Oxidative Mechanisms Utilized by Human Neutrophils to Destroy *Escherichia coli*

By Samuel A. Passo and Stephen J. Weiss

Serum-opsonized bacteria are efficiently ingested and killed by neutrophils within the phagocytic vacuole, where they are exposed to an array of reactive oxygen metabolites and toxic lysosomal components. Although bacteria may be destroyed by oxygen-independent mechanisms alone, many types of bacteria are not killed effectively unless they are attacked by oxygen metabolites. However, the apparent inability of extracellular scavengers, or inhibitors, of oxygen metabolites to gain access to the phagocytic vacuole makes this system difficult to evaluate. Therefore, we investigated the ability of neutrophils triggered with phorbol myristate acetate to destroy unopsonized *E. coli* in a serum-free model system. Neutrophils incubated with phorbol myristate acetate at a cell-to-bacteria ratio of 1:4 caused a >95% reduction in colony-forming units (CFU) of *E. coli* in 60 min at 37°C. Destruction of *E. coli* by the stimulated neutrophils was dependent on neutrophil number, stimuli concentration, and the incubation period. The neutrophil-mediated bactericidal effect was stimulated by superoxide dismutase, but was inhibited by catalase, azide, or compounds known to scavenge hypochlorous acid. Although stimulated neutrophils can generate long-lived endogenous N-chloroamines, these compounds did not play a direct role in destruction of *E. coli* in our model system. However, in the presence of exogenous iodide, endogenous N-chloroamines exerted a powerful bactericidal effect. Finally, neutrophils triggered with opsonized zymosan could also mediate *E. coli* destruction by a qualitatively similar process. Thus, we have demonstrated that neutrophils have the potential to utilize the myeloperoxidase system to generate bactericidal quantities of a species with characteristics similar to, if not identical with, hypochlorous acid.

HUMAN NEUTROPHILS play a pivotal role in host defense by phagocytosing and destroying invading bacteria. Serum-opsonized bacteria are normally killed within the confines of the phagocytic vacuole, where they are exposed to an array of reactive oxygen metabolites and toxic lysosomal components. Although microbes may be destroyed by oxygen-independent processes alone, many types of bacteria are not killed effectively unless they are attacked by oxygen metabolites. Oxygen metabolites implicated in the bactericidal process include superoxide anion (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), the hydroxyl radical (OH$^-$), and products generated by the H$_2$O$_2$-myeloperoxidase-halide system. However, attempts to identify those species responsible for intraphagosomal killing are hampered by the difficulties encountered in manipulating events at the tightly coupled bacterium–phagosome interface. Indeed, neutrophils will destroy opsonized bacteria by oxygen-dependent processes in vitro, even if exogenous enzymes capable of reducing oxygen metabolites are present in the suspending medium. Apparently large molecular weight probes do not gain access into the phagosomal space in sufficient concentrations to impair killing.

As a result of this obstacle, most studies have focused on the bactericidal potential of oxygen metabolites generated by model, cell-free systems rather than those produced by the intact neutrophil. In order to circumvent these difficulties and to gain further insights into the microbicidal process, we have examined the ability of triggered neutrophils to destroy unopsonized *E. coli* by an oxygen-dependent process in a serum-free, model system.

**MATERIALS AND METHODS**

**Neutrophil Preparation**

Neutrophils were obtained from the venous blood of healthy human volunteers. Neutrophil preparations were isolated by Ficoll-Hypaque density centrifugation, followed by dextran sedimentation. The preparations contained greater than 96% neutrophils and 2%–3% eosinophils. Cells were suspended in Dulbecco’s phosphate-buffered saline (GIBCO Laboratories, Grand Island Biological Co., Grand Island, NY; pH 7.4, with 1 mg/ml glucose).

**Bacteria**

*Escherichia coli* (ATCC strain 29552, American Type Culture Collection, Rockville, MD) were maintained on trypticase soy agar and grown in trypticase soy broth (Difco Laboratories, Detroit, MI) aerobically at 37°C to stationary phase overnight under static conditions. Bacteria were harvested by centrifugation at 7,000 g for 10 min at 4°C, washed, and appropriately diluted in Dulbecco’s phosphate-buffered saline (pH 7.4) with 1 mg/ml glucose. Bacterial concentrations were determined spectrophotometrically by measuring the absorbance at 625 nm (A$_{625}$ of 1.0 equals approximately 10$^8$ bacteria/ml). Bacterial concentrations were confirmed by visual counting in a Petroff-Hauser chamber (C.A.H. and Son, Philadelphia, PA) and viable colony counts using the pour plate technique.

