Studies on the defect which causes absence of decay accelerating factor (DAF) from the peripheral blood cells of an individual with the Inab phenotype

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1. We have studied the peripheral blood cells of an individual with the Inab phenotype who is deficient in decay accelerating factor (DAF). 2. In contrast with the situation in paroxysmal nocturnal haemoglobinuria, membranes from peripheral blood cells of the Inab phenotype individual lack DAF, but retain the other glycosylphosphatidylinositol-linked proteins acetylcholinesterase and LFA-3. 3. Unlike normal Epstein–Barr-virus-transformed lymphoblastoid cell lines (EBV-LCL), DAF was not expressed on EBV-LCL derived from peripheral blood lymphocytes of the Inab individual. 4. No differences in the DAF gene of normal and Inab phenotype individuals could be detected by Southern blotting studies. 5. EBV-LCL derived from the Inab individual had a gross reduction in the level of DAF mRNA compared with normal EBV-LCL. 6. Our results suggest that the DAF gene in the Inab phenotype contains a mutation which affects the transcription or processing of DAF mRNA.

INTRODUCTION

Decay accelerating factor (DAF) is thought to play a role in the regulation of complement activation by inhibiting the formation and accelerating the decay of the classical and alternative pathways C3 convertases (Nicholson-Weller *et al.*, 1982; Pangburn *et al.*, 1983). The complete sequence of DAF has been deduced from the nucleotide sequence of cDNA clones (Caras *et al.*, 1987; Medof *et al.*, 1987). DAF, together with other proteins with glycosylphosphatidylinositol-linked membrane anchors (GPI-linked proteins), is deficient from the membranes of peripheral blood cells of patients with paroxysmal nocturnal haemoglobinuria (PNH; Selvaraj *et al.*, 1987).

Individuals with erythrocytes of the rare Inab phenotype have recently been shown to lack DAF (Telen *et al.*, 1988; Parsons *et al.*, 1988; Merry *et al.*, 1989). These cells were originally described as lacking a series of blood group antigens known as Cromer-related antigens (Daniels *et al.*, 1982). The Cromer-related antigens were shown to be located on a sialoglycoprotein of M_r 70000 (now known to be DAF), which was absent from Inab phenotype erythrocytes (Spring *et al.*, 1987).

Inab phenotype erythrocytes are not known to have any associated haematological defects. In particular, and unlike PNH erythrocytes, they are not unusually sensitive to complement-mediated lysis *in vitro* (Merry *et al.*, 1989). However, the first three individuals described as having Inab phenotype erythrocytes also had gut disease (protein losing enteropathy or Crohn's disease, cited in Lin *et al.*, 1988). Two more individuals, who were brother

and sister, have recently been described (Lin *et al.*, 1988) but neither of these individuals has a history of gut disease. The inheritance of the Inab phenotype has been demonstrated in this family (Lin *et al.*, 1988).

In this paper we show that Inab phenotype erythrocytes lack DAF but not the other GPI-linked proteins acetylcholinesterase (AChE) and LFA-3. We also show that DAF mRNA levels are grossly reduced in a cell line derived from the Inab phenotype individual.

MATERIALS AND METHODS

Materials

Five monoclonal antibodies reactive with DAF were used. BRIC 110 and BRIC 128 were described by Spring *et al.* (1987). Details of the production of BRIC 216, BRIC 220 and BRIC 230 will be described elsewhere (D. Hunt, A. B. Gardner, G. Mallinson, E. Yousaf & S. F. Parsons, unpublished work; details on application from S. F. Parsons). Murine monoclonal antibody TS2/9 (anti-LFA-3) was a kind gift from Dr. T. Springer, Harvard University Medical School, Boston, MA, U.S.A. Anti-AChE (AE-1) was from the American Type Culture Collection, Rockville, MD, U.S.A. Inab erythrocytes were obtained from the Red Cross Blood Centre, Tokyo, Japan. PNH erythrocytes were obtained as follows: patient Ba from Professor L. Luzzato, Royal Postgraduate Medical School, Hammersmith Hospital, London, U.K.; patient C1 from Mrs. V. Rawlinson, North-Western Regional Blood Transfusion Centre, Manchester, U.K.; and patients De and Dav from Dr. G. Pasvol, Nuffield Department of Clinical Medicine,

Abbreviations used: DAF, decay accelerating factor; GPI, glycosylphosphatidylinositol; PNH, paroxysmal nocturnal haemoglobinaemia; AChE, acetylcholinesterase; EBV-LCL, Epstein–Barr-virus-transformed lymphoblastoid cell line; RFLP, restriction fragment length polymorphism.

