Semicontinuous Flow Centrifugation for the Pheresis of Immunocompetent Cells and Stem Cells

By Roy S. Weiner, Carol M. Richman, and Ronald A. Yankee

Mononuclear cells from human peripheral blood were purified by semicontinuous flow centrifugation (SCFC) using the Haemonetics model 30 blood cell separator; 64% ± 7% of the mononuclear cells in 600 ml of peripheral blood were collected in a 30-ml volume. Analysis of sequential 5-ml aliquots of the mononuclear cell concentrate revealed that both immunocompetent cells and granulocytic progenitor cells (CFU-C) were proportional to the cell count throughout the buffy coat.

In vitro pheresis of large volumes of human bone marrow resulted in recovery of 63% of the cells, 12% of the hemoglobin, and 84% of the CFU-C in 20% of the original volume. Further centrifugation eliminated 80% of the platelets without loss of cells or CFU-C. SCFC of peripheral blood or bone marrow selectively concentrated mononuclear cells and reduced the contamination by granulocytes and erythrocytes. Large numbers of mononuclear cells can thus be collected for studies in vitro or for cryopreservation and the autologous reconstitution of immunosuppressed or myelosuppressed patients undergoing intensive antitumor therapy.

Semicontinuous Flow Centrifugation (SCFC) of blood has become a well-established technique for harvesting large quantities of platelets from single donors for transfusion to thrombocytopenic patients. More recently, granulocyte collection has been facilitated by SCFC in conjunction with in vitro manipulation of the sedimentation properties of blood. Both platelet and granulocyte preparations obtained by pheresis contain large numbers of mononuclear cells consisting of immunocompetent as well as hematopoietic progenitor cells. The ability to procure high concentrations of these cells may facilitate studies of autologous reconstitution for the support of severely suppressed patients. Specific pheresis techniques designed to harvest mononuclear cells efficiently would provide large numbers of these cells with a high degree of purity in relatively small volumes. Additionally, reducing the granulocyte, erythrocyte, and platelet contamination would facilitate the cryopreservation and storage of these cells to make them available for reinfusion or study at a later time. Likewise, concentration and purification of bone marrow mononuclear cells from large volume aspirates would circumvent the major technical problems in cryopreserving human bone marrow for autologous reconstitution.

We have investigated the use of SCFC to collect and concentrate large numbers of mononuclear cells and have quantitated the yield, purity, and function of immunocompetent cells and hematopoietic precursor cells from peripheral...
blood. In addition, we have studied the usefulness of SCFC to concentrate hematopoietic cells from large-volume bone marrow aspirates.

MATERIALS AND METHODS

Concentration of Peripheral Blood Mononuclear Cells

Normal donors and solid tumor patients with normal hematologic parameters were leukapheresed on the Haemonetics Model 30 semicontinuous cell separator (Haemonetics, Natick, Mass.) using the 225-ml capacity bowl. Acid-citrate-dextrose (ACD) solution A was added to the whole blood at the outflow tubing from the donor's arm at a constant ratio of 1 volume ACD to 8 volumes of blood. The filling rate of the bowl was 60-80 ml/min until the buffy coat reached the shoulder at the top of the bowl. The rate was then slowed to 30 ml/min. When the buffy coat was within 1 cm of the hub of the bowl, the cell collection was begun and continued for 45-60 sec, yielding 20-30 ml of cells. Elimination of platelets from the Haemonetics concentrate was accomplished by diluting the sample with 1.5 volumes of Media 199 and centrifuging at 150 g for 15 min at 15°C in 50-ml conical centrifuge tubes. The top two-thirds of the supernatant was discarded.

Cell counts, differentials and in vitro granulocytic colony-forming assays were performed on aliquots of whole blood obtained prior to pheresis and on the buffy coat samples.

Normal subjects were phlebotomized 600 ml into collection bags containing either 70 ml of ACD solution A or 3000 units of preservative-free heparin (Weddel) in 70 ml of saline. The blood was then separated in vitro by pheresis. The first 30 ml of buffy coat were diverted into a collection bag attached to the platelet port and an additional 30 ml were collected in a second bag attached to the white cell port. The yields of the various cell populations were calculated on the basis of the actual volume of blood required from each donor to fill the 225-ml bowl and generate a buffy coat. In a separate series of in vivo pheneses, serial 5-ml fractions of buffy coat were collected in sterile precalibrated test tubes beginning with a cell-free plasma aliquot and continuing for 10 fractions or 30 ml.

