The Isolation of Reticulocyte-Free Human Red Blood Cells

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We depleted reticulocytes from erythrocytes of both sickle cell disease (SCD) subjects and healthy controls by four methods: fluorescence-activated cell sorting (FACS), Miltenyi immunomagnetic depletion (MACS), a combination of these methods (FACS + MACS) and Percoll density separation. The efficiency of these methods was assessed by new methylene blue staining and manual enumeration of the reticulocytes. FACS sorted erythrocytes from reticulocytes based on size and granularity, as well as the absence of dsDNA staining. MACS depleted reticulocytes from erythrocytes based on the immunoaffinity to CD36 and CD71. Reticulocytes from healthy controls were depleted to ≤0.1% using either the FACS or MACS method (α = 0.1). Reticulocytes from SCD subjects were depleted from 13.6% ± 0.52% to 5.45% ± 0.33% using MACS (n = 2), and from 10.9% ± 0.47% to 2.0% ± 0.2% using FACS (n = 4, α = 0.05). When combining FACS with MACS (n=3), the percentage of reticulocytes was decreased in SCD samples from 13.0% ± 0.51% down to 1.5% ± 0.17% (α = 0.1). Sedimentation through 75% percoll resulted in control and SCD samples being reduced from 0.27%±0.6 (control) and 6.93%±0.8 (SCD) reticulocytes to < 4.8 reticulocytes per million control RBCs and <2.5 per million SCD RBCs. This same method results in <2.1 leukocytes per million control RBCs and <3.7 per million SCD RBCs. We conclude that the percoll density method described here is the most effective method for isolating RBCs for proteomic analysis. Exp Biol Med 232:1470–1476, 2007

Key words: red blood cells; reticulocytes; leukocytes; proteomics

Introduction

Proteomic studies require pure populations of cells because of the exquisite sensitivity of modern mass spectrometers. A minireview covering mass spectrometry and proteomics of the human red blood cell (RBC) can be found in this issue of **Experimental Biology and Medicine**[1]. This review discusses the importance of eliminating reticulocyte and white blood cell contamination in the preparation of proteomic quality RBCs. Human blood contains ~ 5 x 10^6 RBCs per μl. Erythroid preparations contain 0.6 to 2.7 % reticulocytes when isolated from control blood (AA) (1) but is increased to as high as 30% reticulocytes when isolated from subjects that have homozygous (SS) sickle cell disease (2, 3). Several studies have looked at the composition of the human erythrocyte proteome (4–8), one has compared the protein profile of SS versus AA RBC membranes by two dimensional difference gel electrophoresis (2D DIGE) and tandem mass spectrometry (9) and one has looked at the SS versus AA membrane skeleton by ICAT technology coupled to tandem mass spectrometry (10). All of these studies utilizing RBC preparations attempted to diminish any white blood cell contamination, but only one addressed the issue of reticulocyte contamination. The one study that attempted to deplete reticulocytes did so by incubating an RBC preparation at 4°C for 72 hrs (8). This treatment itself can cause profound proteomic changes that may be exacerbated when comparing blood samples from control RBCs versus RBCs from a subject with SCD or other hemolytic anemias. Therefore, the purpose of the current study was to find an optimal technique for isolating reticulocyte free RBC preparations for downstream proteomic studies. These same preparations will be optimal for classical biochemical and physiological studies on RBCs.

Various techniques have been utilized for isolating reticulocytes and we felt that these would be instructive for isolating reticulocyte free RBCs. The original approach was to separate reticulocytes from RBCs by centrifugation (11, 12), based on density differences, leading to the use of

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[1] This invited minireview for **Experimental Biology and Medicine**, by Goodman et al, is entitled "The Human Red Blood Cell Proteome and Interactome". It is currently under review.

