RESEARCH COMMUNICATION

A red cell band 3 variant with altered stilbene disulphonate binding is associated with the Diego (Di^a) blood group antigen

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1. We have shown that the Di^a antigen of the Diego blood group system is associated with the presence of red cell band 3 Memphis, but not all band 3 Memphis samples carry the Di^a antigen. 2. The band 3 Memphis associated with the Di^a antigen was covalently labelled by 4,4'-di-isothiocyanato-1,2-diphenylethane-2,2'-disulphonic acid (H₂DIDS) more readily than was normal band 3 or band 3 Memphis not associated with the Di^a antigen. This altered reactivity with H₂DIDS has previously been noted for a band 3 Memphis sub-type designated variant 2. 3. This is the first example of a band 3 polymorphism associated with an antigenic change in the extracellular region of the band 3 polypeptide and with altered H₂DIDS binding.

INTRODUCTION

Mueller & Morrison (1977) first described a variant of the human erythrocyte anion transporter (band 3) which migrated with a higher *M_r* than normal band 3. The *N*-terminal fragment resulting from Pronase or chymotrypsin treatment of variant red cells gave an *M_r* of 63 000, instead of 60 000 found in normal red cells. The variant band 3 was described as band 3 Memphis, and it has been shown to result from the point mutation Lys-56→Glu (Yannoukakos *et al.*, 1991; Jarolim *et al.*, 1992). This relatively common polymorphism was found in 6–7% of random blood donors (Mueller & Morrison, 1977), and more recent studies (Ranney *et al.*, 1990) have shown that it is particularly prevalent in American Indians (up to 25%) and African Americans (15%), and in the Japanese population (29%; Ideguchi *et al.*, 1992). The variant is not associated with any abnormal red cell function except for a slightly reduced phosphoenolpyruvate transport activity (Iddeguchi *et al.*, 1992). Hsu & Morrison (1985) noted that two types of band 3 Memphis variant could be distinguished (variants 1 and 2). Variant 2 was more readily covalently labelled by 4,4'-di-isothiocyanato-1,2-diphenylethane-2,2'-disulphonic acid (H₂DIDS), but the transport properties of band 3 appeared unchanged in these cells. The frequency of variant 2 was not established.

The Diego blood group system comprises two allelic antigens (Di^a and Di^b). The Di^a antigen was first described by Layrisse and co-workers in 1955 (cited by Race & Sanger, 1975). Subsequent studies have shown that the Di^a antigen is very common in the indigenous peoples of America and South-East Asia, but is rarely found in other populations (Mourant *et al.*, 1976). The Di^a antigen is of clinical significance, since anti-Di^a has frequently been implicated as the causative agent in haemolytic disease of the newborn (Race & Sanger, 1975; Kusnierz-Alejska & Bochenek, 1992).

In this paper we show that the Di^a antigen is associated with the presence of band 3 Memphis variant 2. This is the first evidence linking a blood group antigen with the extracellular portion of the polypeptide chain of band 3.

MATERIALS AND METHODS

Red blood cells

Red cells of known Diego blood group and Di(a–b+) cells of normal and band 3 Memphis type were obtained from the in-house collection of the International Blood Group Reference Laboratory or through the Serum, Cells and Rare Fluids (SCARF) exchange scheme (managed by J. Moulds, Gamma

Fig. 1. SDS/PAGE of membranes from Pronase-treated erythrocytes of different Diego phenotypes

Red cells (10% suspension) were treated with 1 mg of Pronase/ml (from *Streptococcus griseus*; Cambridge Bioscience, Cambridge, U.K.). Membranes were prepared and separated on a gel containing 10% acrylamide and stained with Coomassie Blue as described in the Materials and methods section. The red cell samples were: lane a, control Di(a–b+); lane b, Wal, Memphis Di(a–b+); lane c, Liv, Memphis Di(a–b+); lane d, Esc, Di(a+b–); lane e, Mon, Di(a+b+); lane f, Li, Di(a+b+); lane g, 602, Di(a+b+); lane h, 674, Di(a+b+); lane i, Pet, Di(a+b+); lane j, S3, Di(a+b+); lane k, San, Di(a+b–). The location of the *N*-terminal chymotrypsin fragments of normal (*M_r* 60 000) and Memphis (*M_r* 63 000) band 3 are indicated.

