Ionic calcium content of light dense human red cells separated by percoll density gradients

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Abstract

In this paper we have compared the adequacy of two methods using Percoll density gradients to separate light and dense erythrocytes from fresh human blood. After measuring the distribution of some classical age-markers such as haemoglobin, potassium and creatine contents, it was found that preformed gradients generated more stringent conditions for age-related density separations. Employing such gradients, the free Ca^{2+} content of above sub-populations was assessed with Fura-2, under conditions where the viscosity effect was abolished. In five experiments, the free Ca^{2+} content (mean value $\pm$ 1 S.D.) was 8.4 $\pm$ 2.82 nM and 31.2 $\pm$ 13.0 nM for the 7–10% lightest and densest cells, respectively. These results are discussed in connection to red cell senescence.

Keywords: Light cell; Dense cell; Free Ca^{2+} content; Red cell sub-population; Percoll gradient; Erythrocyte (Human)

1. Introduction

Early studies using atomic absorption spectrometry have shown a two-fold increase in Ca^{2+} content between light and dense human erythrocytes, separated from fresh blood by centrifugation in Percoll gradients [1]. These authors also reported that the heaviest cells took up more Ca^{2+} after being exposed to relatively high Ca^{2+} levels. As there exists a positive correlation between cell age and density [2,3] these results suggest that the red cell Ca^{2+} rises during ageing in vivo.

On the other hand, it is well known that a decrease of both ATP content [4] and activity of some key glycolytic enzymes [5] occurs upon ageing of human red cells. The decline in concentration of phosphorylated compounds thus arising in senescent cells, would lead to a reduction in their Ca^{2+} chelating potential. The possibility then arises of a progressive increase in ionic cellular Ca^{2+} during ageing in vivo. With the interest of investigating such a possibility, the free Ca^{2+} content of human erythrocytes from fresh blood was studied by Fura-2 fluorescence techniques. The cells were separated into light and dense fractions, employing Percoll density gradients that generated stringent conditions for age-related fractionation.

Abbreviations: EGTA, ethylene glycol-bis(β-aminoethyl ether) N,N,N′,N′-tetraacetic acid ; BSA, bovine serum albumin; Pi, inorganic phosphate; PBS, saline phosphate solution; Hb, haemoglobin; AChase, acetyl cholinesterase; G6P dehydrogenase, glucose-6-phosphate dehydrogenase.

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2. Materials and methods

Analytical quality reagents were purchased from Sigma Chemical Co., USA, and British Drug Houses, UK. Percoll was obtained from both Sigma and Pharmacia (Sweden). Fura 2-AM was from Molecular Probes, USA. A stock solution (1 mM) was made in dimethyl sulfoxide, containing 25% pluronic acid and kept at -70°C for not more than two weeks. The pH of all solutions was adjusted at room temperature within ± 0.02 pH units and osmolality to 310 ± 5 mOsm/kg.

Fresh human blood (mainly 0 + group) from 25- to 40-year-old healthy subjects, with no family history of hypertension or diabetes, was employed. The blood was collected in the presence of heparin and processed within 2 h after collection.

2.1. Gradient preparation

Discontinuous gradients were formed using five layers of a basal solution containing 6% meglumine diatrizoate plus 2 mM Tris-HCl (pH 7.4) and Percoll concentrations to obtain densities from 1.080 to 1.150 g/ml. The densities of the three middle layers were calculated for each donor's blood (see below). The loaded gradient (1:9) was centrifuged at $2700 \times g$ for 30 min at 4°C (Sorvall RC-5B, HB-4 rotor, 0.75 rate control).

Preformed gradients were made in a basal solution containing (mM): sodium diatrizoate; 128, glucose; 10, Pi buffer (pH 7.4), 12; plus 2 mg/ml Ca^{2+}-free BSA. Percoll was added to give densities around 1.080 and 1.120 g/ml, according to limits previously estimated (see below). The gradients were self-formed by centrifuging at $26\,500 \times g$ for 10 min at 4°C (Sorvall RC-5B, SS-34 rotor). Two different gradients were run in parallel to separate 5–10% of light and dense cells, respectively. The self-formed gradients were loaded (1:8) with PBS-diluted blood and centrifuged at $2700 \times g$ for 30 min at 4°C (Sorvall RC-5B, HB-4 rotor, 0.75 rate control).

Density of Percoll solutions was checked at room temperature, both by gravimetry and by measuring the refractive index. An Abbe refractometer was used for the latter purpose. In some cases, twin gradients were run containing density markers beads (Pharmacia).

2.2. Preliminary estimation of cell density

Previous tests showed that the overall red cell density from different donors was variable. A linear Percoll gradient of densities between 1.080 and 1.120 g/ml was routinely employed to assess this parameter for each donor's blood. Accordingly, cells were treated as described in (a) or (b).

