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The use of monoclonal antibodies to quantify the levels of sialoglycoproteins α and δ and variant sialoglycoproteins in human erythrocyte membranes

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1. By using radioiodinated monoclonal antibodies we have estimated that there are about 600000 copies of sialoglycoprotein α (synonym glycophorin A) and 80000 copies of sialoglycoprotein δ (synonym glycophorin B) per normal human erythrocyte. 2. Erythrocytes expressing the product of only one α gene contain about 300000 copies of α/cell. 3. Two erythrocyte types containing α–δ hybrid molecules were studied. Those with heterozygous expression of the $(\alpha$–$\delta)^{Mi.V}$ gene contain about 100000 α–δ copies per cell, whereas those with heterozygous expression of the En(UK) gene contain about 80000 α–δ copies/cell. 4. Erythrocyte types containing δ–α hybrid molecules were also studied. About 200000 copies of $(\delta$–$\alpha)^{Dantu}$ were measured in cells with heterozygous expression of the $(\delta$–$\alpha)^{Dantu}$ gene (donor M.P.), whereas about 315000 copies of the putative $(\delta$–$\alpha)^{Dantu}$ hybrid were found on the erythrocytes of donor J.O. [which also have heterozygous expression of the putative $(\delta$–$\alpha)^{Dantu}$ gene]. 5. The erythrocytes of donor M.P. have normal levels of α, whereas those of donor J.O. have only about half-normal levels. It is proposed that the hybrid sialoglycoprotein of donor J.O. is of α–δ–α composition $[(\alpha$–δ–$\alpha)^{Dantu}]$ rather than δ–α and results from a double cross-over analogous to that which gives rise to haemoglobin Parchman.

INTRODUCTION

Purified radioiodinated murine monoclonal antibodies reactive with human erythrocyte surface antigens have previously been used to quantify the number of antigen sites per cell. Four monoclonal antibodies (R10, R18, BRIC 13, BRIC 14) specific for the major human erythrocyte sialoglycoprotein α were used to obtain values for the number of copies of α in the range $(0.3–1.2) \times 10^6$/cell (Merry *et al.*, 1984). These values were of the same order of magnitude as previous estimations of the number of copies of α [250000 (Fairbanks *et al.*, 1971); 500000 (Steck, 1974); 1×10^6 (Gahmberg *et al.*, 1979)]. At least three other sialoglycoproteins (β, γ and δ according to the nomenclature of Anstee *et al.*, 1979) are found in the erythrocyte membrane. We have previously reported the abundance of sialoglycoprotein β (synonym glycophorin C) as 50000 copies/erythrocyte using two monoclonal antibodies (BRIC 4 and BRIC 10, Anstee *et al.*, 1984). The number of copies of sialoglycoprotein δ (synonym glycophorin B) have been estimated relative to α, as 100000 by Dahr *et al.*, (1978), who assumed a value of 250000 for α, and 250000, by Anstee *et al.* (1982*a*), who assumed a value of 1×10^6 for α.

Several unusual human erythrocyte sialoglycoproteins have also been described which appear to be hybrid molecules with either the *N*-terminal portion of the polypeptide derived from α and the *C*-terminal derived from δ (α–δ hybrids) or vice versa (δ–α hybrids) [see Anstee *et al.* (1982*a*) for a review].

In the present study we used radioiodinated, purified monoclonal antibodies in an attempt to define more precisely the number of copies of α and δ per normal erythrocyte. We also attempted to quantify the number of copies of α–δ and δ–α hybrid sialoglycoproteins in several rare erythrocyte phenotypes.

MATERIALS AND METHODS

Details of the production and specificity of the monoclonal antibodies used [NBTS/BRIC 14 (Ridgwell *et al.*, 1983); LICR/R1.3, LICR/R10 and LICR/R18 (Edwards, 1980; Anstee & Edwards, 1982)] have been published previously.

