Volume-Dependent Human Blood Polymorphonuclear Leukocyte Heterogeneity Demonstrated With Counterflow Centrifugal Elutriation

By Roger L. Berkow and Robert L. Baehner

Human peripheral blood polymorphonuclear leukocytes (PMNs) have recently been recognized as a heterogeneous population of cells. Consideration has not been given to the possibility that size may be an additional physical characteristic demonstrating heterogeneity. Using counterflow centrifugal elutriation, we have demonstrated that PMNs can be isolated into at least six volume-dependent frac-

tions. A positive correlation exists for PMN size and superoxide anion release upon stimulation with f-Met-Leu-Phe or phorbol myristate acetate. Total granule contents were also noted to be greater in larger PMN fractions, with a constant percent of release upon stimulation. The implications of these findings are discussed.

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MATERIALS AND METHODS

Materials

Materials utilized in these studies were fMLP, cytochrome C, superoxide dismutase, Micrococcus lysodeikticus, cytochalasin B, egg white lysozyme, dimethyl sulfoxide (DMSO), n-ethylmaleimide, Folin and Ciocalteu's phenol reagent, bovine serum albumin (Sigma Chemical Co, St Louis), guaiaicol (Eastman Kodak, Rochester, NY), Triton X-100 (Research Products International, Mt Prospect, Ill), dextran 70 (Pharmacia, Piscataway, NJ), normal serum albumin 25% (Cutter Biological, Berkeley, Calif), rabbit immunoglobul-

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lin to human erythrocyte membrane (Accurate Chemicals, Westbury, NJ), heat-inactivated fetal calf serum, Hanks' balanced salt solution (GIBCO Laboratories, Grand Island, NY), "Co vitamin B<sub>12</sub>" (Amersham, Arlington Heights, IL), Norit A (Amend Drug and Chemical Co, Irvington, NJ), hydrogen peroxide (Parke-Davis Co, Morris Plains, NJ), phorbol myristate acetate (Consolidated Midland Corp, Brewster, NY), and acid citrate dextrose formula A (Fenwell Labs, Deerfield, IL).

Isolation of Volume-Dependent PMN Fractions

Blood was obtained by venipuncture in accordance with the principles of the Declaration of Helsinki and the Indiana University Committee for Human Investigation and was anticoagulated with 10% acid citrate dextrose formula A. Six percent dextran is added to make a final concentration of 1%, and the blood is allowed to sediment for 60 minutes. The leukocyte-rich supernatant is removed and centrifuged at 400 g and the pellet resuspended in elutriation buffer containing NaCl, 123 mmol/L, KCl, 4.9 mmol/L, Na<sub>2</sub>HPO<sub>4</sub>, 16 mmol/L, 0.01% wt/wt EDTA, and 2% vol/vol heat-inactivated fetal calf serum. This is then loaded into a J-E6 elutriator rotor (Beckman Instruments, Inc, Palo Alto, Calif) calibrated to 1,950 rpm. The elutriator system had been previously sterilized with 70% ethanol, rinsed with sterile distilled water, and primed with elutriation buffer. An initial counterflow rate of 9 to 9.5 mL/min is established during loading by way of a peristaltic pump (Masterflex Pump, Cole-Parmer, Inc, Chicago). This flow rate was seen in preliminary studies to allow elutriation of erythrocytes and platelets while maintaining leukocytes in the separation chamber. After loading, the same counterflow rate is maintained until clearing of the erythrocytes from the separation chamber is noted through the "viewport" in the centrifuge door. Keeping rotor speed constant, the counterflow rate is then increased by 1 mL/min in a stepwise fashion, with 100 mL of effluent collected at each point. This will allow removal of residual erythrocytes, lymphocytes, and small monocytes, and it is generally complete at a flow rate of 13.0 mL/min. No more than 7% of the loaded PMNs is lost during this procedure. The counterflow rate is then increased to 14, 15.5, 17, 19.5, 20, and 21.5 mL/min, with 100 mL of effluent collected as the PMN fractions. These are then centrifuged at 400 g for five minutes, washed twice in 0.9% NaCl, and resuspended in the appropriate buffer for counting, sizing, and functional assay. To minimize differences in time before assay, the earliest collected fractions are assayed first, followed sequentially by the fractions collected later.

