Degranulation and Abnormal Bactericidal Function of Granulocytes Procured by Reversible Adhesion to Nylon Wool

By John C. Klock and Dorothy F. Bainton

Granulocyte bactericidal capacity, chemotaxis, hexose monophosphate shunt activity (before and after phagocytic stimulus), and quantitative nitroblue tetrazolium reduction and enzyme content were examined in cells obtained by filtration leukapheresis (FL) and continuous-flow centrifugation (CFC). A decrease in the bactericidal efficiency of FL-procured cells compared to that of both normal and CFC-procured granulocytes was found; the decrease was 17% with a cell-to-bacteria ratio of 5:1, and 55% with a 1:1 ratio. Moreover, FL-acquired cells were often vacuolated and consistently contained less acid phosphatase and β-glucuronidase than did normal granulocytes. When normal cells were incubated for 1–2 hr with nylon wool, 30% of the total acid phosphatase and β-glucuronidase was released, with no evidence of cell death, thus suggesting degranulation. Similar results were obtained with glass, cotton, or polysulfone plastic fibers. Electron microscopic and peroxidase cytochemical studies of the adherence of normal granulocytes to nylon fibers were also carried out. After 30 min of incubation, cell-to-fiber attachment and cellular aggregation had occurred, although the cells per se appeared normal. After 60 and 120 min, other changes became apparent: (1) a decrease in the amount of cytoplasmic granules; (2) large, intracytoplasmic vacuoles; and (3) extracellular peroxidase on fiber surfaces. We conclude that granulocytes obtained by adherence to nylon fibers show both morphological and biochemical evidence of degranulation and diminished bactericidal capacity, and that these abnormalities may be causally related to decreased granulocyte survival in transfusion recipients.

The advent of practical methods for the collection of large numbers of granulocytes from normal donors1,2 has made clinical studies of normal granulocyte transfusion therapy in infected, granulocytopenic patients feasible. Results of several controlled studies in humans suggest that augmentation of the circulating granulocyte pool may be useful in such situations.3,4 The two most popular procedures for collecting suitably large numbers of granulocytes are continuous-flow centrifugation (CFC)1 and continuous-flow filtration leukapheresis (FL).2 Separation using the CFC technique is possible because of differences in the sedimentation characteristics of white and red blood cells in a centrifugal field; in the FL technique separation is possible because of the property of granulocytes to adhere reversibly to nylon wool fiber.

Studies of granulocytes procured by CFC have shown their in vitro function, as well as their in vivo survival, to be normal.3,5 In vitro studies of granulocytes

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Submitted December 15, 1975; accepted March 3, 1976.

Supported by a fellowship from the Leukemia Society of America (J.K.), Research Career Development Award AM-11902 (D.B.), USPHS Grants AM-16095 and CA-14264, and cancer research funds of the University of California.

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procured by FL, however, have shown their function to be either normal,\textsuperscript{7} or slightly abnormal in bactericidal capacity, chemotaxis, nitroblue tetrazolium reduction, and hexose monophosphate shunt activity.\textsuperscript{3,4,6,8,9} In addition, compared to CFC-procured cells, FL-procured cells have been associated with both an increased incidence of transfusion reaction\textsuperscript{4,6,9} and decreased in vivo recovery.\textsuperscript{6,9}

These observations, plus the finding that FL-procured granulocytes show varying degrees of vacuolization,\textsuperscript{4,9} prompted the current studies. In an effort to define these abnormalities further and to distinguish differences between CFC and FL cells, we have studied bactericidal capacity, chemotaxis, hexose monophosphate shunt activity, nitroblue tetrazolium reduction, and granule enzyme content. Also, after normal granulocytes were separated by the Ficoll–Hypaque (FH) procedure and allowed to adhere to nylon fibers, electron microscopic and cytochemical studies of granule enzyme release were carried out. The data showed that granulocytes procured by FL underwent partial degranulation during the process of adherence and acquired defects in bactericidal function.

**MATERIALS AND METHODS**

**Granulocyte Procurement Methods**

Granulocytes were procured from normal, healthy donors, participants in a clinical study in which the effects of normal granulocyte transfusion in infected, granulocytopenic patients with marrow aplasia or acute leukemia were observed. Our method for procurement by continuous-flow centrifugation was the same as that described by Buckner et al.,\textsuperscript{10} using the Aminco Celltrifuge (American Instrument Co., Silver Springs, Md.) and vein-to-vein technique. Heparin (40 U/min) and ACD (1 ml/min) were the only drugs administered to the subject during donation. No rouleaux-forming agents or corticosteroids were used. Granulocyte yields averaged 3–6 $\times 10^9$ cells per 3-hr donation.

