Improved Isolation of Normal Human Reticulocytes Via Exploitation of Chloride-Dependent Potassium Transport

By Martin P. Sorette, Kathleen Shiffer, and Margaret R. Clark

STUDIES ON human reticulocytes, which constitute approximately 0.5% to 1.5% of red blood cells (RBCs) in normal adult individuals, have been limited by the difficulty of isolating pure populations from normal blood. Since the observation by Key1 in 1921 that reticulocytes are more bouyant than erythrocytes, many studies have used both density-based and cell size-based separation methods to prepare reticulocyte enriched fractions. Centrifugation of peripheral blood on density gradients of bovine serum albumin2 and arabinogalactan3 under isopycnic equilibrium conditions, and sedimentation velocity separations using phthalate esters,4 Ficoll,5 Renografin,6 Percoll,7 combined Percoll-Renografin8 and Percoll-arabinogalactan9 have been used both for erythrocyte separation and reticulocyte enrichment. In some studies, highly purified populations of reticulocytes have been obtained from patients with hemolytic anemia whose whole blood reticulocyte counts may average 15% to 20%. When used to separate normal human blood, however, these methods produced maximal reticulocyte enrichment of only 5% to 20% reticulocytes. Because of the relatively poor resolution of reticulocytes on density gradients, other isolation techniques have been developed to separate reticulocytes from erythrocytes based on their differing surface characteristics. In one study that used differential lectin-mediated agglutination of erythrocytes, a 14-fold enrichment of reticulocytes from a normal blood sample was reported.10 Affinity chromatography on transferrin-sepharose11 and immunomagnetic separation using a monoclonal antibody to the transferrin receptor12 resulted in preparations from normal individuals with reticulocyte counts between 23% to 35% and 98% to 100%, respectively.

Here we describe a rapid, simple method for density separation of normal human blood on discontinuous arabinogalactan gradients that results in selective enrichment of reticulocytes. This method is based on the differential bouyant density of reticulocytes in an isotonic high-potassium (K) medium that approximates the internal cation composition of human RBCs. We hypothesized that chloride (Cl)-dependent K loss from reticulocytes might alter their hydration state during density gradient separation. By suppressing K loss, we obtained highly selected low-density RBC populations from high-K gradients that have reticulocyte counts exceeding those previously reported for other density gradient methods. Subsequent incubation and secondary separation of these fractions in high-sodium (Na) chloride medium yields reticulocyte enrichment comparable to that attained by affinity-isolation techniques.

MATERIALS AND METHODS

Chemicals and reagents. Arabinogalactan (Stractan II) was obtained from the St Regis Paper Co (Tacoma, WA). Ethidium Bromide, EGTA (Ethyleneglycol-bis-(β-amino-ethyl ether) N,N'-Tetra-Acetic Acid), EDTA (ethylenediaminetetraacetic acid), microcrystalline cellulose (Sigma cellulose type 50) a-cellulose, bovine serum albumin, inosine, adenine, glucose and penicillin/streptomycin were purchased from Sigma Chemical Co (St Louis, MO). HEPES (N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid), HEPES Na salt, and PIPES (piperazine-N,N'-bis (2 ethanesulfonic acid) were products of Calbiochem (La Jolla, CA). New Methylene Blue N (Brecher formula) was purchased from J.T. Baker Chemical Co (Phillipsburg, NJ). Reagent grade Na chloride, Na nitrate, K chloride, and magnesium chloride were purchased from Fisher Chemical Co (Fair Lawn, NJ).

Collection of blood samples. After obtaining informed consent under a protocol in accordance with the principles of the Helsinki Declaration and approval by the Human and Environmental
Protection Committee of the University of California, San Francisco, venous blood was collected from healthy normal volunteers into EDTA or acid-citrate-dextrose (ACD) (Vacutainer; Becton Dickinson, Mountain View, CA). Leukocytes and platelets were removed by filtration through a column of cellulose.\textsuperscript{13,14}