**Bactericidal Assay System**

**Serum-independent killing**. A quantity of 10$^7$ *E. coli* was incubated alone or with various numbers of neutrophils in 1 ml of...
Dulbecco's phosphate-buffered saline in the absence of serum. The neutrophils were triggered to generate oxygen metabolites and release lysosomal contents with either the soluble stimulant, phorbol myristate acetate (PMA; Consolidated Midland Corp., Forrester, NY) or opsonized zymosan particles (Sigma Chemical Co., St. Louis, MO) prepared as previously described. The reaction mixtures were incubated at 37°C in either 5-ml round-bottom, polypropylene tubes (Type 2005, Becton Dickinson and Co., Oxnard, CA) with constant rocking or in 24-well tissue culture plates (Falcon 3047) without agitation. All experiments were performed in round-bottom tubes with rocking unless otherwise indicated. Aliquots (0.1 ml) were removed after 0, 30, 60, 90, and 120 min of incubation, the neutrophils lysed with sterile distilled water, the samples vigorously vortexed, and then plated onto trypticase soy agar. In selected experiments, the bacteria were sonicated (6 A, 15-sec pulse with a probe type ultrasonifier, Kontes Co., Vineland, NJ) before plating. The nutrient agar plates were then incubated at 25°C for 24-48 hr to allow visualization of the colony-forming units (CFU).

**Serum-dependent killing.** An aliquot of 10^7 E. coli was incubated alone or with 2.5 x 10^6 neutrophils in 1 ml of Dulbecco's buffer with 10% fresh autologous serum. The samples were then incubated and processed as described above.

Other additions to the bactericidal assay systems included bovine superoxide dismutase (SOD; 2,700 U/mg, Sigma Chemical), bovine catalase (80,000 U/mg, Worthington Biochemical Corp., Freehold, NJ) sodium azide (Fisher Scientific Co., Pittsburgh, PA), taurine, L-alanine, glycine, N-acetyl-L-alanine, N-acetyl-glycine, mannitol (Sigma Chemical Co.), and ethanol. Reagent grade H_2O_2 (30%; stabilizer free) was obtained from Mallincrodt (Paris, KY), and its concentration was determined as described. Superoxide dismutase was assayed according to the method of McCord and Fridovich. Catalase was washed over an XM-100A ultrafiltration membrane (Amicon Corp., Lexington, MA) and assayed before use.

**H_2O_2 Generation by Stimulated Neutrophils**

H_2O_2 generated by neutrophils was quantitated by the method of Thurman et al., as previously described.

**N-Chloramine Production by Neutrophils**

Neutrophils (2.5 x 10^6/ml) were incubated with PMA or opsonized zymosan particles in the presence or absence of oxygen metabolite scavengers or inhibitors for 60 min. Catalase (10 μg) was then added to reduce any residual H_2O_2, the cells pelleted (500 x g for 10 min), and the supernatants assayed for the presence of N-chloroamines, as previously described. All data are expressed as the mean ± 1 SD unless otherwise indicated.

**RESULTS**

**Bactericidal Effect of PMA-Stimulated Neutrophils**

Human neutrophils (2.5 x 10^6/ml) exerted a powerful bactericidal effect when incubated with E. coli (10^7/ml) in the presence of 10% autologous serum (Fig. 1). As expected, neutrophils did not destroy the bacteria in the absence of serum. However, following the addition of PMA (30 ng/ml), 2.5 x 10^6 neutrophils reduced the number of E. coli CFU in a time-dependent fashion in the absence of serum (Fig. 1). Optimal killing was obtained with 30 ng/ml of PMA, and this dose had no effect on E. coli viability. The reduction in CFU by PMA-triggered neutrophils was not due to bacterial clumping, because sonication of the samples before plating had no effect on these results.

![Fig. 1](image_url)

**E. coli Destruction by Stimulated Neutrophils.** E. coli (10^7) were incubated alone, with autologous serum (10%, v/v), or PMA (30 ng/ml) in the absence or presence of neutrophils (2.5 x 10^6) in a final volume of 1 ml in Dulbecco's phosphate-buffered saline (pH 7.4) for the indicated periods of time at 37°C with constant rocking. Results are expressed as the mean CFU ± SE of at least 4 experiments.

