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Density separation of human red blood cells on self forming Percoll[R] gradients: correlation with cell age

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Human red blood cells were density separated on self-forming Percoll[R] gradients. Redistribution of density fractionated red blood cells was studied by recentrifugation on self-forming Percoll gradients. A protocol that avoids centrifugation of red cells prior to removal of white cells and introduces EDTA before red cell pelleting completely avoided redistribution. Dense red cells separated according to this method were senescent on the basis of a biochemical and a physical criterion: the increase in the band 4.1a:4.1b ratio (Mueller, T., Jackson, C.W., Dockter, M.E. and Morrison, M. (1987) J. Clin. Invest. 79, 492–499) and the loss of maximum deformability. Characterization also included the relative content of two surface proteins (complement receptor 1, CR1 (Ripoche, J. and Sim, R.B. (1986) Biochem. J. 235, 815–821); decay accelerating factor, DAF) on density fractionated red cells. Unlike cytoplasmic proteins, these proteins face similar conditions, whether located on circulating reticulocytes or aging red cells. Both components were lost linearly within experimental errors with cell density and were lower by 60 and 40% in dense than light cells, respectively.

Introduction

The phenomenon of cellular aging has been extensively studied on mammalian red blood cells (for reviews see Refs. 1–3). Their mature forms lack protein synthesis and, thus, serve as a model for posttranslational events in cellular aging. Studies in this field rely on techniques to separate young from old red cells: hypertransfusion was used in animal models to suppress erythropoiesis and to generate a population enriched in old cells [4]. In vivo clearance was studied on red cells which were tagged with biotin for subsequent isolation [5]. Studies on human red blood cells relied mainly on density fractionation and assumed a uniform increase in buoyant density with cell age. The density increase is mainly due to a loss of potassium ions and associated water [6]. The techniques used to fractionate cells comprise: (a) high speed centrifugation of red cells at 90% hct in plasma or serum in an angle rotor for 1 h at 30°C [7], or (b) various types of isopycnic centrifugations at low temperatures on density gradients consisting of Percoll [8], Stractan [9–11], or albumin [6,12]. Cohort labeling with [^{14}C]glycine or radioiron and fractionation of cells by density gradients using technique (b) yielded a correlation between red cell age and density [12,13]. In some reports [14] and recent reviews [1,15] the uniform increase in density with cell age was questioned and, thus, the correlation between red cell density and cell age. With regard to human red cells the arguments against a correlation came from cohort labeling experiments with radioiron in which cells were fractionated by method (a). Method (a) suffers from several technical drawbacks: (i) the high viscosity of packed cells inhibits cells from reaching their buyoant density; and (ii) high temperature, pressure, and hematocrit and the long centrifugation times result in noticeable hemolysis during fractionation. Since none of these drawbacks accompanies frac-

Abbreviations: CR1, complement receptor 1 (C3b receptor); DAF, decay accelerating factor; DFP, diisopropyl fluorophosphate; EDTA, ethylenediaminetetraacetic acid; *EI*, elongation index; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate.

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tionation on density gradients, for which a correlation between density and cell age was found [16], the arguments against are weak. An apparently more relevant argument was provided recently by density separation of biotinylated and in vivo aged rabbit red cells that were only enriched by 2–3-fold in dense fractions of a Percoll/hypaque gradient [17]. The enrichment of biotinylated cells in dense fractions might exceed the given value, since the reported data were not corrected for recoveries of biotinylated cells from a population of cells containing a small percentage of biotinylated cells (see Fig. 1 in Ref. 17). Thus, density separation may well be a method to enrich senescent red cells optimally, once its inherent problems are solved. One of these problems is the following: "even the most convincing experiments did show some dispersion in the density distribution of labeled cell cohorts with increasing age" (page 228 in Ref. 1). Such a dispersion can have several reasons: (i) it either means that cells of a certain age have a wide spread of densities, or (ii) dispersion could be caused by contamination with cells of other densities due to clumping, and the effect of high viscosity on equilibration and sampling. We demonstrate by recentrifugation that density fractionation of human red cells on self forming Percoll^R gradients is possible without these imperfections, since fractionated red cells maintain their density.

Separation of cells into subpopulations is only one step, each fraction has to be characterized with respect to cell age. Cohort labeling is impracticable in humans. Thus, various enzyme activities (for reviews see Refs. 15 and 18) and red cell creatine [19–21] have been used as criteria. All these parameters, except for one (pyrimidine 5′-nucleotidase) show a biphasic decay: an exponential decay in activity from reticulocytes to young mature cells and a shallow decay during red cell aging [15,22,23]. Hence, a small increase in reticulocyte numbers can erroneously be taken as evidence for a cell population with increased numbers of young mature red cells. Being aware of this problem we determined established parameters for cell age: maximum deformability and the band 4.1a:4.1b ratio [24]. In addition, the relative content of surface proteins, complement receptor 1 (CR1) [25,26] and decay accelerating factor (DAF) [27] was determined.

