Redundant Contribution of Myeloperoxidase-Dependent Systems to Neutrophil-Mediated Killing of *Escherichia coli*

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Neutrophil microbicidal activity is a consequence of overlapping antimicrobial systems that vary in prominence according to the conditions of the neutrophil-microbe interaction, the nature of the microbe, and its metabolic state. In this study, normal, myeloperoxidase-deficient, and respiratory burst-deficient (chronic granulomatous disease [CGD]) neutrophils killed *Escherichia coli* with equivalent, high efficiencies. Killing by CGD and myeloperoxidase-deficient neutrophils was not augmented by supplements, such as exogenous H$_2$O$_2$ and myeloperoxidase, directed at ameliorating their metabolic defects, suggesting that nonoxidative microbialicidal systems were sufficient for a full microbicidal effect. Neutrophils with an intact myeloperoxidase antimicrobial system (normal or appropriately supplemented deficient cells) were capable of rapidly suppressing *E. coli* DNA synthesis, while unsupplemented CGD or myeloperoxidase-deficient cells were far less effective, indicating that the myeloperoxidase system was active in normal neutrophils. The degree of DNA synthesis inhibition by myeloperoxidase-sufficient neutrophils could account, in a cell-free system, for most of the observed microbicidal activity. While the myeloperoxidase system was active and probably bactericidal, it was not rate limiting for microbicidal activity and appears to have been redundant with other microbialicidal systems in the cell. Rapid and extensive inhibition of bacterial DNA synthesis appears to be an indicator of myeloperoxidase activity in neutrophils.

Phagocytosis of bacteria by neutrophils is typically followed by loss of microbial viability as indicated by the inability to form colonies on nutrient agar. Phagocytosis-associated events introduce preformed microbialicidal proteins, stored in cytoplasmic granules, and reactive oxygen species, such as hydrogen peroxide, generated by a membrane-associated NADPH oxidase. An analysis of neutrophil granule components has identified several antimicrobial proteins, including myeloperoxidase (MPO), bactericidal-permeability-increasing protein, defensins, serprocidins, cathelicidins, and others (22). These proteins optimally exert their antimicrobial effects alone, in combination, or, for MPO, in concert with products of the respiratory burst. Their antimicrobial activities vary with the nature of the microbe, its metabolic state, and the pH, osmolality, and protein composition of the medium in which they function. The assessment of the relative contributions of these possibly redundant systems to the aggregate of neutrophil-mediated events affecting a specific pathogen poses a challenge.

While characterizing the effects of an isolated, neutrophil-related, microbialicidal system consisting of MPO, chloride, and H$_2$O$_2$, we observed a good correlation between loss of bacterial viability and inhibition of microbial DNA synthesis (32). Other oxidative and nonoxidative microbialicidal systems evaluated failed to manifest a similarly close relationship, and the MPO system seemed to possess a measure of specificity for this effect. Model systems serve, at best, to approximate conditions believed to prevail in intact cells. We wished to ascertain whether the concordance between DNA synthesis inhibition and bactericidal effect was also a feature of the microbialicidal activity of intact neutrophils and, if so, whether the MPO system was required. Thus, the effects on *Escherichia coli* viability and DNA synthesis of normal neutrophils were compared to those of neutrophils that lacked the capacity to activate the membrane-associated NADPH oxidase (chronic granulomatous disease [CGD] neutrophils) or that were devoid of MPO (hereditary MPO deficiency).
of fluorescein covalently bound to ingested zymosan particles (11). Samples for
the determination of microbial viability and incorporation of tritiated thymidine
into macromolecular compounds were taken immediately and at 7.5, 15, 30, and
60 min after the addition of neutrophils. Between samplings, tubes were vortexed
continuously at low speed in a 37°C chamber.

Bacterial viability. Phagocytosis and killing were terminated by the transfer of
20 μl of neutrophil-bacterium suspension to 180 μl of 0.1% (wt/vol) Triton X-100
detergent in a microtiter plate well, with mixing by repetitive pipetting. Neutro-
phil lysates were allowed to stand for 2 to 30 min; there was no discernible effect
on bacterial viability. Subsequent serial 1:10 dilutions were made into M63 salts
(34), and 0.1-ml samples of each dilution were plated in molten Trypicase soy
agar. Microbial viability was assessed by enumeration of visible colonies after 18
and amounted to approximately 10% of that associated with fully viable bacteria.

