Effects of Temperature on Granulocyte Preservation

By Jeffrey McCullough, Barbara J. Weiblen, Phillip K. Peterson, and Paul G. Quie

With the increasing use of granulocyte transfusion it is becoming important to determine if granulocytes can be preserved for a few days. If so, the optimum storage conditions must be identified. We studied the function in vitro of granulocytes collected as they would be for transfusion by continuous-flow centrifuge leukapheresis (CFCL) and filtration leukapheresis (FL). Granulocytes collected by CFCL maintained normal ability to phagocytose and kill bacteria after 48 hr and normal chemotaxis after 24 hr of storage at 20°–24°C. Neither 1°–6°C nor 37°C were as effective in preserving chemotactic response. Agitation of the granulocyte suspension during storage caused reduced bacterial killing and chemotaxis. Granulocytes collected by FL functioned very poorly after 24 hr storage at all temperatures studied. These studies suggest that it may be possible to store CFCL granulocytes at 20°–24°C for 24 hr. FL granulocytes should not be stored at all.

TRANSFUSION of granulocytes has gained widespread use in the clinical management of infected granulocytopenic patients. Granulocyte collection is cumbersome, and the logistics of donor scheduling often make it difficult to transfuse granulocytes immediately after collection, thus making storage necessary. It is important to develop techniques for the short-term liquid preservation of granulocytes and to identify the changes that occur in granulocyte function during storage.

Studies in vitro carried out during the development of whole blood anticoagulant preservative solutions indicated that granulocytes remain functionally normal for only a few hours in stored citrated blood.1 However, it appears that granulocytes collected by phlebotomy maintain function in vitro at least during the first 24 hr of storage at refrigerator temperatures.2-7 Granulocytes for transfusion are collected by centrifugation or reversible adhesion to nylon fibers. Immediately after collection, granulocytes obtained from normal donors by continuous flow centrifuge leukapheresis (CFCL) are functionally normal,8,9 while granulocytes collected by filtration leukapheresis (FL) may have a slight but significant reduction in bactericidal capacity.3,10 Because these collection techniques may alter the granulocytes, decisions concerning transfusion of stored granulocytes should be based on studies of cells collected by centrifugation or filtration techniques rather than ordinary phlebotomy.

Our studies of the effect of the storage temperature on chemotaxis, phagocytosis, and bacterial killing by granulocytes collected by CFCL and FL form the basis of this report.
MATERIALS AND METHODS

This study was approved by the University of Minnesota Committee on the Use of Human Subjects in Research. After informed consent was obtained, granulocytes were obtained from normal adult donors by three methods: CFCL, FL, and ordinary phlebotomy.

CFCL was performed as previously described.11 Approximately 9000 ml whole blood were processed through the Celltrifuge (American Instrument, Silver Spring, Md.) during the 3½-hr procedure, and the donor received 4500–5000 units heparin and 400–500 ml sodium citrate hydroxyethyl starch solution. No other medication was given to the donors. The granulocyte concentrate was divided into eight equal parts and stored in Fenwal TA-3 150-ml transfer bags. Additional CPD anticoagulant was added to four of the bags at a ratio of 2.16 ml CPD to 20 ml granulocyte concentrate. One bag of granulocyte concentrate with extra CPD and one without were stored under each of the four temperatures to be described later.

FL was performed as described by Herzig et al.12 Approximately 9000 ml whole blood was processed through the system (Filtration Leukapheresis Set; Fenwal Laboratories, Morton Grove, Ill.) during the 2½-hr procedure and the donor received approximately 15,000 units heparin. Protamine sulfate 50 mg was administered at the end of the procedure. No other medication was given to the donors. The granulocytes were eluted from the filters using 1500 ml ACD plasma saline (pH 6.5) composed of 1000 ml normal saline, 220 ml CPD fresh-frozen plasma, and approximately 50 ml ACD-A. During elution the filters were tapped gently and the eluate concentrated by centrifugation at 200 g for 10 min. The granulocyte concentrate obtained was divided into eight equal portions in Fenwal TA-3 150-ml transfer bags. CPD fresh-frozen plasma was added to four of these bags in a volume equal to the volume of the granulocyte concentrate in each bag. Thus, each bag contained approximately 30 ml granulocyte concentrate in its original eluting solution plus 30 ml CPD fresh-frozen plasma for a final concentration of 60% CPD plasma. One bag of granulocyte concentrate containing the CPD plasma and one containing the original eluting solution (20% plasma) were stored under each of the four temperature conditions.