**Arabinogalactan density gradients.** Arabinogalactan (Stractan II) was decolorized using magnesium oxide\textsuperscript{15} and activated charcoal and purified according to Corash.\textsuperscript{7} For initial experiments at pH 7.4, stock arabinogalactan solutions were prepared that, based on available water, contained HEPES (20 mmol/L), M\textsubscript{6}Cl\textsubscript{2} (1 mmol/L), Na\textsubscript{2}HPO\textsubscript{4} (1 mmol/L), glucose (10 mmol/L), EGTA (0.5 mmol/L), adenine (2 mmol/L), inosine, (12 mmol/L), penicillin (100 U/mL), streptomycin (100 μg/mL). NaCl gradients contained KCl (10 mmol/L), NaCl (114 mmol/L), and KCl gradients contained NaCl (12 mmol/L), KCI (115 mmol/L) added to the stock solution. The pH was adjusted to 7.4, and the osmolality to 290 mOsm using a vapor pressure osmometer (Model 5100 C; Wescor Inc, Logan, UT). For subsequent experiments, NaNO\textsubscript{3} and NaCl gradients contained PIPES, pH 6.8 (20 mmol/L), glucose (10 mmol/L), adenine (2 mmol/L), inosine (12 mmol/L), ouabain (0.2 mmol/L), piretanide (0.1 mmol/L), and either NaNO\textsubscript{3} (165 mmol/L) or NaCl (150 mmol/L). The pH was adjusted to 6.8 and the osmolality to 290 mOsm. Discontinuous gradients were prepared by dilution of stock Stractan with isosmolar buffers of the same composition, using nine layers ranging in equal steps from 1.083 to 1.124 g/mL density. These were layered in 17 mL tubes onto a cushion of at least 1.133 g/mL density. The gradients were centrifuged in a swinging bucket rotor in an ultracentrifuge (Beckman L3-50) for 45 minutes at 74,000g at 20°C. The separated cells were collected from the gradient interfaces using a Pasteur pipet, during which the walls of the tube were washed with buffer to avoid contamination of subsequent layers. The separated cells were washed three times to remove residual Stractan and were resuspended to a defined volume.

**RBCs and reticulocyte density distribution.** RBC distribution in the density gradient was determined by counting the cells in each gradient population with an electronic cell counter (Model Z\textsubscript{40}; Coulter Electronics, Hialeah, FL). Mean cell hemoglobin concentration (MCHC) was determined from a spectrophotometric measurement of hemoglobin as the cyanomet-hemoglobin complex. Manual reticulocyte counts were made on 1,000 cells/sample stained with New Methylene Blue N.\textsuperscript{16} Reticulocyte counts by flow cytometry were performed on ethidium bromide stained samples\textsuperscript{17} using the Becton Dickinson Facscan flow cytometer (Beckton Dickinson, Mountain View, CA). Gating on the light-scattering signal was used to discriminate RBC from particulate debris. Fluorescence was measured with excitation at 488 nm provided by an argon ion laser and detected through narrow-band emission filters at 530 nm (FL1, Green) or 585 nm (FL2, Red). The fluorescence measurements were standardized using fluorescent microspheres (Calibrite; Becton Dickinson).

**Staining for reticulocytes bearing the transferrin receptor.** A mouse monoclonal anti-human transferrin receptor IgG1 raised against MLA 144 Gibbon leukemic cells (Chemicon International Inc, Temecula, CA) was diluted to 10 μg/mL in phosphate-buffered saline (PBS)/1.0% bovine serum albumin. Fifty microliters of washed cells from density gradient fractions were suspended in 200 μL of antibody and incubated for 1 hour at 37°C. Cells were washed three times in PBS and then incubated for 1 hour at 37°C in 200 μL of a goat antiserum IgG-FITC conjugate diluted to 10 μg/mL in PBS. After washing three times, cells were stained in an equal volume of .002% ethidium bromide for 1 hour at room temperature, placed on ice, and immediately analyzed by flow cytometry.

**Quantitation of protein 4.1 a and b components.** RBC ghosts were prepared from separated samples by hypotonic lysis in 40 volumes of ice-cold 5 mmol/L Tris-HCl, 1 mmol/L EDTA, 1 mmol/L diisopropylfluoro-phosphate, pH 8.0. Membranes were solubilized and proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (7% acrylamide) according to the method of Laemmli.\textsuperscript{18} Proteins were stained with Coomasie Brilliant Blue R-250, and the bands corresponding to 4.1α and 4.1β were cut out and the dye eluted in 25% Pyridine and quantitated spectrophotometrically.\textsuperscript{19}
significantly improved isolation of reticulocytes in the low-density fractions of the KCl gradient when compared with the NaCl gradient. Data indicated. Reticulocytes in each fraction were quantitated by flow cytometric analysis of ethidium bromide stained cells. Note the improved isolation of reticulocytes presented as percent reticulocytes (SEM).

Cellulose filtered whole blood from three subjects was separated on NaCl and KCl gradients as shown in Fig 1. Fractions correspond to the densities indicated. Reticulocytes in each fraction were quantitated by flow cytometric analysis of ethidium bromide stained cells. Note the significantly improved isolation of reticulocytes in the low-density fractions of the KCl gradient when compared with the NaCl gradient. Data presented as percent reticulocytes (SEM).