Assays In Vitro

The cell surface and functional characteristics were determined by a battery of in vitro tests. E rosettes, mixed lymphocyte cultures (MLC), and CFU-C activity were measured by techniques described previously. Briefly, E rosettes were determined using sulfhydryl-treated sheep erythrocytes as described by Kaplan and Clark.6 For MLC 2 x 10^5 Ficoll-Hypaque separated cells were incubated with 2 x 10^5 irradiated autologous or allogeneic cells. Results were expressed as mean counts per minute ± SEM of triplicate cultures after 138 hr of incubation followed by a 6-hr exposure to 0.44 μCi of 3H-thymidine (specific activity 6 Ci/mmol; Schwartz Mann Corp.).7 In vitro CFU-C assays were performed using a modification of the method of Iscove.3 '4 Mononuclear cells obtained by Ficoll-Hypaque separation were incubated for 14 days in 10% CO_2 at a concentration of 2 x 10^5 cells/ml in methylcellulose (0.8%), 20% fetal calf serum, and 20% human leukocyte conditioned media. Results were expressed as mean colonies (greater than 20 cells) per triplicate plates ± SEM.

Concentration of Bone Marrow Stem Cells

Normal bone marrow was collected for subsequent autologous reinfusion from 17 adult solid tumor patients by multiple puncture of the posterior superior iliac spines and iliac crests under general anesthesia. Approximately 5 ml of bone marrow per "pull" was aspirated into syringes containing 25 units of preservative-free heparin in 0.5 ml of Hanks' balanced salt solution (HBSS). Four to eight aspirates were taken from each site. The aspirates were pooled, filtered through stainless steel screens, and collected in a 1-liter bag (Fenwal TA10).8 The total volume of bone marrow at completion of the procedure averaged 1110 ml (range 994-1423 ml). Samples were removed for cell counts and functional assays. The bag was connected to the Haemonetics Model 30 using the standard donor set up and the 225-ml bowl. The donor phlebotomy needle was inserted into an injection port connected to an outflow port of the TA10. The connecting tubing from the TA10 was attached to the Haemonetics return bag and
clamped. A 300-ml transfer bag was connected to the platelet port. The filling speed was 60–80 ml/min until the buffy coat reached the shoulder of the bowl. The speed was then slowed to 30 ml/min. When the buffy coat was within 1 cm of the hub, collection was begun and continued for 2–2.5 min or 60–70 ml. The remaining blood and plasma were then returned to the “return” bag. The remainder of the bone marrow aspirate was then used to fill the bowl. When necessary, the cells from the return bag were used to supplement the red cell volume. The procedure was repeated until the entire aspirate had passed through the bowl twice. The collection time for the second pass was 1.5–2 min or 45–60 ml. ACD, using the standard pump and the standard solution, was used only the first time the volume was separated.

Samples were removed from the buffy coat for counts and functional studies, and the remainder was diluted with 1.5 volumes of HBSS and centrifuged at 150 g for 15 min at 15°C in 50-ml conical centrifuge tubes. The top two-thirds of the cell-free platelet-rich supernatant was discarded, and the cells were resuspended in 25% plasma. Final samples were removed for cell counts and functional studies.

RESULTS

Pheresis of Whole Blood In Vitro

The efficiency of SCFC in harvesting mononuclear cells from whole peripheral blood is shown in Table 1. Whole blood drawn into ACD or heparin from four normal subjects was passed through the Haemonetics machine. A mean of 74% of the lymphocytes, 55% of the monocytes, and 67% of the total mononuclear cells were collected in the first 30-ml fraction. The second 30-ml fraction added 7% more lymphocytes and 8% more monocytes. The proportion of granulocytes in the second 30-ml fraction was slightly higher than in the first.

Pheresis of Whole Blood In Vivo

The total yield of mononuclear cells (lymphocytes plus monocytes) obtained from 15 donors undergoing pheresis was 64% ± 7% (mean ± SEM) in a 30-ml volume. The yields were based on the assumption that 600 ml of blood entered the bowl in order to generate the buffy coat (Table 1).

The distribution of the various formed elements in the buffy coat is shown in Fig. 1. The buffy coats generated by pheresis in vivo were collected in 5-ml aliquots beginning with clear plasma. As seen in Fig. 1, the maximum concentration of platelets preceded the maximum concentration of leukocytes. Platelets, however, remained present in high concentrations throughout the leukocyte collection.