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John Radcliffe Hospital, Headington, Oxford, U.K. Normal erythrocyte samples were available from the South Western Regional Blood Transfusion Centre, Bristol.

Restriction enzymes and DNA-modifying enzymes were from Amersham International, Amersham, Bucks.; Pharmacia Biochemicals, Milton Keynes, Bucks.; and Gibco BRL, Paisley, Renfrewshire, Scotland, U.K.

AchE was assayed in whole erythrocytes by the method of Ellman & Calloway (1961). Haemagglutination and methods for trypsin treatment of erythrocytes were carried out as described in Anstee *et al.* (1984). Immunoblotting was as described in Mallinson *et al.* (1986), except that 5% (w/v) bovine milk powder was used as the blocking agent.

Flow cytometry was carried out using a Coulter EPICS C, according to the protocol recommended by the 3rd International Workshop on Leucocyte Differentiation Antigens (Gotch, 1987), using fluorescein isothiocyanate (FITC) (Fab₂) rabbit anti-(mouse IgG) (Dako, High Wycombe, Bucks., U.K.)

Isolation of DAF cDNA clone

Two oligonucleotides were constructed to the cDNA sequence encoding the *N*-terminus of the mature protein and then ligated together using an overlapping complementary oligonucleotide to produce a single long probe (DAF1) which corresponds to nucleotides 168–219 of the cDNA isolated by Caras *et al.* (1987). DAF1 was end-labelled with polynucleotide kinase (Maniatis *et al.*, 1982) and used to screen a λ gt11 human foetal liver cDNA library (Clontech Labs, Palo Alto, CA, U.S.A.). A single clone was isolated which hybridized to DAF1. The cDNA insert (DAFA1) was excised from λ gt11 and subcloned into Bluescript plasmid (Stratagene Cloning Systems, San Diego, CA, U.S.A.). Single-stranded DNA was prepared from the insert-containing plasmid and sequenced using the dideoxy method (Sanger *et al.*, 1977). DAFA1 contained nucleotides 1–1432 of the DAF cDNA clone isolated by Caras *et al.* (1987), but did not contain the 118 bp intron found by these workers to be present in 10% of DAF mRNA. DAFA1 contained an additional 20 nucleotides of the 5' non-coding region of DAF mRNA which were not present in the cDNA clone of Caras *et al.* (1987).

Southern blotting of DNA samples

DNA was isolated from Epstein–Barr-virus-transformed lymphoblastoid cell lines (EBV-LCLs) and Southern blotting was carried out as described in Tanner *et al.* (1988). When oligonucleotide probes were used, they were hybridized using the method of Thein & Wallace (1986).

Northern blotting of RNA samples

RNA was isolated using the guanidine thiocyanate method (Chirgwin *et al.*, 1979). Polyadenylated mRNA was prepared (Aviv & Leder, 1972) and blots were carried out using formaldehyde-containing gels (Maniatis *et al.*, 1982).

RESULTS AND DISCUSSION

Unlike PNH erythrocytes, erythrocytes of the Inab phenotype selectively lack DAF

Erythrocytes of the Inab phenotype were tested with

Table 1. Reactivity of Inab erythrocytes and PNH erythrocytes with monoclonal antibodies to DAF, LFA-3 and AchE

Titration scores were calculated according to Marsh (1972). The strength of agglutination given by each doubling dilution of antibody was assigned a number between 1 and 12 (12 representing maximum agglutination). The sum of the numbers assigned to each set of doubling dilutions represents the titration score for a given antibody with a given cell. Titration scores for anti-AchE were obtained using trypsin-treated erythrocytes, whilst those for anti-DAF and anti-LFA-3 were obtained using untreated erythrocytes.