Estimation of the number of antigen sites

The number of antigen sites were estimated with ^{125}I-labelled monoclonal antibodies as previously described (Merry *et al.*, 1984). R10, R18 and BRIC 14 were purified from ascitic fluid as described previously (Merry *et al.*, 1984). R1.3 ascitic fluid (after dialysis against 0.1 M-potassium phosphate, pH 7.0) was applied to an affinity-purified rabbit anti-mouse IgG column (6 cm × 0.7 cm). The antibody was eluted with 0.1 M-glycine/HCl, pH 2.5, and immediately neutralized with 0.1 M-Tris/HCl, pH 9.5 (yield about 0.7 mg/column

Abbreviations used: the terminology used for the sialoglycoproteins is that proposed by Anstee *et al.* (1979). α, sialoglycoprotein α; δ, sialoglycoprotein δ; β, sialoglycoprotein β; LICR, Ludwig Institute for Cancer Research; BRIC, Bristol Region Transfusion Centre, Immunochemistry Department.

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cycle). The purified R1.3 gave a single Coomassie-Blue-staining band corresponding to IgG on SDS/polyacrylamide-gel electrophoresis under non-reducing conditions (results not shown).

Erythrocytes

Erythrocytes of the Dantu phenotype (J.O.) were provided by Dr. M. Contreras, North London Transfusion Centre, Edgware, Middx., U.K. One example of the phenotype En(a−) (donor E.P.; Walker *et al.*, 1984) was provided by Ms. M. Bergren, Irwin Memorial Blood Bank, San Francisco, CA, U.S.A. and another (donor M. E.-P.) was provided by Dr. W. Wagstaff, Regional Transfusion Centre, Sheffield, U.K. Cells of phenotype Mi.V (Sev and Kay) and St(a+) (Sho) and those of presumed genotype En^a/En (Fis, Cro, Vic and Chi) were donors from the Lancaster Transfusion Centre found in a study by C. Hodson, D. Lee & J. Young (unpublished work). Erythrocytes from Dantu-positive donor M.P. (Tanner *et al.*, 1980) were recovered from storage in liquid nitrogen.

Treatment with proteolytic enzymes

Treatment of erythrocytes with papain was carried out by incubation of a 20% (v/v) cell suspension with papain (BDH Ltd., Poole, Dorset, U.K.) at a final concentration of 0.25 mg/ml in 2 mM-phosphate/1 mM-EDTA/2 mM-cysteine hydrochloride buffer, pH 7.2, for 20 min at 37 °C and washed twice in physiological saline (0.9% NaCl) before use. Treatment with Pronase was performed by incubation of a 10% (v/v) suspension with 1 mg of Pronase (Calbiochem Ltd.)/ml in physiological saline containing 1 mM-$CaCl_2$ for 30 min at 37 °C and washed twice in physiological saline before use.

Immunoblotting

SDS/polyacrylamide-gel electrophoresis of erythrocyte membranes was in a slab containing 10% (w/v) acrylamide with an overlay of 3% acrylamide using the buffer system of Laemmli (1970). The separated membrane components were transferred to nitrocellulose (Bio-Rad Trans-Blot Transfer Medium) at 4 °C for 2.5 h at 250 mA using a Trans-Blot apparatus (Bio-Rad) in transfer buffer [25 mM-Tris/192 mM-glycine/20% (w/v) methanol]. After transfer, the nitrocellulose was immersed in phosphate-buffered saline/0.3% Tween for 30 min at room temperature (Batteiger *et al.*, 1982). The membrane was cut into strips and the strips incubated for 1 h at room temperature in phosphate-buffered saline/0.3% Tween/5% (v/v) normal rabbit serum before incubation with undiluted culture supernatant containing monoclonal antibody R1.3. After two 5 min washes in phosphate-buffered saline/0.3% Tween the strips were incubated (1 h at room temperature) with a 1 in 200 dilution of horseradish peroxidase-conjugated rabbit anti-mouse IgG (Dakopatts, Copenhagen, Denmark) in phosphate-buffered saline/0.3% Tween/5% normal rabbit serum. After two further 5 min washes in phosphate-buffered saline/0.3% Tween and immersion (~ 10 s) in 3,3-diaminobenzidine (0.6 mg/ml; Sigma Ltd., Poole, Dorset, U.K.) in 0.1 M-Tris/HCl, pH 7.6, containing 0.1% H_2O_2, the strips were immersed in distilled water for 5 min and air-dried.

