Granulocyte Concentrate Function During Preservation: Effect of Temperature

By Thomas A. Lane and Bradford Windle

Granulocyte concentrates collected from normal donors are necessarily stored for varying intervals up to the time of transfusion. However, information regarding the fate of collected cells and the optimal mode of storage in vitro in the interval between collection and transfusion is far from complete. We studied granulocyte function during preservation of granulocyte concentrates for up to 72 hr. The initial and most consistent alteration in granulocyte function during storage was failure of random migration and chemotaxis after 24 hr of storage (50% and 81% of normal, respectively). By 48 hr the respiratory burst was decreased by 42%, whereas at 48 hr phagocytic and bactericidal activities were nearly normal. Defects in migration and respiratory burst are not due to delayed activation of these functions but to absolute decreases in maximum rates of migration and oxygen consumption. Comparison of granulocyte concentrate storage at 6°C versus room temperature indicated at 24 hr an improved (p < 0.02) but still abnormal (p < 0.02) chemotactic response with 24°C storage and at 48 hr no difference in migration but a slight advantage in bacterial killing at 6°C storage. These studies show that severe impairment of granulocyte function occurs within 24 hr of collection by centrifugal means; consequently, granulocyte concentrates should be transfused as soon as possible after collection.

With the development of efficient methods of collecting normal granulocytes for transfusion, it has become inevitable that the concentrates will be stored for varying periods of time before transfusion. Since a major goal of the Leukapheresis Unit is to provide large numbers of effectively functioning cells, it is important to ascertain what changes take place in granulocyte function in the interval between collection and transfusion. Accordingly, investigators have shown that cells collected by centrifugal means function normally after collection. In studies of granulocytes stored in whole blood it was found that one of the most highly integrated functions of granulocytes (chemotaxis) was the first to deteriorate in vitro, whereas other granulocyte functions such as bactericidal activity were normal through 72 hr of storage. On the other hand, chemotactic studies of stored granulocytes separated from leukapheresis concentrates have shown both normal and abnormal migration after 24 hr.

The present studies were undertaken using a model system to define alterations that occur in granulocyte functions and storage medium during short-term liquid preservation of granulocyte concentrates and to examine the influence of alternative storage temperatures (6°C versus 24°C) on subsequent granulocyte function.

MATERIALS AND METHODS

We studied granulocyte concentrate storage using buffy-coat preparations from fresh units of blood. Initial studies (to be reported separately) showed that buffy-coat granulocytes functioned similarly to...
granulocytes separated from fresh heparinized whole blood with respect to phagocytosis, oxygen consumption, bactericidal capacity, random migration, and chemotaxis. In addition, in two separate experiments, granulocytes collected from the same donor on the same day by the buffy-coat technique (vide infra) and by the discontinuous-flow centrifuge (Haemonetics, Natick, Mass.) had similar rates of phagocytosis, oxygen consumption, random migration, and chemotaxis after 4, 24, and 48 hr of storage at either room temperature or 6℃.

Within 4 hr of collection, units of fresh donor CPD blood were centrifuged at 3800 rpm on an RC-3 centrifuge for 2 min. The supernatant platelet-rich plasma was expressed into a satellite bag, and the buffy-coat layer was expressed into a second satellite bag. The buffy-coat preparation, consisting of a volume of approximately 45 ml with a granulocyte concentration of $3.3 \pm 0.5 \times 10^4$/ml, was used in further studies. In temperature comparison experiments, equal aliquots of this preparation were transferred aseptically into 150-ml transfer bags (Fenwall, Deerfield, Ill.) and stored at 6℃ or room temperature (24℃).

Cell Preparation

Granulocytes were purified by sedimentation at room temperature for 30 min with one-half volume of 6% hydroxyethyl starch in saline (McGaw Laboratories, Irvine, Calif.). The supernatant leukocyte-rich plasmas were centrifuged for 5 min at 150 g. Erythrocytes were lysed by addition of 5 ml of 0.2% NaCl for 30 sec, followed by 5 ml of 1.6% NaCl. The cells were washed twice in 0.9% NaCl and resuspended in Krebs buffer to a volume of 2 ml. Cell counts and differentials were performed using standard techniques, and unless otherwise indicated the leukocyte suspension was diluted to 107 granulocytes/ml.