Monoclonal antibody	Haemagglutination titration score (% of control)				
	Inab erythrocytes	PNH erythrocytes (4 patients)			
		Ba	Cl	De	Dav
Anti-DAF (BRIC 110)	0	0	0	0	0
Anti-LFA-3 (TS2/9)	92	39	47	60	24
Anti-AchE (AE-1)	62	0	22	4	0

Fig. 1. Immunoblotting of Inab and PNH erythrocyte membranes with anti-DAF and anti-LFA-3

Erythrocyte membrane protein (80 μ g) was applied to each track. Lanes a and h show molecular mass markers (Sigma Chemical Co.), lanes b–d were immunoblotted with anti-DAF (BRIC 128) and lanes e–g were immunoblotted with anti-LFA-3 (TS2/9). Lanes b and e, normal erythrocyte membranes; c and f, erythrocyte membranes of Inab phenotype; d and g, erythrocyte membranes of PNH patient Da.

murine monoclonal antibodies which react with three GPI-linked surface glycoproteins (DAF, AchE and LFA-3). These glycoproteins are known to be deficient in the erythrocytes of patients with PNH (Selvaraj *et al.*, 1987). Haemagglutination tests (Table 1) showed that all the PNH patients gave markedly reduced reactions with monoclonal antibodies against all three proteins, whereas Inab phenotype erythrocytes gave a normal reaction

with anti-LFA-3, a slightly reduced reaction with anti-AchE, but no reaction with anti-DAF. Direct assay of AchE also showed a slightly reduced activity of the enzyme in Inab phenotype erythrocytes (73 % of control), whereas this enzyme was markedly reduced in the erythrocytes of the three PNH patients tested (19 %, 20 % and 0 % of controls respectively for patients Ba, De and Dav). Whether the small reduction in AchE in Inab phenotype erythrocytes has any significance is not clear, since control erythrocytes subjected to the same transportation conditions as the Inab erythrocytes were not available. The extent of the reduction of GPI-linked glycoproteins in different PNH patients is known to vary (Selvaraj *et al.*, 1987).

Immunoblotting with anti-LFA-3 gave a normal pattern with erythrocyte membranes of the Inab phenotype (Fig. 1, lane f), whereas much weaker staining was observed with membranes from PNH erythrocytes (Fig. 1, lane g). The absence of DAF from Inab erythrocytes, which is also shown in Fig. 1, lane c, has already been reported elsewhere (Spring *et al.*, 1987; Telen *et al.*, 1988; Merry *et al.*, 1989).

The above data clearly show that Inab erythrocytes differ from PNH erythrocytes in that they selectively lack DAF.

Absence of DAF from a lymphoblastoid cell line derived from an individual with erythrocytes of the Inab phenotype

PNH is an acquired disorder affecting haemopoietic cells in general. It was therefore of interest to examine other peripheral blood cells of the individual with erythrocytes of the Inab phenotype. An EBV-LCL was generated from lymphocytes of the Inab phenotype donor by EBV transformation because of the difficulty in obtaining regular blood samples from the donor. Untransformed Inab lymphocytes and the Inab EBV-LCL were unreactive with anti-DAF monoclonal antibodies when examined by flow cytometry, whereas normal lymphocytes, granulocytes and EBV-LCL were reactive with the antibodies (Table 2). The reactivity of anti-DAF antibodies was also tested with a variety of human haemopoietic cell lines (Table 2). All the cell lines examined, except the T-cell line MOLT 4, showed evidence for the presence of cell-surface DAF. Northern blot analysis of MOLT 4 polyadenylated RNA confirmed that MOLT 4 cells have little or no DAF mRNA (see Fig. 3b).

Comparison of the DAF gene in normal and Inab phenotype cells

DNA was isolated from EBV-LCLs derived from the Inab phenotype donor and a normal individual. *Hind*III, *Bam*HI and *Eco*RI digests of DNA were examined by Southern blotting and probed with the intact cDNA clone DAFA1 (Fig. 2). The patterns for the *Bam*HI and *Hind*III digests of Inab DNA were clearly different from those observed for normal DNA (Fig. 3a). However, the *Eco*RI digest patterns for the Inab DNA and normal DNA were identical.