Djerassi’s method of continuous-flow filtration leukapheresis\textsuperscript{2} as modified by Schiffer\textsuperscript{9} was employed. The Fenwal leukapheresis pump and Leukopak system (Fenwal, Division of Travenol Laboratories, Morton Grove, Ill.) were used with a vein-to-vein technique. Flow rates during the 3-hr procedure were 60–80 ml/min. Granulocyte yields averaged $15-37 \times 10^9$ cells. A single double-filter set was used after a loading dose of 40 U/kg heparin was given; heparin 80 U/min was continued throughout the procedure for each procurement, and the eluting solution consisted of 250 ml ACD-A, 250 ml human plasma, and 1000 ml isotonic saline with 1 unit heparin/ml. Granulocytes were collected at room temperature by pumping the eluting solution over the filters at 160 ml/min while gently tapping the outside of the fiber packs. The collected cells were then centrifuged (400 g for 15 min) and pooled for transfusion and in vitro testing.

Normal human granulocytes for the in vitro degranulation studies were procured as follows: Fifty milliliters of blood anticoagulated with heparin 10 U/ml were mixed with 5 volumes of normal saline and layered over a Ficoll–Hypaque solution ($D_{20} 1.077$ g/ml) and centrifuged at 400 g for 45 min at 20°C. The supernatant and the mononuclear cell/platelet layer were removed by suction, and the pellet containing polymorphonuclear cells and red cells was diluted with 10 volumes of 0.83% ammonium chloride to lyse the red cells. The granulocytes were collected by centrifugation (80 g for 15 min), washed twice with normal saline, and resuspended in saline at a concentration of 3–10 $\times 10^7$ cells/ml. Time from procurement to testing was less than 2 hr.

**In Vitro Tests of Granulocyte Function**

Tests for granulocyte membrane integrity using trypan blue dye 0.1% were done. Bactericidal capacity was determined according to the method of Hirsch and Strauss,\textsuperscript{11} using *Staphylococcus aureus* 502A and *Escherichia coli* ($1 \times 10^8$ organisms) and 1 or 5 $\times 10^8$ granulocytes in the assay system. Incubations of cells and organisms were carried out in 1–2 ml Hanks’ balanced salt solution and 10%, 15%, of either fresh autologous serum or AB positive serum. To determine the
percentage of surviving bacteria, triplicate aliquots were removed at various time intervals. They were diluted in sterile 0.1% gelatin water, and colony counts on trypticase soy agar, using the pour-plate technique, were then made. Results were expressed as the number of viable organisms seen after 0, 30, 60, and 120 min of end-over-end incubation at 37°C.

Chemotactic ability of granulocytes was determined by a modified Boyden technique, using E. coli lipopolysaccharide as the chemotactic stimulus. Assays were carried out in miniaturized chemotactic chambers (Markit Corp., Chicago, Ill.) fitted with 5-μm pore membranes (Millipore Corp., Bedford, Mass.). An inoculum of 2.5 × 10⁶ cells was placed on the starting side of the filter, and after 3 hr at 37°C and 100% humidity the filters were stained with hematoxylin. Results were expressed as number of granulocytes/10 high power fields migrating 80 μm from the starting side of the filter, divided by number of granulocytes/10 high power fields on the starting side × 100 (as per cent of cells migrating 80 μm). All tests were done in triplicate.

Metabolism of 1-14C-labeled glucose to 14CO₂ was determined after the method of Holmes et al. both in the absence and presence of dialyzed latex particles 0.801-μm diameter. Results performed in triplicate were expressed as cpm/10⁶ cells/15 min and as per cent increase in radioactivity after phagocytic stimulus.

Quantitative nitroblue tetrazolium reduction was performed in triplicate according to the method of Baehner and Nathan. Quantitative assessment of phagocytic ability of granulocytes was determined by the oil red O method of Stossel et al.