Percoll, Ficoll-Percoll, or Ficoll Hypaque gradients for this purpose (13–15). These techniques led to fairly pure reticulocyte preparations but yields of 14–36% (14). This approach was followed by magnetic bead separations that utilized the fact that CD71 is found on reticulocytes but not mature RBCs (16, 17). The purity of these preparations was high, but the yield was again 15–42% (16). Fluorescent dyes and flow cytometry have been used for the enumeration and analysis of stages of reticulocyte maturity (2, 3), but have not been previously utilized for preparation of reticulocyte free RBCs. In the current study we compare new magnetic bead, fluorescence activated cell sorting (FACS), and density based techniques to optimize the isolation of reticulocyte free RBCs. To our knowledge, this is the first attempt to compare and contrast techniques for the purpose of obtaining proteomic quality RBC preparations.

Methods

Preparation of the Red Blood Cell Fraction. Blood was collected from healthy controls (AA) or from SCD patients (SS) in 10 ml Becton Dickinson Vacutainer tubes containing either lithium heparin or potassium EDTA as an anticoagulant. For the experiments involving flow cytometry or immunomagnetic depletion, blood was stored at 4°C for approximately 16 to 24 hrs prior to commencement of any manipulation. To separate the plasma from the cells, the blood was centrifuged (550xg, 20 min, 25°C). A majority of the plasma was removed and 6 ml of phosphate buffered saline solution (153 mM NaCl, 5.64 mM sodium phosphate dibasic, 1.03 mM potassium phosphate monobasic; pH 7.4) was added and combined with the cell fraction. To separate the white blood cells (WBCs) from the erythroid cells, we performed the following common protocol. In a 15 ml polypropylene conical tube, 7 ml of diluted blood was carefully layered on 3 ml of Ficoll-Paque (1.077 density). The gradient was immediately centrifuged in a swinging bucket rotor (400xg, 30 min, 25°C). The plasma and WBC fraction was removed, and the volume was brought to the original value with PBS.

In the experiments involving percoll density separation of cells, the collected blood samples were kept at 4°C and used within 8 hours as described below.

Flow Cytometry Staining and Sorting. Briefly, the cells were diluted to $5x10^6$ cells per ml with RPMI supplemented with 10% fetal bovine serum (RPMI/10% FBS). For staining purposes, one ml aliquots of cells were distributed into polypropylene tubes; to these tubes, 2 µl of Vybrant DyeCycle Green Stain (Molecular Probes) was added. The tubes were wrapped in parafilm, and subsequently in foil, and then incubated in a 37°C water bath for 30 min. Following the incubation, the cells were sedimented (800xg, 15 min, 25°C). The staining buffer was decanted and the cells were resuspended at approximately 10^7 cells per ml in RPMI/10% FBS for cell sorting.

