Polymorphonuclear Leukocytes Prepared by Continuous-Flow Filtration Leukapheresis: Viability and Function

By Michael B. Harris, Isaac Djerassi, Elias Schwartz, and Richard K. Root

Preparation of granulocytes for transfusion in high yield and relatively free of contamination by other cell types has been made possible by the technique of continuous-flow filtration leukapheresis (CFFL). Since previous work suggested that granulocytes collected in this manner may have impaired viability and function, a detailed study of the bactericidal, metabolic, and chemotactic properties of such cells was performed and compared to control cells obtained from the same donors prior to CFFL. The granulocyte percentage of the cell suspensions obtained by CFFL averaged 94.5% ± 1.5% compared to 82.5% ± 1.8% for the controls (p < 0.001) with viability of the PMNs determined by trypan blue exclusion being 97.5% ± 0.9% and 98.2% ± 0.5%, respectively. The phagocytic, metabolic (14C-l-glucose oxidation and protein iodination) and chemotactic properties of both cell types were equivalent in suspensions equalized for granulocyte content. These findings indicate that CFFL technique employed does not impair granulocyte viability or function in vitro. Studies of the in vivo survival and function of CFFL granulocytes are necessary to evaluate their efficacy in combating infection in severely leukopenic patients.

Infection is the major cause of death in patients with leukemia, and the incidence of infection has been shown to increase with the magnitude and duration of granulocytopenia. Granulocyte transfusions have been used as an adjunct to antibiotic therapy in the granulocytopenic patient. Initial studies indicate that they may be of benefit in the treatment of such patients who have infections complicating their clinical course.

One method of preparing granulocytes for transfusion is by continuous-flow filtration leukapheresis (CFFL). This method, developed by Djerassi and co-workers, utilizes the principle that polymorphonuclear leukocytes (PMNs) may be separated from whole blood by their adherence to a nylon-wool filter. Collection of PMNs by the CFFL technique offers several advantages over existing methods for the preparation of PMNs for transfusion, chief among which are much larger yields from normal donors. In 1972, however, Herzig et al. reported that PMNs prepared for transfusion by this method had decreased viability and impaired phagocytic and bactericidal activities. Because
their collection techniques differed somewhat from those employed in the current investigation, we studied the morphologic, metabolic, and functional properties of PMNs prepared for transfusion by this improved method of CFFL. The results of these investigations indicate that the PMNs prepared by this method have normal in vitro characteristics.

MATERIALS AND METHODS

Preparation of Polymorphonuclear Leukocytes

Peripheral venous blood was drawn in heparin (6 U/ml) from the donor prior to granulocyte donation and stored at 4°C. Cells obtained from this blood were used as the control sample in all experiments. In addition, in experiments in which chemotaxis was measured, blood was similarly obtained from normal laboratory personnel.

Granulocytes were then obtained in large quantities by a modification of the method of CFFL described by Isaac Djerassi. Briefly, this method is as follows: the donor is anticoagulated with 15,000 U of heparin, then given 100 mg of solucortef, and blood is drawn by negative pressure from one arm into either cannister 1 or 2 (Fig. 1). From the cannister it is propelled by air pressure to four white cell filters (Leukopak, Baxter, Edison, N.J.; Fig. 1B) through which it drains by gravity into cannister 3 or 4. The blood is pumped from here to the vented bottles (Fig. 1C), from which it returns by hydrostatic pressure into the other arm of the donor. This process is automatically accomplished with the use of a Leukopherator (RAD Instruments, Philadelphia, Pa.) and allows the extraction of PMNs from 9.6 liters of blood within 2 hr. After 2 hr the filters are removed, and four new filters are put into their place for a second 2-hr run.

