HUMAN NEUTROPHILS play a pivotal role in host defense by phagocytosing and destroying invading bacteria.\(^1\) 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 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 \textit{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 \textit{E. coli} in 60 min at 37°C. Destruction of \textit{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 \textit{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 \textit{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.

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.\(^1\)\(^2\) 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.\(^1\)\(^2\) Oxygen metabolites implicated in the bactericidal process include superoxide anion (\(\text{O}_2^-\)), hydrogen peroxide (\(\text{H}_2\text{O}_2\)), the hydroxyl radical (\(\text{OH}^-\)), and products generated by the \(\text{H}_2\text{O}_2\)-myeloperoxidase-halide system.\(^1\)\(^2\) 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.\(^3\) 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.\(^3\) 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.\(^4\)\(^8\) 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 \textit{E. coli} by an oxygen-dependent process in a serum-free, model system.

\section*{Materials and Methods}

\textbf{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.\(^7\) 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).

\section*{Bacteria}

\textit{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 \(10^9\) 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.

\section*{Bactericidal Assay System}

\textbf{Serum-independent killing}

A quantity of \(10^7\) \textit{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.9 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 H2O2 (30%; stabilizer free) was obtained from Mallinkrodt (Paris, KY), and its concentration was determined as described.10 Superoxide dismutase was assayed according to the method of McCord and Fridovich.10 Catalase was washed over an XM-100A ultrafiltration membrane (Amicon Corp., Lexington, MA) and assayed before use.11

H2O2 Generation by Stimulated Neutrophils

H2O2 generated by neutrophils was quantitated by the method of Thurman et al., as previously described.12

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 H2O2, the cells pelleted (500 x g for 10 min), and the supernatants examined for the presence of N-chloroamines, as previously described.13

All data are expressed as the mean ± 1 SE of at least 4 experiments.

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.

The effect of neutrophil concentration on bacterial killing in the PMA model is shown in Fig. 2. The microbicidal effect was directly related to the neutrophil concentration in the range of 10^4–5 x 10^6 cells/ml after a 90-min incubation. In this model, 5 x 10^6 PMA-stimulated cells reduced the E. coli CFU comparably to 2.5 x 10^6 neutrophils in the presence of serum after a 90-min incubation. Thus, although serum-dependent killing may be more rapid and complete, it is clear that PMA-stimulated cells can mediate a powerful microbicidal effect.

Mechanism of E. coli Destruction by PMA-Stimulated Neutrophils

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

*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.
of oxygen metabolites in our model system, experiments were performed in the presence of SOD (to lower the \( O_2^- \) concentration) or catalase (to reduce the generated \( H_2O_2 \)).

As shown in Table 1, SOD significantly stimulated the bactericidal effect of \( 2.5 \times 10^6 \) PMA-triggered 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 had any effect on the bactericidal system (Table 1). If PMA-stimulated neutrophils and \( E. coli \) were coincubated in the presence of catalase (25 \( \mu \)g/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) \( H_2O_2 \) plays a pivotal role in the microbicidal effect.

**Role of \( H_2O_2 \) in bactericidal system.** Although the complete protection of the microorganism by catalase implicates \( H_2O_2 \) in the bactericidal event, \( H_2O_2 \) can potentially react with reduced transition metal complexes to generate \( OH^- \) or be utilized by the myeloperoxidase-chloride system to produce hypochlorous acid (HOCl). 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 \( \pm \) 1.4 \( \times \) \( 10^6 \) to 0.6 \( \pm \) 1.1 \( \times \) \( 10^6 \), whereas the number of CFU in the presence of stabilized neutrophils and 40 mM ethanol or mannitol fell to 0.2 \( \pm \) 0.3 \( \times \) \( 10^6 \) and 0.4 \( \pm \) 0.3 \( \times \) \( 10^6 \), 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 \( H_2O_2 \) for longer periods of time in the presence of a heme-enzyme inhibitor. Indeed, \( H_2O_2 \) could not be detected in the supernates of \( 2.5 \times 10^6 \) PMA-stimulated neutrophils after a 60-min incubation, whereas large quantities of \( H_2O_2 \) accumulated under identical conditions in the presence of azide (see Table 2). In order to examine the bactericidal potential of high concentrations of \( H_2O_2 \) alone, \( E. coli \) were incubated with a bolus of reagent \( H_2O_2 \) for 60 min at \( 37^\circ C \). In the presence of 0.3 mM \( H_2O_2 \) (300 n mole in a final volume of 1 ml), \( E. coli \) remained viable (control 12.0 \( \pm \) 1.0 \( \times \) \( 10^6 \) CFU versus 9.6 \( \pm \) 0.6 \( \times \) \( 10^6 \) CFU; \( n = 6 \)) and reduced the \( H_2O_2 \) concentration to 0.013 \( \pm \) 0.006 mM (\( n = 3 \)). Thus, \( H_2O_2 \) alone did not exert a strong bactericidal effect and was rapidly metabolized by the \( E. coli \). However, when \( H_2O_2 \) (300 n mole) was added to \( E. coli \) in the presence of azide (1 mM), the number of CFU fell to 3.8 \( \pm \) 1.2 \( \times \) \( 10^6 \) (68% reduction; \( n = 4 \)), and 281.7 \( \pm \) 7.2 nmole (\( n = 3 \)) of \( H_2O_2 \) were detected after...
the 60-min incubation. Taken together, these data suggested that neutrophils exerted a bactericidal effect in the presence of azide because (1) H₂O₂ was allowed to accumulate to nonphysiologic levels and (2) bacterial metabolism of H₂O₂ was simultaneously inhibited.