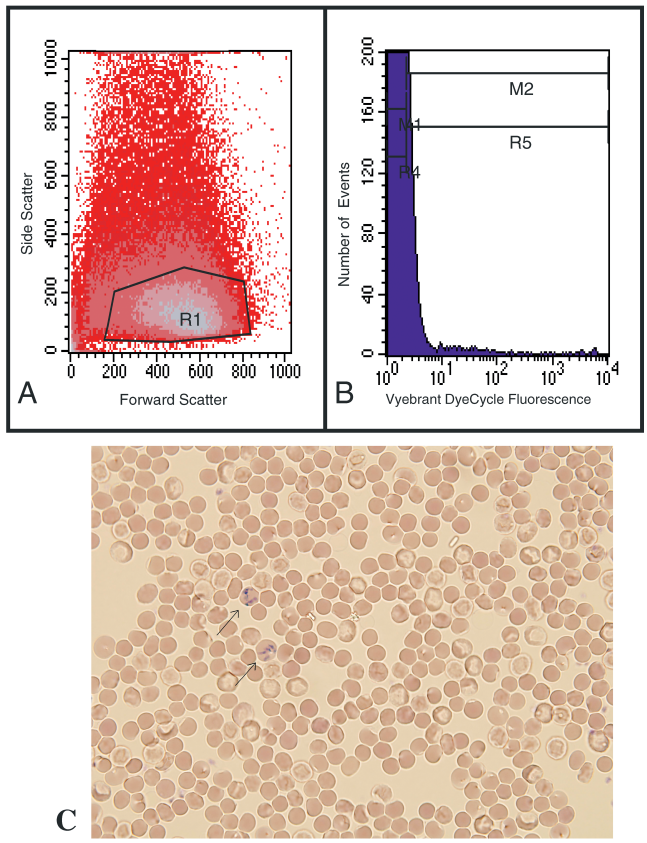

Figure 1. Gating parameters for FACS sorting of erythrocytes from reticulocytes. Panel A. RBCs are gated (R1) based on size and granularity characteristics, as measured by forward scatter and side scatter, respectively. Panel B. Cells from R1 are evaluated for the presence of dsDNA as indicated by staining with Vyebrant DyeCyle. Cells present in the R5 gate were considered positive for dsDNA staining, whereas cells in the R4 gate were considered negative. Cells were collected by the FACSCalibur if they were within both the R1 and R4 gate. Panel C: Reticulocytes stained with new methylene blue (Reticulocyte Stain, Sigma), prior to depletion, are identified with arrows. The blue clusters are reticulum, or clusters of ribosomes and RNA precipitated by the cationic dye.

Flow cytometry sorting was performed on a Becton Dickinson FACSCalibur. As shown in Figure 1A, the desired population was first gated based on size and granularity. This gated population was then assessed and gated for fluorescence. Gated cells which were not fluorescent were captured and assessed for maturity. The sorting was done under exclusion mode, which captures multiple cells at one time as long as a contaminating cell is not present in the collection vicinity. In addition, the cells were sorted at a total event rate below 2000 events per second in an effort to not overwhelm the capabilities of the flow cytometer.

Immunomagnetic Depletion. Diluted blood was run over a Miltenyi Pre-Separation Filter to remove any clumps. For staining purposes, aliquots of $5x10^6$ cells were distributed into 4.5 ml polypropylene tubes. Cells were washed by adding approximately 3.5 ml of FACS buffer (PBS without calcium or magnesium, 2% FBS, 0.1% sodium azide) and centrifuged (800xg, 10 min, 4°C). The

cells subsequently were blocked in 20 μl of human antibody serum (20 minutes, 25°C). PE-conjugated antibodies against CD71 and CD36 (BD Biosciences) were combined in a 1:1 ratio; 10 μl of antibody cocktail was subsequently added to the blocked cells. Following an incubation (5 min, 25°C), the cells were washed with FACS buffer. All stained cells were combined and resuspended in 0.5 ml of MiniMACS Buffer (PBS pH7.4, 0.5% BSA, 2mM EDTA) for immunomagnetic depletion according to the manufacturer's directions. Briefly, the cells were washed in 4 ml of MiniMACS Buffer and resuspended in 80 μl of MiniMACS Buffer per 10^7 cells. Twenty μl of Anti-PE Microbeads (Miltenyi Biotech Inc.) per 10^7 cells were added to the cells. The Microbeads and cells were incubated for 15 min at 4°C protected from light. The remaining beads were then washed away by adding 2 ml of MiniMACS Buffer and centrifuging (800xg, 10 min, 4°C). The cell pellet was resuspended in 500 μl of MiniMACS Buffer for loading onto a MS MACS Column (Miltenyi Biotech Inc.). Prior to addition of the cell suspension, the MS MACS Column was prepared exactly according to the manufacturer's directions. Once the cell suspension was loaded, the column was washed with three aliquots of 500 μl of MiniMACS Buffer. The total effluent was collected; this fraction contained the reticulocyte-depleted erythrocytes.

Percoll Density Separation. The collected blood samples were kept at 4°C and used within 8 hours. All following procedures were carried out at room temperature. Before separation, the blood cells were washed three times in PBS containing 11.9 mM phosphate pH 7.4, 137 mM sodium chloride, and 2.7 mM potassium chloride: the cells were sedimented at 500xg for 10 min and the pelleted cells were resuspended in 10 volumes of PBS. The washed blood cells were finally resuspended in PBS at 50% hematocrit and used in the cell separation experiments.

