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Biochemical characterization of human erythrocytes fractionated by counter-current distribution in aqueous polymer two-phase systems

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Abstract

The fractionation of normal human erythrocytes by counter-current distribution (CCD) in charge-sensitive dextran–polyethylene glycol two-phase systems was confirmed and extended to red blood cells from heterozygous β-thalassaemic patients. The differences between the distribution profiles of normal (homogeneous) and abnormal (heterogeneous) red blood cells reflect their different surface-charge properties. As suggested by the decline of membrane sialic acid released after neuraminidase treatment and the specific activities of two age-dependent enzymes (membrane acetylcholinesterase and intracellular pyruvate kinase) in the distribution profiles (from the left- to the right-hand side fractions), the fractionation seems to be according to red blood cell age. A constancy of the 2,3-bisphosphoglycerate level was observed in ageing red blood cells.

1. Introduction

Several approaches are used to study red blood cell (RBC) ageing. Bleeding and hypertransfusion or ageing of tagged cells are employed *in vivo* (in experimental animals). *In vitro*, the study of cellular ageing relies on the separation of circulating RBCs in subpopulations of various mean age on the basis of differences in physical properties (density, size, volume, surface area, shape, osmotic fragility or surface charge density). Most studies have consisted of comparisons of RBC properties in fractions obtained by centrifugation (or ultracentrifugation) or after centrifugation through phthalate esters or continuous and discontinuous density gradients (gum acacia, Stractan, albumin, dextran, Ficoll, Percoll, etc.) [1–8]. Density fractionation, the most widely used technique, is based on the relationship between increasing RBC age and density. Aged RBCs lose surface area and volume [9] and therefore become denser than young mature RBCs. A question still debatable, whether the two phenomena do in fact correlate throughout the RBC life-span, is in favour of a progressive increase of RBC density with age [8].

Multiple partitions in aqueous dextran–polyethylene glycol (Dx–PEG) two-phase systems, using a thin-layer counter-current distribution (CCD) apparatus [10], is an alternative method for fractionating erythrocytes on the basis of alterations of the negative surface charged properties that occur during ageing [11]. Rat RBC

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^{59}Fe-labelling studies *in vivo* [11,12] and the decline in the CCD fractions of age-dependent enzymes [13–16] have shown in rats the positioning of maturing reticulocytes and ageing RBCs after fractionation in charge-sensitive two-phase systems. These studies were then extended to rat RBCs during animal development (*i.e.*, RBC switching) in order to distinguish between embryonic, foetal and adult RBCs [15]. Finally, this approach was applied comparatively to adult rat and human RBCs on the basis of the decline in specific activity of glycolytic enzymes (HK, PFK and PK) [16]. Our aim in this work was to compare the fractionation of normal and pathological RBCs and to document it as due to changes in cell age. Both β- (or A_2) and F- (or dβ) thalassaemia subgroups affect Mediterranean populations and are encountered in Spain [17]. Mutations in β-thalassaemia, which result in a deficit of β-chain synthesis, account for heterogeneity of these RBCs. The decreased β-chain production leads to an excess of unstable α-chains and intracellular inclusions that interact with the membrane, initiating oxidation reactions that contribute to heterogeneity and abnormality [18–20].

To demonstrate the fractionation according to age, three age-dependent parameters were measured in the fractions: membrane sialic acid released after neuraminidase treatment and specific activity of acetylcholinesterase (EC 3.1.1.7) (AChE) and pyruvate kinase (EC 2.7.1.40) (PK). A non-age-dependent enzyme, bisphosphoglycerate mutase (EC 5.4.2.4) (BPGM), was also measured in an attempt to relate it to 2,3-bisphosphoglycerate (2,3-BPG) levels in the fractions [13,15,16].

2. Experimental

2.1. Cell suspensions and haemolysates

Samples of blood from ten normal adults and five patients with anaemic heterozygous β-thalassaemia (University Hospital) were collected in heparin (10 units/ml). After centrifugation (400 *g* for 10 min) at 4°C, the cells were washed three times with cold 0.15 *M* NaCl, the supernatant and the top layer of packed cells being removed each time. Haemolysates were obtained by hypoosmotic shock with two volumes of stabilizing solution (2.7 m*M* EDTA-Na_2–0.7 m*M* mercaptoethanol), followed by freezing and thawing. RBC suspension and haemolysates were prepared on the day of assay.