**Effect of hypochlorous acid scavengers on neutrophil-mediated *E. coli* destruction.** Model systems consisting of purified myeloperoxidase, H₂O₂, and an oxidizable halide form a powerful microbicidal system. Based on the ability of catalase or azide to protect *E. coli* from PMA-stimulated neutrophils, it seemed likely that the H₂O₂-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 with, HOCI. Amine-containing compounds can rapidly scavenge HOCI, and we have previously used a number of amino acids to inhibit phagocyte-mediated, myeloperoxidase-dependent cytotoxicity. 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 HOCI, then their N-acetyl derivatives (which do not react as rapidly with HOCI) 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 O₂⁻ or H₂O₂ generation, it seems that HOCI, or a species with similar characteristics, is responsible for the microbicidal effects observed in our system.

### Table 2. Effect of Azide on *E. coli* Destruction and H₂O₂ Generation by PMA-Stimulated Neutrophils

<table>
<thead>
<tr>
<th>Additive*</th>
<th>CFU (x 10⁶)†</th>
<th>H₂O₂ (mM)†</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> + N₃⁻ (1.0 mM)</td>
<td>11.6 ± 1.7 (n = 5)</td>
<td>—</td>
</tr>
<tr>
<td>Complete system</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 + N₃⁻ (1.0 mM)</td>
<td>21.7 ± 3.5 (n = 3)</td>
<td>—</td>
</tr>
<tr>
<td>Complete system</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 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. N₃⁻ was determined in samples prepared in a manner identical to that described for the bactericidal assay. Results are expressed as the mean CFU ± SD of the indicated number of experiments.

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

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

<table>
<thead>
<tr>
<th>Additive*</th>
<th>n</th>
<th>CFU (x 10⁶)†</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-acetyllalanine (1.0 mM)</td>
<td>7</td>
<td>1.5 ± 1.1</td>
</tr>
<tr>
<td>Complete system + N-acetylglucine (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 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. 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.
They are capable of oxidizing ble oxidants, whereas N-chloro-L-alanine rapidly disappears when incubated in Dulbecco’s buffer (pH 7.4) at 37°C for 60 min (Weiss and Passo, unpublished observation).

Table 4. N-Chloroamine Generation by PMA-Stimulated Neutrophils in the Presence of E. coli

<table>
<thead>
<tr>
<th>Additive*</th>
<th>n</th>
<th>N-Chloroamine (nmole)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete system</td>
<td>6</td>
<td>33.5 ± 5.0</td>
</tr>
<tr>
<td>+ SOD (10 µg/ml)</td>
<td>6</td>
<td>38.5 ± 6.9</td>
</tr>
<tr>
<td>+ Hi SOD (10 µg/ml)</td>
<td>3</td>
<td>31.3 ± 0.1</td>
</tr>
<tr>
<td>+ catalase (25 µg/ml)</td>
<td>6</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>+ Hi catalase (25 µg/ml)</td>
<td>3</td>
<td>33.1 ± 2.1</td>
</tr>
<tr>
<td>+ N2− (1.0 mM)</td>
<td>6</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>+ ethanol (40 mM)</td>
<td>4</td>
<td>29.1 ± 2.2</td>
</tr>
<tr>
<td>+ manitol (40 mM)</td>
<td>4</td>
<td>30.7 ± 2.2</td>
</tr>
<tr>
<td>+ alanine (1.0 mM)</td>
<td>6</td>
<td>12.3 ± 2.5</td>
</tr>
<tr>
<td>+ glycine (1.0 mM)</td>
<td>5</td>
<td>108.1 ± 6.9</td>
</tr>
<tr>
<td>+ taurine (1.0 mM)</td>
<td>5</td>
<td>161.2 ± 3.1</td>
</tr>
</tbody>
</table>

*The complete system consisted of 2.5 x 10⁶ neutrophils, ~10⁹ E. coli and 30 ng of PMA in a final volume of 1 ml in Dulbecco’s buffer. Samples were incubated alone or with the above additives for 60 min at 37°C. The cells were then pelleted and the N-chloroamine content quantitated as described. Samples treated with azide were preincubated for 5 min at 25°C before PMA was added.

