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Involvement of Superoxide and Myeloperoxidase in Oxygen-Dependent Killing of *Staphylococcus aureus* by Neutrophils

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We have used a quantitative assay that measures independent rate constants for phagocytosis and killing of *Staphylococcus aureus* to investigate the involvement of superoxide and myeloperoxidase in bacterial killing by human neutrophils. To inhibit superoxide-dependent processes, superoxide dismutase was cross-linked to immunoglobulin G and the conjugate was attached to the surface of *S. aureus* via protein A in its cell wall. Myeloperoxidase was inhibited with azide, and myeloperoxidase-deficient neutrophils were used. Adding the NADPH oxidase inhibitor diphenyleneiodonium, to prevent superoxide production, decreased the killing rate to 25%, indicating that oxidative killing mechanisms predominate in this system. The rate constant for killing of *S. aureus* with superoxide dismutase attached was 70% of that for control bacteria linked to inactivated enzyme. Superoxide dismutase had no effect in the presence of diphenyleneiodonium. The rate of killing was decreased to 33% in the presence of azide and to 40% with myeloperoxidase-deficient neutrophils. Superoxide dismutase had no effect in the presence of azide. On the assumption that the oxidative and nonoxidative components of killing can be considered separately, the oxidative rate was decreased by almost half by superoxide dismutase and was about six times lower when myeloperoxidase was inactive. We conclude that myeloperoxidase-dependent processes are strongly favored by human neutrophils as their prime mechanism of oxidative killing of *S. aureus* and that superoxide makes a direct contribution to killing. Our results also suggest that superoxide acts in conjunction with a myeloperoxidase-dependent pathway.

The neutrophil has a variety of oxidative and nonoxidative mechanisms for killing bacteria (10, 29, 46). Oxidative killing involves an NADPH oxidase, which assembles in the phagosomal membrane and converts oxygen to superoxide when the bacteria are ingested (46). An array of antibacterial and digestive enzymes (10) are also emptied from cytoplasmic granules into the phagosome. One of these enzymes is myeloperoxidase, which produces the strong oxidant hypochlorous acid from chloride and hydrogen peroxide (29). Oxidant production is essential for the effective killing of several strains of bacteria (35). However, the inaccessibility of detectors and scavengers makes it difficult to define which particular oxidants are produced in the phagosome and the role that each plays in bacterial killing.

Superoxide produced by neutrophils acts as a precursor of hydrogen peroxide. It is unclear whether this is its only function or if it plays a direct role in bacterial killing. Superoxide itself shows little toxicity to bacteria (1, 28). However, it can form hydroxyl radicals via the iron-catalyzed Haber-Weiss reaction (8) or by reacting with hypochlorous acid (7), and it can react with nitric oxide to produce peroxynitrite (53). Superoxide has also been proposed to aid bacterial killing by oxidizing reducing substrates in the medium surrounding the bacteria (48) or by causing an initial alkalinization of the phagosome (46). It also interacts with myeloperoxidase to enhance hypochlorous acid production (21, 23).

The role of superoxide in bacterial killing has been investigated with superoxide dismutase added to neutrophils plus bacteria (18) and with bacteria that have enhanced superoxide dismutase expression (37, 40, 41, 45). In these studies, elevated superoxide dismutase levels had little effect, but this may be because the enzyme was inaccessible to phagosomal superoxide. The only positive study has been that of Johnston et al. (18), who showed impaired killing of *Staphylococcus aureus* when latex beads coated with superoxide dismutase were coingested with the bacteria. Although this observation was made 20 years ago, it has not been followed up to establish how superoxide acts or to quantify the extent of the effect.

Even though isolated myeloperoxidase-hydrogen peroxide-halide systems kill a wide range of microorganisms efficiently (26, 29), the role of myeloperoxidase in bacterial killing by neutrophils is controversial (46, 49). The main argument against myeloperoxidase involvement is that individuals with chronic granulomatous disease, whose neutrophils do not produce superoxide and its secondary products, suffer from recurrent bacterial infection whereas those with myeloperoxidase deficiency do not (11). However, isolated neutrophils from individuals with chronic granulomatous disease (42) and myeloperoxidase deficiency (30, 33) both kill *S. aureus* more slowly than normal. To explain this apparent discrepancy, greater quantitative understanding of the contribution of myeloperoxidase to oxidative killing is required.