Materials and Methods

Density separation of human red blood cells

Human blood, type O Rh$^+$, was collected in heparin or liquemin and processed within 1 h after collection. Method A: 1 vol. of heparinized blood (usually 400 ml) was mixed at room temperature with 0.5 vol. of PBS (150 mM NaCl, 10 mM $NaKHPO_4$, 0.05 mM EDTA, pH 7.4) containing 0.4 mM DFP. This suspension was passed through a cellulose column [28], equilibrated with the same buffer. The entire procedure was performed in a fume hood. The filtrate was collected on ice into 250 ml tubes which contained within 1/5 of their total volume PBS buffer with 10 mM EDTA. Filtered cells were mixed with this solution, transferred to 40 ml SS34 tubes, and pelleted for 10 min at 2000 rpm ($478 \times g_{bottom}$) using a Sorvall centrifuge. The supernates were aspirated and mixed with 2 M NaOH to hydrolyze residual amounts of the toxic proteinase inhibitor. Pelleted cells were resuspended with a Percoll buffer to a hematocrit of 15%. The Percoll buffer was essentially as described [8], it contained in a final volume of 500 ml: 427 g Percoll, 114 mM NaCl, 0.5% D-glucose, 10 mM phosphate, 0.5 mM EDTA, 30 μg/ml PMSF. The pH was adjusted to 7.4, and the osmolality to 320 to 330 mosmol/kg. The suspension was immediately centrifuged in 40 ml tubes in a Sorvall SS 34 rotor for exactly 20 min at 19000 rpm ($g_{av} = 33000$). A scheme of this procedure is outlined in Fig. 1A.

Method B: Heparinized blood was pelleted at room temperature, and the plasma and buffy coat were removed. Platelet poor plasma was prepared by centrifugation, supplemented with PMSF (30 μg/ml) and returned with an equal volume of PBS to the red cells. The mixture was passed through a cellulose column [28]. The filtrate containing red cells was collected on ice. Red cells were packed, mixed with Percoll buffer, and separated on self forming gradients as described for method A.

Fractionation of Percoll gradients (methods A and B): Self formed gradients, schematically depicted in Fig. 1B, were fractionated into 6 fractions (numbered 0–5). In each experiment corresponding fractions from 16 to 20 gradients were pooled. Fraction No. 1 comprised 8 to 10% and fraction No. 5 4 to 7% of the cells subjected to density separation. Fractionated cells were washed three times with 10 vols. of buffer B (140 mM NaCl, 5 mM KCl, 10 mM phosphate, 0.5 mM EDTA, 0,5 g/l D-glucose, pH 7.4). Samples which were used for creatine determinations were adjusted to 40–50% hct with autologous plasma before adding saponin to solubilize the cells [19]. Samples used for ektacytometry were either resuspended in autologous plasma that was supplemented with 5 mM EDTA or were washed once and resuspended in a buffer containing 40 mM imidazole, 15 mM D-glucose, 1 mM $MgCl_2$, 1 mM phosphate, 100 mM NaCl, 5 mM KCl, 30 mM sucrose, 50 μg/ml PMSF, 200 units/ml penicillin G (pH 7.4). Cell numbers were determined in all mixtures (Ai Cell Counter 134) and ranged from $3 \cdot 10^9$ to $4.5 \cdot 10^9$ per ml. Resuspended cells were kept on ice and ektacytometry was carried out within 12 h from preparation.

Recentrifugation of washed red cells from subpopulations was performed immediately after washing. 6 ml of Percoll buffer was mixed with 0.1 ml of red cells (50–100% hct) and centrifuged for 15 min at 13000

rpm in SS 34 rotors in translucent tubes (1 × 10 cm). A photograph was taken to determine the R_F value of banded red cells and to assess the extent of redistribution.

Ektacytometry

The instrument used to measure red cell deformability was built by Technicon (France). It allows continuous measurement of the diffraction image and of the actual, relative conductivity in the measuring chamber [29]. A signal processor facilitates stepwise recording of the corrected elongation index against the output of the conductivity meter on a XY recorder. The output of the conductivity meter was calibrated in osmolality units (mosm/kg) by measuring the relative output of the conductivity meter for a given osmolality. Osmolality was determined with a 'halfmicro osmometer' from Knauer, Berlin. Deformability measurements were performed by the osmoscan mode as described elsewhere at a shear rate of 796 to 1060 s^{-1} [11]. The shear stress was varied from 107 to 188 dyn/cm^2. The suspending medium contained 14 to 18 g/100 ml (w/v) dextran T 70 (Pharmacia, Uppsala) which was supplemented with 10 mM phosphate, 1 g/l D-glucose, 0.4 g/l sodium azide and NaCl to yield an osmolality of 750 mM for the 'high osmolality buffer'. A continuous gradient was formed with this solution and the 'low osmolality buffer' which contained everything except salt and had an osmolality of 90 to 96 mosmol/kg. Red blood cells kept in buffer or plasma were mixed with a similar dextran solution that had an osmolality of 290 mosmol/kg. This stock suspension contained 1.5 or $2 \cdot 10^8$ red cells per ml. Osmoscan profiles were the same for cells kept in buffer or autologous plasma.

Presentation of ektacytometer data: The osmoscans shown are redrawn from the actual curves and thus lack the scattering of the data points which did not exceed +/− 3–4 line width. The osmolality scale derived from conductivity measurements was not linear and was calibrated every 30 to 50 mosmol/kg. For determinations of the actual osmolalities we linearized the intervals between the calibrated points. Maximum deformabilities were taken from osmoscans. Note that these values were reached at different osmolalities for density-fractionated red cells (see Fig. 4).