Statistics. Viability and DNA synthesis (thymidine incorporation) for sequential
samples were expressed as percentages of the initial values (t = 0) for that
experimental tube. On days when experimental conditions were replicated, re-
sults were averaged and data were considered to represent a single experiment.
Data points in the figures represent the mean ± standard errors of the means
for the numbers of experiments indicated in the legends. For comparison of the
effect of neutrophil cell type on E. coli viability and DNA synthesis, data were
grouped as described in each figure legend and arranged in a two-by-two table
with cell type and enzyme supplement if any (normal, CGD with or without
MPO, MPO-supplemented CGD, and MPO-deficient with or without MPO) along one axis and time
of incubation (7.5, 15, 30, or 60 min) with neutrophils along the other. Percent-
down analysis of variance was used to calculate an F statistic (SigmaStat; Jandel Scientific, San Francisco,
Calif.), and column comparisons (cell type with or without enzyme) were per-
formed by using the Student-Neuman-Keuls test, with P values below 0.05 con-
sidered significant.

RESULTS

Figure 1A shows the decline in viability for E. coli incubated with normal human neutrophils in 10% fresh autologous se-
um. After an hour’s incubation 3% ± 1% of the initial inoc-
um remained viable. E. coli ATCC 11775 is serum resistant, with a doubling time of approximately 1 h in control tubes
containing 10% serum without neutrophils (data not shown).

When neutrophils from patients with CGD or hereditary com-
plete MPO deficiency were substituted for normal neutrophils, there was no significant alteration in either the rate or extent of neutrophil-mediated microbicidal effect. After an hour 5% ± 1% (for CGD neutrophils) and 9% ± 1% (for MPO-deficient neutrophils) E. coli cells remained viable (Fig. 1A). Supplementation of CGD neutrophils with the glucose plus glucose oxidase H₂O₂-generating system (Fig. 1B; viability at 1 h, 3% ± 1%) or of MPO-deficient neutrophils with exogenous MPO (Fig. 1C; viability at 1 h, 11% ± 2%) did not augment their bactericidal activities. Although under some conditions (rough E. coli strain with restricted complement activity during opsonization) lethal effects of CGD nonoxidative microbicidal systems can be reversed by incorporation of 0.1% bovine serum albumin in lysis or culture media (23), this phenomenon was not observed under the conditions employed for Fig. 1 (smooth serum-resistant strain, 10% fresh complement-replete serum; data not shown).

The ability of E. coli cells to incorporate radiolabeled thymidine into TCA-insoluble material was taken as an indicator of DNA synthesis. The decline in DNA synthesis was similar to the decline in viability (Fig. 2), although in comparison with the decline observed with the cell-free MPO system (32), the concordance was more approximate. E. coli DNA synthesis fell to 10% ± 3% of control during the 1-h incubation. In contrast to the normal effects of CGD and MPO-deficient phenotypes on microbicidal activity, there were readily discerned, statistically significant deficits in the capacity of these metabolically deficient neutrophils to inhibit E. coli DNA synthesis. After 60 min, CGD and MPO-deficient neutrophils reduced E. coli DNA synthesis to 49% ± 9% (Fig. 3) and 43% ± 10% (Fig. 4) of the initial values, respectively (corresponding value for normal neutrophils: 10% ± 3%).

Attempts to correct the metabolic defect of CGD cells by supplementing the incubation medium with the hydrogen peroxide-generating system glucose plus glucose oxidase resulted in a dose-dependent restoration of DNA synthesis suppression that approached normal levels (Fig. 3; low glucose oxidase supplement concentration, 1.3 U/ml; P < 0.05 versus normal neutrophils; P < 0.05 versus CGD neutrophils without supplementation). Higher concentrations of glucose oxidase (2.6 to 5.2 U per ml) produced almost complete normalization of CGD cells with respect to suppression of DNA synthesis. Similarly, precoating of bacteria with MPO allowed MPO-deficient neutrophils to manifest a nearly normal suppression of E. coli DNA synthesis (Fig. 4; P < 0.05 versus normal neutrophils; P < 0.05 versus MPO-deficient neutrophils without supplementation).

