Purification and Functional Evaluation of Mature Neutrophils From Human Bone Marrow

By Roger L. Berkow and Robert W. Dodson

Human myeloid maturation proceeds within the bone marrow and results in a mature neutrophil that is released into the peripheral circulation. Previous reports have indicated that neutrophils from bone marrow demonstrate decreased adherence, impaired phagocytosis, and decreased nitroblue tetrazolium dye reduction when stimulated. Due to lack of a suitable method for isolating purified bone marrow neutrophils, these studies have been performed by microscopic techniques. We now report a method for isolating 1 x 10^8 neutrophils [bands plus polymorphonuclear leukocytes (PMNs)] from 10 mL of bone marrow aspirate sample. By means of a discontinuous Percoll-gradient centrifugation through densities of 1.085, 1.095, and 1.10 g/mL a leukocyte-rich suspension of bone marrow can be separated into three leukocyte layers. By combining the lower two leukocyte layers (M2/3), a population of neutrophils consisting of 28% bands and 63% PMNs is seen. When compared with peripheral blood PMNs, these bone marrow neutrophils had a lower alkaline phosphatase activity, decreased ingestion of Oil Red O-coated particles, impaired superoxide release on stimulation with the chemotactic peptide Fmøt-leu-phe (FMLP) or the tumor promoter phorbol myristate acetate (PMA), and less activity of the NADPH-dependent oxidase. These results indicate that morphologically mature neutrophilic cells within the bone marrow exist in a still functionally immature state.

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Neutrophil preparation. Isolation of mature neutrophils from the PB or BM of healthy volunteers was performed simultaneously. Our research was performed in accordance with the Declaration of Helsinki and the University of Alabama at Birmingham Committee for Human Investigations. After appropriate skin cleansing, 10 mL of PB was aspirated from the posterior iliac crest into a syringe containing 1.7 mL of ACD. Slides were prepared for differential counts and the remaining BM was diluted to 20 mL with 0.9% NaCl. This cell suspension was forced through needles with decreasing internal diameter (19, 20, and 21 gauge) to disrupt any remaining fragments. Five milliliters of 6% Dextran (70) in 0.9% NaCl was added to allow erythrocyte sedimentation. After 60 minutes at 25 °C the leukocyte-rich supernatant (LRS) was removed and centrifuged at 400 g at 4 °C; the supernatant was discarded, and the pellet was suspended with 8 to 10 mL of 0.9% NaCl.

For the isolation of neutrophils from PB, the following modifications were made. Whole blood (45 mL) was obtained by venipuncture, anticoagulated with 14.5% ACD, and mixed with 10 mL of Dextran as described. The leukocyte-rich supernatant (LRS) was removed after 1 hour at 25 °C and centrifuged as described. The pellet was suspended in 0.9% NaCl to form a leukocyte suspension.

Four to 5 mL of the leukocyte suspension, containing ~5 x 10⁷ nucleated cells/mL, obtained from BM or PB was layered over the temperature-equilibrated discontinuous Percoll gradient and centrifuged at 700 g at 25 °C for 30 minutes in a swinging bucket rotor in a Beckman TJ-6R centrifuge. The rotor speed was allowed to develop slowly, and no brake was used at the completion of the centrifugation. Three leukocyte bands appeared. These were removed with a polypropylene transfer pipette and washed in 0.9% NaCl. Due to the presence of small amounts of erythrocyte contamination in the BM leukocyte bands, all bands were exposed for 20 seconds at 25 °C to distilled water for lysis of erythrocytes, with reconstitution of osmolarity with 3.5% NaCl. Peripheral blood neutrophils were osmolarity distilled water, according to instructions supplied by Pharmacia. The pH of these solutions was adjusted to 7.4, and then was filter-sterilized. Discontinuous gradients were made by the addition of these solutions to 15-mL polystyrene centrifuge tubes. The bottom layer consisted of 4 mL of the 1.10 g/mL Percoll solution. Two additional layers of 3 mL each with a density of 1.095 and 1.085 g/mL were then carefully applied. All solutions were allowed to come to room temperature prior to centrifugation.