(a) Discontinuous gradients. The linear gradient, already formed in a conical-glass centrifuge tube, was loaded (1:200) with whole blood and centrifuged at $2500 \times g$ for 20 min at room temperature. A major band, diffusing towards bottom and top was currently obtained. The mean cell density was calculated from interpolation into adequate calibration curves. The value thus obtained, and ± 0.005 g/ml, was taken as density for the middle layers of the step gradients. This interval, achieved by trial and error, was required to separate about 10% of both the light and dense cells.

(b) Preformed gradients. The linear density gradient was built into a Wintrobe-type haematocrit tube to increase resolution. It was loaded with blood previously diluted with PBS and centrifuged at $2700 \times g$ for 30 min at 4°C (Sorvall RC-SB, HB-4 rotor, 0.75 rate control). The PBS solution contained (mM): NaCl, 148; $K_2HPO_4 + NaH_2PO_4$, 20 (pH 7.4), and 1 mg/ml Ca^{2+}-free BSA added to prevent clumping. Between 10–15 different bands were generally obtained. In most cases, the Hb concentration showed a bell-shape profile along the gradient. Such a distribution allowed a better estimate of the upper and lower density limits referred to above, to separate about 10% of light and dense cells, respectively.

Fractionation of gradients was made with a low-flow rate (8–30 ml/h) peristaltic pump (Manostat Junior Cassette Pump). The first two fractions of light cells was discarded as it was enriched in reticulocytes. Light and dense cells were washed four times with PBS and kept at 4°C for subsequent analyses. Contamination by reticulocytes generally amounted to 0.2–0.5% in the light cells whilst it was nil in the densest fraction.

Reticulocyte counting was assessed on a Neubauer chamber using Brilliant Cresil Blue (1% methanolic solution) as vital stain.

Yield of fractions was calculated on the basis of fractional Hb content.

OxyHb was measured 540 nm in 0.01 N NH_4OH solutions [6].

Potassium was measured by flame emission at 766.4 nm [7].

ATP and G6P dehydrogenase were assayed enzymatically using commercial kits (SIGMA). Creatine was determined according to Eggleton et al. [8].

Measurements of ionic Ca^{2+} were done on light (L) and dense (D) cells separated by preformed gradients. They were left at 4°C in L(D) solutions, supplemented with: inosine (10 mM), penicillin (10^4 units/ml) and streptomycin (400 μg/ml). These solutions contained (in mM): $MgC1_2$, 0.3; Tris-HCl, 20 (pH 7.55) plus either KCl, 90; NaCl, 63 (L solution) or KCl, 72; NaCl, 81 (D solution).

After standing overnight, the cells were washed, resuspended in the corresponding L(D) solution and finally incubated (10^8 cells/ml) with 1 μM Fura 2-AM for 1 h at 37°C. After four washes with L(D) solutions, they were resuspended in fresh washing medium and kept for fluorescence assays.

Fluorescence measurements were done on 10^7 cells/ml at 21°C, using a Spex Fluorolog 1201 spectrofluorimeter, with quantum correction. Intrinsic fluorescence was routinely subtracted. Excitation spectra were scanned between 250–450 nm and emission was recorded at 515 nm. Bandpass slits of 5 and 10 nm were used for excitation and emission, respectively. Fluorescence was ratioed at 361/389. Manganese (2 mM) was present as quencher of external fluorescence [9]. Free Ca^{2+} concentrations were calculated as described by Grynkiewicz et al. [10], using the K_d values determined in the present work.

Fluorescence intensity was calibrated directly on cells, using Ca^{2+}-EGTA mixtures at pH 7.4 [11] and Triton X-100. The detergent was present at 0.05% (lysed cells) and 0.001% (permeabilized cells) for in vitro and in vivo calibrations, respectively.

3. Results

Discontinuous and self-formed Percoll gradients allowed us to separate light and dense red cells with a yield of about 7–10% (Table 1). The densities (in g/ml) varied between 1.092–1.098 and 1.106–1.107 for the light and dense fractions, respectively. Regardless of the gradient employed, these fractions showed highly significant statistical differences ($P < 0.001$) of Hb, K^+ and creatine contents (Table 1). On the other hand, no major differences in Hb and K^+ contents were found when light or dense cells derived from both gradients were compared with themselves. By contrast, the creatine concentration was statistically different, this difference being highly significant ($P < 0.001$) between the light cells of the different gradients. The greatest creatine concentration (about 200 ± 40 μg/ml cells) was found in the light cells obtained from autoformed gradients.

