A New Method for Isolation of Reticulocytes: Positive Selection of Human Reticulocytes by Immunomagnetic Separation

By Atle Brun, Gustav Gaudernack, and Sverre Sandberg

A method for isolating pure reticulocytes from leukocyte-depleted blood of normal persons is presented. The separation was achieved using an immunomagnetic technique. A monoclonal mouse antibody against human transferrin receptor was bound to magnetic beads conjugated with sheep antimouse antibody. The recovery of reticulocytes from peripheral blood was 15% to 42%. Blood used for isolation of reticulocytes could be stored for 4 days at 22°C without altering the yield of reticulocytes. At 37°C incubation, the reticulocytes matured rapidly and the transferrin receptor was found to have a half-life of 16 hours. The activity of several enzymes and the amount of creatine and hemoglobin A_{2} were measured both in the reticulocytes and peripheral blood. Of the enzymes, porphobilinogen deaminase had the best discriminatory power with a ratio of 8.8 between reticulocytes and peripheral red blood cells. The ratio for creatine was 16.7. The ability to isolate pure human reticulocytes, released after normal erythropoiesis, will offer new possibilities in the study of these cells.

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STUDIES OF HUMAN reticulocytes have been limited by difficulties of separating pure reticulocytes from whole blood. The proportion of reticulocytes in patients with extreme hemolysis rarely exceeds 40% and is more often 15% to 20%. However, in animals a reticulocytosis of 80% or more can be produced by means of phenylhydrazine administration. Therefore, animal red blood cells (RBCs) have often been used in the study of reticulocytes.

Human RBCs increase in density as they mature. This density change has provided the basis for various centrifugation techniques designed to separate a RBC population into fractions of differing mean age. Whereas differential centrifugation was one of the first reticulocyte enrichment procedures reported, more recent techniques use centrifugation with different density gradients. When different reticulocyte enrichment procedures were evaluated, a method with Percoll-Renografin density gradient was found to be the most efficient. However, the relationship between RBC density and cell age has been disputed, and significant proportions of reticulocytes and young cells have been found among cells in the most dense fraction, which should contain the oldest erythrocytes. According to Beutler, undue reliance has in most studies been placed on cell density as a marker of RBC age. It seems, therefore, that the isolation of reticulocytes based on differences in cell density is far from ideal.

The reticulocyte exhibits a large number of transferrin receptors that disappear during maturation. Among the RBCs in peripheral blood, the transferrin receptor appears to be a reticulocyte-specific surface protein. Here we describe a method for separation of human reticulocytes from peripheral blood, based on this difference in membrane proteins. A monoclonal antibody (MoAb) against human transferrin receptor was bound to magnetic polymer particles and used for a rapid, direct isolation of the reticulocytes.

MATERIALS AND METHODS

Chemicals and reagents. Immunomagnetic beads (Dynabeads M-450) coated with affinity purified sheep antimouse immunoglobulin G (IgG) was purchased from Dynal (Oslo, Norway). MoAb ITI-2B4 was directed against transferrin receptor (CD71) and was produced after immunization of a BALB/c mouse with a human T-cell clone (G. Gaudernack, unpublished results, September 1990).

The antibody is of the IgG 2b subclass, and recognizes an epitope on the transferrin receptor closely related to that recognized by the MoAb OKT-9. MoAb OKT-9 was prepared from the cell line OKT-9 obtained from American Type Culture Collection (Rockville, MD). MoAb GG 384 is directed against N-terminal proatrial natriuretic factor (G. Gaudernack, unpublished results, September 1990).

α-Cellulose, microcrystalline cellulose (SigmoidCell Type 50), bovine serum albumin (BSA), dithiothreitol and Hepes (N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid) were obtained from Sigma (St Louis, MO). Porphobilinogen and uroporphyrin were purchased from Porphyrin Products (Logan, UT). Creatine monohydrate, 1-Naphthol, and Diacetyl were from Merck (Darmstadt, FRG). Pyruvate kinase and aspartate aminotransferase enzyme kits were products of Boehringer Mannheim GmbH (Mannheim, FRG).