Neutrophil enzyme content. The cell content of the neutrophil membrane constituent AP was determined by the method of DeChatelet and Cooper using p-nitrophenyl-phosphate as the substrate. The assay time was 30 minutes. The results obtained are expressed as micromoles of p-nitrophenyl phosphate hydrolyzed for each 30 minutes per milligram of protein. Protein content was determined by the method of Lowry and colleagues.

The primary granule constituent myeloperoxidase (MPO) was determined by our previously defined method using the oxidation of guaiacol by myeloperoxidase in the presence of hydrogen peroxide. The change in absorbance was followed at 470 nm at 25 °C. Results are expressed as micromoles of guaiacol oxidized per minute per milligram of protein.

RESULTS

Isolation of bone marrow neutrophils. Figure 1 reveals the appearance of the Percoll gradient before and after centrifugation of the LRS obtained from BM aspirates. Four cell bands appeared (Table I). M1, at the interface of the saline and 1.085-g/mL layer, contained 37% myelocytes, promyelocytes, and blasts, and 30% metamyelocytes with only 15% mature neutrophils (band form plus segmented PMNs). M2, at the interface of the 1.085-g/mL and 1.095-g/mL layers covered ~5 to 8 mm of the gradient. It contained 90% mature neutrophils and accounted for 69.9% of the total mature neutrophils isolated from the combined M2/M3 fraction. Also seen in the M2 layer were 6% metamyelocytes, 2% myelocytes, and 2% mature eosinophils. M3, at the interface of the 1.095- and 1.10-g/mL layers covered ~5 mm, contained 91% mature neutrophils, and accounted for 30.1% of the mature neutrophils in the combined M2/M3 fraction. Also contained in the M3 layer were 1% myelocytes, 2% metamyelocytes, 5% eosinophils, and 1%
nucleated erythroid precursors. M4 was represented as the RBC pellet that contained variable numbers of leukocytes. To obtain the greatest yield of mature neutrophils, the M2 and M3 layers were combined. This combined layer contained 89% mature neutrophils, 4% eosinophils, 5% metamyelocytes, and 2% myelocytes and yielded a mean of 1.2 ± 0.2 × 10^6 mature neutrophils/10 mL of BM aspirate sample. The M2/M3 fraction was used in all further studies and is referred to as BM neutrophils. These were noted to be 99% viable by exclusion of 0.2% (wt/vol) trypan blue and represented 68.9% of the mature neutrophils present in the LRS applied to the gradient.

When the LRS of PB was subjected to the same discontinuous Percoll-density centrifugation, three distinct leukocyte bands were observed that corresponded in position to the BM bands already described. Lymphocytes and monocytes were confined to the PB1 layer. PB2 contained 95% PMNs and 5% band forms and was most frequently of low yield. The PB3 layer was noted to contain 95% PMNs, 4% eosinophils, and 1% band forms. The PB4 layer containing the RBC pellet was noted to have variable numbers of eosinophils with minimal contamination by PMNs. As with the BM, the PB3 and PB2 layers were combined and yielded 1 to 2 × 10^6 mature neutrophils/100 mL of whole blood initially obtained. Viability was always >99%.

**Neutrophil volume.** We previously reported that PB PMNs can be separated into at least six fractions based on differences in mean cell volume.79 To determine if volume variation in PB PMNs could be related to PMN age we assessed cell volume, as described in the Materials and Methods section, in the BM and PB neutrophils. Although the M2 fraction appeared to be slightly larger than PB neutrophils, when the M2/M3 fraction was compared with PB neutrophils, no consistent difference in volume was detectable (data not shown).

