Cryopreservation of Human Granulocytes: Study of Granulocyte Function and Ultrastructure
By Pravatchai Boonlayangoor, Massumeh Telischi, Subhit Boonlayangoor, Thomas F. Sinclair, and Edward W. Millhouse

Human granulocytes can be cryopreserved with dimethyl sulfoxide (DMSO) at −80°C. However, the percent recovery of functional cells has been unsatisfactory to date. Throughout this study we have been able to prepare relatively pure granulocytes (≥ 85%), cryopreserve them with 5%–10% DMSO with and without serum, and store them at −80°C for up to 4 mo. The parameters studied were absolute cell counts and viability determination, myeloperoxidase activity, phagocytosis, candidacidal activity, bactericidal activity, nitroblue tetrazolium reduction, chemiluminescence, and cell morphology by transmission and scanning electron microscopy. Based on our investigation, granulocytes cryopreserved without serum showed an intact membrane of superior integrity as compared with those preserved with serum. At least 50% of the cells recovered were functional after 2 mo of storage, but there was a progressive loss of viability and function on prolonged storage. The property of phagocytosis was the best preserved after storage for 4 mo, whereas myeloperoxidase activity, killing activity, nitroblue tetrazolium reduction, and chemiluminescence were maintained less efficiently. Morphological studies of cryopreserved granulocytes revealed that the nuclear, cytoplasmic, and cell surface architectures were altered by storage. Depletion of nuclear and cytoplasmic material, as well as changes in configuration, were also noted.

Granulocyte Transfusion is becoming widely used in granulocytopenic patients. However, various techniques to preserve mature granulocytes for long periods at low temperatures have met with little success, although there is evidence indicating that human granulocytes can be preserved for some time at certain temperatures. For example, Glasser,1 McCullough et al.,2,3 and Crowley et al.4 report that there was minimal loss of granulocytic activity during a 24–48-hr storage period at 4–6°C. Cavins et al.5,6 described a freezing method using DMSO to preserve granulocytes. A similar method has been reported by Lionetti et al.7,8 and by Graham-Pole et al.9 Nonetheless, the percent recovery of functional cells is still unsatisfactory.

The following studies were undertaken to investigate the function as well as the ultrastructure of granulocytes preserved at low temperatures under certain conditions, which we have found to yield a high degree of recovery of viable granulocytes after up to 2 mo of storage.

Materials and Methods

Blood Collection

Blood for the study of granulocyte function and ultrastructure following storage was collected from healthy volunteer donors according to the standards of the American Association of Blood Banks. Venous blood (450 ml) was collected in 63 ml of citrate phosphate dextrose (CPD) anticoagulant in plastic bags, and the granulocytes were separated within 1 hr after collection. The bags were centrifuged for 6 min at 2000 g (Sorvall, Model RC-3). Theuffy coat layer plus 50 ml of the topmost red cells were transferred to a satellite bag for further isolation of the granulocytes.

Isolation of Granulocytes

The granulocytes were isolated from the buffy coat using the modified method of Mendelson et al.10,11 Buffy coat leukocytes and red cells were layered over a standard Ficoll-Hypaque mixture and the platelets, mononuclear cells, and red blood cells were removed by centrifuging for 35 min at 400 g. The granulocytes were then aspirated, and the cells were suspended in an equal volume of autologous plasma and then mixed with an equal volume of 8% hydroxyethyl starch (HES, Sigma Chemical Co., St. Louis, Mo.) in a 0.9% NaCl solution (pH 7.2) to give a final concentration of 4% HES. The mixed cell–starch solution was drawn into a 50-ml disposable syringe, the syringe was inverted, and the cells then allowed to settle out at room temperature. After 50 min, the granulocyte-enriched supernatant was transferred to siliconized centrifuge tubes and centrifuged at 600 g for 2 min. The cells were then washed twice with Hanks' balanced salt solution (HBSS) without calcium chloride but containing 0.1% glucose and 0.1% EDTA, and resuspended (107 cells/ml) in Roswell Park Memorial Institute medium 1640 (RPMI), RPMI medium with 20% autologous serum, RPMI medium with 4% HES, or RPMI medium with 20% autologous serum and 4% HES.