Membrane preparation and band 4.1a : 4.1b ratio

Red cell membranes were obtained by lysis of density fractionated red cells in 30 vols. of 5 mM phosphate, 1 mM EDTA (pH 7.4) [30]. Membranes were washed once with hemolysis buffer containing 0.4 mM DFP and again without DFP. Packed membranes were made 1% in SDS and 5 mM *N*-ethylmaleimide before freezing at −70°C. These samples were thawed and denatured for 3 min at 100°C in electrophoresis sample buffer containing 40 mM dithiothreitol. Reduced proteins were alkylated by a slight excess of *N*-ethylmaleimide. 20 μg of membrane protein were applied to SDS PAGE containing 8% acrylamide [31]. Gels were stained with Coomassie blue and dried between cellophane. Dried gels were scanned with an Integraph from Bender Hobein, Zurich. The absorbance was integrated for band 4.1a and band 4.1b. The relative integrals were used for calculation of the two types of ratios: (a) 4.1a : 4.1b and (b) 4.1a: (4.1a + 4.1b).

Binding of monoclonal anti-CR1 and anti-DAF antibodies to blotted membrane proteins

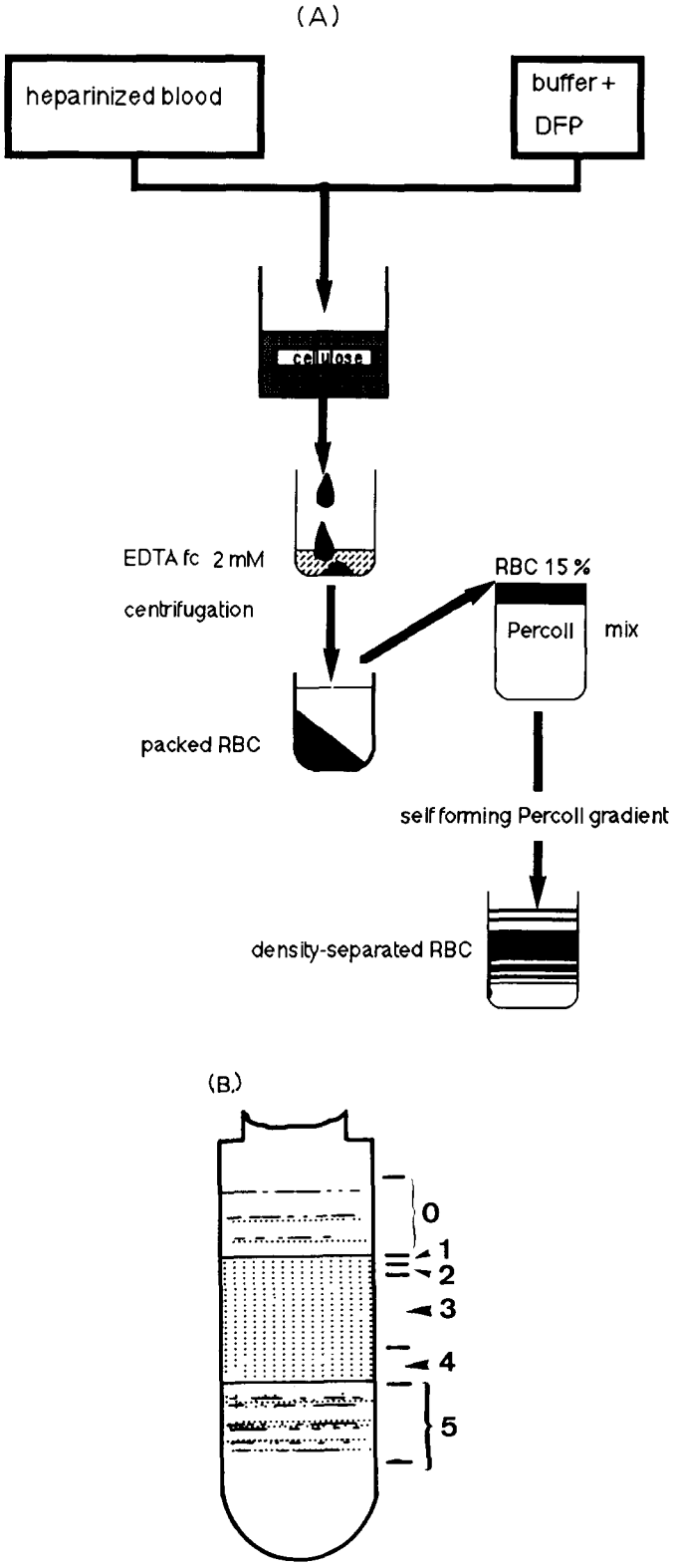
Equal amounts of membrane protein (30 μg) were denatured at 37°C in the absence of reducing agent and were electrophoresed. Reducing agents had to be avoided to preserve CR1 [32] and DAF antigens [33]. SDS PAGE was performed on gels containing 8% acrylamide [31]. Proteins were blotted onto nitrocellulose (Schleicher & Schüll, Feldbach) and blots were blocked, incubated with labeled antibodies, and washed as outlined elsewhere [34]. The following monoclonal antibodies were labeled with $Na^{125}I$ by the chloramine T method [35] (specific activities in the range of (5–16) $\cdot 10^6$ cpm per μg) and were added to blots at (5–10) $\cdot 10^5$ cpm/ml in a buffer containing 1% gelatin and 1% bovine serum albumin: anti-CR1 (J3D3 from M. Kazatchkine) [36], and anti-DAF (IA10 from V. Nussenzweig) [33]. Autoradiographs from washed blots were used to locate immunopositive spots. These areas were cut from the corresponding nitrocellulose, bound radioactivity was measured, and label bound to similarly sized control regions was subtracted. Values from several experiments were normalized as a fraction of 1.0 by assigning this value to the readings for fraction 1 in each experiment.

