Effect of Storage on Normal Neutrophils Collected by Discontinuous-Flow Centrifugation Leukapheresis

By Lewis Glasser

Storage limits for granulocytes have not been defined. The purpose of this study was to determine these limits for neutrophils collected by discontinuous-flow centrifugation and stored at 4°-6°C. The parameters studied were total leukocyte and absolute cell counts, viability measured by dye exclusion, morphology, percentage phagocytic neutrophils, number of Candida organisms ingested per phagocytic neutrophil, candidacidal activity by differential staining, and chemotaxis under agarose. There was a progressive loss of neutrophils on storage that was statistically significant by 48 hr. Phagocytosis was the best preserved function. Microbial killing measured by candidacidal activity was less well preserved. Chemotaxis was the most poorly maintained parameter. There was mild impairment at 24 hr and a severe functional loss at 48 hr. The data suggest the following: (1) the first functions lost on storage are the most highly integrated, i.e., chemotaxis, followed by microbial killing and then phagocytosis; and (2) assuming that these functional losses are irreversible, storage of normal neutrophils used for transfusion should be limited to approximately 24 hr because a severe defect in migration occurs between the first and second days.

GRANULOCYTE TRANSFUSIONS are currently under evaluation in the management of infections in leukemia and solid malignancies, primarily during periods of chemotherapy-induced bone marrow suppression. Continuous-flow centrifugation (CFC), discontinuous-flow centrifugation (DFC), and continuous-flow filtration (CFF) are the methods currently used for harvesting granulocytes. The yield and functional capabilities of neutrophils collected by these techniques have been investigated. Storage of this blood product would enhance its availability, allowing flexibility in its use and providing daily transfusion therapy for the infected neutropenic patient. Storage limits for these cells, however, have not been determined. The purpose of this paper is to define the characteristics of stored granulocytes under certain conditions and to determine the acceptable duration of storage.

MATERIALS AND METHODS

This study was approved by the University of Arizona Human Subjects Committee. Donors were normal healthy individuals. Leukocytes were collected with a Haemonetics separator using hydroxyethyl starch (Volex, McGaw Laboratories, Santa Ana, Calif.) as a sedimenting agent and sodium citrate as the anticoagulant. A 50-ml portion of the processed buffy coat was used for testing. Red cells were allowed to sediment, resulting in some loss of granulocytes from the original material. Aliquots of this leukocyte-rich plasma were stored in plastic tubes, without agitation, at 4°-6°C. Samples were tested immediately following leukapheresis and at 24, 48, 72, and 96 hr. All sequential testing was done from the original granulocyte population. The parameters studied were the total white blood cell count, absolute cell counts, morphology, viability, phagocytosis, microbial killing, and chemotaxis.

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Leukocyte Count

Leukocytes were enumerated in the control specimen using the model D2 Coulter Counter. Subsequent specimens were counted manually to avoid false values secondary to white cell fragments and agglutinated platelets. Differentials, counting 500 cells, were done and the absolute cell counts were calculated. Sixteen donors were included.

Morphology

One hundred neutrophils were evaluated and the percentage of normal cells was recorded. Cytoplasmic and nuclear abnormalities were noted and tabulated separately. Fourteen donors were included.

Viability

Viability was evaluated by dye exclusion using 1% eosin Y. Neutrophils, suspended in Hanks' balanced salt solution containing 0.2% protein, were concentrated to $10.0 \times 10^9$/liter. One part of eosin solution was mixed with two parts of cell suspension and was examined at 2 min with the light microscope using oil immersion. One hundred neutrophils were counted and the percentage of viable (dye-excluding) cells was tabulated. Normal values determined for 46 adults were $95.9\% \pm 2.9\%$ (mean $\pm 1$ SD). Twenty-four experiments were performed.

Phagocytosis

The percentage of neutrophils capable of phagocytosis and the avidity of phagocytic neutrophils were evaluated. Leukocytes were suspended in fresh or fresh frozen donor serum and the absolute neutrophil count was adjusted to $10.0 \times 10^9$/liter. One ml of this suspension was added to a button of Candida albicans containing a predetermined number of organisms, so that the final ratio of yeast to neutrophil was 20:1. Details regarding the culturing of C. albicans have been reported by Lehrer. The reaction mixture was placed on an aliquot mixer and was incubated at 37°C for 30 min. One drop of Zap-oglobin (Coulter Diagnostics, Hialeah, Fla.) was added and smears were immediately prepared using a Larc Spinner (Corning Glass Works, Medfield, Mass.) and stained with Giemsa. One hundred clearly identified neutrophils were counted. Cells in clumps were excluded. Both the percentage of phagocytic neutrophils and the number of organisms ingested per phagocytic neutrophil (phagocytic index) were enumerated. Normal values for 46 adults were $97\% \pm 2.7\%$ (mean $\pm 1$ SD) and $9.23 \pm 2.7$ (mean $\pm 1$ SD), respectively. Eighteen experimental subjects were included.

