and with components α and δ , and, in common with normal δ -component (Dahr *et al.* 1975a), is resistant to trypsin digestion in the intact erythrocyte. Furthermore, radioiodinated wheat-germ lectin binds $\delta^{\text{Mi.III}}$ -component (in membranes separated by SDS/polyacrylamide-gel electrophoresis) in a similar way to normal δ -component (results not shown).

The increase in periodate staining of component $\delta^{\text{Mi.III}}$ suggests that it contains almost twice as much sialic acid as component δ , and this probably gives rise to the higher total sialic acid content of Mi.III/Mi.III cells and heterozygous Mi.III cells. The higher apparent molecular weight of component $\delta^{\text{Mi.III}}$ reflects the increase in sialic acid content and probably that of other sugars as well. These changes are likely

to be a result of differences in the amino acid sequence of parts of the two molecules.

Since component δ (which carries Ss-blood-group antigens in normal erythrocytes) is absent in Mi.III/Mi.III cells, the s-blood-group antigens present on these cells (Chandanayingyong *et al.*, 1977) are likely to be located on component $\delta^{\text{Mi.III}}$. The Mi.III condition has so far shown to be exclusively associated with blood-group-s-antigen activity (Race & Sanger, 1975). The occurrence of the Hil antigen in Mi.III cells suggests that this antigen is present on component $\delta^{\text{Mi.III}}$.

The same results were obtained irrespective of whether the cells examined were reactive with the serum Anek or not (i.e. classified as Mi.III or Mi.VI; Giles *et al.*, 1977). In fact the cells referred to as homozygous Mi.III/Mi.III in the present study would be denoted Mi.III/Mi.VI according to Giles *et al.* (1977) (Chandanayingyong *et al.*, 1977). The reactivity of Mi.VI cells with Anek serum may be a reflection of polymorphism of component $\delta^{\text{Mi.III}}$.

Membranes from Mi.IV erythrocytes

Membranes from the cells of a single donor heterozygous for the Mi.IV condition gave periodate-staining patterns that could not be distinguished from those obtained with membranes from Mi.III heterozygotes (results not shown). Although we have not carried out extensive studies with Mi.IV erythrocytes, the similarity of the gel-electrophoretic pattern with that of heterozygous Mi.III cells suggests that these cells contain a component that is very similar to component $\delta^{\text{Mi.III}}$. Whereas the Mi.III condition is associated with blood-group-s antigen activity, the Mi.IV condition has so far been shown to occur in conjunction with blood-group-S antigen activity (Race & Sanger, 1975). It seems likely that component $\delta^{\text{Mi.IV}}$ is the same as component $\delta^{\text{Mi.III}}$, but carries blood-group-S antigen activity instead of blood-group-s activity. However, unlike Mi.III cells, Mi.IV erythrocytes do not react with anti-Hil serum. Perhaps the expression of Hil antigen is dependent on the Ss status of the abnormal δ -component.

Membranes from M^k/Mi.III erythrocytes and the nature of the M^k condition

The M^k condition is associated with the loss of MN and Ss antigens and has been shown to be accompanied by the absence of component α (Dahr *et al.*, 1977; Anstee & Tanner, 1978; Furthmayr, 1978). On the basis of decreased periodate/Schiff staining of component δ in M^k heterozygotes, Dahr *et al.* (1977) concluded that the M^k condition was also associated with the absence of this component. In our own studies (Anstee & Tanner, 1978) we were unable to confirm this observation unequivocally. Since the

Mi.III condition results in the loss of component δ , the presence or absence of the normal component δ in cells of genotype M^k/Mi.III would unambiguously resolve this problem. Periodate staining of SDS/polyacrylamide gels of membranes from M^k/Mi.III erythrocytes (Fig. 6c) shows that no detectable normal component δ is present. This result is consistent with the suggestion that both component α and component δ are absent in the M^k condition.