We have established an assay that measures individual first-order rate constants for the phagocytosis and killing of *S. aureus* by neutrophils (12). This enables the effects of inhibiting the production or reactions of different oxidants to be quantified. Our previous studies with the NADPH oxidase inhibitor diphenyleneiodonium (DPI) showed that most of the killing is

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TABLE 1. Effect of IgG and IgG-superoxide dismutase on phagocytosis

Bacterium[a]	*n*	Mean rate constant for phagocytosis (min^{-1}) ± SD[b]	$t_{1/2}$ (min)
Control	5	0.110 ± 0.017	6.3
IgG	16	0.127* ± 0.026	5.5
IgG-SOD	16	0.094 ± 0.021	7.4
Inactive IgG-SOD	3	0.100 ± 0.023	6.9

[a] SOD, superoxide dismutase.

[b] Rate constants (k) were determined according to the following relationship (12): rate of phagocytosis = k[extracellular viable bacteria]. *, $P < 0.05$ with respect to other treatments by analysis of variance followed by the Student-Newman-Keuls multiple-comparison method.

oxidative (13). In this study, we have used the assay to quantify the roles of superoxide and myeloperoxidase in oxidative killing.

MATERIALS AND METHODS

Materials. DPI a gift from O. T. G. Jones, Department of Biochemistry, University of Bristol, was dissolved at 1 mM in 10% dimethyl formamide (BDH, Poole, England). Succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SMCC) was purchased from Pierce, Rockford, Ill. All other biochemicals were from Sigma, St. Louis, Mo. *S. aureus* ATCC 27217 (502a) was obtained from the New Zealand Communicable Disease Centre, Porirua, New Zealand.

Preparation of bacteria. Single colonies were transferred from Colombia sheep blood agar plates to nutrient broth (Oxoid no. 1) and incubated overnight at 37°C; they were in stationary phase when used. The bacteria were then centrifuged at 1,000 × *g* for 5 min and resuspended in phosphate-buffered saline (PBS) (10 mM sodium phosphate buffer [pH 7.4], 140 mM NaCl). This was repeated twice before the bacteria were resuspended at the appropriate concentration, as determined by a turbidity measure at 550 nm of 0.2, which corresponded to approximately 10^8 CFU/ml.

Conjugation of superoxide dismutase to IgG. Copper/zinc superoxide dismutase from bovine erythrocytes was used and assayed by its ability to inhibit the reduction of cytochrome *c* by superoxide generated from xanthine oxidase and hypoxanthine (36).

Superoxide dismutase was reacted with iminothiolane to create extra thiol groups on the protein and then linked to amino groups on immunoglobulin G (IgG) with SMCC (52). SMCC was dissolved at 50 mM in dimethyl sulfoxide, and 3 μl was added to 97 μl of purified rabbit IgG (10 mg/ml) in 50 mM sodium phosphate buffer (pH 7.5)–140 mM NaCl. After being incubated for 60 min at 37°C, IgG-SMCC complexes were separated from unreacted SMCC on a 7.5-ml Sephadex G-25 fine column equilibrated with 50 mM sodium phosphate buffer (pH 7.0). Iminothiolane (final concentration, 4.5 mM) was added to superoxide dismutase (10 mg/ml in 50 mM sodium phosphate buffer [pH 7.5]) and incubated for 60 min at 37°C. Superoxide dismutase-iminothiolane complexes were separated from unbound iminothiolane as described for the IgG-SMCC complexes. Samples (500 μl total) of the most concentrated protein fractions of IgG-SMCC (6 μM) and superoxide dismutase-iminothiolane (25 μM) were incubated together for a further 60 min at 37°C to form IgG-superoxide dismutase conjugates. These were stored at 4°C and used within 4 weeks of production. The efficiency of the conjugation was determined by separating IgG plus conjugates from superoxide dismutase on a 25-ml Sephadex G-150 column and determining the amount of superoxide dismutase activity in the IgG fractions. An activity equivalent to 6 μM superoxide dismutase was measured in the 3 μM IgG fraction, indicating on average a 2:1 complex. The conjugate was subsequently used without separation of unbound superoxide dismutase.