Phlebotomy. As a control, granulocytes were collected by ordinary phlebotomy13 of 450 ml whole blood into plastic bags containing 63 ml CPD anticoagulant (Fenwal Quad Pack). The unit of whole blood was divided into four equal parts by transferring 110 ml into each of the three satellite bags. Each portion was then stored under one of the four different temperature conditions.

Temperature conditions during storage. Granulocytes were stored under four temperature conditions: (1) in a standard blood bank refrigerator at 1°–6°C; (2) at room temperature (20°–24°C) without agitation; (3) at room temperature (20°–24°C) with continuous gentle agitation; and (4) in a dry-air microbiologic incubator at 37°C. The effect of agitation during storage at 20°–24°C was investigated because of the beneficial effects on platelet function during similar storage.

Granulocytes were studied within 4 hr of collection and daily thereafter for up to 72 hr. Samples for study were removed from the storage bag through a sterile injection site. Leukocyte counts and pH measurements were made each time the granulocyte concentrate was tested. Granulocyte function was measured in vitro using a bactericidal assay, a phagocytosis assay, and a chemotaxis assay.

The bactericidal assay4 consisted of incubating 1 × 10⁶ granulocytes with 5 × 10⁸ Staphylococcus aureus 502A. Fresh-frozen human serum was used as a standard source of opsonin. The phagocytic mixtures were incubated with end-over-end rotation in plastic tubes to insure maximum contact between bacteria and granulocytes. The number of viable bacteria was determined immediately after bacteria were added to the phagocytic mixture and during incubation by removing aliquots with a calibrated loop. Duplicate samples were diluted in water, and pour plates were made. Following overnight incubation, the number of colony-forming bacteria was determined. Results are expressed as percent bacteria killed after 120 min incubation in the assay system as compared with a normal control:

\[
\text{% normal control killing} = \frac{\text{bacteria killed (%) at 120 min by test granulocytes}}{\text{bacteria killed (%) at 120 min by fresh control granulocytes}} \times 100.
\]

The phagocytosis test was performed as described by Peterson et al.14 S. aureus 502A were grown overnight in Mueller-Hinton broth containing 40 μCi ³H-thymidine. The labeled bacteria were washed three times and resuspended to a concentration of 5 × 10⁸/ml in phosphate-buffered saline (PBS) (pH 7.4). A 0.1-ml portion of bacteria was opsonized for 15 min at 37°C with fresh-
frozen pooled human serum. Granulocytes were isolated by dextran sedimentation of red cells, washed twice with saline, and resuspended to a concentration of \(1 \times 10^7\) cells/ml in Hanks' balanced salt solution (HBSS) with gelatin. The assay mixture consisted of 0.5 ml granulocyte suspension, 0.4 ml gel HBSS, and 0.1 ml labeled opsonized bacteria. The assay tubes were incubated with end-over-end rotation at 37°C, and 100-μl samples were taken at 3, 10, and 20 min for determination of leukocyte-associated bacteria by means of differential centrifugation at 160 g. At 20 min duplicate samples were taken to determine the total bacterial population by placing the samples in water and centrifuging at 1600 g. The resulting pellets were suspended in scintillation fluid and counted in a liquid scintillation counter. The percent bacteria phagocytized at each sampling time was determined as follows:

\[
\% \text{ bacteria phagocytized} = \frac{\text{Average CPM in leukocyte pellet}}{\text{Average CPM in total bacterial pellet}} \times 100.
\]