Respiratory Burst

Singlet oxygen generation was evaluated by the chemiluminescence method of Johnston et al., with minor modifications. The assay was carried out in siliconized glass vials kept in the dark for 24 hr and then exposed only to red light. The assay system consisted of 1 ml of granulocytes (107/μl) and 1 ml of opsonized zymosan (10 mg/ml) (Sigma, St. Louis, Mo.) prepared by the method of Wardlaw and Pillimer. A Packard Tri-Carb model 3375 scintillation counter was allowed to come to room temperature, and the coincidence mode was turned off. Counts were taken in a narrow range in the tritium region. Blank vials were always less than 2000 cpm, and the background counts were subtracted from final readings. Samples in which either opsonized zymosan or leukocytes were omitted resulted in counts no higher than background. Experimental samples resulted in counts 7.5 to 10 times higher than the background or blank-vial counts. All samples were performed in duplicate.

Oxygen consumption was determined using a YSI model 53 biological oxygen monitor (Yellow Springs Instruments, Yellow Springs, Ohio). All sample chambers were siliconized and carefully cleaned and dried prior to each use. Granulocytes (0.5 x 107) in a volume of 0.5 ml were added to 2 ml of Krebs buffer with 2% dextrose and 10% fresh pooled human serum and allowed to equilibrate at 37℃ for 5 min. Progressive changes in oxygen tension were measured using a platinum oxygen electrode embedded in a tight-fitting lucite plunger that was inserted into a sample chamber from which air bubbles were excluded. The signal was amplified to the 100-mV range and recorded continuously. The recorder was set to full scale for each experiment with an electrode in contact with distilled water at 37℃ and equilibrated with room air (PO2 140-150 mm Hg), so that a 20% change in oxygen saturation resulted in a full-scale deviation. The initial measurement determined the oxygen tension of the leukocyte suspension relative to distilled water. The rate of oxygen consumption was determined for 5 min before and for 12 min after the introduction of 0.4 ml of opsonized zymosan particles suspended in Krebs buffer at 10 mg/ml. Samples were run in duplicate, and the variation between duplicate samples was less than 10% in all cases. Oxygen consumption was expressed as microliters of oxygen consumed per 107 cells per hour.

Phagocytosis

At exactly 12 min after the introduction of opsonized zymosan particles into the oxygen monitor cuvette, a sample of the suspension was removed, immediately smeared, and air dried. Slides were stained using Wright's stain and examined for phagocytosis. Two hundred granulocytes were counted. Phagocytosis is expressed as the phagocytic index (number of yeast particles ingested per 200 granulocytes). Yeast particles were considered as ingested only if there was obvious nuclear deformation.
of the granulocyte. Potassium cyanide (0.3 ml of 10-mM solution) resulted in no appreciable difference in oxygen consumption or phagocytosis.

**Microbial Killing**

The bactericidal assay employed a method similar to that of Quie et al.\textsuperscript{13} *Escherichia coli* (ATCC-25922 obtained from the hospital laboratory) were grown for 18 hr in trypticase soy broth and suspended to an optical density of 0.3 at 615 nm. A 1:100 dilution of this suspension contained 3–5 × 10^6 viable bacteria per milliliter. Equal volumes (0.5 ml) of this suspension and granulocyte suspension (10^5 granulocytes/ml) in Krebs buffer with glucose and 10% fresh serum were placed in sterile plastic tubes with caps and incubated at 37°C on a tilting table aliquot mixer for 2 hr. At zero time, at 60 min, and at 90 min, 0.1 ml was removed from each tube and diluted 100-fold in sterile distilled water. Serial 100-fold dilutions were performed, and viable bacteria were quantitated by the standard pour-plate technique. Controls consisted of tubes with granulocytes and serum but no bacteria and tubes with bacteria and serum but no granulocytes.