Attachment of IgG-superoxide dismutase to *S. aureus*. *S. aureus* (200 μl at 10^9 CFU/ml) was pelleted at 1,000 × *g* for 5 min, resuspended in 50 μl of IgG-superoxide dismutase prepared as above, and incubated for 20 min at 37°C and then for a further 20 min after the addition of 130 μl of PBS and 20 μl of IgG-free serum to preopsonize the bacteria. The serum (pooled human serum) was depleted of IgG by mixing with protein A-Sepharose, to prevent it competing with the conjugate for the protein A on the bacterial surface. To assay the amount of attached superoxide dismutase, the bacteria were centrifuged, washed twice, lysed for 4 h at 37°C in 200 μl of PBS containing 50 U of lysostaphin per ml, and then added to the cytochrome *c* assay mixture. Activities were related to that of the soluble Sigma enzyme.

Inactivation of superoxide dismutase. A sample of the IgG-superoxide dismutase conjugate was dialyzed for 1 h against 50 mM sodium carbonate buffer (pH 10.0) containing 25 mM hydrogen peroxide (15). After the conjugate was dialyzed against deionized water, inactivation was completed by dialyzing it for 1 h against 10 mM diethyldithiocarbamate (14) followed by three changes of 50 mM sodium phosphate buffer (pH 7.0). The final product had less than 0.5% of the original activity.

Isolation of neutrophils. Neutrophils were isolated from the peripheral blood of healthy human donors by centrifugation through Ficoll-Hypaque, dextran sedimentation, and hypotonic lysis of contaminating erythrocytes (6). After isolation, the cells were suspended in PBS–1 mM $CaCl_2$–0.5 mM $MgCl_2$–5.6 mM glucose.

Phagocytosis and killing assay. The phagocytosis and killing assay was performed as described previously (12). In brief, bacteria were opsonized with 10% pooled human serum and then added to neutrophils at a bacterium-to-neutrophil ratio of 1:1 (5×10^6 neutrophils per ml) and a final serum concentration of 10%. The tubes were rotated at 37°C, and samples were taken after 10, 20, and 30 min. Extracellular bacteria were separated from neutrophils by centrifugation, and the neutrophil pellets were lysed with saponin to release intracellular bacteria. Samples were spread on blood agar plates, and the numbers of viable extracellular and intracellular bacteria were determined from the number of CFU. Phagocytosis was assessed from the decrease in the numbers of extracellular bacteria, and killing was assessed from the initial increase and subsequent decline in the number of viable intracellular bacteria. The first-order rate constants for these processes were calculated as previously described (12). For bacteria coated with IgG conjugates, opsonization was performed with serum depleted of IgG and subsequent incubation was performed with nondepleted serum.

Characterization of myeloperoxidase-deficient neutrophils. Neutrophils were obtained from a myeloperoxidase-deficient individual and analyzed for superoxide production as cytochrome reduction, for hypochlorous acid production as taurine chloramine formation, and for peroxidase activity by using tetramethylbenzidine, as described previously (19). The cells on two separate occasions produced normal amounts of superoxide (76 ± 11 nmol/10 min/10^6 cells) upon stimulation with 5 mg of opsonized zymosan per ml, but they produced only 5% of control hypochlorous acid levels (1.2 ± 0.5 nmol/10 min/10^6 cells). The total peroxidase activity was 7% ± 2% that of control cells. Activity staining of samples separated on polyacrylamide gels showed that almost all of the peroxidase activity was due to eosinophil peroxidase from contaminating eosinophils (data not shown). This individual would be classified as having complete myeloperoxidase deficiency (38).

Statistics. All results are expressed as the means and standard deviations of *n* experiments. A single analysis of variance for repeated measures was used to compare the ability of neutrophils to kill bacteria under all the different treatment conditions. The Bonferroni *t* test was then used to determine whether there were significant differences between preselected treatments. Differences are described as statistically significant when $P < 0.05$. Analysis of variance was also used to determine significant differences between treatment groups for the amount of superoxide dismutase attached to bacteria and for rates of phagocytosis. The Student-Newman-Keuls multiple-comparison method was then used to test for differences among groups within each analysis. Differences were described as statistically significant when $P < 0.05$. Analyses were done with the software package SigmaStat (Jandel Scientific, San Rafael, Calif.).

