Characteristics of Red Blood Cell Populations Fractionated With a Combination of Counterflow Centrifugation and Percoll Separation


Red blood cell (RBC) fractions were studied after separation of whole blood by means of counterflow centrifugation, Percoll column (Pharmacia, Uppsala, Sweden), and a combination of both separation techniques. Mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), mean corpuscular hemoglobin (MCH), and hemoglobin A1c (HbA1c) were measured in each fraction. From the results it was obvious that the combination of both techniques was the best separation technique of these three.

In order to study characteristics of red blood cell (RBC) senescence, it is necessary to isolate RBC populations of different mean cell age. Most of the investigations have been performed with erythrocytes separated on the basis of differences in cell density. Plain centrifugation, angle-head centrifugation, and the use of several discontinuous gradients, for instance albumin and Stractan, resulted in a more or less efficient separation. Recently the use of Percoll has proven to be an efficient technique. However, it has been suggested that density is not a good criterion of RBC age. Separation on the basis of differences in RBC volume using counterflow centrifugation produces a better separation.

A major drawback in the analysis of RBCs separated on the basis of differences in physical properties is the fact that most separation techniques yield RBC fractions with a small number of RBCs in every fraction. The introduction of flowcytometric methods for the measurement of cell volume (MCV) and hemoglobin concentration (MCHC) has enabled us to analyze these small fractions. The fact that there was no reliable marker of cell age has also hampered many studies. The discovery by Bunn et al that hemoglobin is slowly glycosylated during the RBC life span has led to the use of HbA1c as a marker of cell age. Small amounts of RBCs can be analyzed in that respect by using the high performance liquid chromatography (HPLC) technique.

We describe the results of experiments using these techniques in RBC fractions acquired by a combination of counterflow centrifugation and Percoll separation.

Materials and Methods

Venous blood samples of 20 mL from five healthy volunteers were collected in heparinized tubes (50 U/mL blood).

RBC fractionation according to cell volume was performed using counterflow centrifugation in a Beckmann Model J2-21 centrifuge equipped with the JE-6B system and rotor for elutriation (Beckmann Instruments, Palo Alto, CA). In the elutriation chamber cells are subjected to the centrifugal force of centrifugation and to a centrifugal force, due to the buffer flow toward the center of the rotor. At a given speed of rotation both forces are in balance at a lower flow rate of the buffer for small cells then for large cells. So small cells are carried away with the buffer at a lower flow rate.

The separation was performed at 2,000 rpm and at 20°C. An isotonic buffer containing albumin and glucose (GASP) was used. The composition of the GASP-buffer was: 9 mmol/L Na,HPO,, 1.3 mmol/L NaH,PO,, 140 mmol/L NaCl, 5.5 mmol/L glucose, and 0.8 g/L bovine serum albumin (fraction V; Sigma Chemical Co, St Louis, MO), pH 7.4. Blood was diluted 1:10 with GASP-buffer and 2 mL of this suspension was introduced into the buffer flow to the elutriation chamber. RBCs with increasing volume were eluted from the elutriation chamber at six different flow rates, stepwise increased from 6 to 12 mL/min yielding six fractions.

RBC fractionation according to density was performed by means of a discontinuous Percoll gradient essentially as described by Rennie et al. The gradient was built up in five layers of 2 mL containing 60% (1.096 g/mL), 30% (1.083 g/mL), 40% (1.080 g/mL), 50% (1.075 g/mL), and 70% (1.070 g/mL) Percoll, respectively. Blood was layered on a 7 mL of the SAH-buffer and the elution was started after 15 minutes of centrifugation at 3,000 rpm at room temperature. Four RBC fractions were collected by careful pipetting and extensively rinsed with GASP-buffer to remove any Percoll left.

Separation of RBCs was performed by means of counterflow centrifugation, separation on a Percoll gradient, or a combination of both methods in which the six fractions obtained after elutriation were subsequently separated on the Percoll gradient, yielding 24 fractions.