**Effect of superoxide dismutase and catalase on microbial killing.** In order to assess the potential role

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*This dose of PMA did not alter the number of E. coli CFU, nor did it alter the growth kinetics (turbidimetric analysis) of E. coli suspended in liquid growth medium over a 24-hr period at 37°C.*
OXIDATIVE MECHANISMS IN E. coli KILLING

had any effect on the bactericidal system (Table 1). If PMA-stimulated neutrophils and E. coli were coincubated in the presence of catalase (25 μg/ml) for 60 min and the cells then pelleted by a low-speed centrifugation (500 g for 10 min), all of the microorganisms were recovered in the cell-free supernatant. Based on these data, it appears that (1) E. coli may not be significantly bound or internalized by PMA-stimulated neutrophils, and (2) H₂O₂ plays a pivotal role in the microbicidal effect.

Role of H₂O₂ in bactericidal system. Although the complete protection of the microorganism by catalase implicates H₂O₂ in the bactericidal event, H₂O₂ can potentially react with reduced transition metal complexes to generate OH⁻ or be utilized by the myeloperoxidase-chloride system to produce hypochlorous acid (HOCI). If OH⁻ is generated in toxic quantities in the extracellular space, then high concentrations of compounds known to rapidly react with OH⁻ should impair killing. However, neither ethanol nor mannitol had any protective effect in our system (PMA-stimulated neutrophils reduced the number of CFU from 9.0 ± 1.4 x 10⁶ to 0.6 ± 1.1 x 10⁶, whereas the number of CFU in the presence of stimulated neutrophils and 40 mM ethanol or mannitol fell to 0.2 ± 0.3 x 10⁶ and 0.4 ± 0.3 x 10⁶, respectively; n = 9). In contrast, the addition of azide, a heme-enzyme inhibitor known to block myeloperoxidase activity, increased the number of viable bacteria in the neutrophil system by a factor of 10 (Table 2). Although azide alone had no effect on E. coli viability, it did not completely protect the bacteria from the neutrophils’ bactericidal effect. Because azide inhibits both myeloperoxidase and catalase activity in the neutrophil, E. coli might be exposed to much higher concentrations of H₂O₂ for longer periods of time in the presence of a heme-enzyme inhibitor. Indeed, H₂O₂ could not be detected in the supernates of 2.5 x 10⁶ PMA-stimulated neutrophils after a 60-min incubation, whereas large quantities of H₂O₂ accumulated under identical conditions in the presence of azide (see Table 2). In order to examine the bactericidal potential of high concentrations of H₂O₂ alone, E. coli were incubated with a bolus of reagent H₂O₂ for 60 min at 37°C. In the presence of 0.3 mM H₂O₂ (300 nmole in a final volume of 1 ml), E. coli remained viable (control 12.0 ± 1.0 x 10⁶ CFU versus 9.6 ± 0.6 x 10⁶ CFU; n = 6) and reduced the H₂O₂ concentration to 0.013 ± 0.006 mM (n = 3). Thus, H₂O₂ alone did not exert a strong bactericidal effect and was rapidly metabolized by the E. coli. However, when H₂O₂ (300 nmole) was added to E. coli in the presence of azide (1 mM), the number of CFU fell to 3.8 ± 1.2 x 10⁶ (68% reduction; n = 4), and 281.7 ± 7.2 nmole (n = 3) of H₂O₂ were detected after

of oxygen metabolites in our model system, experiments were performed in the presence of SOD (to lower the O₂⁻ concentration) or catalase (to reduce the generated H₂O₂). As shown in Table 1, SOD significantly stimulated the bactericidal effect of 2.5 x 10⁶ PMA-activated neutrophils after a 60-min incubation. In contrast, small amounts of catalase completely inhibited E. coli destruction by neutrophils, in either the absence or presence of exogenous superoxide dismutase. Neither heat-inactivated SOD nor catalase

Table 1. Effect of Superoxide Dismutase and Catalase on E. coli Destruction by PMA-Stimulated Neutrophils

