Superoxide Anion Release From Blood and Bone Marrow Neutrophils Is Altered by Endotoxemia

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In vivo endotoxin infusion produces neutrophil-mediated acute lung injury and increases superoxide anion release from phorbol myristate acetate (PMA)-stimulated blood neutrophils collected 18–24 hours after the infusion. Because the turnover time of circulating blood neutrophils is only 6–8 hours, it was hypothesized that the prolonged increase in superoxide anion release from peripheral blood neutrophils is associated with increased superoxide anion release from bone marrow neutrophils. To test this hypothesis, two doses of Escherichia coli endotoxin (5.0 and 0.5 μg/kg) were infused into chronically instrumented awake sheep. Blood and bone marrow neutrophils were collected 24 hours after the infusion, and superoxide anion release from unstimulated and PMA-stimulated neutrophils was measured in vitro. Endotoxin infusion produced an increase in pulmonary microvascular permeability, in intravascular activation (degranulation) of blood neutrophils, and in circulating blood neutrophils 24 hours after the infusion. High-dose endotoxin (5.0 μg/kg; n=4) increased superoxide anion release from unstimulated peripheral blood neutrophils (2.25±0.38 times baseline [p<0.05]) and from peripheral blood neutrophils stimulated with 10^{-9} M PMA in vitro (1.46±0.55 times baseline). Low-dose endotoxin (0.5 μg/kg; n=5), on the other hand, did not alter superoxide anion release from peripheral blood neutrophils. Bone marrow neutrophils could not be isolated reproducibly after high-dose endotoxin because of leukoaggregation. Bone marrow neutrophils were isolated after low-dose endotoxin infusion. Stimulation of these cells with 10^{-9} M PMA in vitro resulted in a two- to fourfold increase above control release (p<0.05). Increased superoxide anion release from both peripheral blood and bone marrow neutrophils occurred in the absence of circulating endotoxin, as measured by a Limulus assay. These results show that the prolonged increase in superoxide anion release from peripheral blood neutrophils is associated with an increase in the superoxide anion release from bone marrow neutrophils. Furthermore, the recruitment of affected bone marrow neutrophils into peripheral blood may explain the increased superoxide anion release from blood neutrophils 24 hours after endotoxin infusion. (Circulation Research 1990;67:154–165)

Neutrophils have been implicated as effector cells of the adult respiratory distress syndrome in humans and experimentally induced acute lung injury in large animals. Neutrophils in the alveolar air spaces, where few neutrophils are normally found, have been demonstrated in patients who have died with adult respiratory distress syndrome. Furthermore, neutrophils in the air spaces are assumed to be activated because proteolytic enzymes can be collected from alveolar lavage fluid. In sheep, diverse stimuli such as complement anaphylatoxins,3,4 phorbol myristate acetate (PMA),5 endotoxin,6,7 and air embolization8,9 produce increases in lung lymph flow, lung lymph protein clearance, and in some cases, neutrophil sequestration within the pulmonary circulation. These observations raised the

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question of the role of neutrophils in the pathogenesis of acute lung injury, and more recently, the regulation of neutrophil function under pathophysiological conditions.

Because sepsis is frequently associated with adult respiratory distress syndrome, investigations of the effects of endotoxin on neutrophil function may be of particular importance. A number of in vivo and in vitro studies illustrate alterations in circulating neutrophil function in response to endotoxin. In vivo exposure of neutrophils to a dose of endotoxin that does not cause lung injury "primes" these cells. Doses of complement anaphylatoxin (C5a) or the synthetic chemotactic peptide formyl-norleucyl-phenylalanine (fNLP), which do not produce lung injury, cause endotoxin-primed cells to sequester in the lung. In vitro incubation of neutrophils with endotoxin produces an enhanced superoxide anion release in response to PMA, C5a, or the synthetic chemotactic peptide formyl-methionyl-leucyl-phenylalanine (fMLP). Curiously, neutrophil function also seems to be altered 18–24 hours after endotoxin infusion. This last observation is surprising because the turnover time of circulating neutrophils is approximately 6–8 hours. The short turnover time may imply that prolonged alterations in the function of circulating neutrophils are associated with similar alterations in the function of bone marrow neutrophils that are subsequently released into peripheral blood.

The present investigation tested the hypothesis that endotoxin infusion alters superoxide anion release from peripheral blood and bone marrow neutrophils. Sheep were infused with either 5.0 or 0.5 μg/kg endotoxin to produce acute lung injury. Peripheral blood neutrophils were isolated before, during, and 24 hours after one infusion of endotoxin to measure unstimulated and PMA-stimulated superoxide anion release. Bone marrow neutrophils also were isolated 24 hours after the infusion to measure superoxide anion release. Neutrophil alkaline phosphatase activity was assayed cytochemically throughout each experiment to document degranulation (loss of cytochemical staining), which was used as an index of intravascular neutrophil activation. Our results demonstrate that endotoxin caused 1) intravascular activation of blood neutrophils circulating at the time of the infusion, and 2) alterations in superoxide anion release from peripheral blood and bone marrow neutrophils 24 hours after the infusion.