Preparation of beads coated with transferrin receptor antibody. Purified MoAb antibody (ITI-2B4) against human transferrin receptor (1 mg/mL) was added to immunomagnetic beads (30 mg/mL) coated with sheep antimouse IgG. The coupling was usually performed by mixing 3 mL beads and 45 μL antibody, and the mixture was incubated overnight at 4°C using end-over-end rotation. After incubation, the suspending medium was removed and the beads were washed thoroughly with buffer. Coupled particles were stored as a suspension of 30 mg/mL at 4°C and could be used after several weeks of storage without noticeable loss of binding capacity. Stored beads were washed once before use. For coupling of the antibody OKT-9 to beads, a supernatant from OKT-9 hybridoma culture was used and the amount of antibody was 5 μg/mg beads in the incubation mixture.

Collection of blood samples. Venous blood was obtained from normal adults. Heparin was used as anticoagulant and samples were used within a few hours unless otherwise stated. Reticulocyte counts were performed by counting 1,000 cells on films of RBCs stained with brilliant cresyl blue.

In vitro maturation and storage of reticulocytes. Five milliliters of venous blood from normal adults was drawn in culture bottles.
containing 45 mL blood culture medium (Vacutainer, Ord no. 4954; Becton Dickinson, Rutherford, NJ). The blood, diluted 1:10 in medium, was incubated at 37°C. For storage studies at 4°C and 22°C, 1.2 mL of a CPD-Adenin solution (Fenwal, Traeval Laboratories Ltd, Thetford, England) was added to 8 mL of heparinized blood under sterile conditions.

**Removal of leukocytes.** To avoid interference with monocytes or other leukocytes expressing transferrin receptors, these cells were removed from whole blood essentially as described by Beutler et al.12,13 About 30 mL of whole blood was put on a 1.5- to 2.0-cm high cellulose column (a-cellulose and microcrystallin cellulose) made in a Büchner funnel (diameter 10 cm). The erythrocytes were washed three times and resuspended in buffer (154 mmol/L NaCl, 1 g/L BSA, and 10 mmol/L Hepes, pH 7.4) at a particle concentration of $4.0 \times 10^{12}$ cells/L, unless otherwise stated. The efficacy of white blood cell removal exceeded 99%. It has been shown that cellulose filtering does not affect the age distribution of the RBCs.14

**Isolation of reticulocytes.** Anti-transferrin receptor antibody (ITL-2B4) coupled to beads (see above) was added to leukocyte-depleted, washed erythrocytes. Beads and RBCs were incubated at 22°C with gentle mixing to allow binding of cells with transferrin receptors to the beads. After incubation, the tube was placed in a holder with a cobalt-samarium magnet mounted along the side of the tube (Magnetic Particle Concentrator; Dynal). With occasional, gentle mixing, beads and cells bound to beads were allowed to settle in the magnetic field. The erythrocytes not bound to beads were then discarded while beads with attached reticulocytes were withheld. The tube was rinsed twice with buffer. The magnets were then removed from the tube, and the beads and reticulocytes were resuspended before a second magnetic isolation was performed. The beads and isolated cells were finally suspended in 0.75 to 1.5 mL buffer. Before measurements of enzyme activities and creatine, the cells were quickly freeze-thawed twice (dry-ice and alcohol) and beads were removed by centrifugation. Hemoglobin was measured spectrophotometrically at 540 nm.

**Scanning electron microscopy.** Isolated reticulocytes were collected on a nucloepore filter and fixed in 2% phosphate-buffered glutaraldehyde. The filters with the cells were dehydrated in increasing concentrations of alcohol. Acetone was used as transitional fluid and dehydration was completed with critical point drying (Polaron Equipment Ltd, Watford, England). A Polaron sputter coater E5000 (Polaron) was used to coat the filters with gold. The filters with the cells were dehydrated in increasing concentrations of alcohol. Acetone was used as transitional fluid and dehydration was completed with critical point drying (Eindhoven, The Netherlands) at an accelerating voltage of 25 kV.