<table>
<thead>
<tr>
<th>Additive*</th>
<th>n</th>
<th>CFU (x 10⁶)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td>24</td>
<td>9.2 ± 1.4</td>
</tr>
<tr>
<td>E. coli + neutrophils</td>
<td>19</td>
<td>17.6 ± 4.8</td>
</tr>
<tr>
<td>E. coli + neutrophils + PMA</td>
<td>24</td>
<td>0.8 ± 1.1</td>
</tr>
<tr>
<td>Complete system + SOD (10 μg/ml)</td>
<td>21</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>Complete system + catalase (25 μg/ml)</td>
<td>24</td>
<td>19.7 ± 5.1</td>
</tr>
<tr>
<td>Complete system + SOD/catalase</td>
<td>7</td>
<td>18.4 ± 3.9</td>
</tr>
<tr>
<td>Complete system + HI SOD‡ (10 μg/ml)</td>
<td>3</td>
<td>1.1 ± 1.0</td>
</tr>
<tr>
<td>Complete system + HI catalase‡ (25 μg/ml)</td>
<td>3</td>
<td>0.8 ± 0.4</td>
</tr>
</tbody>
</table>

*The complete system consisted of neutrophils (2.5 x 10⁶/ml), E. coli (~10⁷/ml), and PMA (30 ng/ml) in a final volume of 1 ml in Dulbecco’s buffer. Samples were incubated for 60 min at 37°C, and then processed as described in Materials and Methods.

†Results are expressed as the mean CFU ± 1 SD of the indicated number of experiments.

‡Heat-inactivated (HI) enzymes were prepared by autoclaving the material for 20 min before use.

Fig. 2. Effects of neutrophil concentration on E. coli destruction by PMA-stimulation. E. coli (10⁶) were incubated alone or with the indicated concentrations of E. coli in 1 ml of Dulbecco’s buffered saline (pH 7.4) in the presence of PMA (30 ng/ml) for 90 min at 37°C with constant rocking. Results are expressed as the mean CFU ± 1 SE of 3, 12, and 5 experiments for 1.0, 2.5, and 5.0 x 10⁶ neutrophils.
the 60-min incubation. Taken together, these data suggested that neutrophils exerted a bactericidal effect in the presence of azide because (1) H$_2$O$_2$ was allowed to accumulate to nonphysiologic levels and (2) bacterial metabolism of H$_2$O$_2$ was simultaneously inhibited.

**Effect of hypochlorous acid scavengers on neutrophil-mediated *E. coli* destruction.** Model systems consisting of purified myeloperoxidase, H$_2$O$_2$, and an oxidizable halide form a powerful microbialic system.$^{1,2}$ Based on the ability of catalase or azide to protect *E. coli* from PMA-stimulated neutrophils, it seemed likely that the H$_2$O$_2$-myeloperoxidase-Cl$^-$ system could play a role in our model. We have recently demonstrated that triggered neutrophils can utilize the myeloperoxidase system to generate a chlorinating species with characteristics similar to, if not identical to, those of HOCl.$^{13}$ Amine-containing compounds can rapidly scavenge HOCl, and we have previously used a myeloperoxidase-dependent cytotoxicity.$^{16}$ As shown in Table 3, at a final concentration of 1.0 mM, L-alanine, glycine, or taurine significantly inhibited *E. coli* destruction by PMA-stimulated neutrophils. None of the amino acids alone had any effect on *E. coli* growth or viability. Finally, if alanine and glycine inhibit the bactericidal effect by specifically scavenging HOCl, then their N-acetyl derivatives (which do not react as rapidly with HOCl$^{17}$) should not interfere with killing. Indeed, neither 1.0 mM N-acetyl-L-alanine nor N-acetyl-glycine provided significant protection against *E. coli* destruction by PMA-stimulated neutrophils (Table 3). Because L-alanine, glycine, or taurine do not interfere with O$_2^-$ or H$_2$O$_2$ generation,$^{13,16}$ it seems that HOCl, or a species with similar characteristics, is responsible for the microbialic effects observed in our system.