2.2. Laboratory assessments

RBC count (cells/l), Hb content (g/dl), packed cell volume (PCV) (%), mean cell volume (MCV) (fl), mean cell haemoglobin (MCH) (pg) and mean cell haemoglobin concentration (MCHC) (g/dl) were measured in a Technicon H1 or a STKR Coulter Counter. Hb variants, were detected by standard cellulose acetate electrophoresis with an alkaline (pH 8.5) buffer system (Helena Labs.). An accurate determination of HbA_2 (%), for β-thalassaemia diagnosis, is given by the absorbance (415 nm) of fractions eluted with glycine–KCN developers after DEAE-cellulose anion-exchange microchromatography (Helena Labs.). HbF was determined as a total percentage using a commercialized immune diffusion radial (IDR) method (Helena Labs.).

2.3. Fractionation of RBCs

This was carried out as reported previously [16]. The affinity of phosphate for the Dx-rich bottom phase of charge-sensitive two-phase systems [5% (w/w) Dx–4% (w/w) PEG–0.03 *M* NaCl–isotonic 0.09 *M* sodium phosphate buffer (pH 6.8)] and the partitioning of chloride between both phases gives a relatively higher positive charge to the PEG-rich upper phase than to the bottom phase, thus allowing cell partitioning on the basis of surface charge properties [10,11]. The fractionation of cells differing closely in partition ratio values (total cells in top phase/cells adsorbed at interface), *e.g.*, ageing RBCs, need multiple partitionings to be fractionated. These are carried out in a CCD apparatus (Bioshef MK3; University of Sheffield, Sheffield, UK) with a circular thin-layer unit (60 concentric

cavities) formed by a bottom or stator plate and a top or rotor plate). The experimental conditions were as detailed for human RBCs [11,12,16]. Cell distribution profiles are given by the Hb absorbance values at 540 nm against cavity number. In order to obtain sufficient cells to study the enzyme activity and metabolite levels, some adjacent CCD cavities (with similar partition ratios) were joined in five pools (5–6 cavities) as indicated in Fig. 1.

2.4. Enzyme and metabolite assays

Sialic acid was measured (μg per 10^{10} cells) after neuraminidase (from 1 U/ml *Vibrio cholerae* solution) treatment of RBCs from pooled adjacent CCD fractions (Fig. 1) [21]. Haemolysates were prepared by hypo-osmotic shock of RBCs from pooled adjacent CCD fractions (Fig. 1). The specific enzyme activities (U/g Hb) in haemolysates were measured at 30°C for AChE [22]. PK and BPGM and Hb were determined as described previously [16]. 2,3-BPG was measured with a UV test (Boehringer). Reagents, substrates, cofactors and auxiliary enzymes were obtained, from Sigma and Boehringer.

3. Results and discussion

3.1. Erythrocytic indices

Standard hematological indices were used to study normal cells and to identify thalassaemic RBCs as microcytic hypochromic cells. The mean RBC count was higher in thalassaemic [$(5.7 \pm 1.0) \cdot 10^{12}$ cells/l] than in normal RBCs [$(5.1 \pm 0.4) \cdot 10^{12}$ cells/l]. A mild anaemia was observed, with lower mean Hb (12.2 ± 1.7 g/dl) and PCV ($39.5 \pm 3.1\%$) values in thalassaemic than in normal cells (15.3 ± 1.4 g/dl and $45.1 \pm 3.2\%$, respectively). As a consequence of a lower MCV value in thalassaemic (69.3 ± 6.1 fl) than in normal RBCs (89.3 ± 3.2 fl), the MCH and MCHC indices were characteristically lower in thalassaemic (21.6 ± 3.1 pg and 31.1 ± 0.6 g/dl) than in normal RBCs (30.4 ± 1.8 pg and 34.1 ± 1.5 g/dl). Abnormalities of thalassaemic RBCs, including anisocytosis, poikilocytosis, basophilic stippling and numerous target cells, were also observed by optical microscopy. The increase in the RDW index in thalassaemic cells ($16.1 \pm 1.8\%$) with respect to normal cells ($12.9 \pm 0.6\%$) is in agreement with the anisocytosis of thalassaemia. As a compensation for the defective synthesis of β-chains, the microcolumn chromatographic results show an increase in both Hb A_2 ($3.9 \pm 0.5\%$) and HbF ($5.1 \pm 0.8\%$) with respect to normal RBCs ($2.6 \pm 0.7\%$ and $0.6 \pm 0.2\%$, respectively).