Granulocyte chemotaxis was performed using a modification of the Boyden technique as previously described.\(^5\) A standardized granulocyte suspension consisting of \(8 \times 10^8\) granulocytes suspended in Medium 199 (Microbiological Associates, Bethesda, Md.) was utilized. The chemotactic activity of granulocytes was tested using \(E. coli\) as the chemotactic attractant. Chemotaxis chambers were incubated for 3 hr at 37°C. The filters were then removed, stained with hematoxylin, and examined for the number of granulocytes that had passed from one side of the filter to the other. All tests were run in triplicate, and the number of granulocytes per ten high power fields (HPF) was determined. Results are reported as percentage of a normal control:

\[
\% \text{ normal control chemotaxis} = \frac{\text{No. of test granulocytes/ten HPF}}{\text{No. of fresh normal control granulocytes/ten HPF}}
\]

\(pH\) was measured using a microelectrode immediately after collection and approximately every 24 hr thereafter by removing 5 ml of granulocyte concentrate from the storage bag.

**Controls.** Two different kinds of controls were used in this study. Seven milliliters of whole blood were collected from normal blood donors into heparinized vacutainer tubes and provided granulocytes used as controls. These were always used within 6 hr of collection and were the controls used to calculate the data reported in Table 1. In order to control for the effects of the collection method, 5 ml of granulocyte concentrate were removed from the storage bag, and the pH was measured immediately after collection and approximately every 24 hr thereafter.

**Table 1.** Percent of Normal Control Bacterial Killing and Chemotactic Activity (Mean ± SD) by Granulocytes Collected by Phlebotomy as CPD Whole Blood, by CFCL, and by FL and Stored at Different Temperatures

<table>
<thead>
<tr>
<th>Method of Collection</th>
<th>No. of Granulocyte Concentrates Studied</th>
<th>Storage Temperature (°C)</th>
<th>Bactericidal Killing</th>
<th>Chemotactic Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>0 24 48</td>
<td>0 24 48</td>
</tr>
<tr>
<td>Phlebotomy</td>
<td>5 Fresh</td>
<td>100</td>
<td>84 ± 7†</td>
<td>96 ± 5</td>
</tr>
<tr>
<td></td>
<td>1–6</td>
<td>104 ± 9</td>
<td>91 ± 15</td>
<td>80 ± 11</td>
</tr>
<tr>
<td></td>
<td>20–24</td>
<td>104 ± 6</td>
<td>97 ± 13</td>
<td>87 ± 19</td>
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<tr>
<td></td>
<td>20–24 agitated</td>
<td>104 ± 8</td>
<td>94 ± 12</td>
<td>90 ± 17</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>92 ± 14</td>
<td>27 ± 12*</td>
<td>84 ± 16</td>
</tr>
<tr>
<td>CFCL</td>
<td>5 Fresh</td>
<td>104 ± 7†</td>
<td>96 ± 5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1–6</td>
<td>107 ± 8</td>
<td>106 ± 7</td>
<td>63 ± 16*</td>
</tr>
<tr>
<td></td>
<td>20–24</td>
<td>96 ± 9</td>
<td>91 ± 8</td>
<td>101 ± 17</td>
</tr>
<tr>
<td></td>
<td>20–24 agitated</td>
<td>93 ± 13</td>
<td>83 ± 17*</td>
<td>91 ± 22</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>7 ± 14*</td>
<td>0*</td>
<td>43 ± 17*</td>
</tr>
<tr>
<td>FL†</td>
<td>4 Fresh</td>
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<td>106 ± 11</td>
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<td>NT</td>
<td>29 ± 7*</td>
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<td>20–24</td>
<td>40 ± 18*</td>
<td>NT</td>
<td>22 ± 2*</td>
</tr>
<tr>
<td></td>
<td>20–24 agitated</td>
<td>44 ± 31*</td>
<td>NT</td>
<td>28 ± 7*</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>23 ± 30*</td>
<td>NT</td>
<td>13 ± 10*</td>
</tr>
</tbody>
</table>

NT, not tested.

*Values are significantly (\(p < 0.05\)) different from the corresponding cells tested immediately after collection (fresh).
tion technique (CFCL and FL), normal donors underwent phlebotomy of 450 ml of whole blood and function studies were done in vitro on these granulocytes. These cells are referred to as phlebotomy granulocytes.