Hemoglobin A1c measurements. The relative amount of hemoglobin A1c in each gradient fraction was measured by ion exchange high-performance liquid chromatography (DIAMAT Fully Automated Hemoglobin Analyzer System; Bio Rad, Hercules, CA).

RESULTS

We hypothesized that the CI-dependent K loss from reticulocytes might alter their density during density gradient centrifugation, resulting in less than optimal reticulocyte separation under conditions most commonly used. In an effort to enhance reticulocyte separation, we tested the effect of various factors that influence the activity of Cl-dependent transport. Activation of the transport by reduced pH, and suppression of the transport by reducing the transmembrane K gradient and substituting NO3 for Cl were investigated.

Effect of gradient composition on RBC and reticulocyte distribution. Comparison of the density distribution of total RBCs on high-K and high-Na gradients showed that the major difference was an increase in the proportion of cells in the two lowest density layers of the high-K gradient (Fig 1). When the reticulocyte distributions in gradient populations were determined, it was found that these two least dense populations from the high-K gradient were markedly enriched in reticulocytes (Table 1). In contrast, reticulocytes had a much broader distribution on high-Na gradients and did not show such remarkable enrichment in the lowest-density populations.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Density (g/mL)</th>
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<td>2</td>
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<td>3</td>
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<td>8</td>
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<tr>
<td>9</td>
<td>1.1148</td>
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<tr>
<td>10</td>
<td>1.1195</td>
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If the presence of higher-density reticulocytes on high-Na gradients were caused by Cl-dependent K loss via the KCl cotransport pathway, it should be accentuated by reducing the pH of the NaCl medium to 6.8, the pH optimum for activation of this pathway in cells containing HbC. As expected, incubation and separation of the cells in high-Na medium at pH 6.8 did result in even greater increase and broadening of reticulocyte density (data not shown).

Effect of transient exposure to low pH in ACD anticoagulant. Having found that separation in high-Na medium at low pH resulted in reticulocyte dehydration, we asked whether transient exposure to low pH of blood samples drawn into ACD anticoagulant would affect reticulocyte distribution during subsequent density separation on high-K gradients at pH 7.4. Parallel samples were drawn into ACD (final pH 6.4 to 6.7) and EDTA and were then immediately separated on KCl gradients at pH 7.4. A perceptible shift to higher density was observed in the ACD sample, and the percentages of reticulocytes in the lowest-density layers were reduced (data not shown). When the ACD was adjusted to pH 7.4 before adding the blood, the subsequent density and reticulocyte distribution was identical to that of the EDTA sample. This indicated that it was exposure to low pH, not the effect of an ACD component, that influenced the density distribution. All of the data presented in this report were obtained using samples drawn into EDTA.
Table 2. Reticulocyte Distribution

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<tbody>
<tr>
<td>A</td>
<td>13.0</td>
<td>4.4</td>
<td>3.7</td>
<td>6.5</td>
<td>28.4</td>
<td>64.8</td>
<td>89.9</td>
<td>89.4</td>
<td>99.3</td>
</tr>
<tr>
<td>B</td>
<td>7.1</td>
<td>1.05</td>
<td>2.0</td>
<td>4.1</td>
<td>10.4</td>
<td>33.1</td>
<td>71.3</td>
<td>87.7</td>
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Reticulocyte distribution in cells from one subject that were isolated from low-density fractions of a KCl gradient, incubated in high-NaCl medium, pH 6.8, and resuspended on NaCl gradients. Layers A and B denote the lowest and next lowest density fractions from the original KCl gradient separation. Layers 1 to 9 represent populations recovered from the secondary NaCl gradients. Data presented are percent reticulocytes quantitated by manual counts of 1,000 New Methylene Blue stained cells from each fraction.

Chloride dependence of reticulocyte dehydration in high-Na medium. To further verify that the improved separation of reticulocytes on high-K gradients was caused by suppression of Cl-dependent K loss, the effect of Cl substitution by NO₃ was tested (Fig 2). When low-density cells obtained from a high-K gradient were incubated and recentrifuged on a NaN0₃ gradient at pH 6.8, they returned to the same density as those resuspended on a high-K gradient at pH 7.4. In contrast, a parallel sample resuspended on a NaCl gradient, pH 6.8, yielded a markedly broadened density distribution attributable to cellular dehydration. Quantitation of reticulocytes in each of the density fractions showed that it was the reticulocytes that became dehydrated, thus concentrating in the most dense fractions of the NaCl gradient (Table 2).