**Determination of creatine.** The determination of creatine was performed by a manual procedure essentially as described by Griffiths,27 and slightly modified by Syllm-Rapoport et al.28 To 1 mL of lysed reticulocytes, or 1 mL of a 1:6 dilution of whole blood, was added 1 mL of Ba(OH)$_2$ · 8H$_2$O and 1 mL of ZnSO$_4$. After precipitation for 10 minutes, the mixture was centrifuged (800g, 10 minutes). Two milliliters of the supernatant was mixed with 0.5 mL freshly made 1-naphthol and 0.25 mL diacetyl reagents, and the optical density at 520 nm was read after 20 minutes. Creatine monohydrate was used as standard.

**Enzyme measurements.** The activity of pyruvate kinase and aspartate aminotransferase was determined using reagent kits from Boehringer Mannheim. Porphobilinogen deaminase (uroorphyrinogen I synthase) was determined fluorometrically with porphobilinogen as substrate, as described by Ford et al.24

**Hemoglobin $A_2$ measurements.** The amount of hemoglobin $A_2$ was measured by ion-exchange high performance liquid chromatography (DIAMAT Fully Automated Hemoglobin Analyzer System; Bio-Rad, Richmond, CA).

**RESULTS**

Reticulocytes were isolated from peripheral blood of normal persons (reticulocyte count: 0.2% to 2.0%) by positive selection with immunomagnetic beads. Microscopy of isolated cells stained with brilliant cresyl blue (Fig 1A) showed that 98% to 100% had blue granules typical of reticulocytes. A scanning electron microscopy picture of isolated reticulocytes is shown in Fig 1B. Similar results were found using the commercially available antibody OKT-9. In a control experiment using a different MoAb (GG 384), no RBCs were isolated.

The amount of purified MoAb (in the incubation mixture) for optimal coating of beads was found to be 0.5 μg/mg beads (data not shown) in several experiments. A higher amount of antibody per beads did not increase the efficiency of reticulocyte binding.

The yield of reticulocytes as a function of beads per cell ratio is shown in Fig 2. A large interindividual variation in the maximum reticulocyte yield was observed. The reticulocyte yield at the highest bead/RBC ratio ranged from 15% to 42% if it was expressed as percent recovery from the source. As shown in Fig 2, the yield could not be increased further by increasing the beads/cell ratio beyond a certain level. The variation in reticulocyte yield and the imprecision of the method at two different concentrations of beads are shown in Table 1.

The immunomagnetic beads bound relatively rapidly to the reticulocytes, but incubation for 60 to 90 minutes was necessary to reach a maximum. This optimal incubation time was dependent on the beads/cell ratio used (Fig 3). The concentration of RBCs in the solution during incubation also affected the isolation process. A more dilute cell suspension needed longer incubation time to reach the maximum level of reticulocyte yield. Keeping a constant incubation time (60 minutes) and a constant number of cells in the source, an increasing suspending volume gave a decreasing reticulocyte yield (Fig 4).

Storage of blood at room temperature (22°C) for 4 days had minimal effect on the yield of reticulocytes. However, storing the blood at 4°C increased the reticulocyte yield after 4 days about 40% (Fig 5). In these experiments the hemolysis was less than 2%. To simulate the in vivo maturation of reticulocytes, blood was stored at 37°C in blood culture medium. Under these conditions the amount of reticulocytes that could be isolated decreased with time and was reduced to approximately 50% after 16 hours of incubation (Fig 6). The hemolysis was less than 1%.

Three enzymes (pyruvate kinase, aspartate aminotransferase [GOT], and porphobilinogen deaminase [uroorphyrinogen I synthase]), which have increased activity in reticulocytes,29 and two RBC constituents (creatinine and hemoglobin $A_2$) were measured in the erythrocytes (peripheral blood source) and in the isolated reticulocytes (Table 2). The reticulocyte to peripheral RBC ratio, which indicates the discriminatory power of the parameter, is also given. The enzyme activities were determined within a few hours after isolation of the cells and about 4 to 5 hours after the
Fig 1. Light microscopy (A) and scanning electron microscopy (B) of reticulocytes isolated with immunomagnetic beads. Photomicrographs of isolated cells attached to immunomagnetic beads (see Materials and Methods). The cells for light microscopy were stained with brilliant cresyl blue. The diameter of the beads are 4.5 μm.