Materials and Methods

Physiological Measurements

Adult, male sheep (n = 10, 42±3 kg) were used for the present study. The sheep were surgically prepared for chronic measurement of cardiopulmonary hemodynamics and lung lymph dynamics as previously described. Experiments were started 4 or 5 days after surgery at a time when PMA-stimulated superoxide anion release from circulating neutrophils returned to baseline values. Leukocyte count, differential, and hematocrit were measured when the sheep arrived and daily after the surgical procedure to minimize the incidence of undetected infection. A flow-directed thermodilution catheter (7F, Swan-Ganz, Abbott Critical Care Systems, Chicago) attached to a pressure transducer (Cobe CDX III, Lakewood, Colo.) was floated into a peripheral pulmonary artery (via a Sorenson venous introducer [Abbott Laboratoties, Salt Lake City] placed in the right external jugular vein) for the measurement of pulmonary arterial and wedge pressures. Both pressures were referenced to the level of the left atrium. This catheter was also used for the computer-assisted (model 3500, KMA Inc., Salt Lake City) measurement of cardiac output. An aortic catheter was placed at the time of surgery and fitted with a transducer for the measurement of systemic arterial blood pressure. Lung lymph was collected and weighed in tared centrifuge tubes treated with 100 units of heparin to prevent coagulation.

Lung lymph and 3 ml of systemic arterial blood were collected at 30-minute intervals throughout the experiment. The blood sample was withdrawn at the midpoint of each lymph collection period. The number of leukocytes in whole blood was measured using a Clay Adams automated hemocytometer (Becton Dickinson and Co., Parsippany, N.J.). Leukocyte differentials were determined using Accuscan (Sigma Chemical Co., St. Louis). The blood and lymph samples were centrifuged, and the supernatants were collected and frozen. Plasma and lymph protein concentrations were measured subsequently using an American Optical refractometer (No. 10400, Buffalo). A Limulus lysate assay (E-Toxate kit No. 210-A, Sigma) was used to measure endotoxin in plasma collected 24 hours after infusion.

Endotoxin infusion was started after the attainment of a baseline steady-state period, defined as three consecutive 30-minute periods during which lung lymph flow, pulmonary artery pressure, and cardiac output varied no more than 10% of their respective mean values. Sheep received either 5.0 μg/kg ("high-dose"; n = 5) or 0.5 μg/kg ("low-dose"; n = 5) of Escherichia coli endotoxin (0127:B8, Calbiochem Corp., La Jolla, Calif.) in 60 ml of sterile 0.9% NaCl. The endotoxin was infused through the proximal port of the Swan-Ganz catheter for 30 minutes at a rate of 1.9 ml/min. Physiological measurements were made continuously for 4.25 hours after the infusion and again for 1.5 hours beginning 24 hours after the infusion.

Isolation of Neutrophils

Peripheral blood neutrophils were isolated from 30 ml of whole blood collected in 10% 0.1 M EDTA during the baseline steady state, 15 minutes into the endotoxin infusion period, and 24 hours after the infusion. Theuffy coat was obtained by centrifugation (15 minutes at 900g). Erythrocytes were removed from the buffy coat by 30 seconds of hypo-
tonic lysis. Isotonicity was reestablished by the addition of Hanks’ solution (Difco, Detroit). Leukocytes were pelleted by centrifugation for 5 minutes at 2500g and resuspended in 1.0 ml HEPES buffer containing the following millimolar concentrations: NaCl 140, HEPES 10, KCl 10, CaCl₂ 0.1, MgCl₂ 0.2, NaHCO₃ 11.9, glucose 5.0, as well as 14 μM albumin, pH 7.4.

Neutrophils were isolated from the other buffy coat leukocytes by centrifugation through a discontinuous density gradient. Discontinuous density gradients were constructed from Percoll and autologous plasma. Sufficient plasma was collected, under sterile conditions, from the animals before endotoxin infusion to make all subsequent density gradients. This was done to minimize in vitro exposure of neutrophils to endotoxin. Density solutions containing 80%, 70%, 60%, 50%, 40%, 30%, and 20% Percoll (vol:vol) were mixed. Two milliliters of progressively less dense solutions was gently layered into 15×100 mm plastic test tubes so that the interfaces between each density layer could be identified. Buff coat leukocytes were layered atop the 20% density step. The gradients were then centrifuged for 15 minutes at 4,000g.

Peripheral arterial blood neutrophils from the 80/70% Percoll-plasma interface were washed, and resuspended in HEPES buffer. Neutrophils were greater than 98% viable (measured by trypan blue dye exclusion) and greater than 90% pure. Lymphocytes were the primary contaminating cell in this preparation.

Bone marrow neutrophils were isolated 24 hours after the endotoxin infusion. Sheep were anesthetized with 1 g sodium thiamylal. Under sterile conditions, an incision was made over the dorsal iliac crest, and the underlying muscle was dissected from the crest. Fifteen milliliters of bone marrow was collected into 1,500 units of heparin using a 12-gauge stainless steel needle. Neutrophils were isolated as described above and were collected from the 80/70%, 70/60%, and 60/50% density gradient interfaces.

Four additional sheep with no previous experimental procedures were used as control animals to avoid repeated bone marrow aspiration from the same sheep. Some of these sheep had vascular catheters in place.