Table 1. Estimation of number of α molecules on normal erythrocytes with monoclonal antibodies R10, R18 and BRIC 14

The number of sites per cell were calculated from a Scatchard plot and the cell count. Values in parentheses represent the number of samples tested.

	No. of molecules		
Cell	Antibody... R10	R18	BRIC 14
Normal	620000 ± 70000 (13)	560000 ± 112000 (8)	600000 (3)
En(a−)UK	11000	6000	5000

RESULTS

Estimation of the number of copies of α on normal erythrocytes

The number of copies of α on normal erythrocytes was determined at about 600000 using three monoclonal antibodies with specificities to different epitopes on α (R10, R18 and BRIC 14) (Table 1). En(a−) cells that lack α gave an average of 7000 which was assumed to be non-specific binding (Table 1).

Estimation of the number of copies of α on erythrocytes of presumed genotype En(Fin)/En^a and En(UK)/En^a

Erythrocytes denoted En(a−) do not express α and may arise from two different genetic backgrounds (Metaxas *et al.*, 1977). In the erythrocytes of the phenotype En(a−)Fin (nomenclature of Anstee, 1980) an apparently normal δ is expressed. In the other phenotype, En(a−)UK (nomenclature of Anstee, 1980), there is an expression of an abnormal α–δ hybrid which does not carry the R10 or R18 epitopes (Table 1). Heterozygous cells of presumed genotype En(Fin)/En^a and En(UK)/En^a would therefore only be expected to express half the normal number of α sites as measured by these antibodies. Measurements on erythrocytes from four donors (two donors for each presumed genotype) using R10 and R18 gave a mean value of 313000 ± 40000 (five determinations), which is close to the expected half-normal value (see Table 1 for normal values).

Estimation of the number α–δ sialoglycoprotein molecules in erythrocytes expressing the Miltenberger Class V (Mi.V) phenotype

Erythrocytes of the Miltenberger Class V phenotype express a hybrid sialoglycoprotein molecule denoted '$(\alpha$–$\delta)^{Mi.V}$' (Anstee *et al.*, 1979). Epitopes recognized by monoclonal antibodies R10 and R18 are on $(\alpha$–$\delta)^{Mi.V}$ hybrids (Mawby *et al.*, 1981; Anstee & Edwards, 1982). Erythrocytes homozygous for the Mi.V condition express only the $(\alpha$–$\delta)^{Mi.V}$ sialoglycoprotein (Vengelen-Tyler *et al.*, 1981), whereas erythrocytes heterozygous for the Mi.V condition express normal α, normal δ and the $(\alpha$–$\delta)^{Mi.V}$ sialoglycoproteins.

Values obtained with R10 and R18 for erythrocytes from two donors which are heterozygous for the $(\alpha$–$\delta)^{Mi.V}$ condition were 410000 ± 30000 (four determinations). Subtraction of the average of about 310000 for R10 and R18 binding sites on En(UK)/En^a and En(Fin)/En^a cells