The RBC parameters MCV and MCHC were determined on a Technicon H1 (Bayer-Technicon, Tarrytown, NY). The MCV is a direct measurement. The H1 determines the MCHC in two ways; a conventional method and a direct measurement, determined by the sideway scatter of the flowcytometric signal (CHCM). We used this CHCM, converted to mmol/L. We found an excellent correlation between the CHCM and the manual determination of the MCHC.

The percentage of HbA1c was measured by means of ion exchange HPLC. An ion exchange column, specially selected for
CHARACTERISTICS OF RED BLOOD CELL POPULATIONS

Table 1. Results of the Measurements of HbA1c

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The counterflow centrifugation fractions are shown on the x-axis (fractions 1-6), the percoll fractions on the y-axis (fractions A-D). Results are the results of one typical individual. Results of HbA1c measurements. The HbA1c of whole blood was 4.37%.

The separation of hemoglobin components, was supplied by BIORAD ("HbA1c"; BIORAD Laboratories, Richmond, CA). The equipment for gradient HPLC was obtained from Waters (Division of Millipore, Milford, MA). The elution buffers used were based on a buffer system as described by Jeppsson et al.16 The composition of buffer A was: 20 mmol/L malonic acid and 1 mmol/L NaCN, pH 5.9 in HPLC-grade water. Buffer B had the same composition as buffer A with the addition of 380 mmol/L NaNO3. Sodium cyanide was used as a stabilizer of hemoglobin. Before use the buffers were filtered through a 0.22-μm filter and degassed. The absorbance of the eluent was monitored at 417 nm. The column was held at 30°C.

The hemoglobin components were separated using a simple linear gradient program from 15% buffer B to 26% buffer B in 5 minutes. The column was regenerated during 20 minutes with 15% buffer B.

Statistical analysis was performed with Student's t-test for paired observations. P < .05 was considered significant.

RESULTS

RBC parameters after separation. Counterflow centrifugation yielded six fractions of RBC, Percoll separation four,

Table 2. Results of the Measurements of MCV, MCHC, and MCH

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MCV of whole blood was 87.70 fL

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MCHC of whole blood was 21.31 mmol/L

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<td>2.02</td>
<td>1.96</td>
<td>1.90</td>
<td>1.78</td>
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</table>

MCH of whole blood was 1.87 x 10^11 amol/L

Counterflow centrifugation fractions are shown on the x-axis (fractions 1-6), the Percoll fractions on the y-axis (fractions A-D). Results are the results of one typical individual.

and the combination of both methods 24. Each fraction was analyzed in the H-1 and the HbA1c was determined.

The results of the HbA1c measurements are depicted in Table 1. There was an increase in the counterflow centrifugation fractions from left to right (large cells had a lower HbA1c than small cells). The increase in HbA1c in the
Percoll fractions was only 1.12%, compared with 1.80% in the counterflow centrifugation fractions. The combination produced the lowest and highest values. The lowest value of HbA1c after the combination was 2.32%, far lower than by counterflow centrifugation (3.96%) or Percoll (3.91%). The same applied to the highest value (8.14% vs 5.76% and 5.03%).

The results of the MCV measurements are shown in Table 2. All three techniques, including the Percoll, resulted in a distinct separation of MCV. The combination technique was superior, resulting in an MCV difference of 29.9 fl, in comparison with the Percoll (10.2 fl) and the counterflow centrifugation (15.5 fl). The results of the MCHC measurements (Table 2) show, as expected, an increase in the Percoll fractions, but there was a slight decrease in the counterflow centrifugation fractions. The combination of both techniques showed that the MCHC values in the horizontal rows were equal, but increased in the vertical columns (Table 2). Again, the difference between the lowest and the highest MCHC was larger after
the combination, compared with counterflow centrifugation. The MCH increased in the Percoll fractions, but decreased from left to right in the counterflow centrifugation fractions (Table 2). In the combination there was a clear decrease from left to right, and a slight increase from top to bottom.

The volume-hemoglobin plots of the H-1 confirmed the superiority of the combination. The fraction with younger cells was really distinct from the fractions enriched with older cells, in contrast to either Percoll separation or counterflow centrifugation alone (Fig 1), in which considerable overlap was present. These findings were further strengthened by the analysis of the RBC distribution width and hemoglobin distribution width (Fig 2).