Membranes from Mi.V erythrocytes

SDS/polyacrylamide-gel electrophoresis. Our previous studies on cells heterozygous for the Mi.V condition suggested that these cells contained decreased amounts of component α and increased periodate staining in the region of component β (Anstee & Tanner, 1978). Further investigation of membranes from these cells has shown that, in addition to the increased staining in the region of component β (apparent mol.wt. 40000), another component of apparent mol.wt. 63000 could be

Fig. 6. SDS/polyacrylamide-gel electrophoresis of 'ghosts' from heterozygous Mi.V, M^k/Mi.III and normal erythrocytes

Separation of 'ghosts' containing 150 μ g of membrane protein from (a) normal, (b) heterozygous Mi.V (donor H.S.) and (c) M^k/Mi.III erythrocytes (donor E.Q.) was carried out on tube gels containing 10% (w/v) acrylamide with a 3% overlay, with the buffer system of Laemmli (1970). The gels were stained with the periodic acid/Schiff's-base stain and scanned at 560nm.

resolved by discontinuous SDS/polyacrylamide-gel electrophoresis (Figs. 1a and 6b). Inspection of periodate-stained slab-gel electrophoretograms clearly showed the presence of two further components with a mobility between those of components α_2 and $\alpha\delta$ (apparent mol.wts. 83 000 and 80 000 respectively) and that the staining in the region of component β was broader than in the normal cell samples (Fig. 1a). Quantification of the periodate-staining components of these cells has shown that they also contain a decreased content of component δ [$51 \pm 6\%$ (3)] in comparison with normal control cells. The mobilities of the additional 83 000-, 80 000- and 63 000-mol.wt. components suggested that they may be complexes of a component of the same apparent molecular weight as component β with component α , with itself, and with component δ . Trypsin treatment of intact Mi.V cells resulted in the loss of all the periodate-staining sialoglycoprotein bands except that corresponding to component δ . Our previous results demonstrated that the abnormal component takes up about three times as much periodate stain as normal β -component (Anstee & Tanner, 1978). Lectin-binding experiments with radioiodinated wheat-germ lectin showed that the abnormal component took up more lectin than normal component β and that this lectin also bound to the bands of apparent mol.wt. 80 000 and 63 000 (results not shown). Component β of normal cells does not appear to form any detectable complexes. Thus the component in Mi.V erythrocytes must either be an abnormal component β or a new component that co-migrates with normal β -component.

Our conclusions, developed in more detail below, lead us to suggest that the unusual bands observed in membranes from heterozygous Mi.V cells result from the presence of a new molecule that contains regions related to both α - and δ -components, and we denote this new molecule $(\alpha-\delta)^{\text{Mi.V}}$ at this stage to facilitate discussion of the data.

Lactoperoxidase labelling of Mi.V cells and composition of the unusual complexes. The presence of the unusual periodate-staining components in membranes of heterozygous Mi.V cells was confirmed by lactoperoxidase labelling of the intact erythrocytes (Figs. 4g and 4h). Whereas normal component β was not significantly iodinated in control cells, a labelled band in the region of component β corresponding to $(\alpha-\delta)^{\text{Mi.V}}$ was obtained in the Mi.V erythrocytes. In addition the 80 000- and 63 000-dalton periodate-staining components were clearly labelled. The band corresponding to 83 000 daltons was not consistently resolved from the strongly labelled α_2 -band. The normal δ -, δ_2 - and $\alpha\delta$ -bands were more weakly labelled than in the control. Very weak labelling was also found in a broad area between bands β and δ .

The composition of the abnormal complexes was determined by elution and re-electrophoresis of the

Fig. 7. Re-electrophoresis of radiolabelled bands eluted from electrophoretograms of radioiodinated Mi.V erythrocytes

Heterozygous Mi.V erythrocytes were radiolabelled as described in the legend to Fig. 4. The membranes were separated and the eluted bands analysed as described in Fig. 2. (a) Whole membranes from heterozygous Mi.V erythrocytes (donor L. R.); (b) re-electrophoresis of the $\alpha(\alpha-\delta)^{\text{Mi.V}}$ -band; (c) re-electrophoresis of the $(\alpha-\delta)_2^{\text{Mi.V}}$ -band; (d) re-electrophoresis of band * (see the parenthetic note in Fig. 2); (e) re-electrophoresis of the $\delta(\alpha-\delta)^{\text{Mi.V}}$ -band; (f) whole membranes from normal erythrocytes. For explanation of band †, see the legend to Fig. 9.

bands derived from membranes from lactoperoxidase-labelled Mi.V cells (Fig. 7).