RESULTS

Bacterial killing. Immediately after collection, granulocytes collected by phlebotomy and by CFCL killed 100% and 104%, respectively, of the bacteria killed by control cells (Table 1). Granulocytes collected by FL killed only 84% of the bacteria in the assay. As we reported previously,\(^9\) this was a statistically significant \((p < 0.05, F\) test) reduction. After 24 hr of storage at 1°-6°C or 20°-24°C, phlebotomy and CFCL granulocytes still killed 93%-107% of the bacteria killed by control cells (Table 1). After storage for 24 hr at 37°C, phlebotomy granulocytes functioned well (92% killing), but CFCL granulocytes killed only 7% of the number of bacteria killed by control cells, a significant reduction \((F\) test, \(p < 0.05\)). CFCL granulocytes stored with additional CPD anticoagulant gave results similar to those without. Thus all the studies of CFCL cells were combined and averaged (Table 1).

When granulocytes collected by FL were stored for 24 hr in 60% CPD plasma, bacterial killing was significantly decreased (23%-52%, \(p < 0.05\)) regardless of the storage temperature. As with CFCL granulocytes, killing was poorest by cells stored at 37°C. Granulocytes stored in the eluting solution (20% plasma) killed even fewer bacteria, and these data are not included here. Because of these poor results, FL granulocytes were not studied after 48 hr storage.

After 48 hr storage at 1°-6°C or 20°-24°C, granulocytes collected by phlebotomy (and stored as whole blood) and CFCL concentrates still functioned well, killing 83%-106% of the bacteria killed by control granulocytes (Table 1). There was a significant decrease \((p < 0.5)\) in bacterial killing by CFCL granulocytes stored at 20°-24°C with agitation, and cells stored under other conditions

![Fig. 1. Percentage of bacteria phagocytized by granulocytes collected by CFCL and stored under four different temperature conditions for 48 hr.](image-url)
Phagocytosis. In an effort to determine whether the decrease in bacterial killing that occurred during storage was secondary to impaired phagocytosis or to diminished intracellular killing, phagocytosis of radiolabeled bacteria by stored granulocytes was studied. Only FL granulocytes stored for 24 hr and CFCL granulocytes stored for 48 hr were studied because they had moderately impaired bacterial killing. Results from one of two similar studies of each type of cell are shown in Figs. 1 and 2. No statistical analysis was done, but experience with this assay indicates that differences of 20% from the normal control are significant.

CFCL granulocytes stored for 48 hr at 1–6°C and 20–24°C phagocytized the radiolabeled bacteria as well as did fresh normal control granulocytes (Fig. 1). Thus the slight decrease in bacterial killing by these cells is apparently due to deterioration of intracellular killing mechanisms rather than an inability to phagocytize. CFCL granulocytes stored at 37°C phagocytized very poorly. Whether or not this deterioration in phagocytic ability accounts for the total decrease in bactericidal activity observed in CFCL granulocytes stored at 37°C was not established by these studies.

At all storage temperatures, FL granulocytes stored in 60% CPD plasma had phagocytic activity superior to FL granulocytes stored in the original eluting solution (20% plasma) (Fig. 2). FL granulocytes stored for 24 hr in 60% CPD plasma at either 1–6°C or 20–24°C phagocytized normally, suggesting that the decreased bacterial killing by these cells was due to deterioration of intracellular killing mechanisms. FL granulocytes stored under all other conditions (i.e., 1–6°C, 20–24°C, and 37°C in eluting solution and 37°C in CPD plasma) did not phagocytize normally. Whether or not this loss of phagocytic ability
accounts for all of the loss of bacterial killing by these cells was not established in this study.

**Chemotactic response.** Granulocytes collected by all three methods had normal chemotactic response immediately after collection (Table 1). After 24 hr, phlebotomy granulocytes had no significant reduction (80%–90%) of their chemotactic response regardless of the storage temperature. Granulocytes collected by CFCL retained 101% and 91% of their chemotactic response if stored at 20°–24°C but had significant (p < 0.05) reduction if stored at 1°–6°C or 37°C. Following 48 hr storage, phlebotomy and CFCL granulocytes maintained good chemotactic response when stored at 20°–24°C except for reduced chemotaxis (p < 0.05) by CFCL cells stored with agitation. Neither 1°–6°C nor 37°C preserved chemotactic response well (p < 0.05) for phlebotomy and CFCL cells after 48 hr.