Cryopreservation of Granulocytes

Granulocytes suspended in the above media were preserved with either 5% or 10% DMSO. Six-milliliter aliquots of granulocytes suspended in appropriate medium were placed in polypropylene tubes (17 × 100 mm) and kept in an ice bath prior to the addition of DMSO. To these, equal volumes of cold 10% or 20% concentrations of DMSO were added and mixed with the cell suspensions at a rate of 1.5 ml/min to give final concentrations of 5% and 10%, respectively. Two-milliliter aliquots of granulocyte suspensions were then distributed into polypropylene tubes (12 × 75 mm), frozen at 2–3°C/min to −80°C, and stored at this temperature in a Harris mechanical refrigerator.

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Recovery of Frozen Cells

The granulocyte suspensions were thawed by rapid agitation of the tubes in a 37°C water bath. Just prior to the last ice crystal melting, the tubes were transferred to an ice bath for 2 min and then to a 50-ml tube at room temperature. Washing was initiated immediately, using 2 ml of RPMI medium containing 20% autologous fresh-frozen serum (FFS), which was added in dropwise manner and mixed gently. The cells were allowed to equilibrate for 3 min. Double volumes of this medium were then added in the same manner until a final dilution of 1:15 of the original cell suspension was achieved. This was then centrifuged at 600 g for 2 min, rewashed, and the granulocyte pellet resuspended gently in RPMI medium containing 20% FFS.

Cell Count, Viability Determination, and Myeloperoxidase Activity

Cell counts were performed either by counting the cells in a standard hemocytometer or in a ZBI Coulter counter. Myeloperoxidase (MPO) activity was performed according to the method of Kaplow.11 Viability was determined by trypan-blue dye exclusion as well as by radioactive uptake by the cells. From tubes containing 5 ml of granulocyte suspension, 0.1 ml was removed to an aliquot of trypan-blue for cell counts and viability determination by means of dye exclusion. The cells remaining in each tube were preincubated at 37°C in a water bath for 10 min, and 5 pCi/ml of thymidine-methyl-3H (New England Nuclear Corp., Boston, Mass.) was added to each tube, which was then gently mixed and reincubated at 37°C for 30 min. The method of Booden et al.12 was used to determine the amount of isotope that was taken up by the cells. Since determinations on trypan-blue dye exclusion (DE) indicated that these cells were approximately 95% viable, it was assumed that the amount of isotope taken up in counts per minute (cpm) by the fresh cells would be isotope taken up in counts per minute (cpm) by the fresh cells would be

Phagocytosis and Microbial Killing

Candida albicans, cultured in malt extract medium at 30°C was harvested for phagocytosis and microbial killing after 7 days growth. The cells were washed twice with 0.1 M phosphate-buffered saline (PBS) and resuspended in HBSS containing 0.1% (w/v) gelatin. For the assays of phagocytosis and killing, a yeast suspension containing 2 x 10⁶ Candida cells/ml was prepared in HBSS, and the viability was determined by methylene blue dye exclusion before the initiation of phagocytosis and killing.

Phagocytosis was performed by incubating 1 ml of granulocytes (2 x 10⁶ cells/ml) with 1 ml of Candida suspension at 37°C for 15-60 min, while rotating at 4 rpm. Then, a 0.1-ml aliquot of granulocyte-yeast suspension was taken and a count made under a hemocytometer. Yeast cells attached to the surface of phagocytic cells were considered to be ingested. The phagocytosis was calculated by determining the percent decrease in the initial number of yeast cells by directly counting the number of free yeast cells in the suspension following phagocytosis. The percent phagocytosis index was calculated by converting the percent viable granulocyte cells at various times of storage to 100% by using the following equation:

\[
\text{Percent phagocytosis index} = \frac{\text{Percent viable cells at various storage times}}{\times 100}
\]