Cell Volume Determination

Cell volume measurements were performed on each PMN fraction, isolated as described above, suspended in phosphate-buffered saline (PBS) (NaCl, 123 mmol/L, KCl, 4.9 mmol/L, Na<sub>2</sub>HPO<sub>4</sub>, 16 mmol/L) or Krebs phosphate buffer (PBS + CaCl<sub>2</sub>, 1.0 mmol/L, MgSO<sub>4</sub>, 1.0 mmol/L), by employing a Coulter counter model ZBI connected to a Coulter C-1000 Channelizer (set with a window width of 60) equipped with an XY recorder (Coulter Electronics, Inc, Hialeah, Fla). Results represent the relative cell number with the channel proportional to the cell volume calibrated by standard Coulter latex particles.

PMN Rosettes

Rosetting of PMN fractions with IgG-coated human erythrocytes was performed by the method of Klemper and Gallin.

Superoxide Release

The release of superoxide anion (O<sub>2</sub>·⁻) from each PMN fraction was determined by two methods. First, by our previously described modification of the method of Babior et al, based on the reduction of ferriyochrome C, which allows an endpoint determination of O<sub>2</sub>·⁻ release by the PMN. The second method allows determination of the kinetics of O<sub>2</sub>·⁻ release and utilizes the method described by Cohen and Chovaniec.

Analysis of Granule Contents

Lysozyme activity was determined by our previously described modifications of the change in absorbance at 515 nm of a suspension of Micrococcus lysodeikticus in 0.06 mol/L sodium phosphate buffer upon addition of the sample. Vitamin B<sub>12</sub> binding protein (Vit B<sub>12</sub>BP) content was assayed by the albumin-coated charcoal assay utilizing Co-labeled vitamin B<sub>12</sub> as per the method of Gottlieb et al.

Myeloperoxidase activity was determined according to the method of Paul et al and utilized the reduction of guaiacol by myeloperoxidase in the presence of hydrogen peroxide. The change in absorbance is followed at 470 nm at 25 °C.

Total granule contents were determined by the addition of 0.2% Triton X-100 to a suspension of 5 × 10<sup>6</sup> PMNs/mL. This mixture was shaken vigorously with a vortex Genie (Fisher Scientific Co, Pittsburgh) and then subjected to sonication to ensure complete release of intracellular contents. The mixture was then centrifuged at 1,600 g and the supernatant removed for the appropriate assay.

Granule protein release was determined by incubation of 5 × 10<sup>6</sup> PMNs in Kreb's phosphate buffer with 5 μg/mL of cytochalasin B for five minutes at 37 °C. The appropriate stimulant is then added for an additional five minutes at 37 °C. The reaction is stopped by placing the tubes on ice for five minutes, followed by centrifugation at 1,600 g with the supernatants then utilized for the above assays.

Release of lactate dehydrogenase (LDH) was determined by the method of Wacker et al.

Protein content was determined by the method of Lowrey et al.

Data and Statistics

To normalize data from day to day, the results (nmol O<sub>2</sub>·⁻, enzyme activity, or ng Vit B<sub>12</sub>BP) for each PMN fraction, represented by the flow rate, is taken as a percent of the mean value obtained for all fractions on any given daily experiment run in duplicate or triplicate and expressed as follows:

\[
\text{Percent of mean} = \frac{\text{value obtained from fraction}}{\text{mean value for all flow rates}} \times 100.
\]

Statistical analysis was performed using Student's t test and a linear regression analysis model utilizing a Hewlett Packard desktop computer (Hewlett Packard Co, Corvallis, Ore).

RESULTS

Volume-Dependent Separation of Human PMN

Utilizing the PMN isolation technique of CCE as described in Materials and Methods, it can be seen in Fig 1A that when the whole population of cells is analyzed for volume distribution using the Coulter Channelizer, a fairly uniform distribution of volumes is noted, with a mean volume of approximately 480 to 490 μm<sup>3</sup>. However, if the volume distribution of PMNs isolated by CCE at increasing counterflow rates is assessed in a similar fashion, it can be appreciated in Fig 1B (a representative curve) that the uniform curve noted in Fig 1A can be subdivided into six overlapping
VOLUME-DEPENDENT PMN HETEROGENEITY

Fig 1. (A) A representative volume distribution of CCE-isolated PMN. Cell number is relative. Cell volume is based on Coulter electronics latex calibration particles. (B) A representative recording from the Coulter Channelyzer of the volume distribution of PMNs isolated at increasing counterflow rates, as described in Materials and Methods.

curves that have significantly different mean peak volumes (n = 16; P < .001), ranging from 440.0 to 553.7 μ3. These fractions related to calculated mean diameters of 9.44 to 10.20 μ and calculated mean surface areas ranging from 280 to 326 μ2 (Table 1). The percent of PMN recovery at each increment in CCE counterflow rate is also noted in Table 1. As expected, a normal distribution around the mean flow rates of 17 and 18.5 mL/min is noted. When mean fraction volume is compared to the counterflow rate necessary for the elutriation of that fraction, a correlation coefficient of r = .999, P < .001 is noted. This is the result expected based on the mathematical formula describing elutriation (data not shown).