The band corresponding to 83 000 daltons yielded bands α , α_2 , $(\alpha-\delta)^{\text{Mi.V}}$ and the original undissociated band (Fig. 7b), suggesting that the component contained in it has the composition $\alpha(\alpha-\delta)^{\text{Mi.V}}$. The band corresponding to 80 000 daltons yielded predominantly the $(\alpha-\delta)^{\text{Mi.V}}$ -component and the original 80 000-dalton band (Fig. 7c), suggesting it is $(\alpha-\delta)_2^{\text{Mi.V}}$. The band corresponding to 63 000 daltons yielded the $(\alpha-\delta)^{\text{Mi.V}}$ - and δ -components and the undissociated band corresponding to 63 000 daltons (Fig. 7e), suggesting it is $\delta(\alpha-\delta)^{\text{Mi.V}}$. The apparent molecular weights of the components are consistent with their suggested composition. The strongly labelled band (*, Fig. 7) is not significantly dissociated. A similar band is found in normal cells, although in the latter case it is more weakly labelled (see the legend to Fig. 2).

Abnormal component in Mi.V erythrocytes. Heterozygous Mi.V cells have a low sialic acid content (Luner *et al.*, 1975), reflecting the deficiency of the α - and δ -components. The abnormal component present in these cells took up more periodate stain

than did normal δ -component and was susceptible to trypsin treatment in the intact erythrocytes. The observation that the sialic acid released by trypsin treatment of heterozygous Mi.V erythrocytes is more than that released from heterozygous M^K or heterozygous En(a-) erythrocytes (72, 58 and 63% respectively of that released from normal cells, Dahr *et al.*, 1976b, 1977) is consistent with the increased periodate staining (and thus sialic acid content) of component $(\alpha-\delta)^{Mi.V}$. Component $(\alpha-\delta)^{Mi.V}$ has an apparent molecular weight that is 15000 greater than that of component δ and is able to form complexes with itself, and with components α and δ .

Individuals of genotype M^{Mi.V}/N have an unusual and weak M-antigen activity that is detected in a broad area between components α_2 and β on SDS/polyacrylamide-gel electrophoresis in the system of Fairbanks *et al.* (1971) (Anstee & Tanner, 1978). The electrophoretic mobility of the monomeric form and of the complexes of $(\alpha-\delta)^{Mi.V}$ would be consistent with the presence of the unusual weak M-antigen activity found in M^{Mi.V}/N cells also being located on the abnormal component. M/N^{Mi.V} cells lose blood-group-N activity on trypsin treatment (Anstee & Tanner, 1978), suggesting that component $(\alpha-\delta)^{Mi.V}$ carries the weak blood-group-N antigen activity in M/N^{Mi.V} cells. However, family studies have shown that the Mi.V condition, in common with the Mi.III condition, is associated with s-antigen activity and the presence of the Hil antigen (Crossland *et al.*, 1970; Metaxas *et al.*, 1972). Furthermore, the occurrence of individuals of genotype Ss who are heterozygous for the Mi.V defect suggests that $(\alpha-\delta)^{Mi.V}$ carries blood-group-s antigen activity, and might carry the Hil antigen in these cells.

Since the Mi.V condition is associated with the loss of both normal α - and δ -components, one can ask whether the unusual component is related to either or both of the normal components α or δ . On the one hand, the abnormal component has blood-group M or N antigen activity and has the characteristic trypsin-sensitivity of component α . On the other hand the abnormal component carries the s-antigen activity characteristic of component δ . Thus the abnormal component has molecular features of both component α and component δ .

We suggest that the abnormal component is a hybrid molecule that contains portions of polypeptide that are the same as those present in the normal α - and δ -components. We have designated the abnormal Mi.V component $(\alpha-\delta)^{Mi.V}$ in order to imply the hybrid nature of the molecule. The Lepore haemoglobins are well-documented examples of hybrid molecules containing regions that are normally present in two distinct proteins (Bunn *et al.*, 1977). These hybrid molecules result from chromosomal misalignment with unequal crossing-over between β - and δ -globin genes and elimination of the normal β - and δ -genes. $(\alpha-\delta)^{Mi.V}$ -component may arise by a similar mechanism involving the α - and δ -sialoglycoprotein genes. The absence of the normal α - and δ -sialoglycoproteins in the Mi.V condition would be consistent with this interpretation and would suggest that the α - and δ -genes are arranged as shown in Fig. 8.