RESULTS

Attachment of superoxide dismutase to *S. aureus*. Superoxide dismutase was introduced into neutrophil phagosomes along with ingested bacteria by a novel procedure that is based on the ability of protein A on the surface of *S. aureus* to bind IgG. Superoxide dismutase was conjugated to rabbit IgG by a standard procedure with SMCC and iminothiolane that cross-linked the equivalent of two fully active superoxide dismutase molecules to each IgG. Incubation of these conjugates with *S. aureus* resulted in a 16-fold increase in superoxide dismutase activity over that of control bacteria: 205 ± 66 pmol/10^9 CFU ($n = 10$) compared with 13 ± 10 pmol/10^9 CFU ($n = 7$) for control bacteria ($P < 0.05$). There was no increase in activity when inactivated superoxide dismutase was used (9 ± 4 pmol/10^9 CFU; $n = 3$).

Effect of superoxide dismutase on phagocytosis and killing. A one-step assay that distinguishes the rates of phagocytosis and killing was performed. We have shown (12) that in this procedure, phagocytosis and killing are first order in relation to extracellular bacteria and viable intracellular bacteria, respectively. By sampling at 10, 20, and 30 min, rate constants (k) and half-lives ($t_{1/2} = 0.693/k$) for each step can be calculated (12).

Bacteria coated with IgG-superoxide dismutase complexes were compared with those coated with IgG only, to allow for potential differences due to surface protein. Since IgG links to protein A through its Fab domain, this attachment is not the equivalent of opsonization; therefore, the bacteria were also opsonized with pooled human serum. Bacteria coated with IgG

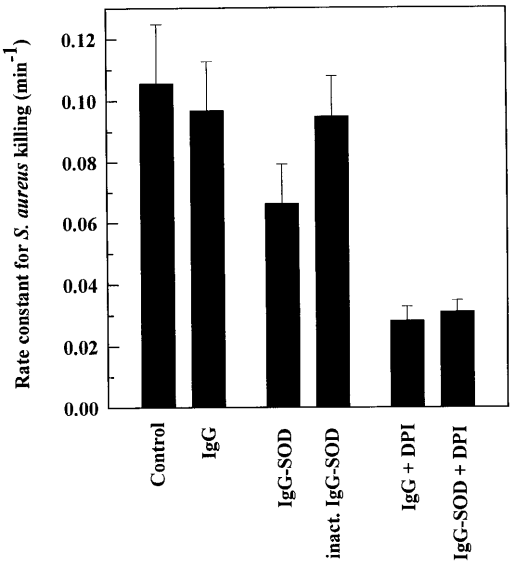

FIG. 1. Effect of IgG-superoxide dismutase (SOD) on bacterial killing. Rate constants (k), were calculated according to the following relationship (12): rate of killing = k[intracellular viable bacteria]. The number of assays was as follows: control unmodified *S. aureus*, $n = 14$; coated with IgG, $n = 16$; coated with IgG-superoxide dismutase, $n = 16$; coated with inactivated IgG-superoxide dismutase, $n = 3$; coated with IgG plus 10 μM DPI, $n = 4$; and coated with IgG-superoxide dismutase plus 10 μM DPI, $n = 4$. Error bars represent standard deviations. Killing of bacteria with IgG-superoxide dismutase was significantly different from all other treatments, and killing in the presence of DPI was significantly different from the control ($P < 0.05$). There was no significant difference in killing between the control bacteria and bacteria coated with either IgG or inactive superoxide dismutase or between killing in the presence of DPI when bacteria were coated with IgG or with IgG-superoxide dismutase.

alone were phagocytosed by neutrophils slightly more quickly (Table 1) but were killed at the same rate as untreated bacteria (Fig. 1). Those coated with the active or inactive superoxide dismutase conjugates were phagocytosed more slowly than were those coated with IgG alone but at the same rate as control bacteria (Table 1). These differences probably reflect small variations in opsonization. The rate constant for killing of *S. aureus* with inactive superoxide dismutase attached was no different from control values (Fig. 1). However, when the superoxide dismutase was active, the rate constant was significantly decreased to approximately 70% of that of all the controls. This corresponds to an increase in the half-life of intracellular bacteria from 7 to 10 min.

Although the objective was to maximize the amount of superoxide dismutase associated with the bacteria, different preparations varied in the amount of active enzyme that was attached. There was no significant decrease in killing rate with increasing attachment (Fig. 2), indicating that the observed effect of superoxide dismutase was maximal.