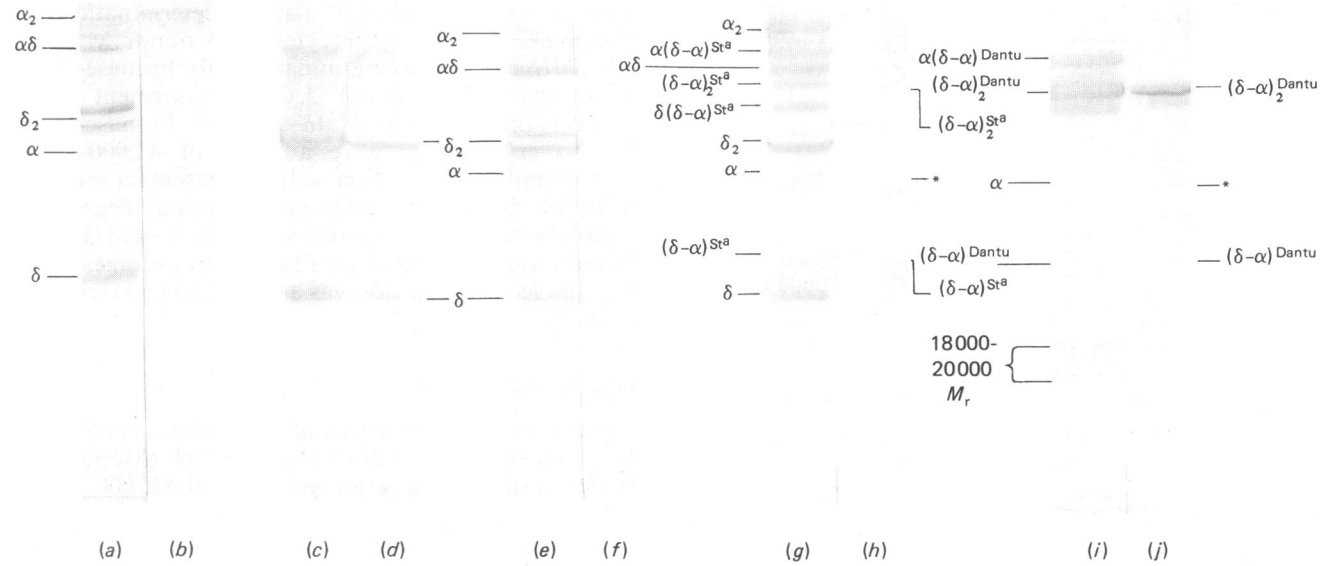

Fig. 1. Reaction of R1.3 with membrane proteins from normal and variant erythrocytes separated by SDS/polyacrylamide-gel electrophoresis

Electrophoresis of solubilized membranes was carried out on an SDS 10%-(w/v)-polyacrylamide slab with a 3% overlay as described in the Materials and methods section. The proteins were then electrophoretically transferred to a nitrocellulose membrane and those bands reactive with monoclonal antibody R1.3 were revealed by reaction with horseradish-peroxidase-conjugated rabbit anti-mouse IgG and diaminobenzidine as described in the Materials and methods section. (*a*) Normal membranes; (*b*) membranes from S−s−U− erythrocytes; (*c*) membranes from En(a−) erythrocytes (donor M.E.-P.); (*d*) membranes from En(a−) erythrocytes (donor E.P.); (*e*) normal membranes; (*f*) Pronase-treated normal membranes; (*g*) membranes from St(a+) erythrocytes (donor Sho); (*h*) membranes from Pronase-treated St(a+) erythrocytes (donor Sho); (*i*) membranes from Dantu-positive erythrocytes (donor J.O.); (*j*) membranes from Pronase-treated Dantu-positive erythrocytes (donor J.O.). The band labelled with an asterisk (tracks *h* and *j*) has an electrophoretic mobility faster than that of normal α and probably corresponds to a complex of δ–α hybrid sialoglycoprotein with residual sialoglycoproteins which remain membrane-bound after Pronase treatment of the erythrocytes. The bands labelled $(\delta\text{–}\alpha)^{Dantu}$ in tracks (*i*) and (*j*) may, in fact, be $(\alpha\text{–}\delta\text{–}\alpha)^{Dantu}$ sialoglycoproteins (see the Discussion section).

(see above) gives a value of about 100000 for the number of $(\alpha\text{–}\delta)^{Mi.V}$ molecules in a heterozygous cell.