Percoll (GE Healthcare, ρ=1.130±0.005 g/ml) was diluted in PBS to an appropriate concentration by addition of 10xPBS (Fisher Scientific) and water. The blood cell density distribution was analyzed in percoll following the manufacturer's protocol: 7.0 ml of sample (the washed blood cells resuspended in PBS at 50% hematocrit) or density markers (GE Healthcare) suspended in PBS were loaded onto a preformed continuous percoll gradient (30 ml) and centrifuged in parallel at 1000xg for 15 min in a bucket rotor; a continuous percoll gradient was preformed by centrifugation of 30 ml of 64% percoll at 20,000 x g for 20 min in a fixed angle rotor. The density zone where red cells were distributed was localized. The percoll concentrations (75–80%) with density values within the selected density zone were tested for cell separation. The washed blood cells resuspended in PBS at 50% hematocrit (~5 ml) were loaded onto a single layer of 75, 76, 77, 78, 79, or 80% percoll of equal volume and centrifuged at 1000xg for 15 min in 15 ml centrifugal tubes in a bucket rotor. Before centrifugation, an aliquot of washed blood cells was saved to determine the reticulocyte count. The cells pelleted through the percoll layer were washed in PBS, and analyzed for reticulocyte count. The reticulocyte count before and after cell separation was determined in parallel using new methylene blue staining as described below.

New Methylene Blue Staining of Reticulocytes and Leukocytes. The depletion efficiency was determined by enumerating reticulocytes following staining with new methylene blue according to the manufacturer's directions. Briefly, 30 μl of blood (or blood cells in PBS at 50% hematocrit) was mixed with 20 μl of Reticulocyte Stain (Sigma-Aldrich). After a brief incubation (10 min, 25°C), 10 μl of stained blood was diluted with 50 μl of RPMI/10% FBS. Five μl was pipetted onto a slide and covered with a coverslip for enumeration. A total of 1,000 cells were counted and presence of reticulum, as shown in Figure 1 Panel C, noted.

After reticulocyte depletion via flow cytometry or immunomagnetic techniques, the cells recovered were pelleted and resuspended in less than 100 μl of RPMI/10% FBS. Fifteen μl of cells was mixed with 10 μl of Reticulocyte Stain, incubated for 10 minutes, and then directly pipetted onto a slide for enumeration. A total of 5,000 erythroid cells were counted and maturation noted.

In the case of reticulocyte depletion via percoll density technique, 30 μl of the sedimented red cells was incubated with 20 μl of stain for 10 min. Ten μl of the incubated mixture was diluted 36 fold with RPMI/10% FBS. Ten μl of the diluted sample was pipetted onto a slide for reticulocyte or leukocyte enumeration. Due to the low counts, all cells pipetted onto a slide were counted.

Statistics. To determine if the reticulocyte count differed before and after the separation schemas, a non-parametric test was chosen. The rationale was two-fold; reticulocytes are considered rare events in the RBC population, and for enumeration purposes, cells are randomly distributed on a slide following a Poisson distribution. Therefore the data sets were analyzed using the nonparametric two-tailed Mann-Whitney U Test. Prior to this test, the percentage data was transformed into arc sin due to the limit of variance on percentages (0–100).

Results

Flow Cytometry. The flow cytometer was first considered and utilized due to its power to sort high numbers of cells at an efficient rate. Although there are many intracellular differences between the reticulocyte and erythrocyte, the use of cells post-sort dictates that the cells cannot be identified with traditional IC protein staining because of the fixation requirement. Therefore, removing reticulocytes from the erythrocyte population needed to be conducted via an intracellular stain which did not require fixation or cell surface staining.