Measurement of Superoxide Anion Release

Isolated neutrophils (0.25×10⁶ neutrophils per 100 μl HEPES buffer) were allowed to settle onto microtiter wells (high affinity, Nunc Inc., Naperville, Ill.) for 30 minutes at 37°C. The HEPES buffer was gently removed after the 30-minute period, and the superoxide anion assay was begun.

The superoxide anion release assay was conducted for 60 minutes using reagent master mixes. Test solutions containing doses of PMA (Sigma) from 10⁻¹¹ to 10⁻³ M and 0.32 mM ferricytochrome c (type III from horse heart, Sigma) were applied to each of four wells. Four wells received ferricytochrome c and HEPES buffer to measure spontaneous superoxide anion release from unstimulated neutrophils. Assay blank wells contained ferricytochrome c, HEPES buffer, and 700 units superoxide dismutase (bovine erythrocytes, Calbiochem) to inhibit ferricytochrome c reduction by spontaneous superoxide anion release. Control wells contained all reagents. These wells demonstrated that superoxide dismutase inhibited (>90%) ferricytochrome c reduction caused by PMA-stimulated superoxide anion release. Changes in the optical density of the reagents by superoxide anion release were measured on a Dynatech MR 600 microtiter plate reader (Chantilly, Va.) set at a test wavelength of 550 nm and a reference wavelength of 670 nm. Superoxide anion release (nmol) was calculated from the change in optical density (O.D.) using the conversion equation of Pick and Mize:¹⁷

\[
\text{nmol superoxide} = \left[\text{(test O.D. } - \text{reference O.D.)} \times 100\right]/6.3
\]

Assessment of Alkaline Phosphatase Activity

The semiquantitative cytochemical staining of alkaline phosphatase activity (Sigma kit No. 86-R) was used as an index of neutrophil degranulation and activation. One milliliter of whole blood was collected in dead space heparin during the baseline steady state, 15 minutes, 4.25 hours, and 24 hours after endotoxin infusion. Ten microliters of each sample was placed on glass microscope slides, smeared, and dried. Smears were fixed for 30 seconds in citrate fixative and washed with tap water. Alkaline phosphatase activity revealed by staining the slides for 10 minutes using 1.0 ml fast red violet alkaline solution, 1.0 ml sodium nitrate, and 1.0 ml naphthol arsenic bismuth alkaline solution in 45 ml distilled water. Slides were then washed with tap water, counterstained with hematoxylin for 90 seconds, and washed again.

Slides were observed using an Axiosplan light microscope (Carl Zeiss, Thornwood, N.Y.). One hundred neutrophils were rated for the intensity of the alkaline phosphatase reaction product (rating scale of 0 to +4). An alkaline phosphatase activity score for each time point was calculated by summing the individual product of each alkaline phosphatase intensity rating and the number of neutrophils so rated.

Data Analysis

Data are reported as mean±SEM. Changes in the circulating leukocyte counts, circulating neutrophil differential, and neutrophil alkaline phosphatase activity were compared with their respective baseline values using a one-way analysis of variance with multiple comparisons (Dunnett’s test). This test was also used to compare superoxide anion release from control neutrophils of each bone marrow fraction to the superoxide anion release from control blood neutrophils. Newman-Keuls multiple range testing was used to compare neutrophil differentials in the control blood isolation fraction and each bone
marrow isolation fraction.\textsuperscript{19} Leukocyte differentials from bone marrow isolation fractions of endotoxin-treated sheep were compared with leukocyte differentials from bone marrow isolation fractions of control sheep by unpaired $t$ tests.\textsuperscript{19} Unpaired $t$ tests were also used to compare the superoxide anion release from bone marrow neutrophils of endotoxin-treated sheep with the superoxide anion release from bone marrow neutrophils of control sheep. Paired $t$ tests were used to compare superoxide anion release from blood neutrophils collected 24 hours after endotoxin with the superoxide anion release from blood neutrophils collected before the endotoxin infusion.\textsuperscript{19} Differences were considered significant at $p\leq 0.05$.

**Results**

Lung lymph dynamics and pulmonary hemodynamics associated with the endotoxin infusions are summarized in Table 1. Endotoxin produced a dose-dependent increase in pulmonary microvascular permeability, defined as an increase in lung lymph protein clearance (lung lymph flow multiplied by the lymph-to-plasma protein concentration ratio). These data are similar to data shown previously.\textsuperscript{6,20}

Neutrophil sequestration was observed in response to in vivo endotoxin infusion\textsuperscript{7} (Table 2). The peripheral blood leukocyte count and neutrophil differential decreased 15 minutes after both high- and low-dose endotoxin (0.75 hours), resulting in an 85–90\% decrease in circulating neutrophils. The decrease in leukocyte count and differential due to high-dose endotoxin persisted for at least 4.75 hours. On the other hand, the leukocyte count approached baseline, and the neutrophil differential was at baseline 4.75 hours after low-dose endotoxin. These data suggest a direct relation between the endotoxin dose and the recovery rate of circulating neutrophils. Interestingly, both doses of endotoxin were subsequently associated with an overshoot in circulating neutrophils (1.5–3.0 times baseline) 24 hours after endotoxin infusion.