Calculation of the percent decrease in phagocytic activity was obtained by the following equation:

\[
\text{Percent decrease in phagocytic activity} = 100\% - \frac{\text{Phagocytosis index at various storage times} \times 100}{\text{Phagocytosis index of fresh control}}
\]

Microbial Killing

Candida albicans activity was performed after 60-min incubation; as described for phagocytosis, another 0.1-ml aliquot of granulocyte-yeast suspension was removed and added to 0.2 ml distilled water containing 0.1% albumin (w/v) and mixed for 30 sec to lyse the phagocytic cells. The number of viable Candida cells was then measured by plating serial dilutions of the lysate in phosphate-buffered saline, pH 7.2, on Sabouraud agar plates. After incubation at 37°C for 24 hr, the number of colony-forming units (CFU) was counted. The percent intracellular killing was calculated by counting the number of CFU in the suspension following incubation and comparing it to the number of CFU percent before the initiation of phagocytosis. The killing index was calculated by subtracting the percent of dead Candida before the initiation of the experiment from the percent of killing. The percentage of decrease in killing activity was then calculated according to the following equation:

\[
\text{Percent decrease in killing activity} = 100\% - \frac{\text{Killing index at various storage times} \times 100}{\text{Killing index of fresh control}}
\]

Bactericidal activity was measured by using a modification of the technique of Steigbigel et al.13 and Wright et al.14 as follows: granulocytes (2 x 10⁶ cells/ml) were incubated with Staphylococcus aureus no. 27712 or no. 9144 (American type culture collection) at a bacteria:granulocyte ratio of 2:1. Aliquots of the incubation mixture were removed after 60 min with a calibrated wire loop and diluted with distilled water to lyse the phagocytic cells. Appropriate dilutions were plated on nutrient agar and the number of CFU was determined after 24 hr. The percent intracellular killing was determined as described above.

Reduction of Nitroblue Tetrazolium

A modification of the method of Baehner and Nathan15 and McCullough et al.1 was used as follows: 2 x 10⁶ granulocytes in 0.5 ml RPMI medium were added to 16 x 10⁶ mm glass tissue culture tubes containing 2 x 10⁶ yeast cells in 0.5 ml HBSS and 0.1 ml of 0.01 M KCN. The mixture was incubated at 37°C in a water bath for 10 min, after which 0.5 ml of a 0.2% solution of NBT in PBS was added to each tube. The tubes were incubated for an additional 30 min in 37°C water bath and the reaction terminated by adding 3 ml ethyl acetate to each tube. After 5 min at room temperature, the optical density of the supernatant was read spectrophotometrically at 535 nm against an ethyl acetate blank.

Chemiluminescence

The chemiluminescent properties of preserved granulocytes were measured by using a modification technique of Stjernholm et al.16 Briefly, 2 x 10⁶ granulocytes and/or yeast cells were incubated in a scintillation vial containing 15 ml RPMI 1640 for 30 min at room temperature in a dark environment. At 5-min intervals, the chemiluminescence produced was summed for 1 min in a Packard-Tricarb liquid scintillation spectrometer operated in the out-of-coincidence mode as described by Stanley and Williams.17

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DRATED, CRITICAL-POINT DRIED, COATED WITH GOLD PALLADIUM, AND THEN POLY-L-LYSINE-COATED COVER SLIP

A 20-μL drop of cell suspension postfixed with 0.5% was placed on a RCA EMU III or RCA EMU IV transmission electron microscope.

The separation of granulocytes from each unit of whole blood yielded approximately 5.8 ± 0.6 × 10^6 cells, and the mean ratio of granulocyte to mononuclear cell and to red blood cell was 82.5:3.2:14.3 by differential cell count. The results in Table 1 demonstrate that there was no significant loss in absolute number of cells during the period of study, however, there was a significant reduction in viability and MPO activity following any period of cryopreservation, including samples that were merely frozen and then immediately thawed. The viability was maintained considerably more efficiently when certain media were employed (Table 2). RPMI medium containing 4% HES and 5% DMSO or RPMI with 5% DMSO were superior to the various other media employed (p < 0.1). Fresh or fresh-frozen serum (FFS) was unnecessary and actually may have been deleterious to the maintenance of viability during cryopreservation. Fresh-frozen serum used in the media containing 5% DMSO, or 5% DMSO and 4% HES, resulted in viabilities of 61.5% and 65.3%, respectively, immediately after freezing and thawing, whereas when FFS was absent from the medium, 78.0% and 81.3% remain viable (Table 2). These differences are significant at the 0.05 level and were consistently observed throughout the period of cryopreservation.