**Cell Migration**

Random migration and chemotaxis were determined using a modification of the migration-under-agarose assay, as described by Chenoweth et al.\textsuperscript{14} Slides were cleaned in ethanol/HCl, coated with 0.2% gelatin (Difco, Detroit, Mich.), air dried, and coated with agarose gel consisting of complete basal Eagle's medium with Earl's salts (Microbiological Associates, Bethesda, Md.), antibiotics, and glutamine (2x) mixed with an equal volume of 0.9% agarose (Marine Colloids, Rockland, Maine) and 0.5% gelatin in distilled water. The gels were allowed to harden for 15 min, after which wells were cut. Each slide contained six columns of wells consisting of three rows each. The central well of each column contained granulocytes, the upper well contained either buffer or a dilution of chemotactic agent, and the lower well contained buffer. Some 3–4 × 10^5 PMN were placed in each central well. The chemotactic agents used in this study were zymosan-activated serum (neat, 1:2, 1:5, 1:10 dilutions) and N-formyl-methionine-phenylalanine (10^-4, 5 × 10^-5, 2.5 × 10^-5, 10^-3 M) (Sigma). Quadruplicate slides consisting of four concentrations of chemotactic agent each were placed in moist chambers and incubated at 37°C in a CO2 incubator for 2 hr, after which the slides were fixed with methanol, stained with Wright's stain, and read under a microscope using an ocular micrometer (100 divisions/mm). Slides were read using the cell front method. Random migration and chemotaxis were expressed as absolute distances in millimeters (A mm × 10^-2) of the moving cell front from the edge of the granulocyte well. In agreement with the experience of others, examinations using the cell density method\textsuperscript{15} revealed similar data.

Measurements of pH and glucose concentration were performed by the hospital laboratory using Radiometer and Technicon AA-2 equipment.

Statistical analyses were performed using the paired t test. In the text, each “experiment” refers to a separate unit.

**RESULTS**

Units were stored at 6°C, and at 24-hr intervals granulocyte function studies were repeated. Results on 10 such units are summarized in Table 1. After 24 hr there was progressive loss of granulocytes in the concentrates, and variable but progressive decreases occurred in all granulocyte functions. Overall percentages of neutrophils, monocytes, and lymphocytes did not change during storage. Granulocyte migration to N-formyl-methionine-phenylalanine was the earliest and the most severely affected function influenced by storage. Although the ratio of peak migration to random migration was not decreased by storage, the absolute distances of both random migration and directed migration were profoundly decreased at only 24 hr of storage at 6°C (to 50% and 61% of control, respectively), with further reductions at 48 hr (to 27% and 33% of control, respectively). Next, chemiluminescence and oxygen consumption were decreased at 48 hr (55% and 58% of control, respectively). It is of note that decreases in oxygen consumption
GRANULOCYTE FUNCTION DURING STORAGE

Table 1. Preservation of Granulocyte Concentrates (10 Units)

<table>
<thead>
<tr>
<th>Hours Storage at 6°C</th>
<th>&lt;4</th>
<th>24</th>
<th>48</th>
<th>72</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMN</td>
<td>3.32 ± 0.5%</td>
<td>2.85 ± 0.3%</td>
<td>3.70 ± 0.5%</td>
<td>2.43 ± 0.4%</td>
</tr>
<tr>
<td>PI</td>
<td>4.6 ± 0.3%</td>
<td>5.5 ± 0.4%</td>
<td>4.0 ± 0.2%</td>
<td>3.0 ± 0.2%</td>
</tr>
<tr>
<td>SOC</td>
<td>72 ± 6%</td>
<td>68 ± 6%</td>
<td>41 ± 4%</td>
<td>37 ± 4%</td>
</tr>
<tr>
<td>CL</td>
<td>12,500</td>
<td>11,500</td>
<td>11,000</td>
<td>10,500</td>
</tr>
<tr>
<td>BCA</td>
<td>62 ± 2%</td>
<td>63 ± 2%</td>
<td>46 ± 2%</td>
<td>37 ± 2%</td>
</tr>
<tr>
<td>CTX</td>
<td>140 ± 19</td>
<td>85 ± 11</td>
<td>36 ± 9</td>
<td>18 ± 9</td>
</tr>
<tr>
<td>pH</td>
<td>7.048 ± 0.010</td>
<td>6.99 ± 0.014</td>
<td>6.950 ± 0.011</td>
<td>6.906 ± 0.021</td>
</tr>
</tbody>
</table>

*Mean ± SE. Numbers in parentheses are percentage of fresh granulocytes; PMN, concentration of granulocytes remaining in bag × 10^5/µl; PI, phagocytic index, particles/PMN; SOC, stimulated oxygen consumption, µl O_2/10^5 PMN/hr at 37°C; CL, chemiluminescence, cpm at 10 min (5 experiments); BCA, bactericidal activity, percentage bacteria killed at 90 min (mean of 12 experiments); CTX, chemotaxis under agarose; a: peak migration, mm × 10^-2/hr; b: random migration, mm × 10^-2/hr.