Circulating neutrophils were stimulated by intravascular endotoxin to release specific granules, of which alkaline phosphatase is a constituent (Table 2). By 4.75 hours, neutrophil alkaline phosphatase cytochemical activity was decreased by both high- and low-dose endotoxin infusion. The first cytochemical evidence of degranulation was associated with high-dose endotoxin as early as 0.75 hours (Table 2). A similar effect was demonstrated in vitro after a 30-minute incubation of naive buffy coat leukocytes on glass slides with 5.0 $\mu$g of endotoxin followed by staining for alkaline phosphatase activity. In these in vitro cytochemical experiments, neutrophil alkaline

### Table 1. Dose-Dependent Effect of Endotoxin on Pulmonary Hemodynamics and Lung Lymph Dynamics in Awake Sheep

<table>
<thead>
<tr>
<th>Endotoxin</th>
<th>Period (n)</th>
<th>Pulmonary artery pressure (cm H$_2$O)</th>
<th>Lymph flow (g/hr)</th>
<th>L/P ratio (total protein)</th>
<th>Lymph protein clearance (g/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>High dose (5 $\mu$g/kg)</td>
<td>Baseline (5)</td>
<td>31±3</td>
<td>6.63±1.60</td>
<td>0.67±0.01</td>
<td>4.89±0.57</td>
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<td></td>
<td>Peak (5)</td>
<td>64±5</td>
<td>10.80±2.65</td>
<td>0.60±0.05</td>
<td>6.44±1.69</td>
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<td></td>
<td>4.75 hr (3)</td>
<td>44±5</td>
<td>45.31±10.27</td>
<td>0.68±0.06</td>
<td>30.81±7.89</td>
</tr>
<tr>
<td></td>
<td>24 hr (3)</td>
<td>27±4</td>
<td>18.94±10.30</td>
<td>0.62±0.06</td>
<td>12.37±7.45</td>
</tr>
<tr>
<td>Low dose (0.5 $\mu$g/kg)</td>
<td>Baseline (5)</td>
<td>29±4</td>
<td>8.12±1.97</td>
<td>0.64±0.03</td>
<td>5.14±1.34</td>
</tr>
<tr>
<td></td>
<td>Peak (5)</td>
<td>54±6</td>
<td>12.87±3.09</td>
<td>0.53±0.04</td>
<td>6.97±1.51</td>
</tr>
<tr>
<td></td>
<td>4.75 hr (5)</td>
<td>38±5</td>
<td>24.79±5.71</td>
<td>0.74±0.07</td>
<td>18.11±5.22</td>
</tr>
<tr>
<td></td>
<td>24 hr (4)</td>
<td>27±3</td>
<td>10.45±2.35</td>
<td>0.61±0.03</td>
<td>6.30±1.37</td>
</tr>
</tbody>
</table>

Values are mean±SEM. L/P ratio, lymph-to-plasma protein concentration ratio.

### Table 2. Whole Blood Leukocyte Count, Percent Neutrophils, and Neutrophil Alkaline Phosphatase Activity in Response to Endotoxin Infusion

<table>
<thead>
<tr>
<th></th>
<th>High-dose endotoxin</th>
<th></th>
<th></th>
<th>Low-dose endotoxin</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WBC</td>
<td>% PMN</td>
<td>NAPA</td>
<td>WBC</td>
<td>% PMN</td>
<td>NAPA</td>
</tr>
<tr>
<td>Baseline</td>
<td>7.0±1.2</td>
<td>39±6</td>
<td>317±19</td>
<td>11.7±1.6</td>
<td>42±3</td>
<td>237±28</td>
</tr>
<tr>
<td>(n=5)</td>
<td>(n=5)</td>
<td>(n=5)</td>
<td>(n=5)</td>
<td>(n=5)</td>
<td>(n=5)</td>
<td>(n=5)</td>
</tr>
<tr>
<td>0.75 hours</td>
<td>2.7±0.3*</td>
<td>8±3*</td>
<td>121±38*</td>
<td>5.2±1.0*</td>
<td>11±2*</td>
<td>248±48</td>
</tr>
<tr>
<td>(n=5)</td>
<td>(n=5)</td>
<td>(n=4)</td>
<td>(n=5)</td>
<td>(n=5)</td>
<td>(n=5)</td>
<td>(n=5)</td>
</tr>
<tr>
<td>4.75 hours</td>
<td>2.4±0.3*</td>
<td>17±6*</td>
<td>106±31*</td>
<td>7.3±1.7*</td>
<td>43±5</td>
<td>179±31*</td>
</tr>
<tr>
<td>(n=3)</td>
<td>(n=3)</td>
<td>(n=4)</td>
<td>(n=5)</td>
<td>(n=5)</td>
<td>(n=5)</td>
<td>(n=5)</td>
</tr>
<tr>
<td>24.0 hours</td>
<td>13.6±4.3</td>
<td>56±3*</td>
<td>250±55</td>
<td>20.2±2.0*</td>
<td>38±10</td>
<td>265±36</td>
</tr>
<tr>
<td>(n=3)</td>
<td>(n=3)</td>
<td>(n=4)</td>
<td>(n=5)</td>
<td>(n=4)</td>
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</table>

Values are mean±SEM. WBC, whole blood leukocyte count; % PMN, percent neutrophils; NAPA, neutrophil alkaline phosphatase activity.