Possible confounders of our study would be if the modified bacteria adhered to the neutrophils but were not phagocytosed or if they agglutinated or lost viability. These possibilities seem unlikely. Others have shown that in a similar assay procedure, up to 10% of the bacteria identified as intracellular could simply be adherent to the neutrophil surface (34). We examined samples at 30 min by electron microscopy. Most of the control bacteria were ingested, with few adherent to the neutrophils. No difference was apparent when the bacteria were coated with superoxide dismutase (results not shown). In the absence of neutrophils, bacteria coated with IgG conjugates produced the same number of CFU per absorbance unit of turbidity as did untreated bacteria (data not shown), indicating that there was no loss of viability or agglutination.

To establish that superoxide dismutase affected the oxidative rather than the nonoxidative component of killing, the NADPH oxidase inhibitor DPI was used. Consistent with our previous observations, at a concentration that inhibits superoxide production by 95% (13), it decreased the rate constant for killing of *S. aureus* to 25% of normal (Fig. 1), giving a half-life of 28 min. In the presence of DPI, bacteria coated with superoxide dismutase were killed at the same rate as were control *S. aureus* organisms, which was significantly lower than with the coated bacteria in the absence of DPI (Fig. 1). Therefore, superoxide dismutase affected only oxygen-dependent bacterial killing.

Effect of azide and myeloperoxidase deficiency on bacterial killing. With azide added to inhibit myeloperoxidase, the mean rate constant for the killing of *S. aureus* was decreased to 33% of the control value. It was decreased to 40% when myeloperoxidase-deficient cells were used (Fig. 3). The difference between these values did not reach significance. They correspond to an increase in half-life of viable intracellular bacteria from 7 to approximately 18 min. The myeloperoxidase-deficient cells contained only 7% of control peroxidase activity, which was mainly from copurifying eosinophils and should make little contribution to overall killing of bacteria. Killing by these cells was not affected by azide, suggesting that there was no contribution by residual peroxidase activity. The rate constant for nonoxidative killing in the presence of DPI was not affected significantly by azide (Fig. 3). It was significantly lower than for azide alone, implying a small oxidative component that does not require myeloperoxidase activity.

Azide and myeloperoxidase deficiency did not affect the rate of phagocytosis (data not shown). Azide did not alter bacterial viability in the absence of neutrophils, and it did not alter killing if the bacteria were preexposed to it and then washed before incubation with neutrophils (data not shown).

Combined effect of superoxide dismutase and myeloperoxidase inhibition. In the presence of azide, there was no significant difference in the rate constant for killing of *S. aureus*

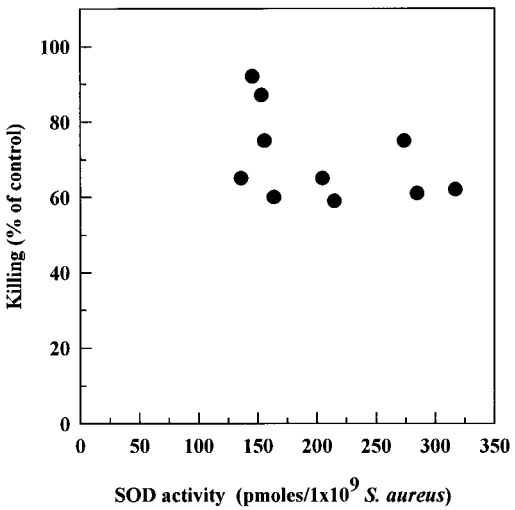

FIG. 2. Relationship between the amount of superoxide dismutase attached to *S. aureus* and the rate constant for the killing of the bacteria by neutrophils. Results are from 10 separate experiments. Analysis of the data by least-squares linear regression gives a line of the form $y = 86.2 - 0.0786x$ ($r^2 = 0.19$). The slope is not significantly different from zero ($P = 0.20$).