Our attempt at reticulocyte depletion utilized a membrane permeable double stranded DNA (dsDNA) stain available from Molecular Probes. The rationale was based on the fact that reticulocytes still retain their mitochondria,

but these organelles are removed as reticulocytes mature into erythrocytes. This theoretical background prompted us to stain the RBC population for the presence of dsDNA. The stain utilized fluoresces only after binding to its target. Preliminary studies with membrane permeable RNA stains yielded less satisfactory reticulocyte depletion (data not shown), so we focused our attention on the dsDNA stain.

The RBC population was sorted using a FACSCalibur instrument. Gating the erythroid population based on size and granularity was straightforward (Figure 1 Panel A), but the gating based on fluorescence was not clearly defined due to the absence of peaks. When visualizing the fluorescence, as shown in Figure 1 Panel B there was a large peak with little to no fluorescence and a scattering of very low levels of higher fluorescence. Knowing the percentage of reticulocytes in the sample, the level of positive fluorescence was taken as the percentage of reticulocytes multiplied times 1.5. For example, if there were 5% reticulocytes in a population, then the positive fluorescence gate would capture 7.5% to 10.0% of the total population. When setting this type of gate the boundary of the gate would slice into the base of the large peak of erythroid cells and continue through the scattered low levels of fluorescence. The exception to this fluorescence gating was with control populations where 1.0% to 5.0% of the population would be selected as "positive". To verify that we were selecting out reticulocytes, we captured the "positively stained" erythroid cells. When counting over 5000 control population cells, we found the sample was enriched over 50% with reticulocytes (data not shown). The enrichment would not be expected to be greater than 50% because the positive cells were not a unique peak but, as described in Methods, were within a population of cells skewed to the right of the unstained population (i.e. greater fluorescence).

As shown in Figure 2, selecting out positively stained cells by FACS was statistically successful for both the control and patient populations ($\alpha = 0.05$ and 0.1, respectively). Furthermore, the reticulocytes from the control samples (n = 3; mean, 0.83% ± 0.13%; range, 0.5% to 1.1%) were depleted to less than 0.1% (mean, 0.07% ± 0.04%; range, 0% to 1.0%; $\alpha = 0.1$). However, depleting the patient samples proved more difficult. We saw a statistically significant reduction in reticulocytes in the patient samples (n = 4; mean, 10.9% ± 0.47%; range, 7.8% to 15.6%), but the total number of reticulocytes remaining averaged 2.0% ± 0.2% (range 0.3% to 3.9%; $\alpha = 0.05$). The remaining reticulocytes may be late stage where they are depleted of mitochondria, but still containing some reticulum.

Magnetic Bead Isolation. A second approach for depleting reticulocytes, from a RBC preparation, is by immunostaining identification and subsequent selection by immunomagnetic strategies. Two cell surface proteins which are present on reticulocytes are the transferrin receptor (CD71) and platelet glycoprotein IV (CD36). Van Schravendijk et al demonstrated very low levels of CD36

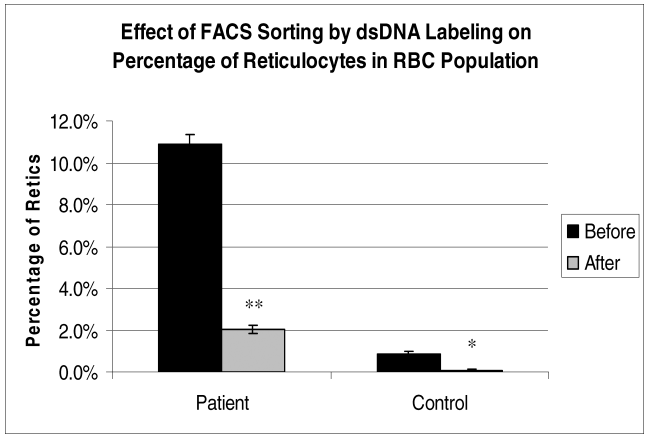

Figure 2. Effect of FACS sorting on the percentage of reticulocytes in RBC populations from SCD subjects and healthy controls as determined by new methylene blue staining. Cells were sorted based on size and granularity, as well as negative dsDNA staining patterns. In the SS samples (n=4), the reticulocytes were reduced from 10.9% ± 0.47% down to 2.0% ± 0.2%. In the control samples (n=3), the reticulocytes were reduced from 0.83% ± 0.13% down to 0.07% ± 0.04%. The reduction in the number of reticulocytes from the SS and AA control samples were significant according to the Mann-Whitney U test.
* $\alpha = 0.1$, ** $\alpha = 0.05$