FL granulocytes showed marked and significant (p < 0.05) loss of chemotactic response after 24 hr storage at all temperatures. Similar results were obtained when FL granulocytes were stored in the 20% eluting solution or in 60% CPD plasma.

The effect of pH on granulocyte function was measured, although the study was not designed to specifically evaluate this. There was a more rapid decrease of pH at 20°–24°C than at 1°–6°C (Table 2). This lower pH may actually be helpful by preventing clumping of the granulocytes. The pH of all FL granulocyte concentrates was lower than the corresponding CFCL concentrates. FL granulocytes stored in 20% plasma had a much lower pH than those stored in 60% plasma (Table 2). Poor chemotaxis, phagocytosis, and bacterial

**Table 2. pH of Granulocyte Concentrates Collected by Three Different Methods and Stored Under Four Different Temperature Conditions**

<table>
<thead>
<tr>
<th>Method of Collection</th>
<th>Storage Temperature (°C)</th>
<th>Storage (hr)</th>
<th>As Initially Collected</th>
<th>With Extra CPD Solution or CPD Plasma*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0 24 48</td>
<td>0 24 48</td>
<td></td>
</tr>
<tr>
<td>Phlebotomy</td>
<td>Fresh</td>
<td>7.2 — —</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1–6</td>
<td>— 7.2 7.1</td>
<td>— 7.2 7.1</td>
<td></td>
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<tr>
<td></td>
<td>20–24</td>
<td>— 7.0 6.9</td>
<td>— 7.0 6.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>20–24 agitated</td>
<td>— 7.0 6.9</td>
<td>— 7.0 6.9</td>
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<tr>
<td></td>
<td>37</td>
<td>— 6.8 6.7</td>
<td>— 6.8 6.7</td>
<td></td>
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<tr>
<td>CFCL</td>
<td>Fresh</td>
<td>7.3 — —</td>
<td>7.0 — —</td>
<td></td>
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<tr>
<td></td>
<td>1–6</td>
<td>— 7.3 7.2</td>
<td>— 7.0 7.0</td>
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<tr>
<td></td>
<td>20–24</td>
<td>— 7.1 7.1</td>
<td>— 6.9 6.7</td>
<td></td>
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<tr>
<td></td>
<td>20–24 agitated</td>
<td>— 7.2 7.2</td>
<td>— 7.0 6.8</td>
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<tr>
<td></td>
<td>37</td>
<td>— 7.1 6.8</td>
<td>— 6.7 6.2</td>
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<tr>
<td>FL</td>
<td>Fresh</td>
<td>6.6 — —</td>
<td>7.0 — —</td>
<td></td>
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<tr>
<td></td>
<td>1–6</td>
<td>— 6.5 6.3</td>
<td>— 7.0 6.9</td>
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<td></td>
<td>20–24</td>
<td>— 6.15 5.8</td>
<td>— 6.9 6.6</td>
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<tr>
<td></td>
<td>20–24 agitated</td>
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<td>37</td>
<td>— 5.9 5.75</td>
<td>— 6.5 6.15</td>
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</tr>
</tbody>
</table>

NA, not applicable.

*Data in these columns refer to storage in extra CPD solution for CFCL cells and 60% CPD plasma for FL cells.
Role of insulin-stimulated protein phosphorylation in insulin action$^{1,2}$

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are reduced at temperatures below 37°C. Because of immobilization of the granulocyte and slowing of metabolic processes that occur near 0°C it might be advantageous to store granulocytes under the standard 1°–6°C temperatures adopted for RBC preservation. The success of this preservation, however, is assayed under physiologic conditions. In the present study, granulocytes were stored at various temperatures but their function was tested in a balanced salt solution at physiologic temperature and pH.