**Degranulation Experiments**

For in vitro tests of degranulation, cells procured by the Ficoll-Hypaque/ammonium chloride technique were used. Granulocytes (3-10 × 10⁶) were incubated in vitro with scrubbed 3-denier nylon fiber (Fenwal), pyrex fiber (Corning Glass Works, Corning, N.Y.), or fiber of similar size made from polysulfone or polyvinyl plastics (Amicon Corp., Lexington, Mass.).

Incubations were carried out at 37°C in sterile 3-ml polystyrene tubes, and enzyme levels were determined on aliquots of the supernatant at 15, 30, 60, and 120 min. All supernatant aliquots were centrifuged at 80 g for 10 min to pellet intact cells. Per cent release of enzyme was calculated in each experiment by arbitrarily defining the enzyme content of an aliquot of whole cells at the start of the incubation as 100%.

Assays for β-glucuronidase were performed according to the methods of Gianetto and deDuve, using phenolphthalein β-d-glucuronide as substrate. Results were expressed as μM substrate cleaved/10⁶ cells/hr. Assay for acid phosphatase was determined after the method of Weaver and Boyle, using β-glycerophosphate as substrate. Results were expressed as μM phosphorus released/10⁶ cells/hr. All enzyme determinations were carried out in the presence of Triton X-100, 0.1% (Sigma Chemical Co., St. Louis, Mo.).

**Electron Microscopic Studies**

Ultrastructural examination of pieces of nylon wool and their adherent granulocytes (isolated by the FH method) was performed after 30, 60, and 120 min of incubation in vitro. The conditions of incubation were the same as those used in the degranulation experiments. Cytochemical staining for myeloperoxidase to quantitate peroxidase-positive azurophil and peroxidase-negative specific cell granules was done according to the methods of Bainton et al.

**RESULTS**

**Function of CFC and FL Cells: Similarities**

The assessment of plasma membrane integrity, using trypan blue, revealed that over 96% of the granulocytes used for in vitro tests excluded the dye. Tests of chemotactic activity, hexose monophosphate shunt activity before and after phagocytic stimulation, and quantitative reduction of nitroblue tetrazolium revealed no significant differences between control cells and cells procured by CFC or FL (Fig. 1).
Function of CFC and FL Cells: Differences

Assays of bactericidal capacity of granulocytes procured by FL revealed a 17\% decrease in killing capacity compared to the killing capacity of control and CFC cells when the cell-to-bacteria ratio was 5:1 (p < 0.001). When the ratio of cells to bacteria was reduced to 1:1, this difference was even more noticeable (55\%, p < 0.001) (Fig. 2). Similar data were obtained using E. coli as the test organism.

Quantitative assessment of phagocytic ability of granulocytes revealed no difference between normal FH-separated, CFC, and FL cells (0.14, 0.24, and 0.22 mg paraffin oil ingested/10^7 phagocytes/min, respectively). This result suggested that a decrease in phagocytic ability could not explain the diminished killing ability of the FL cells.
Table 1. Total Enzyme Content of Granulocytes From Five Donors

<table>
<thead>
<tr>
<th>Source of Cells</th>
<th>Autologous Control (FH)</th>
<th>FL Procured</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid phosphatase</td>
<td>0.022</td>
<td>0.015</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.019</td>
<td>0.012</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.024</td>
<td>0.018</td>
<td></td>
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<tr>
<td></td>
<td>0.022</td>
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<tr>
<td></td>
<td>0.020</td>
<td>0.019</td>
<td></td>
</tr>
</tbody>
</table>

<0.05

| β-Glucuronidase | 0.024       | 0.029       |
|                | 0.028       | 0.019       |
|                | 0.029       | 0.017       |
|                | 0.023       | 0.020       |
|                | 0.028       | 0.018       |

<0.05

*All tests were done in duplicate. Results are expressed as μM substrate released/10⁶ cells/hr.

Studies of Cell Granule Enzyme Content and In Vitro Adherence and Degranulation

Total granulocyte β-glucuronidase and acid phosphatase levels in FL-procured cells from five separate donors were found to be consistently low compared to normal FH-separated cells from the same donors obtained before undergoing leukapheresis (Table 1). In data not shown, CFC-procured cells also showed normal enzyme content.