The dissociation between phagocytosis and resulting oxygen consumption was most impressive at 48 hr, at which time phagocytosis was essentially normal but oxygen consumption was decreased to 58% of control value. Decreases in oxygen consumption were paralleled by changes in chemiluminescence, which is to be expected. Bactericidal activity was essentially normal at 24 hr of storage and only moderately decreased at 48 hr.

Since decrements in granulocyte chemotaxis and oxygen consumption may have been due to delayed responses of stored granulocytes to chemotactic and phagocytic stimuli, we next examined the time courses of chemotaxis and random migration (4 experiments) and oxygen consumption (10 experiments).

In a typical chemotaxis experiment (Fig.1), migration of fresh granulocytes was apparent 60 min after incubation, and the maximum rate of migration was achieved by 90 min (range 60–120 min). In preliminary time courses using fresh neutrophils, migration was only occasionally noted at 30 min, but migration was always apparent by 60 min; hence 60 min was the first time period examined in subsequent experiments. In cells stored for 24 hr at 6°C, neither the time at which migrating cells were first consistently noted (60 min) nor the time at which maximum migration rate was achieved (90 min) was altered. On the other hand, in stored cells the absolute maximum rates of both random migration (0.4 µ/min) and peak directed migration (9.2 µ/min) were decreased as compared with those of fresh granulocytes (1.9 µ/min and 15.5 µ/min, respectively). Similarly, as shown in Table 2, storage for 24 hr at 6°C did not increase either the lag time of oxygen consumption after addition of opsonized zymosan (0.8 min fresh versus 0.7 min stored) or the time at which the maximum rate of oxygen consumption was achieved (1.6 min fresh versus 1.2 min stored). In contrast, the absolute maximum rate of oxygen consumption was decreased (9.6%/min fresh versus 5.6%/min stored, p < 0.01).

In addition, there were progressive decreases in pH (7.048 ± 0.010 to 6.906 ± 0.021) and glucose concentration (356 ± 7 mg/dl to 267 ± 21 mg/dl) in the supernatant plasma of units stored for 72 hr at 6°C.
Fig. 1. Time course of chemotaxis of fresh and stored granulocytes in response to buffer or increasing concentrations of N-formyl-methionine-phenylalanine. Distance migrated expressed as absolute distance in mm $\times 10^{-2}$ (A mm $\times 10^{-2}$)
Table 2. Rate of Stimulated Oxygen Consumption in Granulocytes During Preservation at 6°C

<table>
<thead>
<tr>
<th>Time of Storage at 6°C</th>
<th>Tp* (min)</th>
<th>Tr (min)</th>
<th>Rmax (mL/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;4 hr</td>
<td>0.8 ± 0.1</td>
<td>1.6 ± 0.12</td>
<td>9.6 ± 0.9%</td>
</tr>
<tr>
<td>24 hr</td>
<td>0.6 ± 0.1</td>
<td>1.2 ± 0.1</td>
<td>9.5 ± 0.1%</td>
</tr>
<tr>
<td>48 hr</td>
<td>0.7 ± 0.1</td>
<td>1.2 ± 0.1</td>
<td>5.6 ± 0.6%†</td>
</tr>
</tbody>
</table>

*Tp time in minutes (± SE) from addition of zymosan to initial evidence of oxygen consumption; Tr time from addition of zymosan to maximum rate of fall in oxygen tension; Rmax, maximum rate of decrease in oxygen tension per minute.

†p < 0.01 (10 experiments).