More than 20 years ago James Tullis showed that granulocytes stored at 4°C maintained amoeboid activity for at least 7 days, phagocytosis for 3 days, and oxygen utilization for 4 days. More recently, we showed that bactericidal activity and chemotactic response are well maintained by granulocytes collected by phlebotomy and stored at 1°–6°C as CPD whole blood. Crowley and Valeri found only a small loss of phagocytosis, oxygen consumption, and mobility of granulocytes stored for 24 hr at 1°–6°C, and hexose monophosphate shunt activity remains 90% of normal in granulocytes similarly stored.

We found no loss of bacterial killing, NBT reduction, or oxygen consumption by granulocytes collected by CFCL and stored 24 hr at 4°C. Granulocytes collected by FL had decreased bacterial killing but normal NBT reduction and oxygen consumption. CFCL and FL granulocytes showed slight decreases in chemotaxis after 24 hr storage at 4°C. More recently, Glasser showed that granulocytes collected by discontinuous flow centrifugation (Model 30 Blood Processor, Haemonetics Corporation, Natick, Mass.) do not have a significant loss of number of cells, phagocytic ability, candidicidal activity, or chemotaxis during the first 24 hr storage at 4°–6°C.

In the present study, phagocytosis and bacterial killing by granulocytes collected by CFCL was well maintained for 48 hr at either 1°–6°C or 20°–24°C without agitation, and chemotactic response was well maintained for 24 hr at 20°–24°C but not 1°–6°C. During such storage the mild loss of bactericidal capacity that occurs is due to deterioration of intracellular killing mechanisms, not phagocytic ability. Agitation appears to be detrimental during 20°–24°C storage of granulocytes.

Because granulocytes collected by FL had poor phagocytosis, bacterial killing, and chemotactic responses following 24 hr storage regardless of the temperature, they should not be stored. Contact of the cells with the eluting solution should be minimized, since such contact was detrimental to phagocytosis and bacterial killing.

Bryant et al. and Phelps and Stanislaw found essentially no effect of pH differences between 7.0 and 7.6 on leukocyte migration. However, granulocyte mobility decreased markedly as the pH fell below 7.0. In the present study granulocytes from some concentrates stored at pH 6.7–6.9 functioned well in vitro (assayed at pH 7.4) while others at pH of 7.1–7.3 did not. It is difficult to evaluate these data, since storage temperature was an additional variable. Further studies are underway in our laboratory to evaluate the effects of pH as an isolated variable.

If granulocytes have been preserved optimally they should function well, but in addition normal numbers of cells should be recovered in the circulation and the cells should leave the intravascular space at the normal rate and by the usual mechanisms.
Price and Dale, using a rabbit model, found that granulocytes collected by phlebotomy and stored at either room temperature or 4°C for 24 hr or longer had reduced posttransfusion recovery and were temporarily sequestered. After 24 hr storage at the two temperatures, differences were small; however, by 48 and 72 hr storage, posttransfusion recovery of granulocytes stored at room temperature was better than that of cells stored at 4°C.

No studies of the intravascular kinetics of human granulocytes collected by CFCL or FL and stored at 20°–24°C have been reported. Although the report of Price and Dale indicates that in rabbits 1°–6°C storage produces better recovery in vivo of stored cells, our studies indicate that function in vitro is better maintained when granulocytes are stored at 20°–24°C.

Transfusion of stored granulocytes may have some clinical ramifications. Cell damage during storage may release enzymes and pyrogens into the supernate resulting in febrile reactions in recipients. Stored granulocytes may have altered antigenicity. Finally, obtaining sufficient numbers of granulocytes for transfusion requires careful and diligent technique. Even though the loss of cell number and function seems minimal during storage, this may be enough to interfere with the therapeutic value of the transfusion.

We believe that it may be possible to store granulocytes for up to 24 hr; however, (1) every effort should be made to transfuse granulocytes as soon as possible after collection, (2) if CFCL granulocytes are to be stored, 20°–24°C may be the optimum temperature, (3) contact between granulocytes collected by FL and the eluting solution should be kept to a minimum, and (4) granulocytes collected by FL should not be stored at all.

REFERENCES

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Effects of temperature on granulocyte preservation

J McCullough, BJ Wieblen, PK Peterson and PG Quie