†Results are expressed as the mean nmole ± 1 SD of N-chloroamines generated for the indicated number of experiments.

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 ~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 generate chlorinating species, and we next examined the ability of these cells to mediate a bactericidal effect against unopsonized E. coli. When 2.5 x 10⁶ neutrophils were incubated with opsonized zymosan (0.5 mg/ml) and E. coli (10⁷/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 H₂O₂-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-triggered cells accumulated 281.7 ± 7.6 nmole of H₂O₂ (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 ~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.\textsuperscript{21} Apparently, PMA-stimulated neutrophils destroy a large number of the \textit{E. coli}, whereas a smaller subpopulation incurs sublethal but uncharacterized damage.

An examination of the processes involved in the microbicidal effect revealed that \textit{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.\textsuperscript{3} Our ability to recover all the viable \textit{E. coli} in supernatants from mixtures of PMA-stimulated neutrophils, bacteria, and catalase suggests an extracellular site of \textit{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.

Based on the protective effect of catalase and azide, our attention focused on the role of the myeloperoxidase system in the microbicidal process. Model systems consisting of H\textsubscript{2}O\textsubscript{2}, purified myeloperoxidase, and Cl\textsuperscript{-} form a powerful microbicidal and cytotoxic system whose toxic properties are mediated by an oxidant with characteristics similar to, if not identical with, HOCl.\textsuperscript{1,2,14} Alanine, glycine, and taurine have all been demonstrated to be capable of inhibiting the microbicidal effects of cell-free H\textsubscript{2}O\textsubscript{2}-myeloperoxidase-Cl\textsuperscript{-} systems,\textsuperscript{22} and they similarly inhibited \textit{E. coli} destruction by PMA-stimulated neutrophils. As expected, \textit{N}-acetylated amino acids, compounds that do not react as rapidly with HOCl,\textsuperscript{15} failed to inhibit \textit{E. coli} destruction.

Although the amino acids used in our study can “scavenge” HOCl, this does not mean that the oxidizing potential of the chlorinating species is immediately lost. Both \textit{\alpha} and \textit{\beta}-amino acids can react with HOCl to form the respective \textit{N}-chloroamines.\textsuperscript{23} These compounds are two-electron oxidants whose stability is dictated by the characteristics of the amine-containing moiety.\textsuperscript{24} \textit{\beta}-Amino acid chloroamines are generally quite stable,\textsuperscript{13,23} whereas \textit{\alpha}-amino acid chloroamines spontaneously undergo decarboxylation and deamination at varying rates to form the corresponding aldehyde, CO\textsubscript{2}, NH\textsubscript{3}, and Cl\textsuperscript{-}.\textsuperscript{22,24} \textit{N}-chloroamines and aldehydes have been considered as potential microbicidal agents\textsuperscript{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 \textit{N}-chloroamines or aldehydes are never toxic. Indeed, several \textit{N}-chloroamines can exert powerful microbicidal effects similar to those obtained with HOCl.\textsuperscript{22} Thomas has suggested that \textit{N}-chloroamines have sufficient oxidizing potential to destroy microbes, but only if they can cross the bacterial membrane.\textsuperscript{7} Thus, the stability, size, and/or polar characteristics of \textit{N}-chloroamines may regulate their antibacterial properties.

The microbicidal potential of HOCl has been clearly established in a number of studies,\textsuperscript{6–8,25} and the results are consistent with a direct role for this oxidant in the destruction of \textit{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 \textit{N}-chloroamines.\textsuperscript{18} These endogenous \textit{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 \textit{exogenous} amines to form potentially toxic, lipophilic \textit{N}-chloroamines, which would not be expected to accumulate in cellular systems.\textsuperscript{19} The ability of the neutrophil to generate similar lipophilic \textit{N}-chloroamines from \textit{endogenous} amines is presently unknown, but we cannot rule out the possibility that short-lived \textit{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.\(^{26}\) Although the in vivo concentration of free iodide is extremely low,\(^1\) it is intriguing to note that stimulated neutrophils can deiodinate thyroid hormones and release iodide.\(^1\) \( N \)-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.\(^{27}\) 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.\(^{28}\) 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.\(^1,2\) However, in the single report that has attempted to identify the specific oxygen metabolites responsible for the phagosomal destruction of bacteria by neutrophils,\(^3\) 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.\(^3\) In contrast, Repine et al. found that high concentrations of the \( OH^- \) scavenger, DMSO, modestly inhibited neutrophil-mediated staphylococidal activity.\(^{29}\) 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,\(^{30}\) and it is likely that additional oxidative\(^{12}\) and nonoxidative\(^{11,32}\) 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.

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Oxidative mechanisms utilized by human neutrophils to destroy Escherichia coli

SA Passo and SJ Weiss