The effect of increasing the concentration of DMSO on viability after cryopreservation at various storage time is shown in Table 3. The results reveal that employing 10% instead of 5% DMSO consistently gave a better yield of viable cells. This relationship was

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<td>Fresh control</td>
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<td>70.5 ± 7.8</td>
<td>53.3 ± 4.7</td>
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<td>1 mo</td>
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<td>59.8 ± 3.5</td>
<td>63.5 ± 4.1</td>
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<tr>
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<td>3 mo</td>
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<td>26.3 ± 3.3</td>
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<tr>
<td>4 mo</td>
<td>3.3 ± 0.5</td>
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Each value represents the mean ± SEM from 8 replicate experiments. See text for the experimental details.

Electron Microscopy

Fresh and cryopreserved granulocytes were washed twice with HBSS; fixed with 3% glutaraldehyde-3% formaldehyde in cacodylate buffer, pH 7.4 (Tousimis Research Corp.), for 30–60 min at 4°C; washed with 0.1 M cacodylate buffer, pH 7.4; and postfixed with a mixture of 2% osmium tetroxide (OsO₄) fixative buffer and 0.2 M cacodylate buffer, pH 7.4, for an additional 1–2 hr at 4°C. The cells were then dehydrated in graded ethanol, embedded in Epon 812 following sectioning on a Porter Blum MT2, and collected on formvar-coated grids. These were then stained for 30 min with saturated uranyl acetate and for 3 mm with Reynold's lead citrate. The cells were then examined under a RCA EMUIII or RCA EMU IV transmission electron microscope.

The method used for the scanning electron microscopy study was modification of the technique of Sanders et al. and Kelley et al. A 20-μL drop of cell suspension postfixed with OsO₄ was placed on an 8 mm diameter) and stored in a 4°C for 8 replicate experiments. See text for the experimental details.

RESULTS

Viability Determination and Myeloperoxidase Activity

Separation of granulocytes from each unit of whole blood yielded approximately 5.8 ± 0.6 × 10^6 cells, and the mean ratio of granulocyte to mononuclear cell and to red blood cell was 82.5:3.2:14.3 by differential cell count. The results in Table 1 demonstrate that there was no significant loss in absolute number of cells during the period of study, however, there was a significant reduction in viability and MPO activity following any period of cryopreservation, including samples that were merely frozen and then immediately thawed. The viability was maintained considerably more efficiently when certain media were employed (Table 2). RPMI medium containing 4% HES and 5% DMSO or RPMI with 5% DMSO were superior to the various other media employed (p < 0.1). Fresh or fresh-frozen serum (FFS) was unnecessary and actually may have been deleterious to the maintenance of viability during cryopreservation. Fresh-frozen serum used in the media containing 5% DMSO, or 5% DMSO and 4% HES, resulted in viabilities of 61.5% and 65.3%, respectively, immediately after freezing and thawing, whereas when FFS was absent from the medium, 78.0% and 81.3% remain viable (Table 2). These differences are significant at the 0.05 level and were consistently observed throughout the period of cryopreservation.

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observed both when viability was determined using trypan-blue dye exclusion and when the radioisotope uptake method was utilized. The latter was considered to be the superior method for viability determination since correlation coefficients, between the percent recovery of viable cells using this technique were higher than those obtained using the dye exclusion method when viability and the NBT assay of granulocyte function were compared.