Results

Density fractionation without dispersion

Red blood cells were prepared from whole blood according to method A (Fig. 1A) and were density fractionated as shown schematically in Fig. 1B. Density separated red blood cells were washed and a sample was recentrifuged on self forming Percoll gradients. Recentrifuged red cells maintained their density (Fig. 2A) whether collected from fraction 1, 2, or 5 of the initial Percoll gradient (Fig. 1B). In particular, dense red cells did not show dispersion upon recentrifugation in all four independent experiments (Fig. 2A). Some dispersion or redistribution was, however, noted (Fig. 2B) when red cells were prepared and density fractionated according to method B. This method included a centrifugation step before removing white cells and before inactivation of complement proteinases. Thus, pelleting of red cells under these conditions, must have generated some cellular aggregates which banded at

high density on the original Percoll gradient and redistributed to lower densities upon washing with EDTA-containing buffer and recentrifugation. The qualitative differences between the two protocols were not revealed by the creatine contents of the separated fractions (Fig. 2). Creatine contents varied greatly between donors (Fig. 2) and increased unproportionally in the lightest fractions which were enriched in reticulocytes (Fig. 3A). Thus, characterization should not depend on parameters that change markedly with the reticulocyte content.



Band 4.1a:4.1b ratio and deformability of density separated red cells

In vivo aged red cells obtained by hypertransfusion [24] or by collecting biotinylated cells [37] showed an increase in the relative content of band 4.1a. These relative changes were determined either as a ratio of the protein contents in band 4.1a:4.1b or as a ratio of the protein content in band 4.1a over the sum in the two bands. The increment between ratios for light and dense red cells was largest for red cells separated according to method A (Table I), irrespective of the type of data presentation. In A the increment was 2-times that in B, when given as a band 4.1a:4.1b ratio. The results suggest that light red cells obtained by method B were contaminated by dense red cells and the dense red cells by light ones. Hence, only method A yielded cells that were well separated with regard to this cell age parameter.

Dense red cells obtained by method A were further considered senescent by their decreased maximum deformability. Red cells subjected to shear stress deform to ellipsoids and their diffraction pattern changes from a circular to an ellipsoidal one which can be detected by ektacytometry [11]. When red cells are continually pumped through this measuring device in solutions of changing osmolalities, deformability of the cells can be studied as a function of osmolality (osmoscan). Fig. 4 shows osmoscans from equal numbers of density fractionated red cells. Two parameters decreased significantly with increasing density: (a) the maximum deformability of red cells and (b) the osmolality at which the cells were no longer deformable * (see circles on abscissa).

A compilation of the data from several density separations according to method A shows a significant loss of maximum deformability with decreasing osmolality at zero deformability and thus with density (Fig. 3B). The maximum deformabilities of dense red cells were

Fig. 1. (A) Schematic depiction of the pretreatment of blood for red cell density fractionation according to method A (see Materials and Methods). (B) Schematic depiction of a self-formed Percoll gradient containing red cells at an initial hematocrit of 15%. The majority of red cells are located in the center of the tube and apparently form an continuous zone. Above and below this zone a series of distinct, less populated bands are visible. Fractionation of such gradients into 6 fractions (No. 0–5), was performed as indicated on the right side of the tube. Fraction 0 was heavily enriched in reticulocytes and was not always collected.

not just lower because an insufficient shear stress was applied to cells having increased viscosities. The ratio of the maximum deformabilities of light and dense red cells did not decrease by raising the shear stress from 110 to 150 dyn/cm^2 (Table II, ratio of EI_{max}). A further increase of the external viscosity and, thus, of the shear stress to 188 dyn/cm^2 raised both values and did not eliminate the difference between light and dense red cells (not shown). The ratios of EI_{max} for red cells density separated according to method A were substantially higher than those obtained by separating red cells on Stractan gradients or by the procedure of