Estimation of the number of sialoglycoprotein δ copies on En(a−)Fin erythrocytes and number of copies of the α–δ hybrid sialoglycoprotein on En(a−)UK erythrocytes

Monoclonal antibody R1.3 has been reported to react with the *N*-terminal portion of both α and δ (Anstee & Edwards, 1982). The specificity of this antibody was further investigated by using immunoblotting. Fig. 1 shows the reaction of R1.3 with membranes from normal cells. Binding to α, δ and dimers of α and δ was observed (Figs 1*a* and 1*e*). R1.3 bound to δ, δ_2 and to higher-M_r components that are probably trimers and other oligomers of δ in the membranes of En(a−)Fin cells which lack α (Fig. 1*d*). The specificity of R1.3 binding to δ was clearly demonstrated by the absence of binding in the region of these components in membranes from S−s−U− erythrocytes, which lack δ (Fig. 1*b*).

In order to estimate the number of copies of δ expressed, the specific binding of R1.3 to δ in En(a−) erythrocytes of genotype En(Fin)/En(Fin) was investigated by using radioiodinated purified R1.3; a value of 78000 copies/cell was obtained from a single determination, and this presumably represents expression from both chromosomes. Non-specific binding in this experiment was estimated at less than 600 sites per cell from the reaction with papain-treated cells (papain treatment of normal cells destroys reactivity with R1.3). The cells from the En(a−)UK donor M.E.-P. have the presumed genotype En(UK)/M^k. An α–δ hybrid that has the same M_r as δ and that carries the R1.3 epitope (Fig. 1*c*) is expressed from one chromosome. Neither α nor δ are expressed from this chromosome or from the other chromosome, which carries the so-called 'Mk' gene. A value of 82000 R1.3 binding sites per cell was obtained with these cells. This reflects the level of expression of the hybrid α–δ sialoglycoprotein from one chromosome in this cell and suggests that the hybrid is expressed at about twice the level of δ.

Estimation of the number of copies of δ–α hybrid sialoglycoprotein molecules in erythrocytes expressing either the St^a or Dantu antigens

In addition to normal α and δ, erythrocytes positive for the St^a antigen contain a δ–α hybrid sialoglycoprotein molecule with its *N*-terminus derived from δ and its *C*-terminus derived from α (Anstee *et al.*, 1982*b*). Erythrocytes positive for the Dantu antigen (Contreras *et al.*, 1984) also appear to have a δ–α hybrid sialoglycoprotein. The presence of Dantu antigen correlates with the presence of δ–α cross-over and can

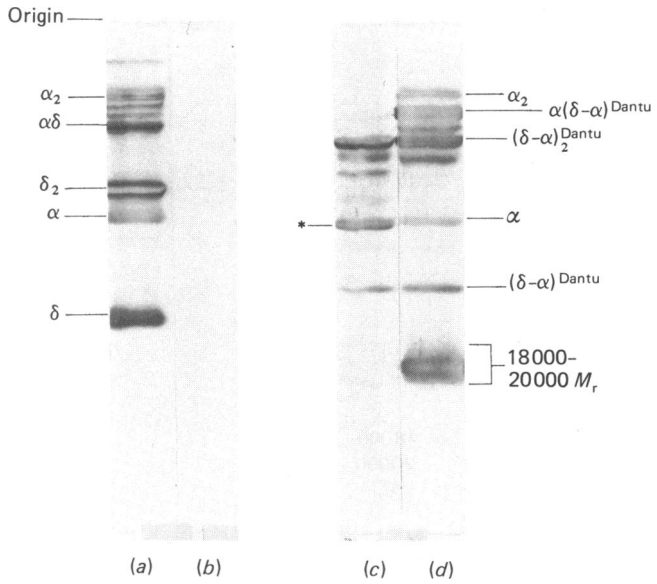

Fig. 2. Reaction of R1.3 with sialoglycoproteins of normal and Dantu-positive erythrocyte membranes (donor M.P.) by immunoblotting