The fractions separated from self-formed gradients were further characterized in terms of some additional age markers such as ATP, G6P dehydrogenase and AChase (Table 3). The ATP concentration and activities of both AChase and G6P dehydrogenase fell within the expected range [12]. A highly significant statistical difference ($P < 0.001$) of these parameters between L and D fractions was obtained. Glucose-6-phosphate dehydrogenase activity was about

Table 1
Composition of light and dense cell fractions separated by discontinuous and self-formed Percoll gradients

	Type of gradient			
	Discontinuous		Self-formed	
Fraction	L	D	L	D
Yield (%)	9 ± 8.2 (3)	8 ± 4.1 (3)	7 ± 3.5 (42)	11 ± 4.0 (37)
Density (g/ml)	1.092 ± 0.0 045 (11) [a]	1.106 ± 0.0074 (12) [c]	1.0980 ± 0.0029 (4) [b]	1.1074 ± 0.00 33 (4) [c]
Hb (mg/ml cells)	409.8 ± 23. 82 (5) [d]	477.0 ± 20.59 (4) [e]	415.7 ± 19.45 (5) [d]	490.2 ± 21.91 (6) [e]
K^+ (μequiv/ g Hb)	269.6 ± 10. 59 (4) [f]	173.4 ± 7.19 (4) [h]	236.6 ± 11.36 (4) [g]	159.9 ± 11.63 (6) [h]
Creatine (μg/g Hb)	195.7 ± 11. 01 (5) [i]	64.2 ± 2.38 (6) [k]	486.6 ± 4.89 (4) [j]	80.6 ± 3.70 (6) [i]

The yield and density of these fractions are also shown. Different letters denote statistical significant differences ($P < 0.01$). Statistical differences between L and D parameters from each gradient were highly significant ($P < 0.001$).

Table 2
Divergence of parametric L/D ratios from self-formed and discontinuous gradients

Cell parameter	Type of gradient		
	Discontinuous (L/D)	Self-formed (L/D)	*P*
Hb	0.859 ± 0.0724 (4)	0.848 ± 0.0647 (5)	> 0.10
K^+	1.555 ± 0.0571 (4)	1.480 ± 0.0872 (4)	> 0.10
AChase	1.526 ± 0.3429 (11)	1.364 ± 0.0304 (4)	> 0.10
G6P dehydrogenase	2.425 ± 0.7433 (3)	2.790 ± 0.5688 (6)	> 0.10
Creatine	3.048 ± 0.0674 (5)	6.037 ± 0.0470 (4)	< 0.0001

The concentrations of Hb, K^+ and creatine as well as the activity of AChase and G6P dehydrogenase, were measured in light (L) and dense (D) cell fractions obtained from self-formed and discontinuous gradients. The L/D ratio of each cell parameter was calculated for the corresponding Percoll gradient, as shown above. *P* denotes the probability derived from Student's *t*-tests.

64 ± 12.5% lower in dense than in light cells from preformed gradients. This decrease was not significantly different from that of 59 ± 2.7% obtained with cells fractionated from discontinuous gradients.

The divergence (ratio spread of light over dense cells contents) of Hb, K^+, AChase and G6P dehydrogenase between self-formed and discontinuous gradients was not statistical significant, whilst that of creatine was highly significant (Table 2).

Fura-2 fluorescence and K_d measurements were considerably affected by altering viscosity. On the one hand, the fluorescence intensity was increased by nearly two orders of magnitude when 0.01% Triton X-100 was added to Fura-2-1oaded cells, that is, when all cells were lysed. Such an increase did not occur when a much lower Triton concentration (0.001%) was used. As under the latter condition, Fura-2 fluorescence spectra changed in the expected way upon addition of Ca^{2+}, we shall consider this condition as one of cell permeabilization.

On the other hand, the K_d values measured in lysed cells were greater than those in permeabilized cells. Thus, in four experiments using the light fraction, a K_d of 142 ± 15.5 and 75 ± 21.5 nM was obtained for lysed and permeabilized cells, respectively. By decreasing the excitation bandpass to 2 nm and increasing to 10 nm that for emission and computing the 361/389 fluorescence ratio, it was found that both in vitro and in vivo Ca^{2+} calibration curves were identical. In addition, the K_d values of the different cell fractions were practically identical under these conditions, being 53 ± 11.2 and 57 ± 9.9 nM in four experiments with light and dense cells, respectively. The above settings were also found adequate to suppress the K_d difference encountered in the presence and absence of 50% sucrose, determined with assays with Fura 2 (penta potassium salt).

Under conditions where the viscosity effect was suppressed, the free Ca^{2+} content of the 7–10% light and dense cells differed statistically with each other, being roughly 8 and 30 nM, respectively (Table 3).