Rosetting With IgG-Coated Erythrocytes

The first clear demonstration of heterogeneity in PMNs was the description by Klempner and Gallin that 80% of peripheral blood PMNs formed rosettes with IgG-coated erythrocytes. Table 1 shows the results of seven experiments in which 400 cell counts were performed on duplicate samples of each fraction, as described above, to assess erythrocyte rosettes. It is noted that no significant difference is appreciated between any of the VDPF, with a range of 83.9% to 87.7% rosetting PMN.

Superoxide Release of VDPF

As shown in Fig 2, when 2.5 × 10^6 PMNs are stimulated with 10^−6 moL/L fMLP for six minutes at 37°C in the presence of cytochrome C, the superoxide release increases in a linear fashion as the counterflow rate of elutriation (and therefore the volume of the PMNs) increases. When the value obtained at each flow rate is expressed as a percent of the mean value for all flow rates, a range of 88.8% to 114.3% is noted (r = .98, P < .01; n = 8). To assess whether the increased volume of the PMNs would totally account for the increasing O_2− release noted in larger VDPF, the values seen in Fig 2 were corrected for differences in PMN volume. It is noted in Fig 3 that although the slope of the curve was less steep, an increasing relationship is still noted, with a correlation coefficient r = .87, P < .05, and a range of percent mean value of 95.0% to 105.9%. In a separate series of four experiments, fMLP-induced O_2− release was expressed both in terms of protein content and PMN number. As can be seen in Fig 4, when O_2− release is expressed by protein content, an inverse relationship compared to the O_2− release value obtained per cell number was obtained. This suggests that the larger volume PMNs have a proportionately greater amount of nonoxidative protein compared to the smaller volume PMNs.

To assess whether the greater oxidative burst noted

<table>
<thead>
<tr>
<th>Flow Rate (mL/min)</th>
<th>Mean Volume (μ3) (n = 16)</th>
<th>Mean Surface Area (μ2) (n = 16)</th>
<th>Mean Diameter (μ) (n = 16)</th>
<th>PMN Recovery (%) (n = 12)</th>
<th>Rosettes With RBC (n = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>14.0</td>
<td>440 ± 1.9*</td>
<td>280.0</td>
<td>9.44</td>
<td>3.9 ± 0.5</td>
<td>85.7 ± 2.8</td>
</tr>
<tr>
<td>15.5</td>
<td>458 ± 1.9*</td>
<td>287.7</td>
<td>9.57</td>
<td>15.5 ± 1.8</td>
<td>87.7 ± 2.6</td>
</tr>
<tr>
<td>17.0</td>
<td>480 ± 2.5*</td>
<td>296.2</td>
<td>9.71</td>
<td>27.1 ± 1.0</td>
<td>86.6 ± 3.0</td>
</tr>
<tr>
<td>18.5</td>
<td>507 ± 2.5*</td>
<td>307.9</td>
<td>9.9</td>
<td>27.5 ± 0.8</td>
<td>84.6 ± 3.4</td>
</tr>
<tr>
<td>20.0</td>
<td>535 ± 3.7*</td>
<td>320.5</td>
<td>10.1</td>
<td>18.9 ± 1.5</td>
<td>84.8 ± 3.6</td>
</tr>
<tr>
<td>21.5</td>
<td>553 ± 3.7*</td>
<td>326.9</td>
<td>10.2</td>
<td>5.9 ± 0.9</td>
<td>83.9 ± 3.3</td>
</tr>
</tbody>
</table>

Values represent mean ± SEM.
*P < .01 for next higher and lower fraction.
in the larger volume PMN fractions was due to an intrinsic difference between the PMN groups or was related to the ligand used, we quantified PMN \( O_2^- \) release stimulated by PMA, which activates the oxidase system by a mechanism different from that of fMLP.\(^3\) It can be seen in Fig 5 that 50 ng/mL PMA elicited a linear increase in percent of mean PMN \( O_2^- \) release as PMN volume increased. A wider range was noted, however, than when fMLP was used as the stimulant, with a range of 71.1% to 133.7% (\( r = .97, P < .01; n = 4 \)).