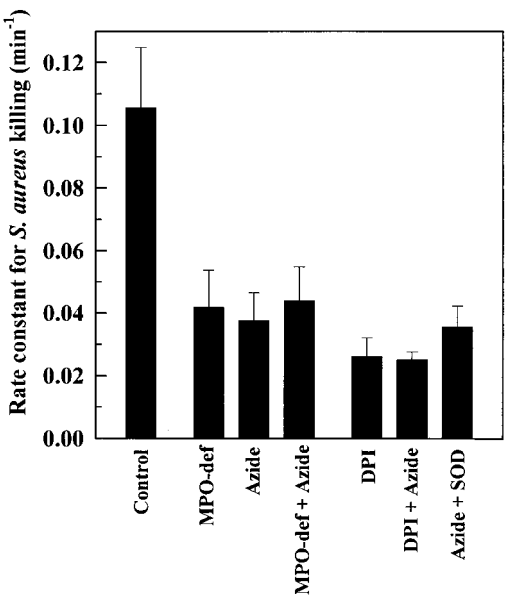

FIG. 3. Effects of azide and myeloperoxidase (MPO) deficiency on rate constants for the killing of *S. aureus*. The number of experiments was as follows: control neutrophils, $n = 14$; myeloperoxidase-deficient neutrophils alone, $n = 4$; neutrophils plus 1 mM azide, $n = 9$; myeloperoxidase-deficient neutrophils plus 1 mM azide, $n = 2$; neutrophils plus 10 μM DPI, $n = 9$; neutrophils plus 1 mM azide and 10 μM DPI, $n = 2$; neutrophils plus 1 mM azide and IgG-superoxide dismutase (SOD)-coated bacteria, $n = 4$. Error bars represent standard deviations, except when $n = 2$, and ranges are shown. The control was significantly different from all treatments, and there was significantly more killing in the presence of azide than in the presence of DPI ($P < 0.05$).

coated with IgG-superoxide dismutase and control bacteria (Fig. 3). These rate constants were significantly lower than with superoxide dismutase alone (Fig. 1). This indicates that superoxide dismutase had no effect on killing when myeloperoxidase was inhibited but suppressed myeloperoxidase-dependent killing.

Distinction between oxidative and nonoxidative killing. To assess the effects of superoxide dismutase and the inhibitors of myeloperoxidase on oxidative killing, we have made the assumption that overall killing is the sum of oxidative plus nonoxidative components and that these are independent first-order processes. It is theoretically possible that mechanisms such as synergy between the two processes or oxidative inactivation of nonoxidative pathways could operate. However, in the absence of published evidence supporting either of these interactions, it seems reasonable to consider the two processes independent. The rate constant for nonoxidative killing, measured in the presence of DPI and unaffected by the inhibitors, was subtracted from the overall rate constants (Table 2). Since the results obtained in the presence of azide and with myeloperoxidase-deficient cells were not significantly different, they have been combined to calculate the rate constant in the absence of myeloperoxidase activity. The mean rate constant for oxidative killing, calculated on this basis (Table 2), was 57% of the control rate when superoxide dismutase was attached to the bacteria and 18% for neutrophils lacking myeloperoxidase activity.

DISCUSSION

Contribution of superoxide to killing. Neutrophils must generate superoxide to kill oxidatively. Whether the superoxide acts solely as a precursor of hydrogen peroxide or plays a direct role in the process is less well established. This is partly a problem of getting enough superoxide dismutase into the phagosome to probe for superoxide dependency. Inhibition of killing has been reported in only one instance, when superoxide dismutase was attached to latex beads that were phagocytosed along with bacteria (18), but this observation has not until now been followed up. We took the novel approach of introducing superoxide dismutase into the phagosome by attaching it to *S. aureus* via an IgG conjugate that bound to protein A on the cell wall. These bacteria were phagocytosed normally but were killed more slowly than were unmodified *S. aureus* cells. This depended on the superoxide dismutase being active, since bacteria with either IgG or IgG linked to inactive enzyme were killed at the control rate. Our results show that the superoxide generated by neutrophils makes a substantial contribution to the oxidative killing of *S. aureus*. Superoxide dismutase caused a 30% decrease in the overall rate of killing. Provided that oxidative and nonoxidative killing mechanisms are independent, this corresponds to a decrease in the rate of oxidative killing to almost one-half of the control rate.

Contribution of myeloperoxidase to killing. A number of studies have shown that *S. aureus* cells are killed more slowly by neutrophils that are myeloperoxidase deficient (25, 30, 33) or are treated with azide (27, 30), cyanide (27, 30), or salicylhydroxamic acid (16). Our study is the first to dissect the oxidative and nonoxidative components and assess quantitatively the contribution of myeloperoxidase to the oxidative process. We found that the rate of killing by myeloperoxidase-deficient or azide-treated cells was substantially decreased, to about 38% of the control rate. Considering the oxidative and nonoxidative components separately (Table 2), these results indicate that oxidative killing is approximately six times faster when myeloperoxidase is active. Myeloperoxidase-dependent processes therefore appear to be overwhelmingly favored by the neutrophil as its prime mechanism for oxidative killing.