"close to the limit of detection" in mature erythrocytes using monoclonal antibodies against CD36 and FACS analysis and suggested "that the number of CD36 molecules per cell is below 500." (18). Joneckis et al demonstrated the presence of 17,500 CD36 copies per positive reticulocyte in SS blood samples (2) and the positive cells are in large part immature stress reticulocytes as demonstrated by Browne and Hebbel (3). The large difference in reported copy number between stress reticulocytes and mature RBCs made CD36 a reasonable marker for the magnetic bead technique where a goal was to diminish reticulocytes from SS blood (2, 3). In addition, approximately 30% of all reticulocytes positive for RNA staining are also positive for CD71 (16, 17) which is not found on mature RBCs. In an effort to maximize depletion, antibodies against both proteins were used concurrently for immunodepletion.

We chose to use the PE-conjugated Microbeads platform. In this platform, we used antibodies against CD36 and CD71 which were conjugated to PE. Since the PE molecule has 16 antigenic sites for binding the anti-PE Microbead, the signal from the original antigen is amplified. Thus increasing the probability that cells expressing antigens in lower quantities will have enough Microbeads attached to be attracted to the magnet and separated from the milieu.

As shown in Figure 3, the reticulocytes were removed from the control sample (n = 3; mean, 0.8% ± 0.13%; range, 0.7% to 0.91%) decreasing to ~0.1% (mean, 0.11% ± 0.05%; range, 0.08% to 1.5%; $\alpha = 0.1$). However, this technique performed worse than the flow cytometry on SS samples (n = 2; mean, 13.6% ± 0.52%) which were depleted down to an average of 5.45% ± 0.33% (range, 4.6% to

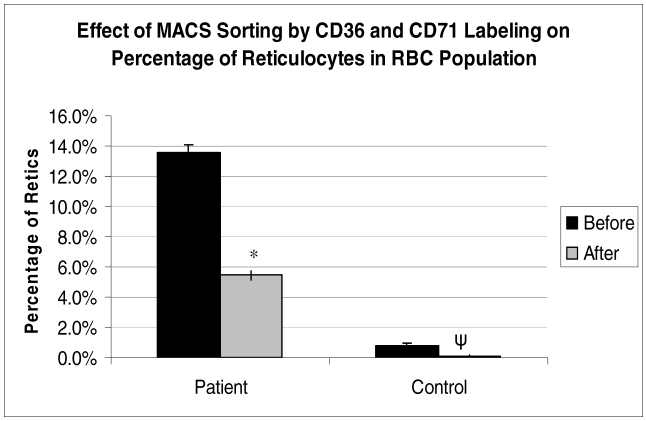

Figure 3. Effect of MACS sorting on the percentage of reticulocytes in RBC populations from SCD subjects and healthy controls as determined by new methylene blue staining. Reticulocytes were eliminated based on the presence of CD36 and CD71. In the SS samples (n=2), the reticulocytes were reduced from 13.6% ± 0.52% down to 5.45% ± 0.33%. In the control samples (n=3), the reticulocytes were reduced from 0.8% ± 0.13% down to 0.11% ± 0.05%. The reduction in the number of reticulocytes from control samples was significant according to the Mann-Whitney U test. The reduction in the number of reticulocytes from patient samples was significant according to the Student's t test.
* $\alpha = 0.1$, $^{\Psi}$ $p < 0.001$