The white blood cells are eluted as soon as the filters are removed. Six hundred milliliters of ACD plasma, adjusted to a pH of 6.5, are forced through each of the filters under a pressure of 250 mm Hg maintained by a blood pressure cuff. While the plasma is passing through the filters each is tapped gently. The effluent is collected in plastic bags and centrifuged at 500 rpm for 20 min at 10°C. From four filters a median yield of $4.0 \times 10^{10}$ (range, $1.4 \times 10^{10}$ to $6.0 \times 10^{10}$).
granulocytes are obtained. The final yield (from eight filters) is approximately $9 \times 10^{10}$ PMNs in a volume of 150-180 ml of plasma.

In the in vitro functional studies performed in the present investigations, erythrocytes were removed from the filtered PMNs and from control blood samples by dextran sedimentation and hypotonic lysis. The leukocytes were concentrated by low-speed centrifugation (150 g for 7 min) and washed twice in modified Hanks’ solution (MHS).

Total and differential cell counts were done by Hemocytometer- and Diff-Quik (Harleco, Philadelphia, Pa.)-stained smears, respectively, and viability was assessed by the exclusion of 0.1% trypan blue dye and by phase microscopy. The cells were then suspended in Hanks’ balanced salt solution (HBSS) to a final concentration of $10 \times 10^6$ viable PMNs/ml for use in the in vitro functional assays.

**Measurement of Phagocytosis**

*Staphylococcus aureus* 502A was radiolabeled with a mixture of $^{14}$C-amino acids by a method described previously. Heat-killed radiolabeled bacteria ($5 \times 10^7$) were then tumbled in $12 \times 75$-mm glass tubes (Becton-Dickinson, Rutherford, N.J.) which contained $5 \times 10^6$ PMNs in a HBSS solution with $10\%$ serum (final volume of 1 ml). Ingestion of bacteria was halted at 8 min (during the linear phase of uptake) by the addition of 2 ml of cold $10\%$ fetal calf serum HBSS solution containing $1 \times 10^{-2} M$ sodium fluoride and by placing the tubes in ice. Noningested bacteria were separated from the PMNs by low-speed centrifugation (500 rpm x 5 min at 4°C). The pellet was washed twice by low-speed centrifugation in $10\%$ fetal calf serum-HBSS, dried overnight, digested with 0.1 N NaOH, and radioactivity was counted in Aquasol (New England Nuclear, Boston, Mass.) as previously described. All tests were run in duplicate.

**Glucose Oxidation**

Oxidation of the first carbon of glucose ($l^{14}$C-glucose) was measured by a previously described method. The only modifications were that yeast ($1 \times 10^8$ per tube) was used instead of polystyrene balls, and that the scintillation fluid was Aquasol (10 ml) with 1 ml of distilled water added. Triplicate determinations were made on each PMN sample.

**Iodination of Protein by PMNs**

The interaction of hydrogen peroxide, iodide, and myeloperoxidase (MPO) was measured by the ability of granulocytes to iodinate protein using slight modifications of methods previously described. Heat-killed baker’s yeast particles were opsonized by incubation at 37°C for 30 min in a 50% mixture of donor serum in HBSS. The yeast were then collected by centrifugation at 3000 rpm for 10 min at 4°C, washed twice with MHS, and resuspended in HBSS to a concentration of $1 \times 10^9$ yeast/ml. Final suspensions in $12 \times 75$-mm glass tubes contained $1 \times 10^9$ yeast, $5 \times 10^6$ PMN, 10 nmoles of Na with 0.2 μCi of Na$^{125}$I in HBSS, with a final volume of 0.85 ml. Sodium azide (1 mM final concentration) was added to duplicate tubes in order to inhibit MPO and provide background counts of non-MPO-mediated iodination. The suspensions were tumbled at 37°C for 60 min at which time the reaction was stopped with 1 ml of a 1 mM solution of sodium thiosulfate in 0.15 M NaOH, and the tubes were placed in ice. The protein was precipitated by the addition of 20% trichloroacetic acid (TCA). The suspension was then centrifuged at 3000 rpm at 4°C for 5 min and the pellet washed three times with 2 ml of cold 10%, TCA. The radioactivity of $^{125}$I in the pellet was determined by placing each tube inside a 18 × 150-mm tube and counting in a NaI crystal well scintillation counter (Baird Automatic, Orelad, Pa.).