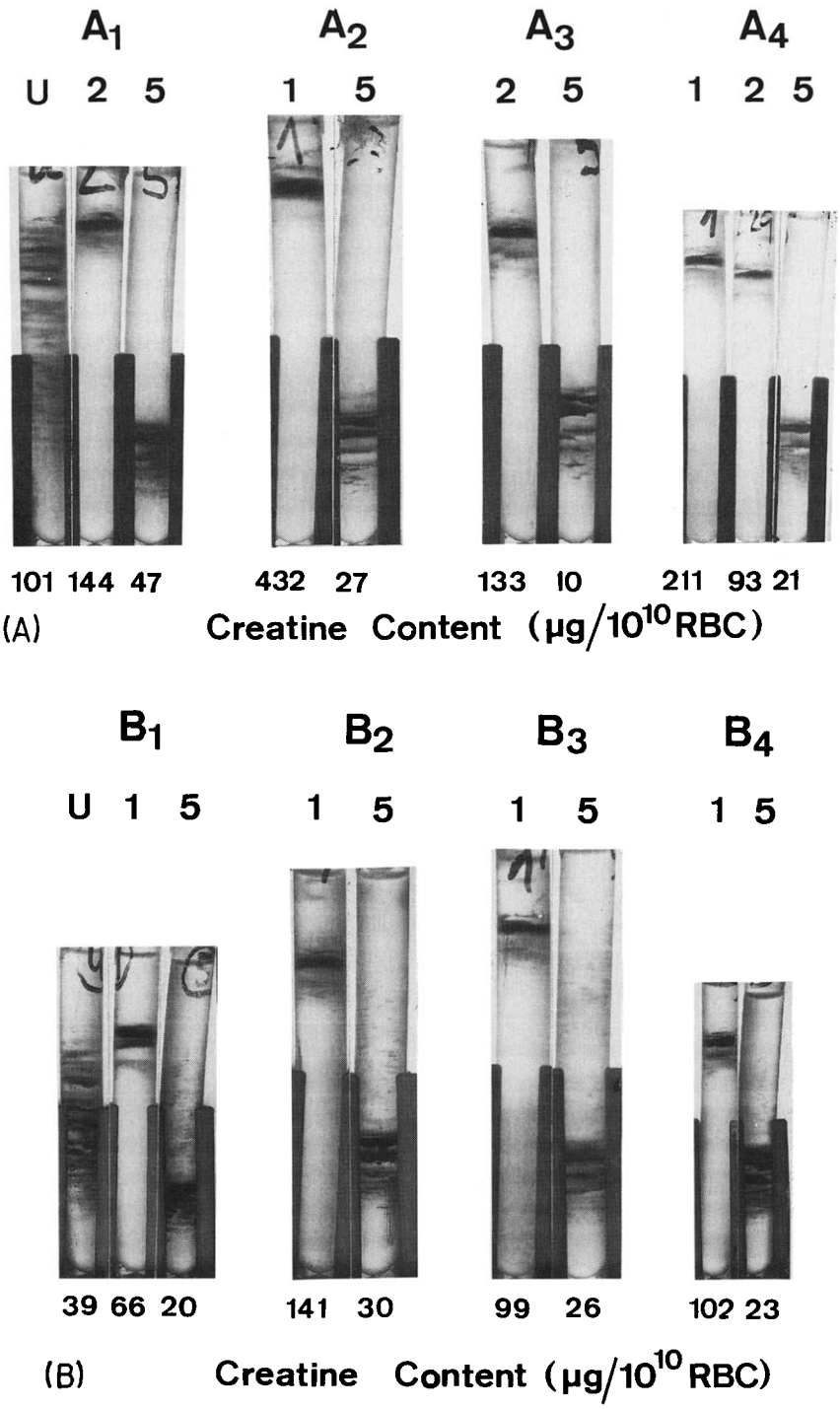


Fig. 2. Recentrifugation of density separated and washed red cells on self forming Percoll gradients at 2% hct. Red cells were obtained from fractions 1–5 of Percoll gradients loaded with red cells prepared according to method A (A), or according to method B (B). For each run the fraction number and the corresponding creatine content is given. Each set of data was from an independent experiment with blood from different healthy donors whose unseparated red cells had the following creatine contents: 101 $\mu g/10^{10}$ RBC in A_1; 42 in A_2; 64 in A_3, and 56 in A_4; 39 in B_1; 60 in B_2; 58 in B_3; and 52 in B_4.

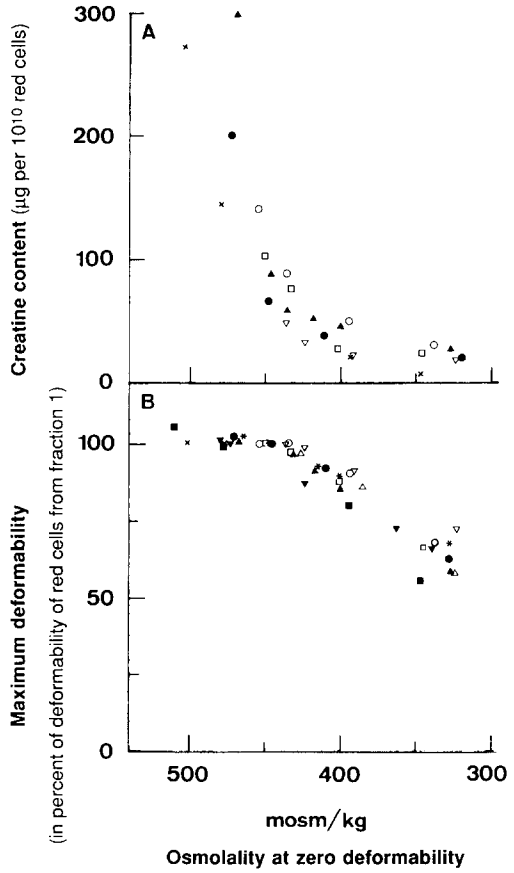


Fig. 3. Creatine contents and maximum deformabilities of density separated red cells as a function of cell density given as osmolality at zero deformability. Creatine contents (A) and maximum deformabilities (B) were determined on red cells, density separated by method A. Creatine contents are given in μg per 10^{10} red cells and maximum deformabilities were normalized to 100% for fraction 1 to better compare ektacytometer readings from different experiments. Data are from six and eight experiments. Identical symbols were used for corresponding creatine contents and maximum deformabilities.

Murphy as calculated from published data (Table II) [38].