The relatively weak M- and N-antigen activity of the abnormal Mi.V component and its intensity of periodate staining suggest that component $(\alpha-\delta)^{Mi.V}$ is less abundant than normal component- α . Re-electrophoresis of the $\alpha(\alpha-\delta)^{Mi.V}$ complex from radio-

Fig. 8. Possible origin of abnormal sialoglycoprotein $(\alpha-\delta)^{Mi.V}$ by unequal crossing-over

Roman letters M, N, S and s and 'Hil' refer to blood-group antigens characteristic of the sialoglycoproteins. N and C denote the N-terminus and C-terminus of individual sialoglycoproteins. NT $_{\alpha}$ and CT $_{\delta}$ refer to the N-terminal portion of component α and C-terminal portion of component δ respectively.

iodinated heterozygous Mi.V erythrocytes was used to estimate the number of $(\alpha-\delta)^{\text{Mi.V}}$ molecules relative to component α . The results of a single experiment gave a ratio $\alpha/(\alpha-\delta)^{\text{Mi.V}}$ of 1.6:1. An analogous decrease in the $\delta\beta$ hybrid relative to β -globin is found in the Lepore situation (Bunn *et al.*, 1977). In fact, since $\delta^{\text{Mi.III}}$ and $(\alpha-\delta)^{\text{Mi.V}}$ components also share the rare antigen Hil, the abnormal Mi.V component is probably more closely related to component $\delta^{\text{Mi.III}}$ than to normal component δ . However, although the Mi.III condition is commonly found in oriental populations, no example of the Mi.V condition is yet known in this ethnic group.

Membranes from S-s- erythrocytes

We reported previously that S-s- cells contained components that could be labelled by lactoperoxidase and that co-migrated with $\alpha\delta$ (PAS-4)- and δ (PAS-3)-components on the SDS/polyacrylamide-gel electrophoresis system of Fairbanks *et al.* (1971) (Tanner *et al.*, 1977). Our interpretation of this result was that S-s- cells contained a component δ that had an altered

Fig. 9. Re-electrophoresis of radiolabelled bands eluted from electrophoretograms of radioiodinated S-s- erythrocytes

S-s- erythrocytes were labelled as described in the legend to Fig. 4. The membranes were separated and the eluted bands analysed as described in Fig. 2. (a) and (d) Whole membranes from normal erythrocytes; (b) whole membranes from S-s- erythrocytes (the offset band of slightly faster mobility than component α is an artifact resulting from a mark on the gel (cf. Figs. 4f and 4l); (c) re-electrophoresis of the band designated $\alpha\omega$ (see also Fig. 4) eluted from S-s- erythrocytes. The band labelled $\dagger$ is a minor component that is occasionally detected on radioautographs of whole 'ghosts'. The band is also detectable in some of the re-electrophoresis experiments (see Figs. 5c, 5d, 7b, 7c and 7d).

structure. The observation that, after lactoperoxidase labelling of Mi.III erythrocytes and subsequent analysis by discontinuous gel electrophoresis, unusual labelled components migrating in the region of normal component δ can be detected (Fig. 4e), suggested that the abnormal component of S-s- cells might correspond to one of these. The abnormal component of S-s- cells (ω in Fig. 4l) has a mobility on discontinuous SDS/polyacrylamide-slab-gel electrophoresis that is significantly slower than that of normal δ -component. Elution and re-electrophoresis of the band in the $\alpha\delta$ region showed the presence of normal component and the abnormal component α , α_2 , but no trace of normal δ -component (Fig. 9c). Although it is distinct from component δ and not detected in normal erythrocytes, the abnormal component, in common with the normal δ -component, also forms a complex with component α , supporting our previous suggestion that it is an altered δ -component. However, it is possible that it is not related to component δ but represents a normal membrane component that becomes susceptible to lactoperoxidase labelling as a result of the surface alterations occurring in S-s-erythrocytes. It is not clear what relationship, if any, exists between the abnormal component in S-s-erythrocytes and the minor components X, Y, Z found in Mi.III erythrocytes.

In previous papers we have described inherited variants involving component α (the blood-group-MN-active sialoglycoprotein). In the present paper we have studied inherited variants involving component δ (the blood-group-Ss-active sialoglycoprotein). It is clear that considerable variation, ranging from total absence to substantial alteration, in both these components can occur in healthy individuals, suggesting that there are few functional constraints on the structure of these glycoproteins. The very wide range of known blood-group-MN and Ss-related polymorphisms (Race & Sanger, 1975) probably reflects this lack of functional constraints. Thus far no variants of components β and γ have been described. It is possible that the structure of these components is more critical for their function in the erythrocyte.

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