Further studies of the interaction of normal FH-separated cells with nylon fiber are shown in Fig. 3. Over a 120-min period, granulocytes placed in contact with nylon fiber released into the supernatant fluid 30% of the two granule enzymes. This release did not represent cell lysis, since lactic dehydrogenase, a nongranule enzyme, was not found in the supernatant during these incubations. In addition, spontaneous release of granule enzymes did not occur when nylon...
When normal FH-separated granulocytes were incubated with normal plasma, with plasma exposed to the nylon fiber for 1 hr at 37°C, or with plasma exposed to nylon fiber and then heated at 56°C for 30 min, less than 5% enzyme release occurred. These results suggest that in this system plasma factors, particularly activated complement components, probably did not act alone to stimulate degranulation.

After incubation of normal FH-separated granulocytes with nylon fiber in the presence of colchicine (10^{-4} M) and hydrocortisone (3 \times 10^{-4} M), agents known to inhibit degranulation, no release of enzymes occurred (Fig. 4), suggesting that an active degranulation process was responsible for the enzyme release.

**Fine-Structural Studies of Adherence and Degranulation**

The previous work (Table 1) had revealed a decrease in the total amount of the two granule enzymes, acid phosphatase and \(\beta\)-glucuronidase, in polymorphonuclear leukocytes (PMN) obtained from the same donor before leukapheresis, as well as from his PMN attached to the filter. To determine if granule...
Fig. 5. (A) A field of granulocytes isolated by the FH procedure and incubated with nylon fiber (NF) for 30 min. Cells adhere to the fiber, occupying much of its surface. Note that the cytoplasmic area next to adherent plasma membrane (pm) is devoid of organelles (double-headed arrows) such as the nuclei (N), peroxidase-positive azurophil granules (ag), peroxidase-negative specific granules (sg), mitochondria (m), and smooth-surfaced endoplasmic reticulum (ser). This broad zone of organelle exclusion may represent an assembly of actin-containing filaments. × 10,800. (B) Arrows show zone of organelle exclusion at higher magnification. Cells were fixed in 1.5% glutaraldehyde, reacted for peroxidase with 3,3’ diaminobenzidine and H2O2 at pH 7.6, post-fixed in OsO4, washed in uranyl acetate, and embedded in Epon. × 22,500.
Fig. 6. Micrograph illustrating appearance of granulocytes, isolated by the FH method, before settling on nylon fibers. No intimate cell-to-cell contact can be seen, although several cells exhibit filopodia (fp). These control cells contain abundant azurophil (ag) and specific (sg) granules, as well as islands of particulate glycogen (gl). Vacuoles (V) are only occasionally present. An occasional eosinophil (E) is present. Specimen preparation was the same as for Fig. 5, except uranyl acetate wash was omitted. x 9900.

Fig. 7. (A) Field of granulocytes adherent to each other at 30 min. Cells adhere not only to the nylon fibers (see Fig. 5) but often to one another (sometimes as many as 10-15) and dangle from cells attached to fibers. Mode of adherence shown is different. Note long filopodia (fp) as well as area of contact (arrow, and in inset B). While plasma membranes (pm) of the two cells are in close apposition, they have not fused, and a fuzzy area of increased cytoplasmic density, characteristic of filopodia, can be seen near them. Specimen preparation as in Fig. 5. (A) x 12,150. (B) x 40,500.
Fig. 8. Field depicting two granulocytes incubated with nylon fiber (NF) for 2 hr. The following changes have occurred: (1) The number of cytoplasmic granules, both azurophils (ag) and specific (sg) has decreased. (2) Large vacuoles (V1-V3) have formed within the cytoplasm. (3) The wide zone, lacking in organelles present in most 30-min cells adhering to the fiber, has narrowed. The granules (arrow) have now moved very close to the adherent plasma membrane (pm). (4) Direct evidence of degranulation is apparent, since the granule enzyme peroxidase (ep) is demonstrable on some of the fiber surface. Specimen preparation as in Fig. 6. × 14,400.

enzymes were released into the extracellular medium, we initiated further studies of isolated PMN that were allowed to settle on nylon filters at various time intervals.

Granulocytes isolated by the FH method appeared relatively normal and contained numerous peroxidase-positive azurophil and peroxidase-negative specific granules, as well as islands of particulate glycogen (Fig. 6). Occasional vacuoles were present. No intimate cell-to-cell contact was observed, and a few cells exhibited filopodia.