Southern blot analysis with *Bam*HI-digested and *Hind*III-digested DNA from individuals in extended families has shown the presence of commonly occurring *Bam*HI and *Hind*III restriction fragment length polymorphisms (RFLPs) in the DAF gene (Rey-Campos *et al.*, 1987; Stafford *et al.*, 1988). In order to ascertain whether the differences between the Inab and normal

Table 2. Reactivity of anti-DAF (BRIC 110) with peripheral blood cells and haemopoietic cell lines by flow cytometry

Similar results were obtained with four other monoclonal DAF antibodies (BRIC 128, BRIC 216, BRIC 220 and BRIC 230) with the exception of BRIC 128 which gave negative reactions with the Daudi and Raji lines. The shape of the fluorescence histogram obtained is indicated by P, positive peak; N, negative peak; or C, complex peak, comprising a continuum of fluorescence from negative to strongly positive.

Cell sample	Positive cells (%)	Histogram type
Peripheral blood cells:		
Normal granulocytes	97	P
Normal lymphocytes	19–68	P
Normal monocytes	43–99	P
Normal platelets	16	N
Inab lymphocytes	5	N
Haemopoietic cell lines:		
K562	98	P
IM9	91	P
U937	75–92	P
HEL	73–92	C
Normal EBV-LCL	81	C
HL60	44–85	C
Raji	22–87	C
Daudi	11–54	C
MOLT 4	5–11	N
Inab EBV-LCL	5	N

DNA were due to RFLPs, Southern blots were probed with restriction fragments of DAFA1 and oligonucleotides representing different portions of the DAF cDNA sequence. The results of these studies are summarized in Fig. 2. Oligonucleotide DAF6 hybridized to two *Hind*III bands in normal DNA (11.5 kb and 7.4 kb), but only to the 7.4 kb *Hind*III band in Inab. The 11.5 kb *Hind*III fragment in normal DNA, which is not present in Inab DNA, clearly results from an RFLP. This polymorphism is identical to that found by Rey-Campos *et al.* (1987) and Stafford *et al.* (1988). The Southern blotting data were not sufficient to order the 4.7 kb, 5.3 kb and 3.3 kb *Hind*III fragments. The Inab DNA has the *Hind*III map shown in Fig. 2(a), while the normal DNA is heterozygous for the *Hind*III maps shown in Figs. 2(a) and 2(b). The *Hind*III map in Fig. 2 which is derived from our data differs from that reported by Stafford *et al.* (1988) in the location of the 9 kb fragment. The origin of this difference is not clear, but this disagreement does not affect the conclusions of the present study.

The Southern blotting results also allowed the construction of the *Bam*HI maps for the Inab and control DNA shown in Fig. 2. The Inab DNA was homozygous for a DAF gene containing two 30 kb fragments (Fig. 2c), whereas the normal DNA was heterozygous for two of the three different polymorphic forms of the DAF gene shown in Figs. 2(d)–2(f).

DAF mRNA in normal and Inab phenotype cell lines

We were unable to detect any deletions in the DAF gene by Southern blotting analysis of Inab phenotype DNA. This suggested that the gene alteration which

Fig. 2. *Bam*HI and *Hind*III maps of the DAF gene

The open box represents the coding region of the DAF cDNA in the cDNA described by Caras *et al.* (1987). The arrow indicates the end of DAF A1. A partial restriction map is given below the cDNA (D, *Dde*I; S, *Sty*I) with the restriction fragments used to probe Southern blots. The regions corresponding to the synthetic oligonucleotides DAF2, 4, 5, 6 and 7 used to probe genomic DNA in dried agarose gels are also shown. The *Hind*III gene maps (a and b) and the *Bam*HI gene maps (c-f) are represented schematically. The positions of the boundaries between the genomic fragments have not been accurately determined with respect to the cDNA sequence. The individual of the Inab gene studied was homozygous for the DAF gene producing the *Hind*III fragments depicted in (a) and the *Bam*HI fragments depicted in (c).