Fig 2. Effects of the number of beads per cell on the yield of reticulocytes. Reticulocytes were isolated from 1 mL peripheral blood from four normal persons. The ratio of beads per RBC was increased from $0.32 \times 10^{-3}$ to $19.2 \times 10^{-4}$, i.e., approximately 0.02 to 2.0 beads per reticulocyte. The yield of reticulocytes is expressed as the amount of hemoglobin in the isolated cell suspension. The number in parentheses represent the percentage of the reticulocytes isolated from the source (percentage of recovery) at highest bead/RBC ratio.

Blood was drawn. Of the parameters examined (Table 2), creatine gave the best discrimination, while porphobilinogen deaminase was the enzyme with the highest discriminatory power. Hemoglobin Aₙ had a ratio of $0.11 \pm 0.02$, which gives an inverse ratio of $8.9 \pm 1.2$.

DISCUSSION

The present work shows that it is possible to isolate a 98% to 100% pure reticulocyte suspension in a single step from leukocyte-depleted peripheral blood of normal persons. The isolation method is based on the fact that transferrin receptors are still present on the cell surface of reticulocytes but have disappeared on mature erythrocytes. The separation was achieved using an immunomagnetic technique. A monoclonal mouse antibody against human transferrin receptor was bound to magnetic microspheres conjugated with sheep antimouse antibody. These immunomagnetic beads bound directly to transferrin receptors on the reticulocytes, which could then easily be separated from the nonreactive cells. MoAb IT1-2B4, made in our laboratory, was used for this study, but the commercially available antibody OKT-9 gave essentially similar results. The exact amount of antibody required for optimal coating of beads will vary with different affinities of the antibodies. Therefore, it is important to

<table>
<thead>
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<th>Beads/RBC</th>
<th>% Recovery of Reticulocytes (mean and range)</th>
<th>CV (%)</th>
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<tr>
<td>$1.3 \times 10^{-3}$</td>
<td>5.1 (1.9-9.1)</td>
<td>7.5</td>
</tr>
<tr>
<td>$10 \times 10^{-3}$</td>
<td>24 (14-37)</td>
<td>9.2</td>
</tr>
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The amount of reticulocytes in the source was 0.8% (1.2% to 0.3%) (mean and range). The reticulocytes were isolated from 20 normal persons at two different beads per cell ratios. Each sample was analyzed twice and the coefficient of variation was calculated using the difference between duplicates.
Fig 3. Effect of incubation time on the yield of reticulocytes. Immunomagnetic beads and RBCs from two normal persons were incubated at 22°C. A low (1.3 x 10^-3, closed symbols) and a higher (10 x 10^-3, open symbols) beads/RBC ratio were used. Mean values of double samples are shown. Other experiments gave essentially similar results.

perform a careful titration to define the optimal amount of antibody per milligram of beads in each individual case.

Immunomagnetic beads have previously been used to remove cells such as neuroblastoma cells and T cells from bone marrow or peripheral blood. A positive selection of pure functionally active subsets of human T cells and megakaryocytes have also been obtained by this technique.

Fig 4. Effect of dilution on the reticulocyte yield. RBCs from a normal person were concentrated to an erythrocyte volume fraction of 0.62 and suspended in 0 to 8 mL buffer. The cells were incubated with immunomagnetic beads at a bead per cell ratio of 10 x 10^-3 beads/RBC (closed symbols), and 1.3 x 10^-3 beads/RBC (open symbols) for 90 minutes. Essentially similar results were found using cells from different persons.