Since storage of granulocytes at 6°C resulted in poor preservation of function at 24 hr, comparison was made between storage at 6°C and room temperature (24°C). On the day of collection, equal aliquots of granulocyte concentrates from the same unit were gently and aseptically placed into each of two 150-ml plasma transfer bags, and bubbles were excluded. The bags were stored unagitated at 6°C or 24°C. Granulocyte function studies are shown in Table 3. After 24 hr, granulocytes stored at 6°C and 24°C had similar rates of decrease in total granulocyte number, phagocytic index, stimulated oxygen consumption, and bactericidal activity, all of which remained nearly normal. As previously noted, both chemotaxis and random migration were decreased after 24 hr of storage at 6°C. Changing the storage environment to 24°C resulted in significant improvement in both random migration (54 ± 8% of fresh at 6°C versus 70 ± 9% of fresh at 24°C, p < 0.01) and directed migration (62 ± 7% of fresh at 6°C versus 80 ± 7% of fresh at 24°C, p < 0.02). Only 2 of 10 units stored for 24 hr at 6°C had normal (> 90% of fresh) migration. This increased to 5 of 10 units stored at 24°C. Nevertheless, as shown in Fig. 2, 4 units of the remainder stored at 24°C had severely impaired migration (46%–68% of normal). At 48 hr there was further reduction in migration at both temperatures and significant differences in random migration between 6°C and 24°C (p < 0.02). Use of zymosan-activated serum as the cytotaxin resulted in similar defects in cells stored at both 6°C and 24°C for 24 and 48 hr.

Table 3. Preservation of Granulocyte Concentrates at 6°C and 24°C (10 Units) as Percentage of Fresh

<table>
<thead>
<tr>
<th>Storage Time/Storage Temperature</th>
<th>24 hr</th>
<th>48 hr</th>
<th>72 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6°C</td>
<td>24°C</td>
<td>6°C</td>
</tr>
<tr>
<td>PMN*</td>
<td>86 ± 9†</td>
<td>89 ± 9</td>
<td>81 ± 14</td>
</tr>
<tr>
<td>PI</td>
<td>120 ± 9</td>
<td>106 ± 4</td>
<td>96 ± 4</td>
</tr>
<tr>
<td>SOC</td>
<td>95 ± 8</td>
<td>93 ± 7</td>
<td>58 ± 6</td>
</tr>
<tr>
<td>BCA</td>
<td>100 ± 2</td>
<td>94 ± 5</td>
<td>90 ± 6†</td>
</tr>
<tr>
<td>CTX</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>DM</td>
<td>62 ± 7†</td>
<td>80 ± 7</td>
<td>32 ± 5†</td>
</tr>
<tr>
<td>RM</td>
<td>54 ± 8§</td>
<td>70 ± 9</td>
<td>30 ± 6§</td>
</tr>
</tbody>
</table>

PMN, concentration of granulocytes remaining; PI, phagocytic index; SOC, stimulated oxygen consumption; BCA, bactericidal capacity; CTX, chemotaxis (N-formyl-methionine-phenylalanine); DM, directed migration; RM, random migration.

†Percentage of fresh granulocytes ± SE.
§p < 0.02.
§p < 0.01.
10.05 > p > 0.02.
Although there was no significant difference in bacterial killing of granulocyte concentrates stored for 24 hr (0.05 > p > 0.02) at 6°C and 24°C, granulocytes stored at 6°C for 48 hr killed more bacteria than those stored at 24°C (p < 0.02). It is also of note that after 60 min of incubation of granulocytes with bacteria, concentrates stored at 6°C continued to kill bacteria, whereas those stored at 24°C did not (p < 0.02 at 60 min, p < 0.01 at 90 min) (Fig. 3).

There was no significant difference between concentrates stored at 6°C and 24°C for up to 48 hr with respect to granulocyte number, phagocytic index, or stimulated oxygen consumption.