Cells adhered either to the fibers directly (Fig. 5A) or indirectly by adhering to other cells (Fig. 7); as many as 10–15 cells could be seen dangling from some areas of the fiber. Two distinct types of contact were demonstrable: (1) cell-to-
Fig. 9. Fields of granulocytes from a 2-hr sample, illustrating the formation of vacuoles and the loss of cytoplasmic granules. (A) Large vacuole (V) within the cytoplasm is partially filled with dense peroxidase reaction product (arrows), indicating that some intracellular degranulation has occurred. x 17,400. (B) Extracellular peroxidase (ep) between the plasma membranes of two granulocytes. Other portions of the membrane (pm) are devoid of enzyme reaction product. x 15,660. (C) Some of the cells are unattached to the filter, revealing extensive loss of peroxidase-negative specific granules. Only a few specific granules are still present, and peroxidase-positive azurophil granules (ag) remain. Long filopodia (fp) are evident. x 9570. (D) Other cells are unattached to the fiber, exhibiting extensive vacuolization (V) and partial depletion of the two granule types. Centrioles (ce) can also be seen. Specimen preparation as in Fig. 5. x 9570.

fiber adherence, with plasma membrane closely contiguous, following the contours of the filter surface (Fig. 5); and (2) cell-to-cell adherence, apparently affected by a mechanism of interdigitating filopodia (Figs. 7A, 7B). Characteristically, in the 30-min specimen, the cytoplasmic region adjacent to the adherent plasma membrane contained no organelles. This broad zone of organelle
exclusion may represent an assembly of actin-containing filaments\textsuperscript{21,22} (Figs. 5A, 5B). After incubation for 120 min, however, this relatively broad zone of organelle exclusion disappeared and granules appeared very near to the partially adhering plasma membrane (Fig. 8). Additionally, peroxidase reaction product was found on some parts of the fiber surface, thereby documenting the extracellular discharge of azurophil granules (Fig. 8). By 2 hr, the cells in both types of attachment displayed evidence of vacuolization and degranulation (Figs. 8 and 9).

DISCUSSION

These experiments were designed to investigate the nature of the morphologic changes observed in granulocytes exposed to nylon fiber and to assess their in vitro function. According to our data, granulocytes procured by FL show both morphological and biochemical evidence of degranulation and, as has been previously noted by others,\textsuperscript{3,4,6,8,9} some degree of functional impairment. Our data also suggest that attachment of the granulocytes to the nylon fiber for 1–2 hr is important in the degranulation process. Attachment to fibers composed of various other materials was also shown to affect granulocytes similarly. The results of plasma incubation experiments suggested that "activated" plasma factors probably do not play a major role in this degranulation process. Also, all of our in vitro degranulation studies were carried out in the absence of plasma.

The possibility that degranulation itself is the cause of diminished bactericidal capacity of FL cells is speculative but represents the most likely explanation of the phenomenon at present. Granule enzymes are undoubtedly important in enabling granulocytes to kill microorganisms.\textsuperscript{23} Furthermore, granulocytes known to be genetically deficient in normal granule formation\textsuperscript{24,25} do not possess normal bactericidal capacity.

With the advent of effective granulocyte procurement methods, granulocyte transfusion therapy is rapidly gaining popularity. In light of this trend, whether such cells will be effective in vivo is an important question. Our data and those of others\textsuperscript{3,4,6,8,9} differ from those of Harris et al.\textsuperscript{7} Although Harris and colleagues have also observed vacuole formation in FL granulocytes, all in vitro functions of their cells were normal. Methodologic differences between our in vitro testing methods or differences in granulocyte procurement techniques may explain these differences.

It is well known that FL cells have decreased in vivo survival and decreased post-transfusion augmentation of the circulating granulocyte pool.\textsuperscript{6,9} FL cells are also associated with a higher incidence of transfusion reaction.\textsuperscript{6,8,9} Whether these effects are related to abnormalities induced by the collection procedure is unknown. However, an infusion of cells that show degranulation and membrane alterations, as well as infusion of released granule contents, might be partly responsible for these untoward effects.

In the future, frequent filter changes to decrease the contact time with nylon fiber, alternative systems made of less deleterious materials, or the addition of corticosteroids or other drugs to the procurement protocol may be useful, but the practical clinical value of these alternatives remains to be determined.
Moreover, the overall clinical cost of the slight to moderate decrease in functional capacity of FL-procured granulocytes weighed against the ability to procure and transfuse larger numbers of cells must be considered. In the meantime, it should be noted that bactericidal defects and morphological changes indicative of active degranulation are observed in FL-procured cells.

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