**Table 2. Effect of Azide on *E. coli* Destruction and H$_2$O$_2$ Generation by PMA-Stimulated Neutrophils**

<table>
<thead>
<tr>
<th>Additive*</th>
<th>CFU (x $10^6$)†</th>
<th>H$_2$O$_2$ (n mole)†</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em></td>
<td>11.5 ± 1.0 (n = 11)</td>
<td>0.0 ± 0.0 (n = 3)</td>
</tr>
<tr>
<td><em>E. coli</em> + neutrophils</td>
<td>11.6 ± 1.7 (n = 5)</td>
<td>—</td>
</tr>
<tr>
<td><em>E. coli</em> + azide</td>
<td>25.3 ± 3.8 (n = 11)</td>
<td>0.0 ± 0.0 (n = 4)</td>
</tr>
<tr>
<td><em>E. coli</em> + neutrophils + azide</td>
<td>21.7 ± 3.5 (n = 3)</td>
<td>—</td>
</tr>
<tr>
<td>Complete system</td>
<td>0.7 ± 0.8 (n = 11)</td>
<td>0.0 ± 0.0 (n = 3)</td>
</tr>
<tr>
<td>Complete system + azide</td>
<td>7.6 ± 0.9 (n = 9)</td>
<td>288.8 ± 13.1 (n = 4)</td>
</tr>
</tbody>
</table>

*The complete system consisted of neutrophils (2.5 x $10^6$/ml), *E. coli* (~$10^7$/ml), and PMA (30 ng/ml) in a final volume of 1 ml in Dulbecco’s buffer. Samples were incubated for 60 min at 37°C, and then processed as described. None of the amino acids alone affected the *E. coli* viability.†Results are expressed as the mean CFU ± 1 SD of the indicated number of experiments.

**Table 3. Effect of HOCl Scavengers on *E. coli* Killing by PMA-Stimulated Neutrophils**

<table>
<thead>
<tr>
<th>Additive*</th>
<th>n</th>
<th>CFU (x $10^6$)†</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em></td>
<td>7</td>
<td>12.1 ± 1.7</td>
</tr>
<tr>
<td><em>E. coli</em> + neutrophils</td>
<td>6</td>
<td>28.4 ± 2.9</td>
</tr>
<tr>
<td>Complete system</td>
<td>7</td>
<td>0.6 ± 1.1</td>
</tr>
<tr>
<td>Complete system + alanine (1.0 mM)</td>
<td>7</td>
<td>16.3 ± 2.3</td>
</tr>
<tr>
<td>Complete system + glycine (1.0 mM)</td>
<td>6</td>
<td>15.0 ± 2.4</td>
</tr>
<tr>
<td>Complete system + taurine (1.0 mM)</td>
<td>5</td>
<td>19.4 ± 2.8</td>
</tr>
<tr>
<td>Complete system + N-acetylglycine (1.0 mM)</td>
<td>7</td>
<td>1.5 ± 1.1</td>
</tr>
<tr>
<td>Complete system + N-acetylalanine (1.0 mM)</td>
<td>7</td>
<td>0.8 ± 0.9</td>
</tr>
</tbody>
</table>

*The complete system consisted of neutrophils (2.5 x $10^6$/ml), *E. coli* (~$10^7$/ml), and PMA (30 ng/ml) in a final volume of 1 ml in Dulbecco’s buffer. Samples were incubated for 60 min at 37°C, and then processed as described. None of the amino acids alone affected the *E. coli* viability.†Results are expressed as the mean CFU ± 1 SD of the indicated number of experiments.
Neutrophils incubated with phagocytosable particles can also utilize the myeloperoxidase system to generate chlorinating species, and we next examined the ability of these cells to mediate a bactericidal effect against unopsonized E. coli. When 2.5 \times 10^6 neutrophils were incubated with opsonized zymosan (0.5 mg/ml) and E. coli (10^7/ml) with constant rocking, no bactericidal effect was observed.

The inability of zymosan-triggered neutrophils to mediate E. coli killing suggested that zymosan might interfere with HOCl-mediated killing. Indeed, E. coli destruction by either reagent HOCl or by a model H_2O_2-myeloperoxidase-Cl^- system could be completely inhibited by opsonized or unopsonized zymosan particles (data not shown). Additionally, zymosan-stimulated cells might generate large quantities of HOCl directly in the phagocytic vacuole, with only small amounts being available for extracellular killing. Although zymosan clearly has the potential to either reduce or limit extracellular, HOCl-dependent killing, we attempted to maximize potential interactions between oxygen metabolites generated by zymosan-triggered neutrophils and the E. coli by decreasing the neutrophil:bacteria ratio to 1:1 and pelleting the cells and bacteria in flat-bottom plates. Under these conditions, we could detect a small but significant degree of E. coli killing (Table 5). As with PMA-stimulated cells, catalase inhibited the bactericidal effect, whereas superoxide dismutase did not protect the E. coli. In the presence of azide (1 mM), zymosan-stimulated cells accumulated 281.7 \pm 7.6 nmol of H_2O_2 (n = 3), which had an intermediate inhibitory effect on E. coli. As expected, taurine completely inhibited killing by the zymosan-stimulated cells. Thus, neutrophils incubated with opsonized zymosan destroyed E. coli by a process qualitatively similar to that observed with PMA-triggered cells.