The correlation between RBC parameters and HbA1c. The relationship between MCV, MCHC, and MCH with HbA1c in one individual for each separation technique is depicted in Figs 3 through 5. Assuming that HbA1c is a reliable marker of RBC age it can be seen that there is a highly significant relation between MCV and RBC age.
This relationship was found in all three separation techniques. In Fig 6 the relationship between HbA1c and MCV is shown for the means of five individuals. These results confirmed the good correlation between MCV and RBC age.

With regard to the MCHC the results are somewhat different. In the Percoll separation there was, as expected, a large increase in MCHC, but only a small increase in HbA1c. Even more surprising was the observation that we found a slight decrease in MCHC in the counterflow centrifugation fractions with increasing HbA1c. The combination showed a clear correlation between these two parameters.

The MCH decreased in the counterflow centrifugation fractions with an increasing HbA1c, while it increased in the Percoll fractions.

The results of the means of five individuals showed identical relationships (data not shown).

DISCUSSION

The results of these investigations show that the method chosen to study the relationship between RBC parameters
and RBC age has a profound influence on the results that are obtained. Historically, most investigations have been performed with a separation technique based on differences in cellular density. Our results show that it is possible to obtain fractions with a widely differing MCHC and a differing MCV with this method, but with only a small increase in HbA1c. Furthermore, there was an unexplained increase in MCH in the Percoll fractions. It is impossible that the hemoglobin content of RBCs increases during cell life. These results concur with the doubts that others have expressed about the validity of separation according to density.18 The results of the HbA1c measurements after counterflow centrifugation confirm the findings of Van der Vegt,9 which found that volume separation is superior compared with density separation. These results are not in agreement with Vaysse et al,17 who concluded that counterflow centrifugation is a very questionable procedure for isolating age-defined RBCs. Some of the discrepancies between their study and ours can probably be explained by the very inferior separation according to density that they obtained by plain centrifugation. The finding that there was no change in MCHC after counterflow centrifugation was somewhat surprising since Van der Vegt has shown previously that fractions isolated at the slowest pump speed were
enriched with old cells. The combination of both methods yielded the best separation in all measured parameters (MCV, MCHC, MCH, and HbA1c). Furthermore, this was the only technique that completely separated fractions with younger cell from fractions with older cells. It has been demonstrated previously with the use of 59Fe that the specific activity is at first high in the fractions with a high MCV and low MCHC. After 40 days the specific activity increases in the fractions with a low MCV, and high MCHC and a high HbA1c.10

In each separation there was a close correlation between the RBC mean volume (MCV) and RBC age (HbA1c). The relationship between RBC hemoglobin concentration, (= RBC density, MCHC), hemoglobin content (MCH), and RBC age yielded different results in the three separation techniques.

Assuming that the combination is superior, it can be concluded that during the life of the RBC, there is a steady decrease in volume and hemoglobin content and an increase in hemoglobin concentration. This leads to the conclusion that during the life of the RBC there is a steadily ongoing loss of water and hemoglobin, in which the loss of water is proportionally larger than the loss of hemoglobin. However, we must keep in mind that although we have achieved a superior separation technique, we are still dealing with probably very heterogenous fractions. The differences in MCV and MCHC in the most extreme fractions in the separations (Tables 1 and 2) are large and it can be seen in the RBC distribution width and in the hemoglobin distribution width that there is no overlap in these fractions. However, in the individual measurements we found in all fractions an RBC distribution width of ±10%, and a hemoglobin distribution width of ±6 mmol/l. Because our separation is performed on the basis of differences in physical properties of the RBC and there is no evidence that all RBCs of a given cell age have identical MCV and MCHC, the variation in cell age in every fraction is probably much larger.

REFERENCES

Characteristics of red blood cell populations fractionated with a combination of counterflow centrifugation and Percoll separation

FH Bosch, JM Werre, B Roerdinkholder-Stoezewinder, TH Huls, FL Willekens and MR Halie