**Bactericidal Activity**

The ability of the PMNs to kill *S. aureus* 502A was determined by a modification of a method described previously. *S. aureus* 502A was grown in trypticase soy broth for a 4-hr period, spun down at 3000 rpm for 10 min, washed once in MHS, and resuspended in HBSS to a final concentration of $1 \times 10^9$ bacteria/ml. Bacteria ($1 \times 10^9$) and $5 \times 10^6$ PMNs were added to a 10%, homologous Serum HBSS Solution (final volume, 1 ml) and tumbled at 37°C. Either 10 μl or 100 μl Eppendorf micropipettes were used to sample each tube at 20, 30, and 60 min. Each sample was placed in 10 ml of distilled water and agitated in order to lyse the PMNs; after
10 mm 10-μl and 100-μl samples were plated by pour-plate technique in trypticase soy agar. At 20 min lysostaphin (10 U) was added to all the tubes in order to kill extracellular bacteria. The zero time bacteria count was determined by immediately plating a cell-free control; this cell-free control was incubated for 60 min (without the addition of lysostaphin) and plated, to be certain that only cell-associated killing of bacteria took place. Duplicate tests were run on each cell sample.

Chemotaxis

Chemotaxis of PMNs was determined using 51Cr-labeled cells by the method of Gallin et al. In this method two 5-μ Millipore filters (Millipore Corp., Bedford, Mass.) are placed into a chemotactic chamber separated into upper and lower compartments. The chemotactic stimulus is generated from 0.1 ml human serum by activation with Escherichia coli endotoxin (E. coli 0127:B8 lipopolysaccharide B; Difco Labs, Detroit, Mich.). The chemotactic stimulus is placed in the lower chamber and 51Cr-labeled PMNs are layered on top of the filters in the upper chamber. At the end of a 3-hr incubation period, the radioactivity of both filters is determined, with the radioactivity of the lower filter representing the PMNs that had passed through the upper to the lower filter. With a chemotactic stimulus in the lower chamber, the radioactive counts on the lower filter are markedly increased above unstimulated controls, representing active chemotaxis of PMNs through the upper filter.

RESULTS

Differential Cell Counts and Viability

Polymorphonuclear leukocytes comprised 94.5% of the total white blood cells prepared by CFFL as compared to 82.5% for the control (p < 0.001). This difference is due mainly to the lower percentage of lymphocytes in the CFFL preparations (Table 1).

Viability, as determined by trypan blue exclusion, was similar for both CFFL and control PMNs (Table 1). The appearance of the granulocytes under phase microscopy was also similar; the granulocytes in both groups adhered to glass well, had fine nuclear detail, and had minimal vacuolization.

Metabolic Activities

The oxidation of 14C-l-glucose and the myeloperoxidase-mediated conversion of iodide to a protein-bound form were similar for both CFFL and control cells (Table 2). The minor differences observed are not statistically significant.

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**Table 1. Viability and Differential Counts of Leukocytes Prepared by CFFL Technique**

<table>
<thead>
<tr>
<th>Collection Method</th>
<th>Per Cent Viable</th>
<th>Per Cent PMN</th>
<th>Per Cent Lymphocytes</th>
<th>Per Cent Monocytes</th>
<th>Per Cent Eosinophiles</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFFL</td>
<td>97.5% ± 0.9</td>
<td>94.5 ± 1.5</td>
<td>3.9 ± 1.5</td>
<td>0.9 ± 0.5</td>
<td>0.7 ± 0.3</td>
</tr>
<tr>
<td>(6)</td>
<td>(7)</td>
<td>(7)</td>
<td>(7)</td>
<td>(7)</td>
<td>(7)</td>
</tr>
<tr>
<td>Control</td>
<td>98.2 ± 0.5</td>
<td>82.5 ± 1.8</td>
<td>13.7 ± 1.5</td>
<td>0.7 ± 0.2</td>
<td>1.9 ± 0.5</td>
</tr>
<tr>
<td>(9)</td>
<td>(11)</td>
<td>(11)</td>
<td>(11)</td>
<td>(11)</td>
<td>(11)</td>
</tr>
</tbody>
</table>

*CCFL leukocytes compared to those obtained from whole heparinized blood from the same donor and from laboratory controls with erythrocytes removed from all samples by dextran sedimentation and hypotonic lysis as described in text.
†Per cent of PMNs that excluded 0.1% trypan blue dye.
‡Mean ± SE.
§Value significantly different from control (p < 0.001), two sample t-test.