3.2. Fractionation profiles

The reproducibility of the distribution profiles for normal RBCs was as shown previously under identical conditions [11,12,16]. An apparent homogeneous curve is usually observed. A representative profile is then shown (Fig. 1, Control). RBCs are located all along cavities 20–50. Cells with affinity for the top phase are found as fast-moving cells towards the right-hand side (pooled CCD fraction 5) whereas cells with affinity for the interface are found as slow-moving cells towards the left-hand side (pooled CCD fraction 1). This is due to the partition ratio of the ageing RBC subpopulation which, owing to the subtle differences in surface charge properties, are very close to each other. The relationship between RBC age and partition ratio (surface charge) seems to be similar to that between RBC age and density [8]: the RBC distribution is overall a normal curve, composed of the integral of a multitude of normal distribution curves of cells of progressively increasing age and density. Each cohort of cells ages and increases in density at a constant rate. The effect is exerted on the entire population, which become homogeneously distributed along the fractions with regard to density.

Such a progressive relationship between age and any other physical property does not exist for heterogeneous RBCs (*e.g.*, thalassaemic RBCs), which are then distributed as containing several populations. Representative profiles for two thalassaemic RBC samples are given in Fig. 1. The distribution curves are now generally

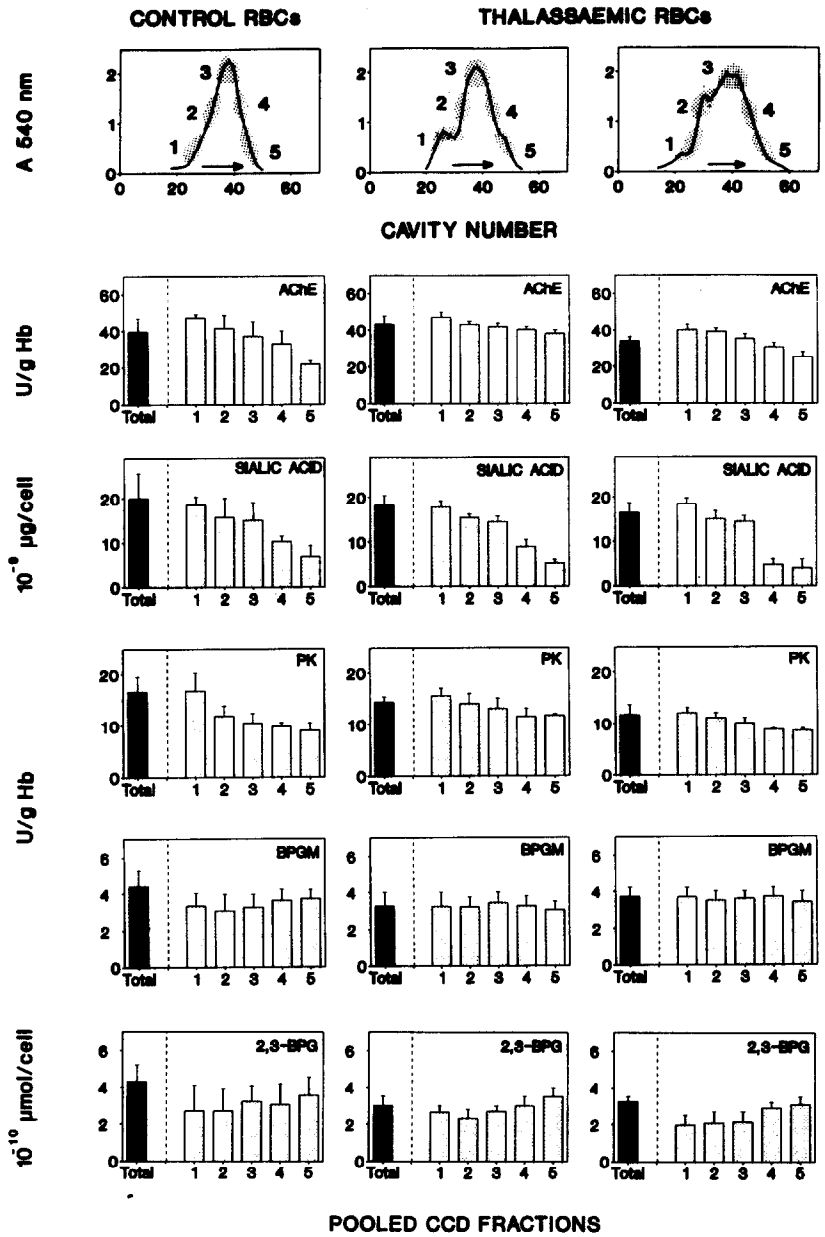

Fig. 1. Fractionation by counter-current distribution of human (normal and thalassaemic) RBCs. CCD profiles (top) show the Hb absorbance value (cell number) for each cell population. Acetylcholinesterase (AChE); sialic acid; pyruvate kinase (PK); bisphosphoglycerate mutase (BPGM); 2,3-bisphosphoglycerate (2,3-BPG). Whole RBC populations (total). Pooled CCD fractions (1–5) were prepared from adjacent cavities, as shown in the CCD profiles. Bars represent the mean ± S.D. for four separate CCD runs and quadruple measurements.

broader (between cavities 15 and 55) and clearly more heterogeneous. This is in agreement with a higher RDW index (see above) and a non-progressive relationship between RBC age and surface properties. Reproducibility was then not possible for RBCs from different patients.

3.3. Changes in age-dependent constituents

Although initial studies indicated that human RBCs of different age were not subfractionated by CCD, ^{51}Cr-labelling studies *in vitro* had shown that fractionation of human RBCs also reflects surface changes during ageing [11,12]: young human RBCs have a slightly lower partition value than the whole population and would be distributed towards the left-hand side of the CCD profile. Older RBCs, which have a higher partition value than the population as a whole, would be distributed towards the right of the CCD profile. Such a