Neither supplementation of media with glucose plus glucose oxidase nor precoating of bacteria with MPO had discernible effects on bacterial viability or DNA synthesis in the absence of
DISCUSSION

MPO-deficient neutrophils lack the enzyme that catalyzes the H$_2$O$_2$-mediated oxidation of chloride to the potent microbicidal HOCl. Neutrophils from individuals with CGD are unable to provide the H$_2$O$_2$ substrate for MPO and are similarly unable to synthesize HOCl. Despite these metabolic deficiencies, both CGD and MPO-deficient neutrophils were able to kill *E. coli* as efficiently as normal neutrophils under the in vitro conditions employed in this study. The nonessential role for MPO was emphasized by the observation that reconstitution of CGD and MPO-deficient cells with H$_2$O$_2$ and MPO, respectively, failed to produce an incremental microbicidal effect (Fig. 1B and C). The observation of normal neutrophil killing of *E. coli* by CGD neutrophils is concordant with several (9, 37, 38) but not all (8, 13, 14, 16) other studies. The bases for the discrepancies among studies have not been explicitly characterized but may relate to incubation conditions that permit significant secretion of MPO, with attendant extracellular antimicrobial activity of the MPO-mediated antimicrobial system (25). In any case, it is clear that under some incubation conditions, such as those employed in this study, the nonoxidative microbicidal systems of neutrophils are sufficient to produce a normal microbicidal effect against *E. coli*.

Since there was no requirement for a functional MPO system in mediating bactericidal activity, one might ask whether this particular system is sufficiently active to engender an antimicrobial effect. An evaluation of neutrophil-mediated inhibition of bacterial DNA synthesis identified a major metabolic effect of the MPO system on *E. coli* cells. Normal neutrophils suppressed *E. coli* DNA synthesis nearly as extensively as they diminished survival (Fig. 2). In CGD and MPO-deficient cells, there was a defect in suppression of DNA synthesis that could be reversed by supplements expected to reconstitute their MPO systems (Fig. 3 and 4). Rapid inhibition of *E. coli* DNA synthesis is thus a clearly discernible biochemical effect of the neutrophil MPO system. The degree of suppression would be sufficient, in a cell-free system, to account for most of the microbicidal effect (32). Thus, while not detectable by virtue of its microbicidal effects, the MPO system appears to be active at “physiological” levels of opsonization, phagocytosis, and respiratory burst function.

In a cell-free MPO antimicrobial system, suppression of bacterial DNA synthesis was evaluated by comparison of the system with equally bactericidal oxidative (acetaldehyde–xan-
The MPO system was distinct from the other two in the rapidity and extent of suppression of bacterial DNA synthesis. Similar to inhibition by the cell-free MPO system, rapid neutrophil inhibition of *E. coli* DNA synthesis is relatively specific to the MPO component of the antimicrobial armamentarium. Limited inhibition of bacterial DNA synthesis by CGD cells indicated that the respiratory-burst-independent microbicidal systems were insufficient to produce the effect. In accord with these observations, lysates of rabbit neutrophils incubated under conditions where the MPO system should be inactive exhibited minimal effects on bacterial DNA and protein synthesis while producing substantial bactericidal effects (4). Impaired inhibition of bacterial DNA synthesis by MPO-deficient cells indicated that non-MPO oxidative microbicidal systems, such as those dependent on superoxide, hydrogen peroxide, and hydroxyl radicals, were also insufficient to produce major early suppression of *E. coli* DNA synthesis.

These findings should not be taken to suggest that the MPO system is broadly irrelevant to neutrophil-mediated antimicrobial effects. The importance of MPO and other oxidative systems has been demonstrated for neutrophil-mediated killing, in vitro, of *Staphylococcus aureus* (9, 10, 12, 13, 26, 27), *Burkholderia* (formerly *Pseudomonas*) cepacia (15, 35), and aspergillus (24, 26, 36) and candida (2, 6, 19, 20) species. The importance of oxidative antimicrobial mechanisms is further emphasized by the clinical importance of the above pathogens in afflicting CGD patients. While it should be noted that these patients are also susceptible to serious *E. coli* infections, there is no reason to presume a lesser-than-normal susceptibility of these patients to this moderately virulent organism.

The diverse elements of the antimicrobial armamentarium of neutrophils constitute an overlapping, broad-spectrum antimicrobial system in which the relative contribution of each component varies according to the species and growth phase (1, 18, 21, 29) of the pathogen encountered, as well as the metabolic microenvironment (e.g., aerobic or anaerobic) in which the phagocyte-microbe encounter takes place (5). We conclude that, for *E. coli*, there is a substantial redundancy of neutrophil bactericidal systems and that, in our system, participation of the MPO system was probably sufficient but not necessary for the observed microbicidal effects.

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