Microbial Killing

This property was assessed by determining neutrophilic candidacidal activity using differential staining. The experimental procedure was identical to that described for phagocytosis. One hundred phagocytic neutrophils were counted and the number of decolorized Candida organisms was recorded. The candidacidal index was defined as the number of decolorized yeast organisms per phagocytic neutrophil. Normal values for 46 adults were $1.26 \pm 0.73$. Eighteen experiments were included.

Chemotaxis

Neutrophil migration under agarose was used to assay chemotaxis. Two wells, 2.5 mm in diameter, were placed 2.5 mm apart in an agarose gel. Neutrophils were suspended in Hanks' balanced salt solution containing 1% gelatin and a standard number ($1.0 \times 10^5$) placed in one well. The other well was filled with fresh-frozen-thawed AB serum. Dishes were incubated overnight at 37°C in a 5% CO$_2$ atmosphere with humidity (25%, 30%). After incubation, they were fixed with alcohol and formalin and stained with Giemsa. Migration was scored by counting a representative number of neutrophils migrating between the two wells in four consecutive high-power fields ($\times 400$) using a 10-mm ocular grid. All the cells within or touching the grid were counted. Only one grid was counted in each field. The final score was the total number of neutrophils in the grid area of all four fields. Determinations were done in quadruplicate and averaged. The score of the control specimen (cells tested immediately postleukapheresis) was
arbitrarily considered 100% migration for that donor. Scores from samples tested after storage were compared to the control value and expressed as a percentage. The variability of the procedure, determined from 30 duplicate specimens, was 11.3 ± 1.9% (mean coefficient of variation ± SE). Thirteen subjects were studied.

Statistics
Data were analyzed using the difference of means paired t test.

RESULTS
The results are summarized in Table 1.

Leukocyte Count
The total white blood cell count decreased gradually on storage and was statistically significant at 48 hr (p = 0.017). There was no significant loss of neutrophils at 24 hr. On subsequent storage the decrease in the total leukocyte count was due primarily to a loss of neutrophils.

Morphology
Alterations in the morphology of neutrophils involved both the cytoplasm and nucleus. After 24 hr of storage, an average of 9% of the neutrophils showed cytoplasmic vacuolization. This finding was the earliest and most extensive abnormality. Nuclear degenerative changes occurred later and were extensive by 96 hr. The nuclei showed swelling, loss of demarcation between chromatin and parachromatin, hyperchromatism, and loss of segmentation producing a single round homogeneous hyperchromatic nuclear mass. Some cells had lost their cytoplasmic integrity and appeared as nuclei with satellite neutrophilic granules. Smudge cells increased from 0.1/100 white cells in control specimens to a mean of 32/100 white cells at 96 hr. The cells of origin could not be identified. Minor cytoplasmic aberrations included prominent basophilic granules of uncertain derivation and rare patchy pale blue areas simulating Döhle bodies. The average mean decrease of intact neutrophils was 15% for each 24 hr of storage. However, individual variation was considerable and increased with the duration of storage.

Viability
After DFC leukapheresis, the mean viability of neutrophils was 94.4%; this decreased approximately 12% for each 24 hr of storage (Table 1) and was statistically significant after 24 hr (p < 0.001).

Table 1. Summary of Experimental Data on Neutrophils Stored at 4°–6°C

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>WBC (10⁶/liter)</th>
<th>PMN (10⁶/liter)</th>
<th>Normal Morphology (%)</th>
<th>Viability (%)</th>
<th>Phagocytic Neutrophils (%)</th>
<th>Phagocytic Index</th>
<th>Candidalcidal Index (%)</th>
<th>Chemotaxis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>41.7 ± 4.9</td>
<td>19.7 ± 2.7</td>
<td>96.1 ± 1.2</td>
<td>94.4 ± 0.8</td>
<td>96.6 ± 0.4</td>
<td>7.98 ± 0.52</td>
<td>1.37 ± 0.18</td>
<td>100</td>
</tr>
<tr>
<td>24</td>
<td>41.4 ± 5.1</td>
<td>19.5 ± 2.8</td>
<td>84.0 ± 2.0</td>
<td>84.6 ± 1.5</td>
<td>92.9 ± 1.0</td>
<td>8.25 ± 0.56</td>
<td>1.10 ± 0.11</td>
<td>87 ± 9</td>
</tr>
<tr>
<td>48</td>
<td>38.7 ± 5.2</td>
<td>17.7 ± 3.2</td>
<td>68.7 ± 2.7</td>
<td>69.9 ± 3.0</td>
<td>84.1 ± 2.2</td>
<td>7.99 ± 0.42</td>
<td>0.97 ± 0.14</td>
<td>26 ± 5</td>
</tr>
<tr>
<td>72</td>
<td>34.5 ± 5.0</td>
<td>14.0 ± 3.0</td>
<td>54.6 ± 3.8</td>
<td>58.0 ± 2.9</td>
<td>73.3 ± 3.5</td>
<td>7.73 ± 0.33</td>
<td>0.85 ± 0.13</td>
<td>5 ± 1</td>
</tr>
<tr>
<td>96</td>
<td>29.2 ± 5.6</td>
<td>9.9 ± 4.0</td>
<td>33.3 ± 7.0</td>
<td>46.0 ± 4.2</td>
<td>47.1 ± 8.6</td>
<td>5.88 ± 0.48</td>
<td>0.42 ± 0.09</td>
<td>0 ± 1</td>
</tr>
</tbody>
</table>