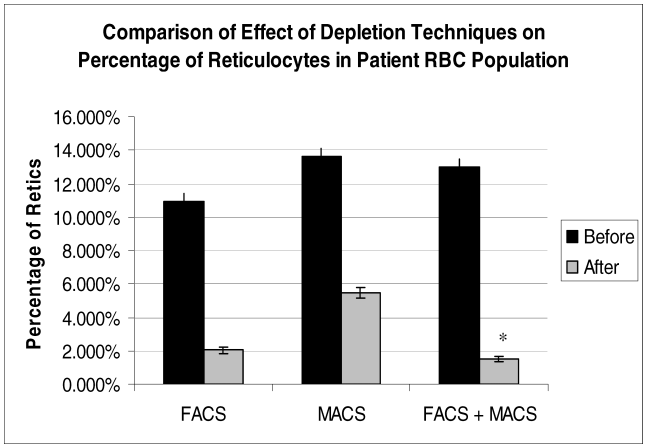

Figure 4. Comparison of the effect of FACS, MACS, and FACS plus MACS combined on the percentage of reticulocytes in RBC populations from SCD blood as determined by new methylene blue staining. Cells were sorted based on size and granularity, as well as negative dsDNA staining patterns. In the SS samples (n=4) subjected to FACS, the reticulocytes were reduced from 10.9% ± 0.47% down to 2.0% ± 0.2%. In the SS samples (n=2) subjected to MACS, the reticulocytes were reduced from 13.6% ± 0.52% down to 5.45% ± 0.33%. When the SS samples were subjected to MACS then FACS, the reticulocytes were reduced from 13.0% ± 0.51% down to 1.5% ± 0.17%. The number of reticulocytes remaining after combining MACS and FACS methods was significantly different than the number remaining after either of the methods individually according to the Mann-Whitney U test.
* $\alpha = 0.1$

6.3%). Note, the Mann-Whitney U test requires at least 3 experiments and only 2 experiments were conducted for patient samples with this technique. However, this depletion was significant according to the parametric Student's t-Test ($p<0.001$). The inability to deplete reticulocytes further could have been due to the presence of reticulocytes lacking both CD71 and CD36 and/or insufficient antibody to remove all of the stress reticulocytes.

Two Prong Approach: Immunomagnetic Depletion and Flow Cytometry Sorting. To determine if combining these two approaches would allow us to bring SS samples down to below 0.1% reticulocytes, we performed flow cytometry sorting on immunomagnetically depleted samples. We did not perform this technique on control samples given the fact that the individual methods brought these reticulocyte counts down to ≤0.1%. We were able to deplete the reticulocytes from the SS samples (n = 3; mean, 13.0% ± 0.51%; range, 7.6% to 16.2%) down to 1.5% ± 0.17% (range, 0.7% to 2.9%; alpha = 0.1). As shown in Figure 4 this combined depletion method was statistically superior to either method when working with SS samples (alpha = 0.1), but was not sufficient for proteomic studies where our goal was to deplete to ≤ 0.1%.

A Density Based Method. The FACS method and the magnetic bead immunodepletion method were both able to lower reticulocytes within the AA control RBC samples to ≤0.1%. However, applied to the blood samples with higher reticulocyte count derived from SCD patients, the two techniques individually or coupled together were not capable of depleting reticulocytes to these levels. We therefore tried to optimize a density based technique, with the goal of rapid isolation of large numbers of RBCs that were reticulocyte free.

A Percoll based density separation technique was used to deplete reticulocytes from the RBC population. The technique exploits the difference in floating densities of different cell types. We looked for a Percoll concentration that would allow most RBCs, but not reticulocytes, to be pelleted through a single isopycnic percoll layer with a resulting reticulocyte count of 0.1% or less. For that purpose we analyzed the cell density distribution in blood samples, using a continuous percoll gradient, and localized the density zone where control red cells were distributed. The percoll concentrations (75–80%) with density values within the selected density zone were tested for cell separation. The blood cells loaded onto a single layer of 75, 76, 77, 78, 79, or 80% percoll were sedimented and the RBC population pelleted through the percoll layer were analyzed for reticulocyte count. Having determined the reticulocyte count before the cell separation, the reticulocyte depletion was assessed. Based on the reticulocyte depletion results, we selected 75% percoll as an optimal density medium allowing few reticulocytes, but maximal number of RBCs, to be sedimented through percoll layer (data not shown).