Electrophoresis of solubilized membranes was carried out on an SDS 10%-(w/v)-polyacrylamide slab with a 3% overlay as described in the Materials and methods section. The proteins were then electrophoretically transferred to a nitrocellulose membrane and those bands reactive with monoclonal antibody R1.3 were revealed by reaction with horseradish-peroxidase-conjugated rabbit anti-mouse IgG and diaminobenzidine as described in the Materials and methods section. (*a*) Normal erythrocyte membranes; (*b*) Pronase-treated normal erythrocyte membranes; (*c*) Pronase-treated Dantu-positive membranes (donor M.P.); (*d*) Dantu-positive membranes (donor M.P.). The band labelled with an asterisk (track *c*) corresponds to that shown in Fig. 2 (*h*, *j*) and its possible origin is described in the legend to Fig. 2.

occur in two phenotypes. In the first phenotype to be described (donor M.P.; Tanner *et al.*, 1980) the inheritance of the hybrid sialoglycoprotein [designated $(\delta-\alpha)^{Ph}$ by Tanner *et al.*, 1980] was in association with normal levels of α and absence of δ. The cells of the original donor M.P. were subsequently shown to be Dantu-positive by Contreras *et al.* (1984), and so the hybrid molecule should now be designated $(\delta-\alpha)^{Dantu}$. In the second phenotype, the hybrid is inherited with reduced levels of α and the absence of δ (Contreras *et al.*, 1984). Quantitative estimation of the number of copies of α in these two phenotypes confirmed that the erythrocytes of donor M.P. have normal levels of α (585000 in a single determination using R18), whereas those of the Dantu-positive donor J.O. have approximately half the normal level of α (233000; mean of two determinations, one with R10 and one with R18).

R1.3 clearly binds to the abnormal hybrid molecules in St(a+) and Dantu-positive erythrocytes (Figs 1 and 2). The hybrid molecules are more resistant to digestion with Pronase on intact erythrocytes than are α or δ, so it is possible to define conditions whereby only the hybrid molecules remain intact (Figs. 1*h* and 1*j* and Fig. 2*c*). At a Pronase concentration of 1 mg/ml, partial degradation of $(\delta-\alpha)^{St^a}$ was observed (Fig. 1*h*), whereas $(\delta-\alpha)^{Dantu}$ appeared to be very resistant to digestion (Figs 1*j* and 2*c*). Estimation of the number of R1.3 epitopes remaining on Pronase-treated erythrocytes expressing these hybrids gave a value of 205000 (single determination) for Pronase-C treated erythrocytes from donor M.P. and 315000 (mean of two determinations) for Pronase-treated erythrocytes from donor J.O. Measurement of the number of molecules of $(\delta-\alpha)^{St^a}$ on Pronase-treated St(a+) erythrocytes gave a value of 43,000 (single determination). This latter value represents a minimum value in view of the evidence for partial degradation under the conditions of pronase treatment used (Fig. 1*h*). Pronase-treated normal cells bound an average of 3300 R1.3 molecules, and this was assumed to be non-specific binding.

DISCUSSION

Our previous estimations of α in normal erythrocytes gave values of $(0.3–1.2) \times 10^6$ copies/cell (Merry *et al.*, 1984). In the present series, estimates of 550000 ± 70000 were obtained with monoclonal antibody R10 and 560000 ± 112000 with monoclonal antibody R18 (Table 1). The increase in precision of measurement may be accounted for by the use of affinity-purified antibodies and the use of high concentrations of labelled antibody (around 200 μg/ml), which increases the reliability of the extrapolation of the Scatchard plot used to estimate the number of binding sites. This increased reproducibility allowed us to quantify the level of α in erythrocytes of presumed genotype En(UK)/En^a and En(Fin)/En^a, and values in the region of half that for normal erythrocytes were found.