In order to test the distribution of immunocompetent cells and hematopoietic progenitor cells in the SCFC buffy coat, sequential 5-ml fractions were analyzed.

| Table 1. Yield of Cells from Pheresis of Whole Blood In Vitro* (Cells ± SEM x 10⁶) |
|-------------------------------|-----------------|-----------------|
| Pre                           | First 30 ml     | Second 30 ml    |
| Lymphocytes                   | 1.01 ± 0.17     | 0.69 ± 0.05     |
|                               | (74% ± 19%)†    | (7% ± 1%)       |
| Monocytes                     | 0.38 ± 0.09     | 0.18 ± 0.05     |
|                               | (55% ± 23%)     | (8% ± 2%)       |
| Granulocytes                  | 2.93 ± 0.43     | 0.25 ± 0.11     |
|                               | (8% ± 3%)       | (10% ± 2%)      |

*Volume necessary to generate the buffy coat using the 225-ml bowl was 594 ± 11 ml.
†The numbers in parentheses are the per cent ± SEM yield based on the total cells in the volume of blood pheresed.
with respect to E rosette, MLC, and CFU-C function. Table 2 shows the distribution of leukocytes in the buffy coat and the functional characteristics of the mononuclear cells. Hematopoietic progenitor cells and immunocompetent cells were found in all fractions. Monocytes and lymphocytes were distributed similarly throughout the buffy coat, and selective pheresis of either element was not possible under the conditions used in these experiments. Likewise, no functional separation of stem cells from immunocompetent cells was observed.

In order to confirm that the buffy coats were functionally representative of the mononuclear cells in whole blood, we compared the concentration of CFU-C per $2 \times 10^5$ Ficoll–Hypaque separated mononuclear cells from whole blood and from the SCFC-derived buffy coat samples from the same donors. The results shown in Fig. 2 show a linear relationship between the whole blood and buffy coat CFU-C concentration (slope = 0.95).

### Bone Marrow Stem Cell Concentration In Vitro

Table 3 shows the results of SCFC separation of bone marrow from 17 patients. A mean volume of 1110 ml of bone marrow aspirate was concentrated to a buffy coat volume of 198 ml. Further centrifugation at 150 g for 15 min resulted in a platelet-poor concentrate with a mean volume of 150 ml. Thus 14% of the original volume contained 63% of total cells, 12% of the hemoglobin, and 20% of the platelets; 84% ± 18% of the granulocytic progenitor

<table>
<thead>
<tr>
<th>Fraction</th>
<th>WBC ($\times 10^3$/mm$^3$)</th>
<th>Differential (%)</th>
<th>E Rosette (%)</th>
<th>MLCT AAx</th>
<th>MLCT ABx</th>
<th>CFU-C$^t$</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>1.0</td>
<td>1.0</td>
<td>51</td>
<td>32.5</td>
<td>5.3</td>
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<tr>
<td>2</td>
<td>4.1</td>
<td>66</td>
<td>58</td>
<td>618</td>
<td>14212</td>
<td>28</td>
</tr>
<tr>
<td>3</td>
<td>5.6</td>
<td>51</td>
<td>18</td>
<td>705</td>
<td>16003</td>
<td>39</td>
</tr>
<tr>
<td>4</td>
<td>4.2</td>
<td>41</td>
<td>51</td>
<td>1050</td>
<td>23020</td>
<td>34</td>
</tr>
<tr>
<td>5</td>
<td>4.6</td>
<td>15</td>
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<td>1337</td>
<td>2620</td>
<td>41</td>
</tr>
<tr>
<td>6</td>
<td>29.9</td>
<td>8</td>
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<td>2963</td>
<td>21980</td>
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<tr>
<td>7</td>
<td>19.1</td>
<td>10</td>
<td>51</td>
<td>2747</td>
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<td>51</td>
<td>2747</td>
<td>26230</td>
<td>24</td>
</tr>
</tbody>
</table>

*Granulocytes, eosinophils, basophils.
$^t$Mean cpm ± SEM of triplicate cultures of $2 \times 10^5$ Ficoll–Hypaque separated responding cells incubated with $2 \times 10^5$ irradiated autologous cells (AAx) or allogeneic cells (ABx).
$^t$Mean colonies in triplicate plates ± SEM per $2 \times 10^5$ Ficoll–Hypaque separated mononuclear cells.
Fig. 2. The relationship between concentration of CFU-C in Ficoll-Hypaque separated whole blood and CFU-C in Ficoll-Hypaque separated Haemonetics Buffy coat (y = 0.95x).

cells in the original marrow aspirate were recovered in the final concentrate. The residual volume of marrow aspirate contained less than 10% of the original CFU-C.

The SCFC preferentially eliminated mature granulocytes from the bone marrow aspirate and concentrated myelocytes, erythroid precursors, and lymphocytes (Fig. 3). Approximately 90% of the lymphocytes were recovered in the buffy coat, whereas only 40% of the mature granulocytes were recovered. The recovery of granulocyte precursors, with the exception of myelocytes, did not differ from total cell recovery and thus were neither concentrated nor eliminated.

DISCUSSION

Mononuclear cell pheresis is of interest because it permits collection and concentration of immunocompetent cells and hematopoietic precursor cells. Lymphocytes collected in large numbers maintain immunocompetence after cryopreservation. Cryopreserved normal lymphocytes can thus be used as standard reference cells in a variety of in vitro tests of cellular immunity. Autologous or syngeneic cells can be collected for future immunologic reconstitution and, eventually, for specific adoptive immunotherapy.