**Neutrophil enzyme content.** Based on histochemical staining, it has long been established that the AP content of BM neutrophilic cells is less than that of PB cells.10-13 This has also been demonstrated by the observation that biochemically measured neutrophil AP decreases in the PB after administration of corticosteroids to release the BM neutrophil pool.38 Figure 2 shows that Percoll-purified BM PMNs isolated as described in the Materials and Methods section have 45% ± 4.4% of the biochemically measured AP activity as is noted in the PB neutrophils (P < .001).

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**Table 1. Percentage of Cell Types in Bone Marrow Fractions**

<table>
<thead>
<tr>
<th>Layer</th>
<th>F</th>
<th>My</th>
<th>MM</th>
<th>B</th>
<th>P</th>
<th>Eos</th>
<th>Ly</th>
<th>O</th>
<th>Yield P + B (% from LRS)</th>
</tr>
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<tbody>
<tr>
<td>M₁</td>
<td>37</td>
<td>30</td>
<td>11</td>
<td>4</td>
<td>ND</td>
<td>14</td>
<td>4</td>
<td>14.6</td>
<td></td>
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<tr>
<td>M₂</td>
<td>2</td>
<td>6</td>
<td>33</td>
<td>57</td>
<td>2</td>
<td>ND</td>
<td>ND</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>M₃</td>
<td>1</td>
<td>2</td>
<td>12</td>
<td>79</td>
<td>5</td>
<td>ND</td>
<td>1</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>M₁/₂</td>
<td>2</td>
<td>5</td>
<td>26</td>
<td>63</td>
<td>4</td>
<td>ND</td>
<td>ND</td>
<td>68.9</td>
<td></td>
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<tr>
<td>LRS</td>
<td>14</td>
<td>16</td>
<td>16</td>
<td>31</td>
<td>2</td>
<td>6</td>
<td>15</td>
<td>100.0</td>
<td></td>
</tr>
</tbody>
</table>

F, bone marrow fraction; My, myelocyte; MM, metamyelocyte; B, band form; P, polymorphonuclear leucocytes; Eos, eosinophil (mature); Ly, lymphocyte; O, other (erythrocyte precursors, myeloblasts); ND, none detected; LRS, leucocyte-rich supernatant. Results are the mean of nine separate separations.

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Koeffler and co-workers, using the HL-60 promyelocytic leukemic cell line, showed that synthesis of the primary granule constituent myeloperoxidase decreased as the cell matured.13,39 This may imply that BM neutrophils, being less mature than circulating neutrophils, should have greater MPO activity than PB neutrophils. Figure 2 reveals no difference between these populations in MPO activity, which indicates that MPO synthesis is complete by the time the neutrophilic cells reach the band form stage of development, and degradation of activity does not accompany further maturation.

**Phagocytosis by bone marrow neutrophils.** Altman and Stossel reported that neutrophilic cells within the BM have a decreased capacity to ingest particles compared to neutrophils from PB.8 These data are supported by the observation of decreased phagocytosis by leukemic myeloid cells.24,25 Figure 2 shows that when ORO-coated lipopolysaccharide particles that had been opsonized with human serum were used, BM neutrophils ingested 59.5% ± 4.6% of the milligrams of Oil Red O as did PMNs from PB (P < .01).

**Release of superoxide anion.** By examining whole BM samples, Zakhireh and Root showed that immature neutrophils from BM had decreased reduction of nitroblue tetrazolium dye, indicating a decreased oxidative capacity.8 No study has directly evaluated the superoxide release of mature neutrophils isolated from BM.

Table 2 shows that BM neutrophils released 23.3%, 34.9%, and 24.0% of the O₂⁻ release by similar numbers of PB neutrophils, in a 5-minute assay, when 10⁻⁴, 10⁻⁻, and 10⁻⁴ mol/L of FMLP, respectively, was the stimulant. To determine if the decrease in O₂⁻ release observed was due to a delay in initiation of the respiratory burst or due to an altered maximal velocity and duration of O₂⁻ release we assessed the kinetics of O₂⁻ release. Figure 3 shows that in a representative experiment the lag time to initiation of the respiratory burst was similar for both BM and PB neutrophils. BM
neutrophils, however, demonstrated a slower maximal rate of 
\( \text{O}_2^- \) production regardless of the concentration of FMLP
used as the stimulant. Also apparent from Fig 3 is that the duration of 
\( \text{O}_2^- \) release (time to reach plateau) was the same for both BM and PB
neutrophils. These data indicate that the slower \( \text{O}_2^- \) release noted in BM neutrophils stimulated 
with FMLP was not overcome by increasing the agonist
concentrations and that the initiation and termination of the
respiratory burst was the same for BM and PB neutrophils.