Number in parenthesis denotes number of samples.
Table 2. Results of Iodination and Glucose Oxidation by CFLL and Control Cells

<table>
<thead>
<tr>
<th></th>
<th>CFLL (3)* (Mean ± SE)</th>
<th>Control (3) (Mean ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose oxidation†</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonphagocytizing yeast</td>
<td>32.6 ± 6.9</td>
<td>25.7 ± 4.1</td>
</tr>
<tr>
<td>Phagocytizing yeast</td>
<td>188.8 ± 2.3</td>
<td>173.6 ± 35.9</td>
</tr>
<tr>
<td>Protein iodination‡</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phagocytizing yeast</td>
<td>2.573 ± 0.711</td>
<td>2.798 ± 0.868</td>
</tr>
</tbody>
</table>

*Number of experiments (three different donors).
†Nanomoles of glucose oxidized by 5 x 10⁶ PMN/60 min.
‡Nanomoles of TCA precipitable iodide/5 x 10⁶ PMN/60 min.

Functional Activities

The rates of phagocytosis at the midpoint of the ingestion phase of S. aureus 502A (bacteria:cell = 10:1) were equivalent (Table 3). The slight increase in phagocytosis associated with the CFLL cells is not statistically significant. Similarly, intracellular killing of S. aureus 502A at the end of a 60-min incubation period was similar for control and filtered leukocytes (96.5% killed by the CFLL PMN compared to 95.3% killed by the control PMN).

Chemotaxis

Previous adherence of cells to nylon wool or preparation times of up to 8 hr before assay did not affect chemotactic responses (Table 4). There was a greater number of PMNs on the lower filter for CFLL cells; however, the differences proved not to be significant (p > 0.2 for CPM on lower filter, p > 0.2 for per

Table 3. Phagocytic and Bactericidal Activities of CFLL and Control PMN

<table>
<thead>
<tr>
<th></th>
<th>CFLL (3)* (Mean ± SE)</th>
<th>Control (3)* (Mean ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phagocytosis†</td>
<td>27.1 ± 1.5%</td>
<td>23.0 ± 1.2%</td>
</tr>
<tr>
<td>Bactericidal‡</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>8.9 x 10⁶ ± 3.8 x 10⁶</td>
<td>8.2 x 10⁶ ± 2.5 x 10⁶</td>
</tr>
<tr>
<td>30</td>
<td>1.8 x 10⁶ ± 0.9 x 10⁶</td>
<td>2.6 x 10⁶ ± 1.2 x 10⁶</td>
</tr>
<tr>
<td>60</td>
<td>6.3 x 10⁵ ± 3.3 x 10⁵</td>
<td>8.4 x 10⁵ ± 4.4 x 10⁵</td>
</tr>
</tbody>
</table>

*Number of experiments (three different donors).
†Per cent uptake of added S. aureus 502A at the end of an 8-min incubation period by 5 x 10⁶ PMN/ml.
‡Number of bacteria per milliliter surviving. The inoculum size was 1.8 x 10⁷ bacteria/ml. Lysostaphin was added at 20 min.