**DISCUSSION**

We have demonstrated that PMA-triggered human neutrophils can destroy large numbers of unopsonized E. coli in a model in vitro system by a process dependent on both cell concentration and time. At a neutrophil-to-bacteria ratio of 1:4, killing was maximal after a 90-min incubation, with >95% of the original inoculum of E. coli destroyed. Although these results are based on the CFU assay, they may well represent an underestimate of the true extent of damage. When bacteria incubated with PMA-stimulated neutrophils were plated on trypticase soy broth agar, we found that \sim 10%-30% of the surviving organisms grew abnormally and formed microcolonies. Interestingly, Hassan and Fridovich reported that E. coli injured by an oxidative process mediated by paraquat also grew as microcolonies and suggested that a...
mutagenic alteration had taken place. 21 Apparently, PMA-stimulated neutrophils destroy a large number of the E. coli, whereas a smaller subpopulation incurs sublethal but uncharacterized damage.

An examination of the processes involved in the microbicidal effect revealed that E. coli destruction by PMA-stimulated neutrophils was enhanced by exogenous SOD and completely inhibited by catalase. The addition of small concentrations of SOD or catalase in the fluid phase would not be expected to affect bacterial killing if it occurred within the confines of the phagocytic vacuole. 3 Our ability to recover all the viable E. coli in supernatants from mixtures of PMA-stimulated neutrophils, bacteria, and catalase suggests an extracellular site of E. coli destruction. Thus, in this model, the absence of tight effector cell–bacterial coupling provides us with a simple and malleable system to investigate the bactericidal potential of oxygen metabolites generated by human neutrophils.

Table 5. Bactericidal Potential of Zymosan-Triggered Neutrophils

<table>
<thead>
<tr>
<th>Additive*</th>
<th>n</th>
<th>CFU (x 10^6)t</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td>7</td>
<td>2.8 ± 0.8</td>
</tr>
<tr>
<td>E. coli + neutrophils</td>
<td>6</td>
<td>2.7 ± 0.4</td>
</tr>
<tr>
<td>E. coli + neutrophils + zymosan (0.5 mg/ml)</td>
<td>7</td>
<td>1.1 ± 0.4</td>
</tr>
<tr>
<td>Complete system + SOD (10 µg/ml)</td>
<td>6</td>
<td>1.3 ± 0.4</td>
</tr>
<tr>
<td>Complete system + catalase (25 µg/ml)</td>
<td>6</td>
<td>2.4 ± 0.5</td>
</tr>
<tr>
<td>Complete system + N₂O₃ (1.0 mM)</td>
<td>5</td>
<td>1.9 ± 0.4</td>
</tr>
<tr>
<td>Complete system + taurine (10 mM)</td>
<td>5</td>
<td>3.3 ± 0.9</td>
</tr>
</tbody>
</table>

*The complete system consisted of neutrophils (2.5 x 10⁶), E. coli (~2.5 x 10⁶), and opsonized zymosan particles (0.5 mg) in a final volume of 1 ml in flat-bottom plates. The samples were then centrifuged (500 g, 5 mm) and incubated for 60 min at 37°C. The mixtures were processed as described in Materials and Methods.

†Results are expressed as the mean CFU ± 1 SD of the indicated number of experiments or as the mean percentage of the control number of CFU.

Directed by the characteristics of the amine-containing moiety, 22 β-Amino acid chloroamines are generally quite stable, 13,23 whereas α-amino acid chloroamines spontaneously undergo decarboxylation and deamination at varying rates to form the corresponding aldehyde, CO₂, NH₃, and Cl⁻. 22–24 N-chloroamines and aldehydes have been considered as potential microbicidal agents 1,2,22 but, in our study, the expected chlorinated products of alanine, glycine, or taurine did not exert an observable bactericidal effect under the conditions studied. These results do not allow us to conclude that N-chloroamines or aldehydes are never toxic. Indeed, several N-chloroamines can exert powerful microbicidal effects similar to those obtained with HOCl. 22 Thomas has suggested that N-chloroamines have sufficient oxidizing potential to destroy microbes, but only if they can cross the bacterial membrane.7 Thus, the stability, size, and/or polar characteristics of N-chloroamines may regulate their antibacterial properties.