Exoplasmic proteins in density separated red cells

As red blood cells age most of their enzymes lose activity biphasically mainly by endogenous proteolysis [39] early and by various protein modifications [40] throughout their lifespan. Hence, internal enzyme activities are not linearly related to red cell age. In contrast to this, exoplasmic membrane components, once inserted in the membrane, are exposed – though for different times – to the very same milieu whether localized on reticulocytes or mature red cells and should provide more reliable cell-age parameters than intracellular ones. CR1 and DAF represent two externally located components that differ in the type of anchorage to the membrane [26]. Two groups have earlier shown by anti-CR1 antibody binding to intact erythrocytes that dense red cells contain less CR1 than light ones [41,42]. Antibody reactivity with red cell antigens could, however, be impaired by seclusion of CR1 or inaccessibility on dense red cells because of their increased stickiness [43]. Therefore, we quantified the number of red cell-associated CR1 and DAF, by studying binding of ^{125}I-iodinated anti-CR1 [36] and anti-DAF [33] to electro-blotted polypeptides from membranes of density fractionated red cells (Fig. 5). Binding of both types of antibodies decreased with increasing density of the corresponding red cell subpopulation. Binding of anti-CR1 decreased irrespective of whether CR1 existed as one or two polypeptides. Furthermore, binding of both antibodies to the parent antigen decreased in all experiments without giving rise to antigenic fragments of smaller size. Quantitation of bound antibodies revealed a 40–50% loss of DAF and a 50–60% loss of CR1 antigens (Fig. 6).

Discussion

The best resolution into dense and light red cells was obtained by density centrifugation of red cells pretreated according to method A. Recentrifugation of red cell subpopulations demonstrated the quality of separation: no redistribution of cells occurred upon recentrifugation and the dense fractions obtained were indeed senescent as judged from an increase in the band 4.1a:4.1b ratio and the loss of maximum deformability. The 4.1a to 4.1 total ratio increased from 0.44 ± 0.04 to 0.61 ± 0.04 for fraction No. 1 and No. 5, respectively. These numbers are somewhat higher than those reported for hypertransfused mice (0.28 for normal and 0.54 for hypertransfused animals [24]) or in vivo aged, biotinylated rabbit red cells (0.3 for control and 0.47 for in vivo aged rabbit red cells [17]). Higher numbers could originate from species differences rather than the use of a Neville type of SDS PAGE [31], since

TABLE I

Relative content of protein band 4.1a

Density separation by method	No. of experiments	Ratio 4.1a:4.1b		Ratio 4.1a:4.1a+4.1b	
		light cells	dense	light cells	dense
A	4	0.79 ± 0.14	1.56 ± 0.23	0.44 ± 0.04	0.61 ± 0.04
B	5	0.98 ± 0.12	1.32 ± 0.1	0.49 ± 0.03	0.57 ± 0.02

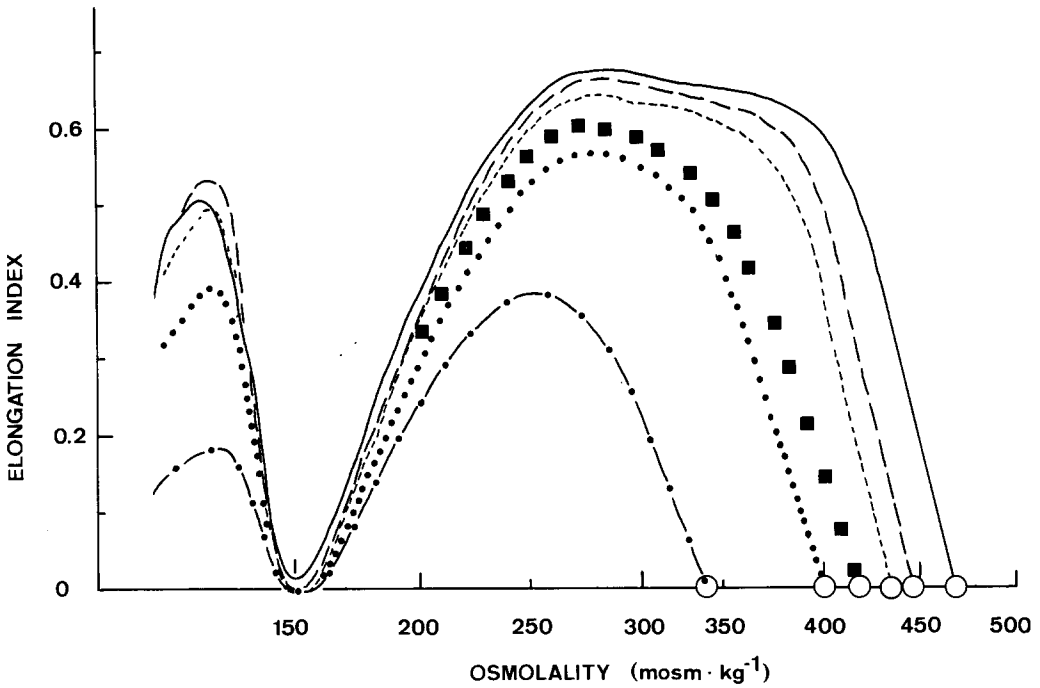


Fig. 4. Osmoscans obtained by ektacytometry from density separated red cells. Equal numbers of washed red cells from fraction 0, 1, 2, 4, 5, and unseparated cells were used for deformability measurements by the osmoscan mode. The shear stress in these experiments was 143 dyn/cm^2. The circles on the abscissa give the osmolality at zero deformability. The following symbols are used: ——— fraction 0; — —, fraction 1; – – – –, fraction 2; ■, unseparated cells; ●, fraction 4; ●–●–●, fraction 5.

almost identical data have been reported for human red cells, density separated on Stractan gradients (0.46 ± 0.03 for fraction No. 1 and 0.65 ± 0.03 for the densest red cells, comprising 1% of the red cell population [44]). The increment of this ratio from density fraction No. 1 to No. 5 suggests a successful enrichment of old cells by density separation according to method A.