Abbreviations used: H₂DIDS, 4,4'-di-isothiocyanato-1,2-diphenylethane-2,2'-disulphonic acid; DIDS, 4,4'-di-isothiocyanatostilbene-2,2'-disulphonic acid.

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Fig. 2. [³H]H₂DIDS labelling of band 3 of red cells of different Diego types

Red cells (20% suspension) were labelled using 2 μM- or 5 μM-[³H]H₂DIDS (200 mCi/mmol; HSC Research & Development, Toronto, Canada) as described by Hsu & Morrison (1985). The cells were then treated with 1 mg of chymotrypsin/ml and membranes were prepared and separated by SDS/PAGE using gels containing 8% acrylamide as described in the Materials and methods section. The protein-stained gels were treated for fluorography using Amplify (Amersham International, Amersham, Bucks., U.K.), dried and exposed to pre-flashed X-Ray film (Kodak X-OMAT-AR) for 18 weeks at -80 °C. The locations of the *N*-terminal chymotrypsin fragments of normal (*M_r* 60000) and Memphis (*M_r* 63000) band 3 is indicated. (a) Membranes from cells labelled with 2 μM-[³H]H₂DIDS and treated with chymotrypsin. Lanes 1-7 are a fluorograph of a dried-down gel: 1, control Di(a-b+); 2, Lac, Di(a+b-); 3, Wal, Memphis Di(a-b+); 4, Alh, Memphis Di(a-b+); 5, Liv, Memphis Di(a-b+); 6, Mon, Di(a+b+); 7, Pet, Di(a+b+). Lanes 8-14 are a photograph of the same dried-down Coomassie Blue-stained gel shown in lanes 1-7. (b) Membranes from cells labelled with 5 μM-[³H]H₂DIDS and treated with chymotrypsin. Lanes 1-6 are a fluorograph of a dried-down gel: 1, control Di(a-b+); 2, Lac, Di(a+b-); 3, Wal, Memphis Di(a-b+); 4, Alh, Memphis Di(a-b+); 5, Mon, Di(a+b+); 6, Pet, Di(a+b+). Lanes 7-12 are a photograph of the same dried-down Coomassie Blue-stained gel shown in lanes 1-6.

Biologicals, Houston, TX, U.S.A.). Additional Di(a+b-) samples (Esc and San) were obtained from Dr. G. Garratty, American Red Cross Blood Services, Los Angeles, CA, U.S.A., and Dr. M. Reid, New York Blood Center, New York, NY, U.S.A.

Proteinase digestion of red cells

Red cells were digested with proteinases, and erythrocyte membranes were prepared as described previously (Jenkins & Tanner, 1975). SDS/PAGE was done using the method of Laemmli (1970).

Anion transport assays

Before assay, red cells were washed with Hepes-buffered Krebs saline (120 mM-NaCl, 4.8 mM-KCl, 5 mM-MgSO₄, 1.2 mM-KH₂PO₄, 2 mM-EGTA, 25 mM-Hepes, pH 7.4), suspended in Hepes-buffered saline containing 5 mM-glucose and incubated at 37 °C for 2 h. 4,4'-Di-isothiocyanatostilbene-2,2'-disulphonic acid (DIDS) titration of sulphate influx was done as described by Schofield *et al.* (1992) using Di(a+b-) cells from donor Esc and control normal cells. The assays were done at 10% haematocrit using an equal number of cells of each type. When the sulphate transport activity was plotted against DIDS concentration, the

two cell types gave superimposable straight lines showing that the transport activity and total number of reversible and irreversible DIDS binding sites were the same in the two samples (see Schofield *et al.*, 1992).