Peripheral blood stem cells have been shown to increase after myelosuppressive therapy in man. Previous studies have shown that during stem cell rebound it is possible to collect these cells in sufficient numbers for cryopreservation and study of autologous hematopoietic reconstitution. The buffy coat gen-

Table 3. Haemonetic s Separation of Human Bone Marrow (Mean ± SEM)

<table>
<thead>
<tr>
<th></th>
<th>Volume (ml)</th>
<th>Total Cells (x 10^10)</th>
<th>Total Hb (g)</th>
<th>Total Platelets (x 10^11)</th>
<th>Total CFU-C* (x 10^6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial aspirate</td>
<td>1110 ± 37</td>
<td>2.0 ± 0.14</td>
<td>94 ± 8</td>
<td>1.3 ± 0.15</td>
<td>3.02 ± 0.75</td>
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<tr>
<td>Haemonetics</td>
<td>200 ± 15</td>
<td>1.2 ± 0.08</td>
<td>12.1 ± 1.5</td>
<td>1.1 ± 0.15</td>
<td>0.3 ± 0.05</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>150 ± 11</strong></td>
<td><strong>1.1 ± 0.11</strong></td>
<td><strong>12.1 ± 1.5</strong></td>
<td><strong>3.0 ± 0.05</strong></td>
<td><strong>2.04 ± 0.46</strong></td>
</tr>
<tr>
<td>Final concentrate†</td>
<td>14 ± 1.1</td>
<td>63 ± 2.9</td>
<td>12 ± 0.8</td>
<td>20 ± 5.5</td>
<td>84 ± 18</td>
</tr>
<tr>
<td>Recovery† in final concentrate (%)</td>
<td></td>
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*CFU-C: colonies greater than 20 cells in methylcellulose (see text).
†Final concentrate: values obtained after the Haemonetics concentrate was depleted of platelets (see text).
‡Final concentrate/initial aspirate x 100; recoveries were calculated for each sample.
erated by SCFC of peripheral blood contains both immunocompetent and hematopoietic precursor cells. Each buffy coat can yield up to $1 \times 10^9$ mononuclear cells with a blood loss of 30 ml and requires only 20 min. We have taken up to eight buffy coats during a single session. Since hemoglobin loss is minimal, pheresis can be repeated frequently. Semicontinuous flow pheresis is well tolerated by both normal subjects and patients. The donors experience perioral tingling during reinfusion of the ACD-blood, but this depends on the rate of reinfusion. Lower concentrations of ACD appear to lessen the clinical symptoms of hypocalcemia and permit more rapid reinfusion of the residual blood. Since the bowls and all tubing are in a sterile disposable kit, there is minimal risk of donor contamination.

Under the conditions reported here there was no clearcut separation within the buffy coat of monocytes and lymphocytes, or of immunocompetent cells and stem cells. Further studies using agents which change the sedimentation properties of blood may provide a means of selective pheresis of specific cell types.

The application of SCFC to in vitro separation of human bone marrow has been prompted by major technical problems in processing bone marrow for cryopreservation and storage. Controlled-rate cooling requires efficient heat exchange, which is difficult to obtain for large-volume containers. Concentration of the bone marrow by simple centrifugation is unacceptable since the contaminating granulocytes and platelets are preserved poorly and contribute to severe clumping, resulting in cell loss upon thawing. Although bone marrow stem cells can be separated by density gradient centrifugation, this procedure requires a primary separation step and is cumbersome. SCFC, however, efficiently reduces the volume and the erythrocyte and granulocyte contamination of the bulk bone marrow while retaining 84% of the granulocytic progenitor cells. The granulocyte precursors remaining in the bone marrow concentrate are a function of the large volume of buffy coat required to recover the stem cells. Pilot experiments in which bone marrow buffy coat has been collected in sequential 30-ml aliquots indicate that as many granulocytic progenitor cells are present in the second 30-ml sample as are present in the first 30-ml sample. The first 30 ml, however, is less contaminated by polymorphonu-
clear cells, bands, and metamyelocytes, and more closely resembles the peripheral blood buffy coat. We have elected to accept the impurity of the larger sample in order to increase the yield of stem cells. The additional step of slow-speed centrifugation further reduces the volume and eliminates 80% of the platelets. The final specimen can be cryopreserved in small containers or purified further by physical or immunologic techniques.

Semicontinuous flow centrifugation for the specific purpose of concentrating mononuclear cells from the peripheral blood and bone marrow represents a useful technique with broad clinical and research potential. In vitro manipulation of the sedimentation properties during pheresis may permit the selective harvest of functionally defined mononuclear cell populations.

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