Fig. 3. Southern blot analysis of genomic DNA derived from the Inab individual (a) and Northern blot analysis of polyadenylated RNA derived from cells of Inab phenotype (b, c)

(a) Lanes 1, 3 and 5 contain control DNA, whereas lanes 2, 4 and 6 contain DNA derived from the Inab individual. DNA was digested with the following restriction enzymes: lanes 1 and 2, *Eco*RI; lanes 3 and 4, *Hind*III; lanes 5 and 6, *Bam*HI. Band sizes are given in kb. The blot was probed with DAF A1 and washed in 1 × SSC (0.15 M-NaCl/0.015 M-sodium citrate) containing 1 % SDS at 65 °C. Poly(A)⁺ RNA was obtained from the following sources in (b) and (c): lane 1, K562 cells; lane 2, MOLT 4 cells; lane 3, an EBV-LCL derived from the individual of the Inab phenotype; lane 4, an EBV-LCL derived from a normal individual. A portion of 5 µg of poly(A)⁺ K562 and MOLT 4 RNA was used per track; and 10 µg of poly(A)⁺ RNA from the EBV-LCLs was used per track. Band sizes are given in kb. (b) The blot was probed with DAF A1 and washed in 1 × SSC containing 1 % SDS at 65 °C. (c) The blot used in (b) was deprobed by repeated washings in 0.01 × SSC containing 0.01 % SDS at 100 °C and reprobed with a mouse actin cDNA clone (Humphries *et al.*, 1981). The blot was washed in 1 × SSC containing 1 % SDS at 65 °C.

results in the absence of expression of the DAF protein in the Inab phenotype causes a transcriptional, translational or post-translational defect. To distinguish between these possibilities, we examined the levels of DAF mRNA in cells derived from the Inab phenotype individual. Because of the difficulty in obtaining adequate amounts of peripheral blood cells from the Inab individual, the EBV-LCL derived from the Inab donor was used as a source of mRNA. These cells were shown to be deficient in the expression of the DAF protein in the experiments described above (Table 2). An EBV-LCL derived from a normal individual was used as a control source of mRNA. A Northern blot of polyadenylated RNA from the control cell line was probed with DAF cDNA and this sample contained substantial amounts of the different DAF mRNA species (Fig. 3b). The multiple DAF mRNA species found in normal cells are known to result from the use of at least two of the four polyadenylation signals in the 3' non-coding region of the protein, and also from the presence of fully spliced mRNA and mRNA retaining one intron which gives rise to related, but different, proteins (Caras *et al.*, 1987). In contrast with the control sample, only very small amounts of the DAF mRNAs could be detected in the polyadenylated RNA from the Inab cell line (Fig. 3b), and these corresponded with the major DAF mRNA species found in the normal cell line. To ensure that the Inab RNA sample was not degraded, and contained the same amount of total mRNA as the normal cell line, the same blot was subsequently probed with actin cDNA (Fig. 3c). Both normal and Inab samples gave the same actin mRNA bands and these were of equal intensity.

Although they are present in drastically reduced abundance in the Inab cell line, the sizes of the DAF mRNAs present in the Inab polyadenylated RNA are similar to those of the major DAF mRNAs found in normal cells. This confirms the absence of any major deletions in the exons of the DAF gene, and is consistent with the results of the Southern blotting experiments. The deficiency of DAF mRNA probably results from a point mutation or small deletion in the DAF gene which was not detected in our Southern blotting studies.

There are several possible reasons for the gross reduction of DAF mRNA in Inab phenotype cells. The mutation in the DAF gene of the Inab individual studies could cause the alteration of a transcriptional control element which results in decreased levels of transcription of the gene. Alternatively, a mutation in a sequence of the DAF gene which is required for correct splicing of DAF RNA transcripts could result in the decrease of the amount of fully processed mRNA, together with the turnover of incompletely spliced or improperly spliced transcripts in Inab phenotype cells. A further possibility is that the defect leads to an unstable DAF mRNA. It is clear that more detailed studies on the DAF gene in the Inab phenotype will be necessary to establish the exact nature of the defect.

The deficiency of DAF mRNA in Inab phenotype cells contrasts with the normal mRNA levels of this protein in PNH cells (Stafford *et al.*, 1988). Stafford *et al.* (1988) suggest that in PNH there is a post-translational defect which results in the defective incorporation of GPI-linked proteins into the membrane. The latter mechanism clearly does not account for the absence of DAF in Inab phenotype cells.

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