Isolation of reticulocytes from normal persons gave a large variation in the reticulocyte yield (Fig 2 and Table 1). Because there was only a small variation in the reticulocyte concentration in peripheral blood and since the variation is not due to imprecision of the method (Table 1), this difference in reticulocyte yield must be explained by factors affecting the binding of reticulocytes to beads. It has previously been shown that during reticulocyte maturation in the peripheral blood the transferrin receptor is rapidly lost or reduced to a large extent before the reticulocyte completely matures. Reticulocytes can be identified in automated systems by fluorescent RNA staining or an immunofluorescent method with antitransferrin receptor antibody. When both methods are applied to the same specimen, it has been shown that the antitransferrin antibody technique identifies only the newly released reticulocytes. The ratio between reticulocytes positive for transferrin receptor by immunofluorescent staining and reticulocytes positive for RNA staining has been shown to be approximately 0.3. Our findings of 15% to 42% reticulocyte recovery are in accordance with these results and indicate that reticulocytes isolated with this

Fig 5. Effect of storage time and temperature on the reticulocyte yield. To 8 mL of heparinized blood we added 1.2 mL CPD-adenin solution. The blood was stored at room temperature (22°C) and at 4°C. At the time indicated, the reticulocytes were isolated as described in Materials and Methods. The error bars represent the standard deviation from six different experiments.

Fig 6. Effect of in vitro incubation at 37°C on the reticulocyte yield. Five milliliters of venous blood from normal adults were drawn in culture bottles containing 45 mL blood culture medium. Reticulocytes were isolated at time indicated. Each point represents the mean value of double samples and is given as percent of the values before incubation. The data are plotted on a logarithmic scale. Essentially similar results were found using cells from different persons.
The ratio between reticulocytes and peripheral RBCs was calculated for each measurement. Results from 10 different experiments are presented as mean ± SD.

Abbreviation: Hb, hemoglobin.

**Table 2. Measurements of Different Constituents in Reticulocytes and Peripheral RBCs From Normal Persons**

<table>
<thead>
<tr>
<th></th>
<th>Pyruvate Kinase (U/g Hb)</th>
<th>Aspartate Aminotransferase (U/g Hb)</th>
<th>Porphobilinogen Deaminase (µmol/a x g Hb)</th>
<th>Hb A\textsubscript{1c} (%)</th>
<th>Creatine (µmol/g Hb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peripheral RBCs</td>
<td>13.1 ± 1.7</td>
<td>7.8 ± 1.4</td>
<td>37 ± 6</td>
<td>4.7 ± 1.5</td>
<td>0.9 ± 0.2</td>
</tr>
<tr>
<td>Reticulocytes</td>
<td>83 ± 16</td>
<td>23 ± 3</td>
<td>319 ± 88</td>
<td>0.6 ± 0.08</td>
<td>14.3 ± 2.7</td>
</tr>
<tr>
<td>Ratio</td>
<td>6.4 ± 1.0</td>
<td>3.0 ± 0.5</td>
<td>8.8 ± 2.1</td>
<td>0.11 ± 0.02</td>
<td>16.7 ± 2.5</td>
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The amount of creatine and hemoglobin A\textsubscript{1c} and the activity of pyruvate kinase, aspartate aminotransferase (GOT), and porphobilinogen deaminase (uroporphyrinogen I synthetase) were measured in leukocyte-depleted peripheral blood and isolated reticulocytes as described in Materials and Methods. The ratio between reticulocytes and peripheral RBCs was calculated for each measurement. Results from 10 different experiments are presented as mean ± SD.

**immunomagnetic method** represent the newly released (youngest) reticulocytes. The reticulocyte recovery is therefore expected to depend on the relative amount of this subset of young reticulocytes present in peripheral blood. The fact that a subset of reticulocytes is isolated with this method may be an advantage or disadvantage depending on the experimental interests of the investigator.

In Fig 2 the curve with the lowest reticulocyte yield levels off at 3 beads per 1.0 x 10\textsuperscript{3} RBCs, whereas the top curve reaches the plateau at about 12 beads per 1.0 x 10\textsuperscript{3} RBCs. To assure an optimal recovery of reticulocytes from peripheral blood with a normal reticulocyte count, 10 to 12 beads per 1.0 x 10\textsuperscript{3} RBCs is therefore recommended.

One interesting observation is that the yield of reticulocytes remains nearly unchanged when the reticulocytes are stored at 22°C and that the yield increases when stored at 4°C (Fig 5). Normally 20% to 25% of the transferrin receptors are located at the cell surface, and one could speculate that storage at 4°C, which will decrease the metabolism of the cell, could increase the percentage of transferrin receptors at the cell surface due to interference with the internalization or recycling of the receptors. The possibility to isolate reticulocytes after several days may enhance the utility of the method because blood can be transported for some time before isolation of reticulocytes. Whether this storage will affect constituents of the cells is presently under investigation.