The microbicidal potential of HOCl has been clearly established in a number of studies, 6,8,23 and the results are consistent with a direct role for this oxidant in the destruction of E. coli by the triggered neutrophil. However, we have recently demonstrated that stimulated neutrophils can utilize the myeloperoxidase system to generate a class of long-lived oxidants with characteristics identical to those of N-chloroamines. 18 These endogenous N-chloroamines are potentially bactericidal, but we were unable to demonstrate any direct microbicidal properties attributable to the oxidants that accumulated in the supernate fraction of stimulated neutrophils. Thomas et al. have demonstrated that neutrophils can chlorinate certain exogenous amines to form potentially toxic, lipophilic N-chloroamines, which would not be expected to accumulate in cellular systems. 19 The ability of the neutrophil to generate similar lipophilic N-chloroamines from endogenous amines is presently unknown, but we cannot rule out the possibility that short-lived N-
chloroamines play a role in the bactericidal event. Nonetheless, the long-lived $N$-chloroamines that accumulated in our system were markedly bactericidal in the presence of free iodide. $N$-chloroamines can readily oxidize iodide to iodine, triiodide, and possibly other toxic iodide oxidation products. Although the in vivo concentration of free iodide is extremely low, it is intriguing to note that stimulated neutrophils can deiodinate thyroid hormones and release iodide. $N$-chloroamines could represent a stable reservoir of oxidizing equivalents that could accumulate at sites of infection and mediate microbicidal effects long after infiltrating neutrophils ceased generating oxygen metabolites.

Taken together, our data indicate that triggered neutrophils destroyed E. coli by utilizing the myeloperoxidase system to generate a microbicidal oxidant with characteristics similar to, if not identical with, HOCl. Whether HOCl is released from the neutrophils in sufficient quantities to mediate a direct bactericidal effect or is generated at the bacterial membrane by bound myeloperoxidase and released $H_2O_2$ has not been determined. Nogueira et al. recently demonstrated that macrophages incubated with opsonized zymosan could destroy extracellular T. cruzi if the organism was first coated with eosinophil peroxidase. It should be noted, however, that Selvaraj et al. reported that HOCl scavengers did not protect bacteria from a model $H_2O_2$-myeloperoxidase-Cl$^-$ system if the microbes were first coated with myeloperoxidase. Apparently the exogenous scavenger cannot effectively compete with the high, local concentration of HOCl generated at the bacterial surface.

Does the myeloperoxidase system play an important role in more physiologic models of neutrophil-mediated bacterial killing? A variety of indirect evidence suggests that the myeloperoxidase system plays an important role in intraphagosomal killing, including the inhibitory effects of heme-enzyme inhibitors, the halogenation of internalized microbes, and the abnormal rate of bacterial destruction by neutrophils isolated from individuals with myeloperoxidase deficiency. However, in the single report that has attempted to identify the specific oxygen metabolites responsible for the phagosomal destruction of bacteria by neutrophils, there was no evidence to suggest a role for the myeloperoxidase system. Both SOD and catalase-coated latex beads or OH$^-$ scavengers inhibited the killing of opsonized Staph. aureus or E. coli, and Johnston et al. concluded that the OH$^-$ was the primary species responsible for the bactericidal effect. In contrast, Repine et al. found that high concentrations of the OH$^-$ scavenger, DMSO, modestly inhibited neutrophil-mediated staphylocidal activity. Thus, the multiple processes responsible for intracellular bacterial killing remain to be clearly identified.

In this study, the MPO-$H_2O_2$-Cl$^-$ system was the major bactericidal mechanism, but nonoxidative processes may synergistically increase killing, and it is likely that additional oxidative and nonoxidative processes come into play as the distance between the cell and microbe is decreased. Simple manipulations of our model system and the study of other microorganisms should allow us to clearly assess additional processes utilized by the neutrophil in antimicrobial defenses.

REFERENCES

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Oxidative mechanisms utilized by human neutrophils to destroy Escherichia coli

SA Passo and SJ Weiss