**Phagocytosis**

The results of phagocytosis of *C. albicans*, shown in Fig. 1, reveal that this function by either fresh or cryopreserved granulocytes remained a rapid process. At 15-min incubation, the majority of the engulfment of *C. albicans* has occurred; after 30-min incubation, the phagocytosis declined but the percent phagocytosis increased until it reached the maximum at about 1 hr. After 1 hr, fresh granulocytes had phagocytized approximately 92% of the yeast, while granulocytes cryopreserved for 4 mo phagocytized only about 15%.

The percentage of phagocytosis by cryopreserved granulocytes slowly decreased from the initial time of storage till about 2 mo, and then markedly decreased (Fig. 1). When the phagocytosis index is examined (Fig. 2), the ability of granulocytes to engulf *C. albicans* appears to slowly decrease, as shown by the percentage of decrease in phagocytic activity. For example, at 2 and 4 mo storage, the phagocytosis index is 77% and 58%, respectively, and the decrease in phagocytic activity is 17% and 38%, respectively, when using fresh granulocytes as a standard control.

**Killing Activity**

The results shown in Table 4 and Fig. 3 reveal that after 60-min incubation with *C. albicans* and *S. aureus*.
stimulated by 0.02, respectively (Table 5). Similarly, granulocytes were capable of reducing NBT to some degree during the period of storage at -80°C, but the interaction of these cells resulted in a steady increase in OD535, which represents about 27.63% from the reading obtained with fresh granulocytes (0.72 ± 0.02).

Chemiluminescence

Figure 4 reveals that bioluminescence was evoked in granulocytes upon phagocytic challenge, although upon prolonged storage it was markedly decreased. For example, the bioluminescence of granulocytes preserved with DMSO at -80°C, for instance, after 4 mo of storage at -80°C granulocytes interacting with C. albicans in the NBT reduction test resulted in a reading of 0.20 ± 0.01 OD535, which represents about 27.63% from the reading obtained with fresh granulocytes (0.72 ± 0.02).

Table 5. Reduction of Nitroblue Tetrazolium (NBT) by Granulocytes Preserved With DMSO at Various Times of Storage at -80°C

<table>
<thead>
<tr>
<th>Granulocytes</th>
<th>OD</th>
<th>Percent</th>
<th>OD</th>
<th>Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh</td>
<td>0.38</td>
<td>100.0</td>
<td>0.72</td>
<td>100.0</td>
</tr>
<tr>
<td>Frozen-thawed</td>
<td>0.36</td>
<td>94.4</td>
<td>0.67</td>
<td>93.2</td>
</tr>
<tr>
<td>1 wk</td>
<td>0.30</td>
<td>79.6</td>
<td>0.55</td>
<td>76.8</td>
</tr>
<tr>
<td>1 mo</td>
<td>0.28</td>
<td>74.6</td>
<td>0.48</td>
<td>66.0</td>
</tr>
<tr>
<td>2 mo</td>
<td>0.24</td>
<td>63.2</td>
<td>0.42</td>
<td>57.9</td>
</tr>
<tr>
<td>3 mo</td>
<td>0.18</td>
<td>47.4</td>
<td>0.29</td>
<td>40.6</td>
</tr>
<tr>
<td>4 mo</td>
<td>0.11</td>
<td>28.8</td>
<td>0.20</td>
<td>27.6</td>
</tr>
</tbody>
</table>

*Fresh C. albicans reduce NBT yielding an OD535 = 0.08 ± 0.01. Each value represents the mean ± SD from 4 replicate experiments. See text for the experimental details and calculation.

Gradually lost their capacity to respond to this stimulus by NBT reduction. For instance, after 4 mo of storage at -80°C granulocytes losing activity of C. albicans, and C. albicans in the NBT reduction test resulted in a reading of 0.20 ± 0.01 OD535, which represents about 27.63% from the reading obtained with fresh granulocytes (0.72 ± 0.02).
Fig. 5. Transmission electron micrograph of fresh granulocyte showing cell processes, segmented nucleus, and numerous neutrophilic granules. Original magnification ×12,000.