To evaluate the influence of the initial rate of \( O_2^- \) release and the time to onset of the respiratory burst after the addition of the stimulant (lag time) on the increased \( O_2^- \) release noted above, we measured the kinetics of \( O_2^- \) production as described by Cohen and Chovaniec.\(^2\) As can be seen in Fig 6, the initial rate of change in absorbance at 550 nm increases linearly with increasing PMN fraction size (or counterflow rate) when 100 ng/mL PMA was used as the stimulant. A range of 81.5% to 118.1%, when expressed as the percent of the mean value, is noted (\( r = .99, P < .01; n = 4 \)). However, the lag time to onset of the burst was not different between the PMN fractions.

When \( 10^{-7} \) mol/L fMLP was used as a stimulant, a very rapid (less than ten seconds) lag time was noted that was not different among the VDPF (data not shown). The initial change in absorbance at 550 nm, as seen in Fig 7, did, however, increase in a linear fashion among the smaller VDPF, but reached a maximal initial rate among the larger PMN fractions. Resting PMN \( O_2^- \) release was never different among the VDPF (data not shown).
Evaluation of Granule Contents and Release

The total content and release upon stimulation of granule contents can be noted in Table 2. Lysozyme, contained in both the azurophilic and specific granules, is noted to have a range of 93.5% to 106.4% when expressed as the percent of the mean value for the smallest to the largest fractions. Although this represents a slowly rising total enzyme activity, a positive correlation was noted between lysozyme content and the PMN size group \((r = .99, P < .01)\). The percent release of lysozyme upon stimulation with \(10^{-6}\) mol/L fMLP, however, was not different among the six PMN fractions.

Myeloperoxidase (MPO), which is contained exclusively in the azurophilic granules, was noted to have a range of total enzyme activity in the PMN fractions of 66% to 134.6% when each fraction was expressed as the percent of the mean activity from all fractions, with a very steep increase noted among the middle three fractions. A correlation coefficient \(r = .97\) was noted for the relationship of MPO content to PMN fraction size. The percent release of MPO upon stimulation with \(10^{-6}\) mol/L fMLP was not noted to be significantly different between the increasingly larger VDPF.

Vitamin B\(_{12}\) binding protein, which is contained exclusively in the specific granules, followed a slightly different pattern, demonstrating a linear increase in total content among the smallest four fractions, with a maximal content noted among the largest PMN fractions. Again, percent release of vitamin B\(_{12}\) binding protein upon stimulation with \(10^{-6}\) mol/L fMLP was not significantly different among the VDPF. In all studies, release of cytoplasmic LDH was always less than 4%.

A graphic representation of total granule contents (the value of activity at each flow rate is expressed as a percent of the mean activity of all flow rates) of the VDPF is noted in Fig 8, which reveals the striking relation of MPO activity among the fractions as well as the plateau of vitamin B\(_{12}\) binding protein content among the largest fractions.

**DISCUSSION**

PMN heterogeneity has been demonstrated in a variety of ways.\(^{13-21}\) However, consideration has not been given to the possibility that size may be an additional physical characteristic that can subdivide

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**Table 2. Granule Contents and Release**

<table>
<thead>
<tr>
<th>Counter Flow Rate</th>
<th>Lysozyme (n = 3)</th>
<th>Myeloperoxidase (n = 3)</th>
<th>VitB(_{12})BP (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Percentage of Mean Total Content</td>
<td>Percentage of Release</td>
<td>Percentage of Mean Total Content</td>
</tr>
<tr>
<td>14.0</td>
<td>93.5 ± 3.8</td>
<td>40.9 ± 2.12</td>
<td>66.0 ± 4.0*</td>
</tr>
<tr>
<td>15.5</td>
<td>95.1 ± 1.3</td>
<td>40.5 ± 1.0</td>
<td>75.9 ± 3.9</td>
</tr>
<tr>
<td>17.0</td>
<td>97.5 ± 5.4</td>
<td>39.6 ± 1.7</td>
<td>81.9 ± 2.6*</td>
</tr>
<tr>
<td>18.5</td>
<td>101.1 ± 4.5</td>
<td>37.6 ± 0.9</td>
<td>102.8 ± 2.4*</td>
</tr>
<tr>
<td>20.0</td>
<td>104.5 ± 2.9</td>
<td>38.8 ± 1.1</td>
<td>131.8 ± 2.3</td>
</tr>
<tr>
<td>21.5</td>
<td>106.4 ± 3.7</td>
<td>39.9 ± 2.0</td>
<td>134.6 ± 4.3</td>
</tr>
</tbody>
</table>

Values represent mean ± SE of the mean of triplicate samples. ND, not done.