To determine if the impaired \( \text{O}_2^- \) release by BM neutrophils was unique to the agonist used or due to an intrinsic cellular
defect, we assessed the respiratory burst induced by the
tumor promoter, PMA, an agonist that activates neutrophils by a different biochemical mechanism than that of
FMLP.\(^{40,44}\) Figure 4 shows a representative experiment
which revealed that, as with FMLP, BM neutrophils stimulated 
with PMA have a slower rate of \( \text{O}_2^- \) release (48% to
60% of PB neutrophils), regardless of the concentration of 
stimulant used. The maximal rate of \( \text{O}_2^- \) release of BM and 
PB neutrophils is noted in the inset to Fig 4. Although the duration of 
\( \text{O}_2^- \) release of BM neutrophils stimulated with
PMA was slightly longer than for PB neutrophils (as shown in Fig 4),
allowing the reaction to proceed for times up to 30
minutes did not alter our findings. Lag time to initiation of 
\( \text{O}_2^- \) release was not significantly different between BM and 
PB neutrophils, 58.5 \pm 7.9, 84.25 \pm 18.0 and 194 \pm 12.5
seconds v 58.5 \pm 7.9, 76.5 \pm 19.5 and 218 \pm 18.2 s at 5, 2.5
and 1 \times 10^{-8} \text{ mol/L} of PMA, respectively, for BM and PB
neutrophils (M \pm SEM, n = 5). In addition, we also observed a similar slowing of \( \text{O}_2^- \) release when serum-
treated zymosan particles were used as the stimulus (data
not shown).

Because activation of the NADPH-dependent oxidase
represents the final pathway for the reduction of molecular oxygen to \( \text{O}_2^- \) by all neutrophil agonists,\(^{32,40,44}\) we measured the activity of this enzyme system in cell-free neutrophil
sonicates in the presence of arachidonic acid and NADPH.

Figure 5 shows that BM neutrophils contained only 47% of
the NADPH-dependent oxidase activity as did PB neutrophils
(4.40 \pm 0.4 vs 9.36 \pm 1.20 nmol \( \text{O}_2^- \)/min/mg of cell
protein for BM and PB neutrophils, respectively, M \pm SEM,
n = 9).

Based on these results, it is likely that a deficiency of
NADPH oxidase activity in BM neutrophils is the cause of the
slower rate of \( \text{O}_2^- \) release noted. To determine if the 26%
band forms in the BM neutrophil suspension or a subpopulation
of BM-segmented PMNs were not effectively producing \( \text{O}_2^- \) on stimulation, we assessed the reduction of nitroblue
tetrazolium dye on addition of PMA (2.5 and 5.0 \times 10^{-8}
\text{ mol/L}) by BM neutrophils that were adherent to glass slides.

We observed that in the adherent cells, band forms still
represented 25% to 30% of neutrophilic cells. Although BM
neutrophils were noted to have fewer blue-black granules per
cell than did PB neutrophils, 96% of BM neutrophils
demonstrated reduction of the dye (data not shown).