Hybrid α–δ sialoglycoproteins are thought to arise by chromosome misalignment with unequal crossing-over between the α and δ genes at meoisis, in a manner analogous to that which gives rise to the haemoglobin Lepore (reviewed by Anstee *et al.*, 1982*a*). Two types of α–δ hybrid have been described: that which occurs in erythrocytes of the Mi.V phenotype $[(\alpha-\delta)^{Mi.V}]$ and that which occurs in En(a−)UK erythrocytes. We obtained values of approx. 100000 for $(\alpha-\delta)^{Mi.V}$ and 80000 for the α–δ hybrid in En(a−)UK erythrocytes. These values are substantially less than the value of 300000 obtained for α on En(Fin)/En^a and En(UK)/En^a erythrocytes.

By using monoclonal antibody R1.3 we obtained a value of 80000 for δ on En(a−)Fin erythrocytes. Assuming that expression of δ is normal in these erythrocytes, this can be taken as an estimate of δ in normal erythrocytes.

Hybrid δ–α sialoglycoproteins are thought to arise in a manner analogous to the anti-Lepore type haemoglobins [see Anstee *et al.* (1982*a*) for review]. Marked differences in the estimates of $(\delta-\alpha)^{St^a}$ and $(\delta-\alpha)^{Dantu}$ were obtained in the present study and this may well be due to underestimations of the abundance of $(\delta-\alpha)^{St^a}$, owing to its increased susceptibility to Pronase relative to $(\delta-\alpha)^{Dantu}$. This difference in Pronase-susceptibility presumably reflects differences in the cross-over points in the hybrids (Ridgwell *et al.*, 1984). Of particular interest are the different values obtained for the Dantu-positive hybrids on the erythrocytes of donor M.P. compared with those of donor J.O. (205000 and 315000 respectively).

The results obtained for the Dantu phenotype cells of donor J.O. show that there is only half the expression of