distribution was confirmed in rats and humans on the basis of the decline in the specific activity of three age-dependent glycolytic enzymes (HK, PFK and PK) [16]. However, in rats (*in vivo* ^{59}Fe-labelling plus enzyme changes), the fractions to the left contain older RBCs whereas the fractions to the right are progressively enriched in younger RBCs. This means that the positioning of ageing RBCs is reversed in humans and rats (is species specific). An additional important point is that reticulocytes (normally present as a minor subpopulation in the original RBC samples) are distributed in the cell fractions (from both species) located further to the left, as a consequence of their lower partition ratio [11,12,14]. The number of reticulocytes in the CCD fractions was not detected after staining with cresyl brilliant blue [16].

Specific acetylcholinesterase activity

The mean value for the specific activity of AChE (U/g Hb) in whole normal RBCs (total bars, Fig. 1) agrees with that given by others [22] and with the value found in whole thalassaemic RBCs. A decline in activity for normal RBCs is observed from pooled CCD fraction 1 (or fraction 2) to fraction 5, which means that the youngest RBCs (highest enzyme activity) are distributed towards the left-hand side fractions and older RBCs (with decreasing activity) towards the right-hand side of the profile. Although not always significant for different thalassaemic RBCs (Fig. 1), such a decline seems also to be observed from pooled CCD fraction 1 (or fraction 2) to fraction 5.

Sialic acid

Measurement of sialic acid is based on the hypothesis that desialylation during ageing is responsible for the recognition and subsequent phagocytic sequestration of senescent RBCs. Sialic acid, the final sugar residue in the carbohydrate chain of membrane RBC glycoproteins and glycolipids and the main factor responsible for negative charges, is released by neuraminidase from the receptor domain of the glycophorins on the outer surface of the membrane. A loss of surface charge density (decrease in partition ratio) and a loss of sialic acid are then a reflection of the RBC ageing process in peripheral blood [1,2,5–7,23].

In the whole normal RBC population (total bars, Fig. 1), the mean value for the concentration of sialic acid per cell is similar to that given by others [21], and similar to that measured in thalassaemic RBCs (Fig. 1). A marked decrease in the content of sialic acid is observed from pooled CCD fraction 1 (or fraction 2) to fraction 5, in both normal and abnormal RBCs. In general, such a decrease is more pronounced than the decrease in AChE activity. Using density fractionation, the decrease of sialic acid per normal RBC (from the least to the densest fractions) is around 10–15% in several mammalian species [1]. From our data, the sialic acid content in pooled CCD fractions is at least 20% higher in the left-hand side fractions, indicating a fractionation according to age.