All data are expressed as the mean ± SEM.
Abbreviations: n, number of donors; PMN, polymorphonuclear leukocytes.
Phagocytosis

This property was the best preserved function of the stored neutrophil even though there was a gradual and statistically significant decrease in the percentage of the neutrophil population participating in phagocytosis throughout the period of storage (Table 1). At 24 hr a mean of 93% of the neutrophils ingested organisms \((p = 0.006)\) and at 96 hr approximately half still retained this ability. Those cells that did take in yeast had a normal capacity and the phagocytic index did not show a statistically significant change until 96 hr \((p = 0.005)\).

Microbial Killing

The candidacidal index had a mean control value of 1.37. It decreased with each day of storage and was statistically significant at 24 hr \((p = 0.02)\). There was a marked contrast between the number of organisms ingested per phagocytic neutrophil, which remained essentially constant throughout the first 72 hr of storage, and the progressive decline in the number of organisms killed per phagocytic neutrophil (Table 1).

Chemotaxis

Migration of neutrophils under agarose toward a chemotactic attractant was the most severely affected function studied. At 24 hr the number of migrating neutrophils showed a mean decrease of 13%. Individual variation was considerable. Some stored samples showed migration equal to control specimens. One sample preserved poorly and retained only 25% of its original function. The reason for this variation was not investigated. At 48 hr there was severe impairment with a mean 74% functional loss. Minimal activity was present at 72 hr. A representative experiment is shown in Fig. 1.

DISCUSSION

Few studies are available on the preservation of stored neutrophils. These have been performed primarily on whole blood collected in a variety of anticoagulants.\(^6\)\(^-\)\(^12\) Other studies include leukocytes stored in plasma\(^13\) and serum-enriched Hanks' balanced salt solution.\(^14\) There are no studies on cells stored following leukapheresis by CFC, DFC, or CFF.

Both the quantitative and functional aspects of stored neutrophils were investigated in this study. No attempt was made to assess the effects of the hydroxyethyl starch on the survival of the collected cells during storage. The total white blood cell count gradually fell and the decrement was statistically significant at 48 hr (Table 1). Degeneration of neutrophils accounted for the major portion of the decrease. Cytoplasmic vacuolization was the earliest alteration followed by nuclear degenerative changes. In this study, the data suggest that viability and abnormal morphology have a close relationship (Table 1), making it unlikely that this is a reversible phenomenon.

The experimental data indicate that the most highly integrated functions are the first lost on storage. Phagocytosis was the best maintained function and probably represented the dying gasp of the neutrophil. The progressive decline in the percentage of neutrophils ingesting \textit{Candida} was similar to results ob-
Fig. 1. Representative experiment of migration of neutrophils under agarose. (A) Control showing migration of leukocytes out of the well toward a chemotactic substance. (B) Neutrophils stored 24 hr show excellent migration. (C) At 48 hr there was 26% migration. (D) At 72 hr there was 3% migration. x22.

Leukocytes capable of ingesting yeast did so with normal gluttony until 96 hr. Bacterial assays have shown normal preservation of microbial killing at 72 hr. However, some loss of candidacidal activity occurred at 24 hr. This difference may reflect alternative neutrophil killing mechanisms. Chemotaxis was poorly maintained during storage. After 24 hr there was only a mild functional loss. However, severe impairment occurred at 48 hr. These results are remarkably similar to those obtained on neutrophils.
in stored whole blood using a modified Boyden technic. This severe deterioration, if irreversible, limits the storage of neutrophils for transfusion. Therefore, it is recommended that the storage of normal granulocytes collected by DFC be limited to approximately 24 hr.

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REFERENCES

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