Under normal conditions the maturation time of the reticulocyte is estimated to be about 3 days, with 1 day spent in the peripheral blood, but the literature is scant on this point. Our in vitro storage studies at 37°C give indirect evidence of a rapid decline in the number of transferrin receptors which nearly disappear after 2 days. A half-life for the transferrin receptor of 16 hours can be calculated (Fig 6). This is equivalent to the results recently reported by Noble et al\textsuperscript{33} on in vitro maturation of rat reticulocytes. The complete maturation of reticulocytes in vitro will probably exceed 2 days because transferrin receptors are lost before the reticulocytes loose their RNA content and mature to erythrocytes.

Some of the enzymes in erythrocytes show a decline in activity during the life-span of the RBC. Studies of age-related enzymes in RBCs have, until now, been hampered by an inability to obtain pure reticulocytes. Therefore, the enzyme activity in reticulocytes has been calculated by extrapolating data from density gradient centrifugations to 100% reticulocytes. With this new method it is possible to measure the enzyme activity in pure reticulocyte suspensions. The difference in activity between reticulocytes and peripheral RBCs is given as a ratio (Table 2). The ratio of pyruvate kinase, calculated from our measurements, is higher than previously reported values.\textsuperscript{34,35} The result for aspartate aminotransferase is in accordance with earlier observations.\textsuperscript{36,37} Of the three age-related enzymes studied, porphobilinogen deaminase showed the greatest decrease in activity (ratio 8.8). The literature is very scant on information of the age-related aspects of this enzyme.\textsuperscript{38} This enzyme, in addition to hexokinase,\textsuperscript{39,40} is probably one of the best enzyme markers for a young RBC population.

The formation of hemoglobin A\textsubscript{1c} is a slow process proceeding continuously throughout the life-span of the erythrocyte.\textsuperscript{40,41} It has been reported that patients with anemia due to considerable RBC destruction have low HbA\textsubscript{1c} values, and a correlation between glycosylated hemoglobin and 3\textsuperscript{1}Cr-tagged RBC survival has also been shown.\textsuperscript{42} Using the immunomagnetic method, the concentration of hemoglobin A\textsubscript{1c} in peripheral RBCs was found to be 8.9 times higher than the concentration in reticulocytes (Table 2). This makes hemoglobin A\textsubscript{1c} one of the best indexes of RBC aging.

The creatine concentration in RBCs is a good indicator of RBC age,\textsuperscript{41,42} but the concentration in reticulocytes released from the bone marrow under normal conditions has, until now, not been measured. Because stress erythropoiesis gives a higher creatine concentration in the RBCs compared with a normal condition with timely released cells,\textsuperscript{43,44} it is not possible to use patients or animals with reticulocytosis as a source of reticulocyte enrichment procedures to estimate this content. Applying the immunomagnetic isolation technique, we have measured the concentration of creatine in normal reticulocytes to be 14.3 ± 2.7 µmol/g hemoglobin (4.9 ± 0.9 mmol/L). In peripheral RBCs the creatine content was 0.9 ± 0.2 µmol/g hemoglobin (0.31 ± 0.07 mmol/L), which is in accordance with other reports.\textsuperscript{45,46} The ratio between reticulocytes and peripheral RBCs is therefore 16.7 (Table 2). These results confirm and provide additional evidence that creatine is the marker of RBC age that has the best discriminatory power.

This reticulocyte isolation method has a potential application in blood typing serology. In transfusion medicine the separation of autologous and transfused RBCs is valuable in determining the RBC group phenotype of a patient who is developing antibodies to donor RBCs. For this typing, reticulocyte-enriched fractions after density gradient centrif-
uation have been used. However, these fractions are contaminated with mature RBCs including transfused cells. Isolation of a pure reticulocyte suspension from a transfused patient would guarantee autologous RBCs and therefore improve the quality of such blood typing.

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