* $p\leq 0.05$, one-way analysis of variance and Dunnett's multiple comparison test.
phosphatase activity decreased from 332±10 to 168±15 (n=4; p≤0.05, paired t test). Twenty-four hours after the infusion of either dose of endotoxin, alkaline phosphatase cytochemical activity of peripheral blood neutrophils increased to near baseline values, suggesting that circulating neutrophils were not activated and had a full complement of granule contents.

Figure 1 summarizes the in vitro superoxide anion release data for peripheral blood neutrophils. Superoxide anion release from blood neutrophils collected 15 minutes into the infusion of high- or low-dose endotoxin was similar to their respective baseline release (Figures 1A and 1B). Twenty-four hours after the infusion of high-dose endotoxin, however, unstimulated release and 10⁻¹¹ M PMA-stimulated release were increased 2.25±0.38 and 3.76±0.68 times baseline, respectively (p≤0.05, Figure 1C). Release in response to 10⁻¹⁰ and 10⁻⁹ M PMA also tended to be greater than baseline (2.12±0.50 and 1.46±0.55, respectively). There was no change in the maximal PMA-stimulated release; therefore, superoxide anion release from unstimulated blood neutrophils and blood neutrophils stimulated with low doses of PMA in vitro was increased 24 hours after high-dose endotoxin. This subthreshold release response occurred in the absence of circulating endotoxin, as measured by the Limulus assay. In contrast to the 24-hour data from the high-dose endotoxin experiments, low-dose endotoxin was not associated with altered superoxide anion release 24 hours after the infusion (Figure 1D).

The increased superoxide anion release observed with low doses of PMA in vitro and in vivo high-dose endotoxin was apparently due to an increase in the sensitivity of blood neutrophils to PMA. An index of sensitivity, the half-maximal dose for PMA-stimulated superoxide anion release, was extrapo-
lated from semilogarithmic plots of Figures 1C and 1D. High-dose endotoxin caused a 3.5-fold decrease in the half-maximal PMA dose from 1.8 to 0.5 nM (baseline and 24 hours, respectively). Low-dose endotoxin did not alter the half-maximal dose (1.5 and 1.8 nM at baseline and 24 hours, respectively).

Another piece of supportive evidence for the hypothesis that spontaneous release of superoxide anion had little effect on PMA-stimulated release is shown in Figure 2. The kinetics of superoxide anion release in response to $10^{-7}$ M PMA were identical before and 24 hours after high-dose endotoxin (Figure 2). The maximal amounts of superoxide anion released were also similar before and after endotoxin. Therefore, with a maximal stimulating dose of PMA, the maximal amount and the kinetics of superoxide anion release were independent of spontaneous release.

We next adapted and applied techniques to isolate bone marrow neutrophils from sheep as the prelude to studying endotoxin-induced alterations in bone marrow neutrophils. A problem we encountered was formation of bone marrow leukocyte aggregates. These aggregates were disrupted with washes of Hanks' solution and heparin before the cells were placed on discontinuous density gradients; otherwise, the aggregated leukocytes (including neutrophils) did not travel through the gradient. One problem persisted throughout our study; namely, too few neutrophils were collected to routinely perform complete PMA dose-response curves for the superoxide anion release assay. This problem was particularly evident in sheep exposed to high-dose endotoxin. Therefore, we selected three conditions that reasonably bracketed the complete in vitro dosage scale: unstimulated (no PMA), and stimulated with $10^{-9}$ M PMA and $10^{-7}$ M PMA.

The characteristics of normal bone marrow neutrophils were identified first. Segmented neutrophils were found in the 80/70% Percoll-plasma interface.
(fraction) of both bone marrow and blood. In addition, segmented neutrophils were found in the 70/60% and 60/50% fractions from bone marrow. The purity of bone marrow segmented neutrophils was 73±3% in the 80/70% fraction, 27±3% in the 70/60% fraction, and 21±7% in the 60/50% fraction. Band neutrophils also were present in the bone marrow fractions, accounting for an average of 17–34% of the neutrophil differential in each fraction. Immature neutrophils (promyelocytes, myelocytes, and metamyelocytes), which were not observed in the 80/70% fractions of bone marrow or blood, comprised 22±10% and 24±7% of the leukocytes in the bone marrow 70/60% and 60/50% fractions, respectively. The percentage of lymphocytes tended to be greater in the bone marrow 70/60% and 60/50% fractions (15% and 28%, respectively) than in the bone marrow 80/70% fraction (8%). Eosinophils represented less than 2–3% of the leukocyte differentials of the bone marrow and blood neutrophil fractions. However, the eosinophil differential data probably were biased by our screening procedure for accepting sheep because we rejected sheep that had circulating eosinophil differentials of 5% or greater.

Technical difficulties arose when bone marrow neutrophils were to be isolated 24 hours after the high-dose endotoxin infusion. Bone marrow samples were collected from four sheep; however, irreversible aggregation of bone marrow leukocytes precluded the isolation of neutrophils from two of them. In general, the purity of the bone marrow fractions for those two sheep was comparable with that seen in the fractions from the low-dose endotoxin–treated sheep (see below).