In contrast to this, recentrifugation of red cell fractions prepared according to method B showed redistribution of material from high to lower densities and only a small increment in the band 4.1a:4.1b ratio. Since both sets of results were obtained with the same type of density gradient, the relocation of cells in case B were neither due to a high viscosity within the gradient nor due to a technical error in aspiration. The difference was introduced by the pretreatment of red cells.

While removal of white cell contaminations and

TABLE II

Deformability of light and dense human red blood cells [a]

Type of density gradient	Range of shear stress (dyn/cm^2)	No. of Expt.	Ratio of EI_{max} light/dense	Light red cells			Dense red cells		
				EI_{max}	osmolality at EI_{max} (mosmol/kg)	osmolality at $EI = 0$ (mosmol/kg)	EI_{max}	osmolality at EI_{max} (mosmol/kg)	osmolality at $EI = 0$ (mosmol/kg)
Percoll	105–115	4	1.51 ± 0.15	0.65 ± 0.05	⩾ 278 ± 11	449 ± 4	0.43 ± 0.06	242 ± 9	334 ± 10
Percoll	140–150	5	1.65 ± 0.11	0.75 ± 0.10	⩾ 299 ± 12	473 ± 25	0.45 ± 0.08	249 ± 7	336 ± 10
Stractan (calculated from Ref. 11)	170		1.25 1.19	0.69 0.89		450	0.55 0.75		323
'Murphy' (from Ref. 38)	100 250		1.31 1.26	0.55 (*DI*) 0.58 (*DI*)			0.42 (*DI*) 0.46 (*DI*)		

[a] Data from cells separated on Percoll were derived from osmoscans as shown in Fig. 4, those from Stractan gradients were calculated from osmoscans published by Clark et al. [11]. The values for the osmolality at EI_{max} represent the lowest possible value, when curves showed a plateau. 'Murphy' refers to cells separated according to Ref. 7, Deformabilities of these cells were determined by rheoscopic techniques by Pfafferot et al. [38] and are given as deformability indices (*DI*). We calculated the ratio from their Fig. 3.

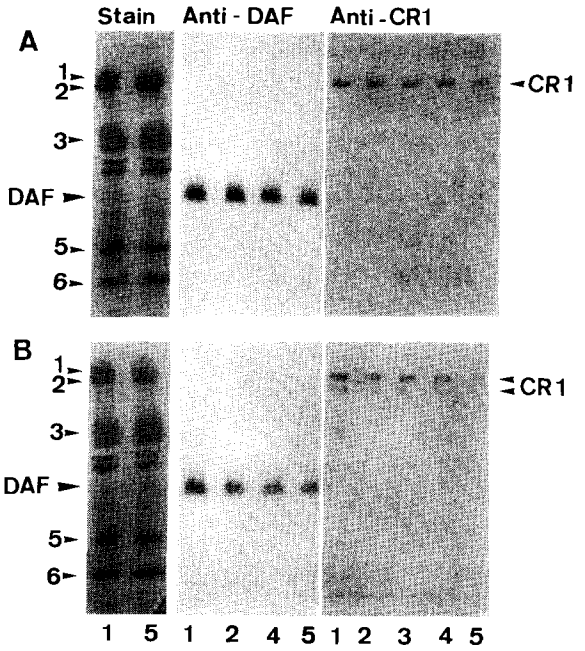


Fig. 5. Binding of ^{125}I-iodinated anti-DAF and anti-CR1 monoclonal antibodies to blotted membrane proteins from density separated red cells. Equal amounts of membrane protein (30μg) from density separated red cell subpopulations from two different donors (A and B) with different allotypes for CR1 were electrophoresed on SDS PAGE in the absence of reducing agent. Electrophoretically spread proteins were blotted onto nitrocellulose and either incubated as indicated or stained. The numbers to the left refer to the red cell membrane polypeptides, those at the bottom to the number of density fractions (see Figs. 1 and 2).

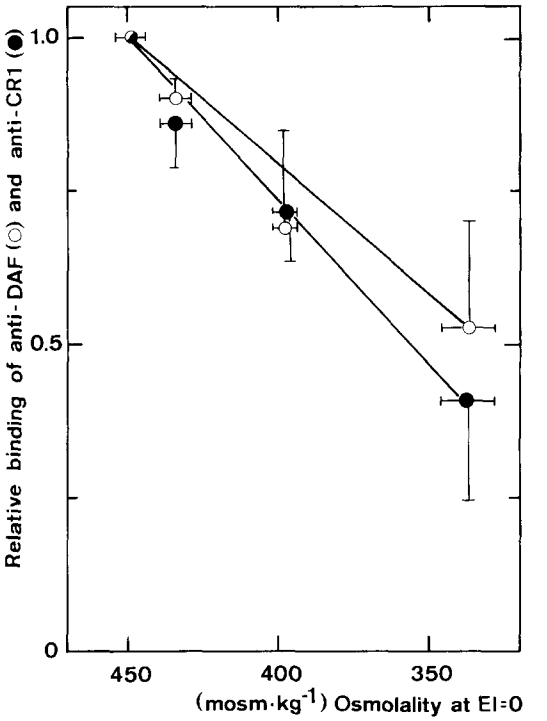


Fig. 6. Binding of anti-CR1 and of anti-DAF antibodies to their corresponding antigen was determined and normalized as outlined. Binding data are plotted against the osmolality at zero deformability of the corresponding red cell subpopulation. Results from three independent experiments with two determinations were averaged and the standard deviations are given with respect to antibody binding and the osmolality value at $EI = 0$.