Seventy five percent percoll (refractive index n^{25}_{D} 1.3490±0.0001, corresponding to 1.1021±0.0004 g/cm^3 density) was used in further reticulocyte depletion experiments with control and SCD patient blood samples. Three control samples and three samples derived from SCD patients were analyzed. Each SCD patient sample was

Table 1. Effect of Percoll Density Based Depletion on the Reticulocyte Count in RBC Populations

	Reticulocyte content in RBC population	
Blood samples	Before depletion %	After depletion ppm
AA control 1	0.2	<2.5
AA control 2	0.3	<2.0
AA control 3	0.3	<4.8
SS patient 1	6.0	<1.9
	6.0	<1.7
SS patient 2	7.3	<1.3
	7.3	<1.4
SS patient 3	7.5	<2.5
	7.5	<1.8

Table 2. Effect of Percoll Density Based Depletion on the Leukocyte Count in RBC Populations

	Leukocyte content in RBC population	
Blood samples	Before depletion %	After depletion ppm
AA control 4	0.27	<1.8
AA control 5	0.50	<1.8
	0.50	<2.1
SS patient 4	0.83	<3.6
	0.83	<3.7
SS patient 5	0.70	<2.6
	0.70	<3.5

analyzed twice. In these nine experiments the reticulocyte count was determined both before and after reticulocyte depletion. The results are presented in the Table 1. As Table 1 demonstrates, the reticulocytes were efficiently depleted from both the control samples (with relatively low initial reticulocyte count) and the patient samples (with higher initial reticulocyte count). In both cases, the reticulocyte count was decreased to less than 4.8 per million control RBCs (ppm) and less than 2.5 ppm SCD RBCs.

In addition to reticulocytes, leukocyte depletion was also determined in RBC populations sedimented through the 75% percoll layer. Leukocyte counts were determined before and after cell separations, on two control samples and two samples derived from SCD patients. All but one sample were analyzed in duplicate. As Table 2 demonstrates, the leukocytes were efficiently depleted from both the control and the patient samples. The leukocyte count was decreased to less than 2.1 ppm RBCs in control samples and less than 3.7 ppm RBCs in the Sickle Cell Disease samples.

Discussion

We have tested FACS, MACS, a combination of FACS and MACS, and a refined percoll density separation technique for the depletion of reticulocytes from RBC preparations. All of the techniques can lower reticulocytes from control RBCs to $\leq$0.1%. However, only the percoll density approach allowed us to lower reticulocytes to fewer than five in a million RBCs derived from SCD blood samples. Thus, the reticulocyte depletion method based on percoll density separation technique proved to be highly efficient. The method is essentially a one step procedure decreasing the reticulocyte count in the red cell population to less than 5 ppm even in the samples with high initial reticulocyte count. The method is fast, inexpensive, and allows treatment of several milliliters of blood sample at once with high yield of purified erythrocytes. While the focus of this study was on reticulocyte depletion, we also measured the number of white blood cells remaining after percoll sedimentation. The level was less than 2.1 leukocytes per million control RBCs and less than 3.7 leukocytes per million SS RBCs Therefore, RBCs isolated by our technique are proteomic quality.

While all of the approaches defined in our study will have value in specialized circumstances, for the reasons described above, we find the percoll density approach to be of the greatest value for future proteomic studies. Because we were able to lower reticulocytes to less than 4.8 and 2.5 ppm in control and sickle cell disease blood samples respectively, this indicates that this method will be optimal for proteomic protein profiling studies where control and high reticulocyte containing hematologic disease samples are being compared.

The authors thank James Jackson who enumerated the number of reticulocytes in RBC preparations, by new methylene blue staining, in several of our early studies.

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