RESULTS AND DISCUSSION

Expression of the Di^a antigen is correlated with the presence of band 3 Memphis

Red cell band 3 from several different individuals representing the three Diego phenotypes (a+b-, a+b+, a-b+) was examined by SDS/PAGE of membranes derived from Pronase-treated erythrocytes. Pronase cleaves band 3 at a similar site to chymotrypsin, and yields a 63000-*M_r* *N*-terminal fragment from band 3 Memphis-containing erythrocytes and a 60000-*M_r* *N*-terminal fragment from normal band 3. Erythrocytes from seven unrelated Di(a+b-) donors contained only band 3 Memphis (for example, see Fig. 1, lanes d and k). Erythrocytes from eleven unrelated Di(a+b+) donors contained both band 3 Memphis and normal band 3 (for example, see Fig. 1, lanes e-j). Erythrocytes from 37 unrelated Di(a-b+) donors were examined. Thirty contained only normal band 3, while seven contained

Table 1. Labelling of normal and Memphis band 3 by [³H]H₂DIDS in red cells of different Diego phenotypes

The protein-stained gels and autoradiographs shown in Fig. 2 were scanned, and the areas of the peaks for the *N*-terminal chymotrypsin fragments of normal band 3 (*M_r* 60000) and Memphis band 3 (*M_r* 63000) were measured. The specific radioactivity of the labelled band 3 fragments in each cell sample was calculated by dividing the peak area of the band in the autoradiograph by the area of the corresponding Coomassie Blue-stained band. The column marked 'Ratio' in the Table is the ratio of the specific radioactivity of the 63000-*M_r* band over the specific radioactivity of the 60000-*M_r* band. Con, control normal Di(a-b+) sample; n.t., not tested.

Red cells	Specific radioactivity in band 3 fragment labelled using:					
	2 μ M-[³ H]H ₂ DIDS			5 μ M-[³ H]H ₂ DIDS		
	60000- <i>M_r</i> band	63000- <i>M_r</i> band	Ratio	60000- <i>M_r</i> band	63000- <i>M_r</i> band	Ratio
Con Di(a-b+)	0.27	—	1.0	0.41	—	1.0
Lac Di(a+b-)	—	0.73	2.7	—	0.92	2.2
Wal Di(a-b+)	0.27	0.29	1.1	0.53	0.56	1.1
Alh Di(a-b+)	0.18	0.17	0.94	0.42	0.51	1.2
Liv Di(a-b+)	0.28	0.28	1.0	n.t.	n.t.	n.t.
Mon Di(a+b+)	0.29	0.72	2.5	0.52	1.25	2.4
Pet Di(a+b+)	0.20	0.64	3.2	0.51	1.27	2.5

both band 3 Memphis and normal band 3 (for example, see Fig. 1, lanes c and d). These results show that, while the Di^a antigen is always associated with band 3 Memphis, not all band 3 Memphis individuals carry the Di^a antigen. It is also clear that the normal band 3 or the band 3 Memphis (if present) in Di(a-) erythrocytes carries the Di^b antigen.

The Di^a antigen is associated with band 3 Memphis variant 2

Hsu & Morrison (1985) showed that two types of band 3 Memphis (variants 1 and 2) could be distinguished by differences in their covalent reactivity with H₂DIDS. Variant 2 was more heavily labelled at low concentrations of H₂DIDS. We examined the reactivity of band 3 in erythrocytes of the different Diego phenotypes labelled using 2 μ M- or 5 μ M-[³H]H₂DIDS and treated with chymotrypsin. The results clearly show that band 3 Memphis in the Di(a+) erythrocytes is more heavily labelled by H₂DIDS than either normal band 3 or the band 3 Memphis in Di(a-) erythrocytes (Fig. 2). This is most clearly seen when the labelling of Di(a+) samples heterozygous for the band 3 Memphis variant (Fig. 2a, lanes 6 and 7) is compared with that of Di(a-) samples heterozygous for band 3 Memphis (Fig. 2a, lanes 3-5) and when 2 μ M-H₂DIDS is used.