**DISCUSSION**

In a previous publication, we reported that PB PMNs
could be separated, by counterflow centrifugal elutriation,
into at least six fractions that had increasingly larger mean
peak volumes. We demonstrated that the larger PMN fractions
had a greater oxidative burst on stimulation with either
FMLP or PMA. In addition, larger PMNs were shown to
have a greater content of the primary granule constituent
myeloperoxidase than did smaller PMNs.\(^{37}\) Further studies
have shown that larger PMNs have a greater phagocytic
capacity but lower alkaline phosphatase activity per milli-
gram of protein than that of smaller PMNs.\(^{44}\) To determine
if volume-dependent PMN fractions were related to the age
of the PMN or to the time of release from the BM PMN

**Table 2. Release of \( \text{O}_2^- \) by PB and BM Neutrophils With
FMLP as Stimulant**

<table>
<thead>
<tr>
<th>Concentration (mmol/L)</th>
<th>PB</th>
<th>BM</th>
</tr>
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<tbody>
<tr>
<td>1 \times 10^{-8}</td>
<td>21.9 \pm 2.8</td>
<td>5.1 \pm 1.0*</td>
</tr>
<tr>
<td>1 \times 10^{-7}</td>
<td>18.3 \pm 246</td>
<td>6.4 \pm 3.4*</td>
</tr>
<tr>
<td>1 \times 10^{-6}</td>
<td>7.3 \pm 3.0</td>
<td>1.8 \pm 0.6</td>
</tr>
</tbody>
</table>

FMLP, F-met-leu-phe; PB, peripheral blood; BM, bone marrow.

Nanomoles of \( \text{O}_2^-/2.5 \times 10^8 \) neutrophils/5 minutes; M \pm SEM; n = 3
to 9.

* \( P < .05 \) v PB, paired t test.

Fig 3. SOD-inhibitable reduction of ferricytochrome C of 2.5 \times 10^8 bone marrow (BM) or
peripheral blood (PB) neutrophils incubated with the stated concentration of F-met-leu-phe (FMLP),
and measured in a double-beam spectrophotometer as the change in absorbance at 550 nm at 37°C.
FMLP is added at time 0. Lag time and maximal rate are determined as described by Cohen and
Chovancie."
storage pool, it was necessary to develop a technique for isolating large quantities of mature BM neutrophils.

Although many studies have examined the isolation of various colony-forming cells from BM, few have attempted to purify mature neutrophils from this storage pool. Early attempts to concentrate mature BM neutrophils have been reported by Evans and co-workers. In their study, 8 \times 10^7 metamyelocytes, bands, and PMNs with 85% purity were recovered from a 15-mL BM sample by means of sequential centrifugation in 15.6% and then 20% Ficoll. Because no differential counts were reported, the number of bands and PMNs in this preparation cannot be determined. Sato and colleagues used discontinuous gradients of Ficoll and sodium metrizoate to isolate postmitotic neutrophils with >70% purity. No further breakdown of this neutrophilic population was given. Using continuous Percoll gradients, Olofsson and colleagues determined the densities of the various neutrophilic cells from human BM and showed that metamyelocytes and mature neutrophils had densities of 1.0799 and 1.0864 g/mL, respectively. By adapting the data reported in these studies, we developed the method previously described for isolating purified BM neutrophils.
The results presented here indicate that morphologically mature neutrophils, ie, bands plus PMNs, can be isolated in sufficient purity and quantity from small volumes of BM to allow biochemical studies. Several points concerning the separation must be noted. First, in the preliminary work, we found that the density of the uppermost layer was crucial to preventing the contamination of the M2 layer with myelocytes and metamyelocytes. A decrease in density of this layer by only .003 to .005 g/mL led to a considerable contamination of M2. Second, as shown in Table 1, we found that the M2 layer had a higher percentage of band forms as compared with the M3 layer, suggesting that cell density increases as the band form neutrophils mature to PMNs. Third, the layering within the Percoll gradient of the PB neutrophils isolated at the same time as BM neutrophils revealed that most PMNs recovered from the M2 layer (data not shown) and thus were demonstrating a somewhat higher band mean density than were the mature neutrophils isolated from the BM. This indicates a continued progression to higher density as the PMN matures in the peripheral circulation. Fourth, the presence of variable RBC contamination in the M2 and M3 layer required the use of a hypotonic lysis procedure to ensure the removal of these elements. Last, despite repeated manipulations of the gradients, we were unable to separate the band forms from the PMNs within layer M2.