*Significantly different from next larger fraction.
the circulating human PMNs. Using CCE, heterogeneity based on size has been demonstrated for red blood cells, platelets, and monocytes. Employing this method, we found that human peripheral blood PMNs can be subdivided into at least six groups with significantly different mean peak volumes. A positive linear relationship exists between PMN size and cumulative O$_2^-$ production when either fMLP or PMA is used as the stimulant. This increase is not due solely to differences in PMN volume. In addition, the initial rate of O$_2^-$ release increases linearly as PMN volume increases when PMA is utilized as the stimulant; however, a maximal initial rate is noted among the largest fractions when fMLP is the stimulant. Granule contents are also noted to be different between the VDPF, with total lysozyme content slowly increasing linearly and myeloperoxidase content having a dramatic linear increase as PMN volume increased. Total vitamin B$_{12}$ binding protein content, however, increases in a linear fashion among the smallest PMN fractions, with a plateau of maximal content in the largest fractions. Percent release of granule contents upon stimulation with fMLP was always noted to be constant among the VDPF; although due to larger total quantities present, the absolute amounts of granule contents released increased with PMN size.

The implications of these data are several. The demonstration that circulating PMNs are heterogeneous with respect to size, and that increasing size has a positive correlation with increasing oxidative burst and granule contents, emphasizes in yet an additional way that PMNs are not homogeneous. The possibility of enrichment of the larger VDPF for a previously defined more active PMN must be considered. That the VDPF showed no difference in rosetting to IgG-coated erythrocytes probably indicates that our largest fractions were not enriched with the more active PMN population of Klemmner and Gallin. Furthermore, in a study by Seligmann et al evaluating PMN subpopulations, PMA produced a homogeneous response with respect to membrane potential change upon stimulation, whereas fMLP produced a heterogeneous response, with a responding and nonresponding population seen. If, in our system, the VDPF were enriched for the responding fraction as described by Seligmann et al, then no difference should have been noted for the oxidative response of the fractions to PMA. To better define the question of whether or not size is an additional characteristic of a previously defined, more active fraction, further studies of VDPF for membrane depolarization, surface binding of the chemotactic peptide and PMA, adherence to surfaces, and directed migration are currently in progress.

The nature of the oxidative responses of the VDPF must be considered next. It is possible that within larger PMN fractions, the greater surface area allowed for greater cell–cell interaction upon stimulation, with a resulting prolongation of the oxidative burst as described by Dahinden et al for PMNs adherent to plastic surfaces. However, our data, as seen for fMLP (Fig 3), did not normalize for volume. It must be realized that membrane surface area may be a more appropriate correction factor. Due to membrane ruffling and folding, which occur in PMNs, the true surface area cannot be accurately determined by geometric methods. Studies to analyze membrane constituents are planned to answer this question.

It has been reported by others that myeloperoxidase plays a role in the termination of the PMN oxidative burst. Our observation that total MPO content showed a very marked increase as PMN size increased, as well as the fact that sodium azide, an inhibitor of MPO, was present in our endpoint O$_2^-$ assay, makes earlier termination of the oxidative burst by MPO in the smaller PMN fractions an unlikely cause for the volume-dependent differences in the total O$_2^-$ release.

In regards to the plateau of the initial rate of fMLP-induced O$_2^-$ release of the larger VDPF, it is interesting to note the striking similarities between this curve (Fig 7) and the percent of mean total vitamin B$_{12}$ binding protein among the VDPF (Fig 8). It is possible that specific granule contents are important in controlling this aspect of the fMLP-induced oxidative burst, as implicated by Borregaard and Tauber. It must also be concluded that a greater initial rate of O$_2^-$ release among the larger VDPF plays more of a role in the greater total oxidative burst when PMA is used as the stimulant, but that factors other than initial rate of release are important when fMLP is used.

Finally, the significance of size heterogeneity of circulating PMNs must be considered. Based on
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Volume-dependent human blood polymorphonuclear leukocyte heterogeneity demonstrated with counterflow centrifugal elutriation

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