platelets is most efficient from heparin anti-coagulated blood [28], the subsequent procedures required removal of calcium ions to prevent clumping, coagulation, and complement activation. Both procedures fulfilled the first requirement. In addition, the pretreatment according to method A avoided a major cause of clumping, namely pelleting of red cells until white cell contaminations and calcium were eliminated. Method B, on the other hand, included pelleting prior to these precautions. We think that the latter protocol must have generated red cell aggregates which banded at a high density initially. These aggregates included some cells of lower density at a frequency sufficiently low to leave the overall density of the aggregate like that of the predominant dense cells. Incorporation of light cells in multicellular aggregates of dense cells may have two possible reasons, the notorious stickiness of dense red cells [45] and the conditions chosen in method B. Washing of these dense fractions with buffer B containing 0.5 mM EDTA apparently dissociated these aggregates and gave rise to cellular redistribution. Thus, it is unlikely that the use of DFP in method A and of PMSF in method B caused the difference between results obtained by the two methods. Our earlier fractionation protocol [8] yielded qualitatively similar results as method A, most likely because it was based on blood that was anticoagulated in citrate (CPD-adenine) and included a washing step in buffer B prior to density centrifugation. Thus, method A is the procedure of choice, if blood was collected in heparin or liquemin, since it allowed efficient removal of white cells and avoided clumping before centrifugation. The data demonstrate that density separation permits fractionation according to cell age, provided that a number of precautions are taken.

* Earlier osmotic gradient ektacytometer studies have shown that the position of the hypertonic arm of the deformability profile (see Fig. 4) depends essentially on the intra-cellular viscosity and thus the cellular density [11]. The authors found that the osmolality at which deformability reached a particular value along the descending arm was linearly related to 1 over the mean corpuscular hemoglobin concentration. These authors used as a parameter the osmolality at which *EI* was equal to one-half that of the normal maximum. Our parameter is an extrapolation of the hypertonic arm of the profile (extrapolation starts from half its height) to the base line (the osmolality at zero deformability). It correlated linearly with the density of fractionated red cells (not shown). The main reason for choosing this parameter as a substitute for an averaged density was that the deformability profile of very dense and oxidatively stressed red cells occasionally did not even reach half maximum of the normal value.

The results also show that fractionation of a density gradient does not guarantee that all cells from a particular fraction have a uniform density. Washing in EDTA containing buffer and recentrifugation were required to demonstrate their actual density. Recentrifugation of cells separated according to method B showed redistribution of a small fraction of cells collected as dense. It apparently did not result in redistribution of cells collected as light. This was surprising, since the band 4.1a:4.1b ratio suggested that also light fractions were contaminated by red cells of higher density. A close examination of recentrifuged material from light cells revealed instead a broader distribution of the uppermost bands and R_F values that were 10% higher as in corresponding fractions obtained by method A. Recentrifugation on density gradients has not been used to assess the quality of density separation in any study which applied the protocol of Murphy, where high viscosities within the red cell column further reduce the chances for a given cell to reach its corresponding density.

Red cell creatine and with it practically all red cell enzyme activities show an exponential decay from reticulocytes to mature cells and a shallow decay during aging of mature cells. This and the donor to donor variability of creatine contents make it an unprecise parameter for red cell age, though it is an excellent parameter for reticulocytosis [19]. In contrast to this, maximum deformability is a reliable indicator of cell age, it decreased with increasing density from fraction 1 to 5. In fact, it linearly decreased for cells having densities corresponding to an osmolality at zero deformability of 450 to 320 mosmol/kg (compare changes of creatine contents and deformabilities in Fig. 3). The shear stress (shear rate times viscosity in Poise) used to induce deformability was 107 to 150 dyn/cm^2 in this study. This shear stress is slightly lower than the one recommended for rheoscopic deformability studies by Meiselman's group [38], but within the optimal range for ektacytometry (> 75 dyn/cm^2 [29]). Increasing the shear stress by raising the external viscosity beyond 18 centipoise did not eliminate the difference in the maximum deformability between dense and light red cells, similar to data reported elsewhere (Fig. 3 in Ref. 38). This strongly suggests that deformability of dense red cells is impaired by 30 to 40%.

The relative content of CR1 and of DAF decreased linearly with density within the limits of two standard deviations. While this was known for CR1 from CR1 determinations on intact red cells [42], the loss of DAF has not been described in detail [33]. CR1 and DAF were quantified from bound monoclonal antibodies to electrophoretically spread membrane polypeptides. This approach was used to avoid incomplete detection of antigens as it may have occurred in binding studies on intact erythrocytes. It evidently yielded similar results as determination of CR1 on density separated, intact red cells [42]. These authors also found a steady loss of CR1 with density on cells fractionated on preformed Percoll gradients. Note that their protocol also avoided red cell pelleting before removal of white cells and used EDTA-anticoagulated blood.

The loss of DAF antigens from aging red cells appears somewhat smaller than that of CR1. This was unexpected for a protein that is anchored by phosphatidylinositides [46] for which shedding was considered a likely process. The mechanism by which the two proteins are released or cleaved is yet unclear. None of our immunoblots revealed proteolytic cleavage products that reacted with either one of the monoclonal antibodies. This does not rule out a loss of antigens by proteolysis, since the antibodies recognize an outer portion of the antigens [33,36]. On the other hand, even a loss of up to 60% of CR1 could be explained by vesiculation along with a small portion of the red cell surface, since CR1 is preferably organized in patches [47] and red cells lose a few percent of their membrane [48] in form of vesicles [49,50], primarily from young cells [51,52].

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