Bone marrow neutrophil isolations were performed with minimal difficulties from sheep infused with low-dose endotoxin. Some samples tended to aggregate, but we were able to disaggregate them by adding Hanks’ solution and heparin. The percentage of neutrophils found in the bone marrow isolation fractions was shifted toward more immature forms 24 hours after low-dose endotoxin infusion. The percentage of segmented neutrophils in the 80/70% fraction was 43±12%, whereas band neutrophils comprised 22±9%. In contrast, the percentage of segmented neutrophils in the 70/60% fraction was 47±8%. Band neutrophils and immature neutrophils made up 23±4% and 8±3%, respectively, of the cell count in the 70/60% fraction. Somewhat surprisingly, the percentage of the three subpopulations of neutrophils in the 60/50% fraction 24 hours after low-dose endotoxin was the same as in the control 60/50% bone marrow fraction. Lymphocyte differentials in the three bone marrow fractions from the low-dose endotoxin experiments were unchanged from the control sheep bone marrow.

Leukocyte differentiation into other granulocyte types may have been induced by endotoxin. For instance, differentiation to the eosinophilic line of granulocytes was evident as a three- to fourfold increase (7–11%) in the percentage of these cells in the 70/60% and 60/50% bone marrow fractions. Despite this apparent increase in bone marrow eosinophil differential, eosinophils were not found in smears or the 80/70% fraction of whole blood after low-dose endotoxin.

Superoxide anion release from matched blood and bone marrow neutrophils collected from control animals is shown in Figure 3. Less than 1.0 nmol of superoxide anion per 250,000 neutrophils per 45 minutes of incubation was spontaneously released from unstimulated neutrophils, regardless of the source. Stimulated superoxide anion release from neutrophils collected from progressively less dense

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**Figure 3.** Comparison of superoxide anion release (mean±SEM) from blood and bone marrow neutrophils collected from control sheep. Release values were obtained 45 minutes into superoxide anion assays (n=4). Superoxide anion release from neutrophils of each bone marrow fraction was compared with superoxide anion release from blood neutrophils by a one-way analysis of variance with multiple comparisons (Dunnett’s test). *Represents a statistically significant difference from blood neutrophil superoxide anion release. PMN, neutrophil; PMA, phorbol myristate acetate; UNS, unstimulated.
gradient fractions decreased in a stepwise fashion in response to 10^{-9} M PMA. After stimulation with 10^{-7} M PMA, superoxide anion release from neutrophils of the 60/50% bone marrow fraction was 50% less (p<0.05) than that seen from neutrophils of the 80/70% and 70/60% bone marrow fractions, as well as from the 80/70% fraction of peripheral blood. The half-maximal PMA dose for superoxide anion release from control neutrophils of the 80/70% bone marrow fraction was 3.0 nM, which was the same as control peripheral blood, whereas that for neutrophils of the 70/60% and 60/50% fractions was 5.0 and 10.0 nM, respectively.

In the two high-dose endotoxin–treated sheep in which neutrophils were successfully isolated from bone marrow, the pattern of superoxide anion release was different from that measured in control sheep (Figure 4). Unstimulated superoxide anion release was increased three- to 10-fold in neutrophils isolated from the 80/70%, 70/60%, and 60/50% bone marrow fractions from the high-dose endotoxin–treated sheep compared with the control sheep. Similar increases were measured at PMA doses of 10^{-11} and 10^{-9} M in all three fractions. As was the case with peripheral blood neutrophils collected 24 hours after high-dose endotoxin, superoxide anion release from bone marrow neutrophils stimulated with 10^{-7} M PMA in vitro was similar before and 24 hours after the endotoxin infusion.

Bone marrow neutrophils from low-dose endotoxin–treated sheep showed a similar pattern of enhanced superoxide anion release in the absence of PMA and in response to 10^{-11} and 10^{-9} M PMA (Figure 4). (Sufficient numbers of neutrophils were obtained for the 10^{-11} M PMA dose in two of the five sheep in this group.) In contrast to the high-dose endotoxin data, however, this pattern of enhanced superoxide anion release did not extend to neutrophils from the 60/50% fraction. Once again, release stimulated by 10^{-7} M PMA in all three fractions was similar before and after the low-dose endotoxin infusion.

**Discussion**

The purpose of this study was to identify the effects of in vivo endotoxin infusion on the function of blood and bone marrow neutrophils in a model of acute lung injury. The data on neutrophil sequestration and the development of dose-dependent acute lung injury are in agreement with other investigations. This study further demonstrates that in vivo administration of endotoxin produces two effects on the function of various neutrophil populations: First, endotoxin causes intravascular activation of neutrophils circulating at the time of the infusion; second, endotoxin alters the in vitro superoxide anion release characteristics of neutrophils isolated from blood and bone marrow 24 hours after the infusion. From the second effect, we hypothesize that superoxide anion release is altered in a population of neutrophils not directly exposed to endotoxin in vivo.