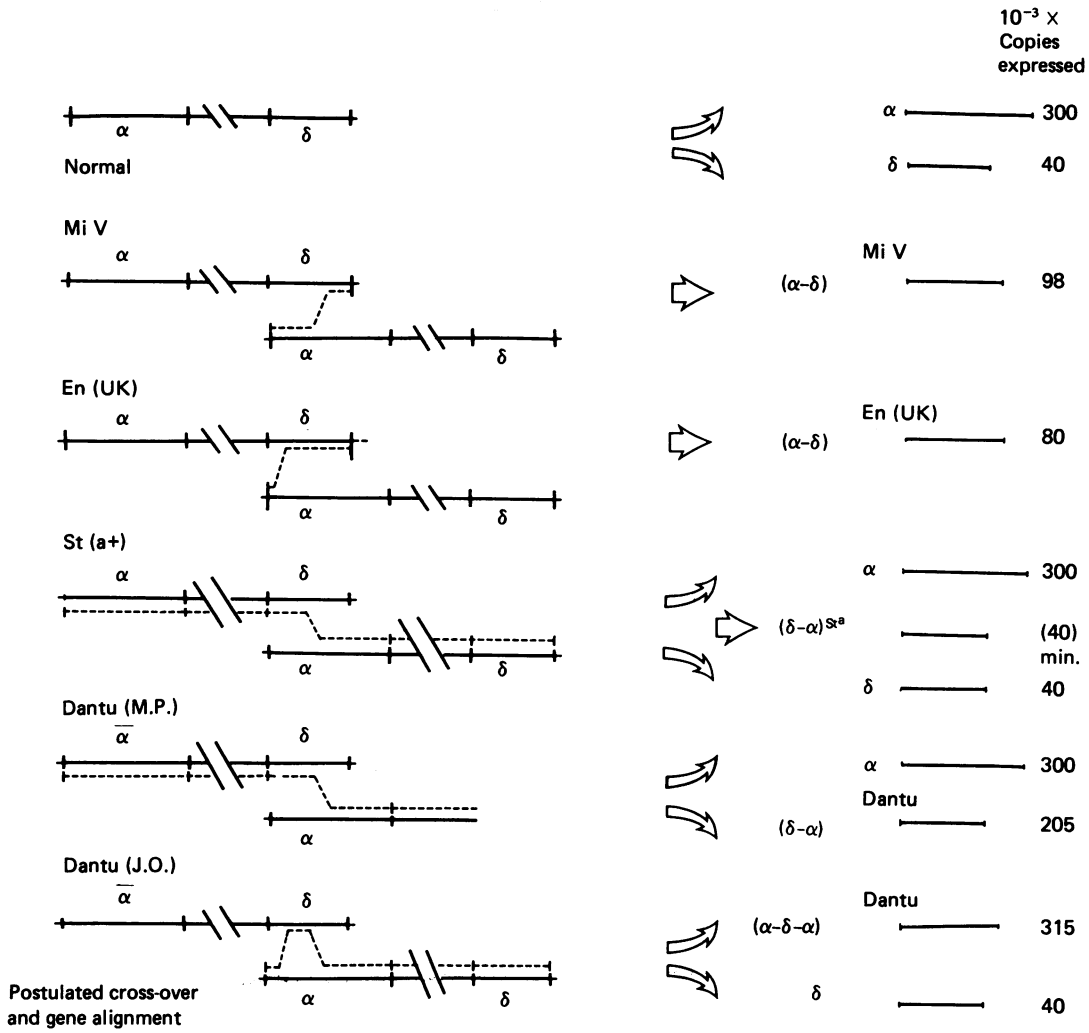

Fig. 3. Postulated cross-over events and number of gene copies produced from normal and abnormal sialoglycoprotein genes

The hypothetical cross-overs between chromosomes giving rise to hybrid sialoglycoprotein genes are indicated on the left. The continuous line on the left represents a portion of the chromosome carrying genes for α and δ which are presumed to be in close proximity with a connecting, untranslated sequence of unknown length. Regions coding for α and δ are indicated. The broken lines represent the composition of the chromosomes produced as a result of unequal cross-over events between misaligned chromosomes. The estimated number of copies present per cell are shown on the right.

α, whereas α is expressed normally in cells of the St^a and Dantu (donor M.P.) phenotypes. Normal α expression would be expected from the gene alignment in δ–α hybrids (Fig. 3). This lack of α expression in the cells of donor J.O. is, at present, unexplained. The very high level of hybrid molecules found (315000) is comparable with the level of normal α expressed by one gene, and this is in contrast with the levels of α–δ and δ–α hybrids expressed in the other erythrocyte types studied which are intermediate between α and δ. One hypothesis, consistent with both these findings, is that the Dantu-positive hybrid molecule of donor J.O. arises from a double cross-over that has taken place from the *N*-terminus of α to δ and then from δ back to the *C*-terminal of α (Fig. 3). One consequence of such a double cross-over would be the production of a chromosome lacking α but expressing an α–δ–α gene, an event consistent with the inheritance of the Dantu antigen in cells of this type (Contreras *et al.*, 1984). An analogous hybrid (haemoglobin Parchman) has recently been described between δ- and β-haemoglobins (Adams *et al.*, 1982). One prediction of this hypothesis would be the existence of Dantu-positive (α–δ–α) molecules carrying an M antigen that would be Pronase-resistant on intact erythrocytes.

These results, which are summarized in Fig. 3, illustrate the value of monoclonal antibodies in providing information on the level of expression of normal and abnormal sialoglycoprotein genes. The finding that expression of α–δ and δ–α hybrid sialoglycoproteins is intermediate between that of α and δ is of considerable interest, since expression of Lepore and anti-Lepore type haemoglobins is also found to be intermediate between the expression of β- and δ-globin genes respectively (Modiano & Pepe, 1983). Those authors concluded that the expression of haemoglobin genes is regulated by sites within both 5′ and 3′ untranslated sequences of DNA. It is also evident that factors other than the 5′ initiation sequence are of importance in the regulation of immunoglobulin-gene transcription and which are thought to affect the access of the RNA polymerase to

the DNA template (Perry, 1984). It therefore seems that similar factors may affect sialoglycoprotein-gene expression in the erythrocyte membrane.

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