Reticulocyte maturation and KCl cotransport activity. Next, we asked whether the level of KCl cotransport activity was correlated with two indices of reticulocyte maturation: RNA content and expression of the transferrin receptor. To address this question, a reticulocyte enriched fraction from the high-K gradient was resuspended on the transferrin receptor. To address this question, a reticulocyte enriched fraction from the high-K gradient was resuspended on the transferrin receptor. To address this question, a reticulocyte enriched fraction from the high-K gradient was resuspended on the transferrin receptor. To address this question, a reticulocyte enriched fraction from the high-K gradient was resuspended on the transferrin receptor. To address this question, a reticulocyte enriched fraction from the high-K gradient was resuspended on the transferrin receptor.
IMPROVED ISOLATION OF RETICULOCYTES

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DISCUSSION

Several density-based or rate-sedimentation techniques for separating reticulocytes from erythrocytes, and for separating erythrocytes into populations of differing mean cell age, have been developed.29 Our study differs from

these in showing that highly enriched populations of reticulocytes can be obtained from normal blood in a one-step density separation on gradients containing a high-K concentration to approximate the internal cation composition of RBC. This method results in isolated populations of up to 80% reticulocytes, a much greater enrichment of normal reticulocytes than previously achieved using high-Na medium or other density-based methods.25 For even greater reticulocyte enrichment, low-density populations isolated on KCl gradients are incubated and recentrifuged in NaCl medium, which promotes selective increase in density of the youngest reticulocytes. The most dense fractions from the secondary separation are almost all reticulocytes. Although affinity based techniques also yield highly enriched reticulocyte populations, they only select fibronectin or transferrin receptor-bearing reticulocytes, and recovery of the cells after isolation in an undamaged state is problematic.

We have shown that the improved isolation of reticulocytes in high-K medium is attributable to the suppression of KCl loss and cell dehydration via the K-Cl cotransporter, which is active in reticulocytes but is suppressed upon erythrocyte maturation.26 It should be noted that even if high-K gradients are used, reticulocyte separation is less effective if the blood is drawn into ACD anticoagulant. This is caused by transient activation of KCl cotransport resulting in irreversible dehydration of some reticulocytes. This mechanism of pH effects on RBC-density-distribution differs from that discussed by Walter et al.27 They found that separation at low pH resulted in an increase in volume and a decrease in the density of mature rat RBC. Although this would cause increased overlap in density between reticulocytes and erythrocytes, the swelling of mature cells would be largely reversible with return to pH 7.4, whereas the KCl cotransport mediated dehydration of reticulocytes would not.

The distribution of putative markers for aging of mature RBCs HbA1c and protein 4.1a was consistent with the reticulocyte distribution, showing low levels of both markers in reticulocyte-enriched cell populations. There was a progressive increase in both markers with increasing cell density, but this tended to plateau in the last few most dense populations. This suggests that although there may be a general increase in average cell age with increasing density of layers from the KCl gradients, the very highest density cells may not represent a highly enriched population of the oldest cells.

These studies show a convenient, highly effective approach for selective separation of reticulocytes from mature erythrocytes in normal blood samples via exploitation of their Cl-dependent K transport function. We anticipate that its capacity to provide improved recovery of reticulocytes in free suspension will facilitate investigation of a wide variety of fundamental biological questions.

ACKNOWLEDGMENT

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Fig 4. (A) The relative proportion of hemoglobin A1c increased with increasing mean cell density in high-K gradients. The low proportion of HbA1c in the highly selected reticulocyte-enriched fractions is consistent with a young mean cell age. (n = 4, mean ± SEM). (B) Quantitation of protein 4.1a and 4.1b from the same gradient populations shows a progressive increase in the 4.1a component though the lowest-density fractions in the gradient. There was no consistent further increase of the 4.1a component in the highest-density populations. (N = 4, mean ± SEM).

fractions throughout the high-K gradients, we assayed two properties that have been proposed to reflect aging of mature RBCs: the percentage of Hb in the A1c form, and the relative proportions of the a and b components of protein 4.1. As has been previously shown for other density gradient separations,23,24 the relative proportion of hemoglobin in the A1c fraction increased with increasing mean cell density (Fig 4A). Quantitation of protein 4.1a and b performed on the same blood samples showed that there was a significant increase in the 4.1a component through the least dense 10% of the cell distribution, but this difference was less pronounced throughout the rest of the density separated population. Neither HbA1c nor the 4.1a component was particularly increased in the highest-density populations (Fig 4B).
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