Evidence for intravascular neutrophil activation (degranulation) was provided using a cytochemical stain for alkaline phosphatase activity. Alkaline phosphatase is an enzyme contained in the specific granules of mature segmented neutrophils. Specific granules are bacteriocidal in function and, at neutrophil activation, are released before azurophilic (primary) granules. We found that alkaline phosphatase cytochemical activity within blood neutrophils

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**Figure 4.** Comparison of superoxide anion release (mean±SEM) in response to different phorbol myristate acetate (PMA) dosage in vitro for bone marrow neutrophil populations isolated from control sheep (open bars; n=4) and sheep infused with either low-dose endotoxin (dotted bars; n=5, except for the 60/50% fraction, for which n=4; †represents n=2 for the 10^{-11} M PMA data; therefore, standard error bars not shown) or high-dose endotoxin (hatched bars; n=2, therefore, standard error bars not shown). Release values were obtained 45 minutes into the superoxide anion assay. *Represents a statistically significant difference from the matched superoxide anion release for control neutrophils (unpaired t test). PMN, neutrophil; UNS, unstimulated.
decreased acutely after endotoxin infusion. Furthermore, endotoxin applied directly to neutrophils in vitro also reduced alkaline phosphatase activity. The persistent decrease in the alkaline phosphatase activity of segmented neutrophils in conjunction with the recovery of neutrophil differential and leukocyte count at 4.75 hours suggest that neutrophils sequestered in the pulmonary circulation during the infusion were activated and subsequently released contents of specific granules into the circulation. Demargination of sequestered, activated neutrophils is suggested in the low-dose endotoxin–treated sheep by recovery of the neutrophil differential at 4.75 hours—a time when alkaline phosphatase activity was still decreased. The recovery of alkaline phosphatase activity at 24 hours in conjunction with increased circulating neutrophil count suggests that the neutrophils that were recruited to peripheral blood were not activated and that they possessed a full complement of specific granules.

Our finding of altered superoxide anion release from circulating neutrophils 24 hours after endotoxin infusion is in agreement with results from other investigations. Superoxide anion release from rabbit neutrophils, which are “primed” (defined as an enhanced response to stimuli that produce neutrophil activation), occurs with a greater sensitivity to PMA 18–24 hours after single in vivo endotoxin infusion or after daily, consecutive infusions. Similarly, PMA-, C5a-, or fMLP-induced superoxide anion release is enhanced where neutrophils are exposed to endotoxin in vitro. In contrast, 24 hours after in vivo endotoxin infusion, superoxide anion release is decreased in response to C5a stimulation, whereas it is not altered in response to fMLP. The decreased response to C5a may be explained by receptor down-regulation; however, the number of fMLP receptors is paradoxically increased. We suggest that endotoxin alters membrane receptors, intracellular transduction mechanisms, and ultimately, the bacteriocidal capabilities of various neutrophil populations.

Support for the hypothesis that altered superoxide anion release occurs in a population of circulating neutrophils not directly exposed to endotoxin is provided by the circulatory time of neutrophils in peripheral blood, estimated to be 6 to 8 hours. If three to eight neutrophil half-lives occur in 24 hours, at most, 6% of the circulating population of neutrophils at 24 hours was originally present during the endotoxin infusion in our experiments. Thus, it is unlikely that alterations in superoxide anion release 24 hours after endotoxin can be attributed to prolonged effects on neutrophils circulating at the time of the infusion.

In another study, we observed alterations in circulating neutrophil superoxide anion release 24 hours after infusion of zymosan-activated plasma but not PMA or air bubbles. In contrast to endotoxin, zymosan-activated plasma produced a 40–60% decrease in 10-7 M PMA-stimulated superoxide anion release without an effect on spontaneous release. Although the effects seen 24 hours after endotoxin and zymosan-activated plasma on neutrophil superoxide anion release are opposite (priming versus down-regulation, respectively), alteration of neutrophil function 24 hours after infusion may be a generalized response to selected stimuli associated with activation of neutrophils and induction of tissue injury. One hypothesis to explain these results is alteration in intracellular transduction mechanisms of neutrophils developing within bone marrow.

To begin to address this hypothesis, we characterized control bone marrow neutrophils on the basis of their distribution within discontinuous density gradients and their superoxide anion release characteristics. Segmented neutrophils of the 80/70% fraction from bone marrow may represent a readily releasable pool because segmented and band neutrophils are present (together representing 90% of the leukocyte differential in the 80/70% fraction), whereas immature neutrophils are not. This fraction also probably contains blood neutrophils obtained coincidentally with the bone marrow sample. We cannot exclude this possibility because the density characteristics and differential are similar to those of the 80/70% density fraction of peripheral blood. Another indication that the segmented neutrophils of the 80/70% fraction may be a releasable pool is that their superoxide anion release characteristics are nearly identical to those of blood neutrophils. Segmented neutrophils of the bone marrow 70/60% and 60/50% fractions, on the other hand, apparently represent different (less mature) subpopulations of segmented neutrophils because they are hypodense compared with neutrophils of the blood and the 80/70% density fraction of bone marrow. The notion that the segmented neutrophils in the two hypodense fractions may be from blood contamination is made less tenable by the greater percentage of band and immature neutrophils in the 70/60% and 60/50% bone marrow fractions (50–56%). Interestingly, 10-7 M PMA-stimulated superoxide anion release from neutrophils of the 70/60% fraction of bone marrow is similar to that from blood neutrophils, whereas release from neutrophils of the 60/50% fraction is 50% of blood neutrophil superoxide anion release.