The autoradiographs were scanned and the specific radioactivity of the labelled band 3 in each of the samples was measured (Table 1). When labelled using 2 μ M-H₂DIDS, the band 3 Memphis associated with the Di^a antigen bound 2.5-3.2 times the amount of radioactive H₂DIDS bound by either the band 3 Memphis or the normal band 3 associated with the Di^b antigen. This increase was reduced to 2.2-2.5 times when labelling was done using 5 μ M-H₂DIDS (Table 1). The band 3 Memphis variant 2 samples studied by Hsu & Morrison (1975) showed a similar increased labelling by H₂DIDS compared with that of band 3 Memphis variant 1 or normal band 3, and this differential labelling was also reduced when the higher concentration of H₂DIDS was used. These results strongly suggest that band 3 Memphis variant 2 is characterized by the presence of the Di^a antigen. The Di(a+b-) donor Lac (Fig. 2a, lanes 2 and 9; Fig. 2b, lanes 2 and 8) is the father of the Di(a+b+) donor Mon (Fig. 2a, lanes 6 and 13; Fig. 2b, lanes 5 and 11), demonstrating the inheritance of both the Di^a antigen and the abnormal H₂DIDS binding with the Memphis variant in this family.

The sulphate transport activity of one Di(a+b-) red cell sample (Esc) was examined and no difference from normal erythrocytes was observed in this sample, which was homozygous for the Memphis variant 2 (results not shown). Titration of the

sulphate transport activity with DIDS showed that this sample contained the same total number of reversible and irreversible DIDS binding sites as normal red cells (results not shown). Hsu & Morrison (1985) found no difference from normal in the anion transport activity of cells heterozygous for the band 3 Memphis variant 2. They also showed that the total (reversible and irreversible) binding of H₂DIDS to these heterozygous cells was the same as for normal cells, and suggested that the covalent reaction of H₂DIDS occurred more readily in the band 3 Memphis variant 2, probably because of a change in the reactivity of the H₂DIDS-binding lysine in the protein.

The abnormal electrophoretic mobility of Memphis band 3 on SDS/PAGE results from the single amino acid substitution Lys-56→Glu in the *N*-terminal cytoplasmic region of the molecule (Yannoukakos *et al.*, 1991; Jarolim *et al.*, 1992). The similar change in electrophoretic mobility of the *N*-terminal portion of variant 1 and variant 2 makes it likely that the abnormal mobility results from the same mutation in the cytoplasmic domain of both variants. The concomitant occurrence of the Di^a blood group antigen at the cell surface and abnormal H₂DIDS binding properties clearly indicates that the Memphis variant 2 has an additional change at the extracellular surface of band 3.

Stilbene disulphonates are widely used anion transport inhibitors, and there is considerable interest in elucidating the relationship between the site of binding of these inhibitors and their effect on anion transport (see, for example, Bartel *et al.*, 1989; Garcia & Lodish, 1989; Wood *et al.*, 1992). The altered H₂DIDS binding properties of band 3 Memphis variant 2 have previously only been studied in cells heterozygous for the variant, and it would be desirable to carry out further studies on the nature of the altered H₂DIDS binding in cells homozygous for variant 2. Hsu & Morrison (1985) noted that the detection of red cells homozygous for the variant by their altered H₂DIDS binding properties is both difficult and laborious. The presence of the Di^a antigen on band 3 Memphis variant 2 should greatly facilitate detection of the homozygous red cells. In addition, the data available on the frequency of the Di^a antigen in different geographical locations (Mourant *et al.*, 1976) will be very helpful in choosing appropriate populations for screening for homozygotes, since the Di^a antigen is very rare in most Caucasian populations. The determination of the amino acid change(s) in band 3 Memphis variant 2 which give rise to the Di^a antigen and its altered reactivity with H₂DIDS binding should throw further light on the surface structure of band 3 and the mechanism by which the stilbene disulphonates inhibit anion transport.

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