Fig. 6. Transmission electron micrograph of frozen granulocyte after 2-mo storage. The nucleus shows swelling and appears as a band; degeneration of nuclear chromatin and cytoplasm are also seen. Original magnification ×12,000.
Electronmicroscopy

Transmission and scanning electron microscopes were employed to evaluate structural damage to granulocytes after cryopreservation. The results, shown in Figs. 5–9, revealed that the nucleus, cytoplasm, and cell surface architecture of cryopreserved granulocytes has been altered. Figures 5 and 8 show transmission and scanning electron micrographs of fresh control cells in which the granulocytes display a fairly uniform appearance of the nucleus, cytoplasm, and microvilli on the cell surface. The nucleus is rich in chromatin material and the cytoplasmic material still abundant. The microvilli are characteristically short, stubby, and evenly distributed over the cell surface. Immediately after thawing, the nucleus showed swelling and degeneration of nuclear chromatin and cytoplasmic material (Fig. 6). Upon prolonged storage, the typical segmented shape of the nucleus was lost. It appeared to “round up” and had lost much of its chromatin. There was a considerable amount of cytoplasmic vacuolization, and some cells had lost their integrity, appearing as a circular nuclei surrounded by fading cytoplasm (Fig. 7). The architecture of the cell surface appeared as a knob-like microvilli or as broader than the microvilli of fresh cells (Fig. 9).

DISCUSSION

Attempts to cryopreserve mature human granulocytes have been reported for more than a decade now, but it is only recently that these cells have been recovered reasonably efficiently. Lionetti et al. reported that optimal recovery of viable and functioning leukocytes from storage at −80°C depended on the addition of 5% DMSO and 4% residual concentration of HES.

Our results have confirmed the observation that the addition of 4% HES to the freezing medium results in slightly better recovery of functioning cells than if DMSO is used alone. However, while consistently higher recoveries were obtained when HES was incorporated into the freezing mixture, the viability and the functional state of these cells was shown to be more markedly dependent on the length of storage at −80°C than on the medium employed. Greater than 50% recovery was obtained for up to 2 mo with or without HES, after which the viability and functional...
integrity of the cells declined rapidly. The rate of loss of normal function, however, was nearly identically similar for cells preserved in 5% DMSO alone or in medium with 5% DMSO and 4% HES (Table 2). This suggests that the function of HES is to protect the cells either during the initial period of freezing or during the thawing or post-thaw handling of the cells. Surprisingly, in view of the fact that Graham-Pole et al. found that the addition of fetal calf serum was beneficial to granulocytes, autologous FFS was not beneficial in our study. We cannot yet explain the detrimental effects of human serum on preservation. Dankberg et al. have speculated that the low molecular weight of serum substances may cause a shift in the freezing rate, thus producing less favorable conditions for preservation.

Aside from relative measures of viability, the phagocytic and MPO activities of the cryopreserved cells were least effected during storage. However, maintenance of these functions may not be of significant value, since marked impairment of NBT reduction and chemiluminescence occurred. This was accompanied by a corresponding loss of microbial killing activity. These observations suggest that the decrease in killing activity seen is due to injury of the NADPH oxidase system. This interpretation is supported by the existence of significant correlation between the decrease in bactericidal activity and chemiluminescence. Such injury might occur during freezing, storage, or thawing as a result of osmotic stress, thermal shock, or membrane-shearing forces due to ice formation. Electron micrography of fresh and frozen cells appears to support this concept, since both the nucleus and the cytoplasm were obviously degenerated. Alternatively, some initiating factor present in the cell membrane may be lost during cryopreservation. Harvath and Anderson have noted the existence of normal phagocytic function in the absence of activation of oxidative metabolism by particulate stimulus. However, since the respiratory responses were normal when soluble agents were used to activate the cells, they have suggested the requirement for another as yet not well understood mechanism for initiating oxidative metabolism. Since we only used particulate stimuli in our study, we are unable to determine whether the defect in cryopreserved granulocytes is due to the loss of normal respiratory constituents or to the loss of another essential mechanism that may play a role in granulocyte function.
REFERENCES

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