We used the aforementioned characteristics of normal bone marrow neutrophils to identify endotoxin-induced alterations in bone marrow neutrophils. A hematological effect was an apparent mobilization of neutrophils from the bone marrow of the endotoxin-treated sheep. For example, with low-dose endotoxin, a 40% reduction in the differential of segmented neutrophils in the 80/70% fraction (43±12% versus 73±3% in the control 80/70% fraction) was matched by a near doubling of the segmented neutrophil differential in the 70/60% fraction (47±8% versus 27±3% in the control 70/60% fraction). Likewise, there was a 30–40% reduction in the number of band neutrophils in the 70/60% and 60/50% bone marrow fractions from the low-dose endotoxin–treated sheep.
compared with the corresponding control bone marrow fractions. In other words, there was a reduction in the total proportion of neutrophils (segmented, band, and immature) in each of the isolation fractions (27%, 6%, and 13% decrease in the 80/70%, 70/60%, and 60/50% fractions, respectively). Another indication that endotoxin affected granulocyte maturation in bone marrow is found in the eosinophil differential from the density fractions. The percentage of eosinophils was three to four times greater in each fraction (7–10%) than in the corresponding control bone marrow fractions (2–3%). Therefore, the shift in eosinophil and neutrophil differential among the three bone marrow fractions supports our hypothesis that endotoxin affected the maturation of granulocytes.

Endotoxin infusion also affected superoxide anion release from bone marrow neutrophils. In particular, release was primed for neutrophils that either were not stimulated in vitro or that were stimulated with low doses of PMA in vitro. There were clear indications that the extent of neutrophil priming at 24 hours was a function of endotoxin dose. In the high-dose endotoxin–treated sheep, priming was observed in neutrophils from all three isolation fractions from bone marrow, including the most hypodense, least functionally mature cells in the 60/50% fraction. Priming also was apparent in blood neutrophils in the sheep given high-dose endotoxin. In sheep given low-dose endotoxin, by contrast, priming at 24 hours was apparent only in the 80/70% and 70/60% fractions of bone marrow neutrophils. The experimental design may well have contributed to this dose-dependent effect in that obtaining blood and bone marrow neutrophils at some point between 5 and 24 hours after low-dose endotoxin may have demonstrated a more generalized priming response in this group of sheep.

Cytokines may play a role in the bone marrow responses we observed, especially those induced by endotoxin. It is known that endotoxin acutely increases the release of granulocyte/macrophage-colony stimulating factor (GM-CSF) from stromal cells in culture (adherent bone marrow cells consisting of macrophages, endothelial cells, fibroblasts, and adipocytes). GM-CSF in turn enhances phagocytosis, degranulation, and superoxide anion release from neutrophils in response to some (e.g., C5a, fMLP, and leukotriene B4) but not all (e.g., PMA) stimuli in vitro. The long-term effect of GM-CSF on bone marrow neutrophils that may occur after endotoxemia has not been elucidated. Endotoxemia is also associated with the expression of granulocyte/macrophage-colony stimulating activity (GM-CSA) from circulating lymphocytes, endothelial cells, and monocytes. GM-CSA increases the differentiation of bone marrow neutrophils as well as the release of granulocytes from bone marrow in response to endotoxemia. Furthermore, monocytes and macrophages may induce GM-CSA by releasing GM-CSF and a newly defined GM-CSA enhancing protein at endotoxin stimulation. Another cytokine associated with endotoxemia is tumor necrosis factor released from macrophages. Tumor necrosis factor can induce and even potentiate neutrophil bacteriocidal responses, including toxic oxygen radical release. Furthermore, interleukin-1, released by phagocytizing mononuclear cells, potentiates the production of GM-CSF from monocytes, macrophages, and bone marrow stromal cells. Interleukin-1 can also directly activate neutrophils to release toxic oxygen radicals. Specific granule release and oxygen-dependent metabolism occur in neutrophils stimulated with leukocytic pyrogen. Finally, interleukin-3 may also be an important cytokine in endotoxemia because it is synergistic with other cytokines in promoting granulocyte proliferation from pluripotent and early progenitor cells in bone marrow. Thus, the neutrophil responses associated with endotoxin infusion may be accounted for by cytokines induced by endotoxin, in addition to the direct actions of endotoxin on neutrophil function.

The present study illustrates that alterations in blood neutrophil function, occurring 24 hours after endotoxemia, are associated with similar alterations in the function of bone marrow neutrophils. The physiological and pathophysiological significance of a peripheral blood and bone marrow population of primed neutrophils remains to be determined. Because neutrophils are an important component of both host defense against bacteria and host tissue injury, the implications of altered neutrophil development and function may extend to mechanisms of host defense, the pathophysiology of acute lung injury, and adult respiratory distress syndrome.

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**References**


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F Cerasoli, Jr, P J McKenna, D L Rosolia, K H Albertine, S P Peters and M H Gee

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