To relate the decline of these (and other) membrane constituents to the membrane loss during cell ageing, the data must be expressed per unit membrane area as a loss of surface area (and volume) with RBC age was shown in density fractionation studies. Thus, the general conclusion is that sialic acid may not be decreasing during the ageing process [1,2,9]. The calculation of sialic acid per unit membrane area cannot be made here (surface area measurements in the pooled CCD fractions seems to be affected by the polymers). However, taking into account the basic differences between both tech-

niques (surface charge against density fractionation), the decrease in sialic acid per cell observed in the pooled CCD fractions (Fig. 1) is clearly in favour of the fractionation of RBCs according to age.

Specific activity of pyruvate kinase and bisphosphoglycerate mutase and 2,3-bisphosphoglycerate level

The incorrect assumption that certain enzymes (*e.g.*, PK) were not age-dependent enzymes came from a poor or imperfect relationship in gradient fractionation between RBC age and density, mainly owing to the presence of reticulocytes in the density fractions. While the light fractions represent a mixture of reticulocytes and young mature RBCs, the dense fractions represent predominantly old RBCs. As reticulocytes have higher enzyme activities than RBCs (*e.g.*, 300-fold for PK), the decline in enzyme level with increasing density appears to be more rapid in the uppermost fractions of the gradient and less rapid in the lowermost fractions. The biphasic disappearance sometimes observed, *e.g.*, for pyrimidine-5-nucleotidase activity, depends on both the RBC ageing and reticulocyte maturation processes [8]. The situation seems to be different in the CCD fractions mainly because RBCs seem not to be mixed with reticulocytes.

The specific activities of PK and BPGM (IU/g Hb) are apparently higher in normal than in thalassaemic whole RBCs (total bars, Fig. 1). The decline in PK activity is also higher in pooled CCD fraction 1 (or fraction 2) than in fraction 5, in both normal and thalassaemic RBCs. This decline was previously observed for both human [16] and rat [13–15] CCD fractions. In all instances, no significant variation was observed in BPGM activity. Both results have now been confirmed for human RBCs and extended to thalassaemic RBCs (Fig. 1). A non-significant variation in the level of 2,3-BPG is observed in any of the pooled CCD fractions. As 2,3-BPG synthesis is directly related to BPGM activity and indirectly related to PK activity, the PK/BPGM ratio was proposed as an index of 2,3-BPG formation. This was confirmed in reticulocyte populations from anaemic rats (and their CCD fractions) at different stages of reticulocyte maturation [13,14], and also in peripheral rat RBCs (and their CCD fractions) at different stages (RBC switching) of animal development [15]: the inverse relationship between 2,3-BPG and the PK/BPGM ratio was shown in all reticulocytes and switching RBCs. The slightly higher 2,3-BPG level in rat (1.15 ± 0.15 μmol/μmol Hb) than in human (0.96 ± 0.08 μmol/μmol Hb) whole RBCs [24] was also related to a lower PK/BPGM ratio in rats than in humans [16]. The higher PK/BPGM ratio observed in rat reticulocyte-rich populations also accounts for a lower 2,3-BPG level in reticulocytes than in circulating RBCs [13]. In all these situations, the lower PK/BPGM ratio is due to a decrease in PK and a simultaneous increase in BPGM.

However, a significant variation in the PK/BPGM ratio was not found in the CCD fractions from adult rat [13,15] and humans [16]. This is confirmed here (Fig. 1): a decline in PK activity without a parallel increase in BPGM activity is shown in the pooled CCD fractions from normal and thalassaemic RBCs. The reason for a non-significant variation in the PK/BPGM ratio, observed during rat or human RBC ageing [16] and thalassaemic RBCs (Fig. 1), is due to the standard deviations of the values for BPGM and PK in CCD fractions. When they are divided to give the PK/BPGM, the error bars for the ratio should be marked. As a consequence, the PK decrease is not enough by itself to explain the variations in 2,3-BPG. This is a different situation to those above described (reticulocyte maturation; RBC switching). Therefore, the maintenance of a constant 2,3-BPG level during the ageing process, necessary for the oxygenation of RBCs all along the life-span, can now be explained on the basis of a fairly constant BPGM activity, in addition to the non-variation of phosphoglycerate kinase [16]. The maintenance of the stability of these enzymes may be the mechanism the RBCs use to keep an appropriate level of 2,3-BPG. For this reason, these enzymes have to be kept away from the normal age-related decay suffered by most enzymes.

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