As stated previously, earlier studies using microscopic techniques revealed decreased AP activity, ingestion of particles, and reduction of molecular oxygen in BM neutrophils.\(^7\) Biochemical studies using the HL-60 cell line demonstrated similar findings.\(^15\) The results of the present study reveal that morphologically mature BM neutrophils have only 45% of the biochemically measured AP activity, 60% of the phagocytic capacity, and a slower \(O_2^-\) release as compared with PB neutrophils, confirming previous studies using cytochemical techniques.\(^7\) These results also confirm the validity of using these parameters as indicators of neutrophil maturity and age. In addition, that the present findings do not correlate with those noted for volume-dependent PMN fractions indicates that volume heterogeneity among PMNs is probably not a reflection of PMN age as previously suspected.\(^17\)

The development of the ability to release superoxide anion is required if the mature neutrophil is to kill bacteria effectively. The formation of this reduction product of molecular oxygen, in the PMN, on the activation of an NADPH-dependent oxidase. This oxidase has been shown to be activated by differing mechanisms dependent on the stimulus used.\(^40\) As shown in the Results section, BM neutrophils demonstrated a slower rate of \(O_2^-\) release with a similar lag time and duration of \(O_2^-\) production as compared with PB neutrophils. This slower rate was not overcome by higher concentrations of stimulants, indicating that the results are not due to an altered threshold for stimulation. In addition, the slower rate of \(O_2^-\) release of BM neutrophils occurred regardless of the stimulant used. These results suggest that the existence of a subpopulation of nonresponding cells or an alteration of the \(O_2^-\) producing mechanism of these cells in some way. The results obtained for nitroblue tetrazolium dye reduction indicate that our findings are not due to a lack of oxygen-reductive capability by the BM bands or a subpopulation of the BM-segmented forms, although the intensity of nitroblue tetrazolium reduction was somewhat less in all BM neutrophils than in PB neutrophils. The data clearly indicate, however, that BM neutrophils contain only 47% of the NADPH oxidase activity contained in PB neutrophils and imply that this deficiency is responsible for the decrease in \(O_2^-\) release noted in the BM neutrophils.

Although release of mature neutrophils from BM undoubtedly occurs during the stress of severe bacterial infection, it is questionable if the deficits in function seen in the present study would lead to a physiologic deficiency of host defense. First, it is noted that females who are heterozygous for the gene coding for chronic granulomatous disease do not have significant bacterial infections despite having, on the average, only 50% of the superoxide-generating capacity of normal persons.\(^34\) Second, during bacterial infections, the neutrophils are exposed to various endogenous and bacterial products that may actually augment their responses to subsequent exposures to stimulants; thus, BM neutrophils may have their responses augmented or "primed" on being released during times of infection.\(^35\) This is supported by the observation of an increased PMN oxidative burst in patients with active bacterial infections.\(^34\)

In conclusion, we report an effective method for the isolation of large numbers of morphologically mature (bands and segment) neutrophils from small quantities of BM aspirate samples. We confirmed earlier reports demonstrating decreased AP activity, phagocytosis, and oxidative burst of BM neutrophils. We extended these studies to demonstrate that BM neutrophil \(O_2^-\) release was slower, but with a similar lag time and duration of release, as compared with PB neutrophils, regardless of whether FMLP, PMA or serum-treated zymosan was used as the stimulus. Moreover, we demonstrated that this was not due to the increased percentage of neutrophilic band forms or a subpopulation of cells with an inability to produce \(O_2^-\) in the BM neutrophils. Finally, we are first to demonstrate a decreased NADPH oxidase activity in morphologically mature BM neutrophils as the cause of the deficient stimulated oxidative burst.

ACKNOWLEDGMENT
We appreciate the expert secretarial help of Jo Lacey.

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