Table 4. Chemotaxis of PMNs Prepared by CFLL Technique

<table>
<thead>
<tr>
<th>Collection Method</th>
<th>cpm/10⁶ PMN on Lower Filter</th>
<th>Per cent Chemotaxis</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFLL</td>
<td>3021 ± 901</td>
<td>26.1 ± 2.5</td>
</tr>
<tr>
<td>Donor</td>
<td>2553 ± 351</td>
<td>18.2 ± 2.8</td>
</tr>
<tr>
<td>Laboratory control</td>
<td>2432 ± 353</td>
<td>18.4 ± 3.1</td>
</tr>
</tbody>
</table>

*Results of four experiments.
†Per cent chemotaxis = CPM lower filter / CPM upper filter + CPM lower filter
‡Mean ± SEM.
cent chemotaxis when CFFL compared to donor, and \( p > 0.1 \) when compared to laboratory control).

**DISCUSSION**

When compared to other methods of collecting granulocytes for transfusion, CFFL is a relatively simple, fast, and inexpensive method.\(^6\) Cell yields from a single normal donor are five- to tenfold greater than reported for the technique of continuous-flow centrifugation (CFC). For example, with our technique of CFFL, a median yield at the midpoint of collection was \( 4.0 \times 10^{10} \) (range, \( 1.4 \times 10^{10} - 6.0 \times 10^{10} \)). This figure represents the count after only four filters (9.6 liters of blood filtered) and is usually less than half of the final total at the end of a donation.\(^1^3\) The median harvests of PMNs using the method of CFC are reported to be anywhere from \( 1.75 \times 10^{10} \) to \( 4.4 \times 10^{10} \) per donation.\(^1^4 - 1^6\) Furthermore, with the use of CFFL very low percentages of lymphocytes are obtained, and only Benbunan et al.\(^1^6\) have obtained comparable percentages with the use of CFC.

With these advantages CFFL might be the method of choice in the preparation of PMNs for transfusion. However, it is important that the functions of the PMNs separated by this method are not impaired. The studies of Herzig et al.\(^7\) indicated that the in vitro functions (and in vivo survival) of PMNs separated from whole blood by a CFFL technique were impaired. Since their collection method differed in some respects from ours, the present study was performed using assays similar to those employed by Richard K. Root in the studies of the cells prepared by Herzig and associates. Additionally, these investigations represent the first study of the chemotactic responses of these cells using a highly reproducible and sensitive technique.

Our in vitro investigations indicate that CFFL-prepared PMNs respond normally to a chemotactic stimulus, ingest and kill staphylococci at normal rates, and they form and utilize the important microbicidal substance hydrogen peroxide normally. Thus, a brief period of adherence of PMNs to a nylon-wool filter or to each other on the filter does not cause detectable alterations in those granulocyte functions important to the normal inflammatory response and to antimicrobial activity. Further, preservation of the PMNs at \( 4^\circ C \) for periods up to 8 hr did not cause a measurable impairment of chemotactic responses.

The better viability and function of the PMNs in the present study compared to those of Herzig et al. might be explained by the differences in the methods of CFFL employed. When eluting the granulocytes from the filter, Herzig et al. used between 1000 and 1500 cc ACD plasma or a 20% ACD saline solution per filter as well as periodically occluding the exit tubing in order to increase the pressure in the filter (thereby stopping flow). We used only 600 ml of ACD plasma (75 ml ACD/500 ml plasma) per filter and maintained a constant flow and pressure by using an inflatable cuff around the bag of plasma. Secondly, Herzig et al. concentrated the PMNs by centrifuging at 2500 rpm for 5 min at \( 20^\circ C \), compared to 500 rpm (60 g) for 20 min at \( 10^\circ C \) in our experiments. Finally, the method described differs from Djerassi's original description\(^6\) in that it is now semiautomated. This allows for a faster collection, thus shortening the time the PMNs remain on the filter.
The present investigations indicate that PMNs prepared by the described CFFL technique can be obtained in high numbers, with little contamination by other cell types and good preservation of in vitro functional activities. In order to effectively enhance the inflammatory response in granulocytopenic patients, they must be demonstrated to have adequate in vivo survival and functional activities. Studies are now underway to evaluate the best method of administration, the kinetics of their survival in the circulation, their entry into areas of inflammation, and their ability to enhance survival from infection in granulocytopenic subjects beyond that achieved by antimicrobial therapy alone.

REFERENCES

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