**DISCUSSION**

These studies constituted an initial attempt to define the chronology of granulocyte function deterioration in vitro using a model system. During short-term liquid preservation, granulocyte migration and chemotaxis were the first and most
GRANULOCYTE FUNCTION DURING STORAGE

Fig. 3. Time course of killing of *E. coli* by fresh granulocytes and cells stored at either 6°C (open circle, dashed line) or 24°C (closed circle, solid line) for 24 and 48 hr.

severely affected functions. Decrements in chemotaxis were not due to delay in the time to achievement of maximal response to the chemotactic stimulus; rather, the absolute magnitude of the maximal rate of migration was decreased. This is reflected by a parallel decrease in random migration. The fact that both random migration and chemotactic response to agents with different receptors (*N*-formylmet-phe and C5a) were equally affected is compatible with the hypothesis that in stored granulocytes there is an underlying metabolic defect not limited to selective loss of cell membrane receptors for the chemotactic agents. This is currently being studied. In addition, the normal phagocytic responses at 24 and 48 hr with a distinctly abnormal chemotactic response suggest that changes in granulocyte membrane deformability alone are not responsible for the altered chemotactic response. Decreased chemotaxis was the most consistently observed defect of all functions examined. In a recent report, Steigbigel et al. have also shown that granulocyte chemotaxis is the most labile function during storage, and other investigators have also noted defects in granulocyte migration after storage, although not as severe as those we reported.

The second most unstable granulocyte function in these studies was the stimulation of oxygen consumption (respiratory burst) in response to a standard phagocytic stimulus. After 48 hr of storage, both oxygen consumption and chemiluminescence in response to phagocytosis were markedly decreased. These decreases cannot be ascribed simply to alterations in phagocytic capacity, since at 48 hr phagocytosis
itself was nearly normal. The dissociation between phagocytosis and the respiratory burst at 48 hr of storage is compatible with an underlying structural or metabolic derangement within the cells; for example, the membrane oxidase system itself may be structurally altered, its relationships to other membrane proteins may in some way be disrupted, or generation of cofactor (presumably NADPH)\textsuperscript{17} may be impaired by alteration in any one of several steps in glycolytic metabolism.

It was anticipated that the organism used in bactericidal studies, a catalase-positive \textit{E. coli}, would allow early recognition of decrements in oxidative pathways of bacterial killing. Consequently, it is of interest that at 48 hr, when the respiratory burst was decreased by more than 50\%, bacterial killing was decreased by only 10\% in cells stored at 6\(^\circ\)C. This supports the data of Johnston et al.\textsuperscript{18} which show substantial reserve capacity in oxidative killing mechanisms.

An attempt was made to improve on the disappointing results of storage at 6\(^\circ\)C by altering the temperature of storage to 24\(^\circ\)C. McCullough et al.\textsuperscript{7} have reported an advantage for cells stored at 24\(^\circ\)C in terms of granulocyte migration. Such an advantage was also observed in these studies. Although storage at 24\(^\circ\)C resulted in nearly normal function in 50\% of units, the remainder still had grossly abnormal chemotaxis; consequently, it can be anticipated that even with 24\(^\circ\)C storage at least 40\% of granulocyte concentrates will migrate poorly after 24 hr of storage.

At 48 hr of storage the major consistent difference observed was an advantage for cells stored at 6\(^\circ\)C in terms of overall bacterial killing (\(p < 0.02\)). Granulocytes stored at 24\(^\circ\)C for 48 hr killed fewer bacteria and were not as capable of prolonged bactericidal activity as cells stored at 6\(^\circ\)C. This is indicated by the failure of cells stored at 24\(^\circ\)C to inhibit growth of \textit{E. coli} after 60 min in vitro. There were insignificant differences in absolute number of granulocytes, phagocytic index, and pH between cell concentrates stored at the two temperatures. The lower glucose concentration of cells stored at 24\(^\circ\)C presumably indicates more active glycolytic metabolism.

In conclusion, these studies indicate that in order to best preserve in vitro function, granulocytes should be kept at room temperature rather than 6\(^\circ\)C in the interval between collection and transfusion. Even when stored at room temperature, at least 40\% of units will have severely reduced random granulocyte migration and chemotaxis after 24 hr. Previously reported associations between altered granulocyte function in vitro and susceptibility to infection\textsuperscript{19-21} suggest that in vitro functions provide useful approximations of in vivo efficacy. It would therefore seem prudent to transfuse even room-temperature-stored granulocyte concentrates as soon as possible after collection.

It is unlikely that further efforts at improving in vitro granulocyte function during storage will be successful until the mechanisms underlying rapid in vitro deterioration are known. Studies to elucidate the mechanisms resulting in deterioration of migration and of the respiratory burst are currently in progress in this laboratory.

REFERENCES

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