4. Discussion

The present work has studied the adequacy of two methods involving Percoll density gradients for separating light and dense erythrocytes from fresh human blood: discontinuous and autoformed gradients. The distribution of some age-related markers such as Hb,

Table 3
ATP, Ca^{2+} and some enzymatic activities of light and dense cells fractions separated by self-formed Percoll gradients

Cell parameter	Fraction		
	L	D	*P*
ATP (μmoles/g Hb)	2.22 ± 0.202 (7)	1.34 ± 0.292 (7)	< 0.001
AChase (μmoles/min × g Hb)	19.5 ± 0.257 (6)	14.3 ± 0.391 (6)	< 0.001
G6P dehydrogenase (μmoles/min × g Hb)	2.79 ± 0.226 (6)	1.00 ± 0.563 (6)	< 0.001
Free Ca^{2+} (nM)	8.4 ± 2.82 (5)	31.2 ± 13.0 (5)	0.005

K^+ and creatine has been compared in these fractions. Previous autoformed gradients appeared to generate more stringent conditions, as can be inferred from the greatest creatine content difference found in cells fractionated with these gradients. By contrast, Hb or K^+ showed the same distribution within either light or dense cells from either gradient. Although creatine has been considered an excellent reticulocyte marker since these cells are heavily enriched in this compound, there is a marked decrease in creatine concentration upon cell maturation [3,13]. The highest creatine concentration found on light cells from autoformed gradients cannot be attributed to reticulocyte contamination, which was generally lower than 0.5%. Therefore, it seems most likely that autoformed gradients have allowed a more stringent separation of light cells, with closely related density values, not attained with discontinuous gradients.

The main problem encountered when correlating cell separation by density gradients with age, arises from the fact that not all dense cells are old [14]. This point has been addressed recently by Lutz et al. [3], who pointed out that density separation permits reliable fractionation according to cell age, provided that a number of precautions are taken, such as use of heparin as anticoagulant, no Ca^{2+} in the medium and minimal cell stressing conditions.

The cells used in the present work were subjected to minimal stressing conditions, which introduced a minimal perturbation of cell density. In addition, Ca^{2+}-free BSA and Pi were added to both cell suspension and gradient solutions to avoid clumping of cells, thus preventing an altered density pattern. The above statement can also be inferred from the fact that both the content and distribution of some age-related markers used, such as G6P dehydrogenase and ATP content, are well correlated with a distinct alternative separation method, involving differential lectin binding [12,15]. Moreover, G6P dehydrogenase activity was about 64% lower in dense than in light cells. Such a difference is coincident to that found with both the complement receptor 1 and the decay accelerating factor in density separated light and dense human red cells using self-forming Percoll gradients [3]. The latter parameters are modernly considered good markers for red cells ageing.

In agreement with earlier results [9], a marked alteration of Fura-2 K_d values was found by increasing viscosity. However, the viscosity effect could be neglected following Busa approach [16]. Thus, by both decreasing to 2 nm the excitation slit and increasing to 10 nm that for emission and computing the fluorescence ratio 361/389, it was found that in vitro and in vivo Ca^{2+} calibration curves were identical. These findings clearly reveal that the viscosity artifact was abolished under above conditions. Such a control was of paramount importance since the internal viscosity increases as the red cell ages [17].

The human red cell Ca^{2+} is very low. There is no consensus for the free Ca^{2+} concentration in these cells and estimates differ widely with the method employed. Thus, using a non-fluorescent method on a whole population of human red cells, free internal Ca^{2+} was about 30 nM [18]. By contrast, fluorescent methods have reported values on similar populations ranging from 78 nM using Fura-2 [9] to 130 nM ionic Ca^{2+} [19]. The latter was obtained with Fluo-3. In the present paper employing Fura-2 and after correcting for intrinsic red cell fluorescence, we found values (5–30 nM, for light and dense cells respectively) that agreed with those obtained by Lew et al. [18]. If this correction is not done, our values (about 80 and 150 nM for light and dense cells, respectively; results not shown) fall within the range reported by David-Dufilho et al. [9], that is, there is an overestimation of cell-free Ca^{2+}.

Our corrected values are in close agreement with that reported recently for the whole red blood cell population of normotensive human subjects, i.e., 23 $\pm$ 3 nM [20]. By contrast, they are almost one order of magnitude lower than those measured by ^{19}F-NMR spectroscopy on light and dense cells fractions separated by density centrifugation at 37°C [21]. The latter is the only work to our knowledge, that has addressed the subject of determining free Ca^{2+} in subpopulations of human red cells. Although these studies suffer from the drawback of having employed separation protocols likely to alter cell density [3], they showed that the ionic Ca^{2+} content increased in the heaviest cells. Such results were confirmed in the present work.

Taking into account that red cell ageing is well correlated with increasing density [3], the finding of a raised Ca^{2+} content in dense cells, strongly suggests that Ca^{2+} homeostasis alters during senescence. The increased free Ca^{2+} would result in an altered cell

physiology, leading in turn to the prompt splenic sequestration of senescent cells.

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