Mechanisms of oxidative killing. The bactericidal agent most likely to be responsible for myeloperoxidase-dependent killing, on the basis of evidence obtained with the isolated enzyme, is hypochlorous acid (29). However, alternative reactions catalyzed by myeloperoxidase cannot be excluded.

Superoxide is likely to contribute to the killing of *S. aureus* through the generation of more toxic species rather than being directly bactericidal (1, 28). Our results support an interaction with a myeloperoxidase-dependent process. Evidence for this is the lack of effect of superoxide dismutase on killing in the presence of azide. Also, the 43% decrease in the rate constant for oxidative killing seen in the presence of superoxide dismutase compared with the more than 80% decrease in the absence of myeloperoxidase (Table 2) implies that a myeloper-

TABLE 2. Calculation of the rate constants for oxidative killing of *S. aureus* by neutrophils

Bacterium[a]	Rate constant (min^{-1})[b]	
	$k_{overall}$	$k_{oxidative}$
Control	0.103	0.078
+IgG	0.097	0.072
+IgG-SOD	0.066	0.041
No MPO activity	0.039	0.014

[a] SOD, superoxide dismutase; MPO, myeloperoxidase.

[b] Results are taken from Fig. 1 and 3. The rate constant for oxidative killing ($k_{oxidative}$) was calculated on the assumption that $k_{overall} = k_{nonoxidative} + k_{oxidative}$, where $k_{nonoxidative}$ is the rate constant determined in the presence of DPI (0.025 min^{-1}).

oxidase-independent component would be too small to account for the entire effect of superoxide dismutase.

The mechanism could involve superoxide reacting directly with myeloperoxidase or combining with hypochlorous acid to enhance killing. Possibilities include oxidizing phagosomal reducing species to allow hypochlorous acid greater access to bacterial targets (48) or reacting with hypochlorous acid to generate hydroxyl radicals (7). However, myeloperoxidase-dependent hydroxyl radical production by neutrophils, while detectable, accounts for less than 1% of the superoxide generated (8, 43), and the hydroxyl radical may not be an efficient antimicrobial agent in the phagosome (51).

Our previous studies suggest two ways in which superoxide might enhance bacterial killing by myeloperoxidase. The first is by increasing hypochlorous acid production in the phagosome through preventing reversible inactivation of the enzyme. Superoxide has been shown to have this effect both with purified myeloperoxidase (21) and with stimulated neutrophils (23) under conditions in which it prevents the accumulation of inactive compound II. The second way is by reacting with the ferric form of the enzyme to give compound III, which then participates in bacterial killing. This could be via a hydroxylating species, as superoxide-dependent hydroxylation has been demonstrated with isolated myeloperoxidase (24). Compound III formation has been shown when neutrophils are stimulated (50), and the following calculations based on reaction rates and estimates of the concentrations of superoxide and myeloperoxidase indicate that it should be formed in the phagosome.

One million neutrophils contain approximately 30 pmol of myeloperoxidase (2) and can produce 10 nmol of superoxide per min upon stimulation. Our assumptions are that maximal stimulation occurs upon ingestion of 20 bacteria, and therefore 5% of the myeloperoxidase and superoxide is released into each phagosome. The volume of the phagosome is assumed to equal that of a bacterium (5×10^{-16} liter). Steady-state superoxide concentrations can be calculated from the following equation: $R = k_d[O_2^-]^2 + k_m[O_2^-][MPO]$, where k_d for dismutation of superoxide (pH 7.4) is 10^5 M^{-1} s^{-1} (36) and k_m for reaction of myeloperoxidase (MPO) with superoxide (O_2^-) to give compound III is 10^6 M^{-1} s^{-1} (20). For conditions as above, the rate of superoxide generation (R) = 0.02 M s^{-1} and [MPO] = 3.3 mM, giving a steady-state [O_2^-] of 5 μM, which is about 80 times lower than if all the superoxide decayed by dismutation. A large proportion of the superoxide, therefore, should react with myeloperoxidase. Altering the initial assumptions changes this proportion, but unless much larger amounts of superoxide are generated in a bigger phagosome, along with lower concentrations of myeloperoxidase, compound III formation remains favored over spontaneous dismutation.

In apparent contrast to our findings, others have shown that superoxide dismutase enhanced the extracellular killing of *Escherichia coli* by neutrophils stimulated with phorbol myristate acetate (31) and had no effect on *E. coli* killing by purified myeloperoxidase and xanthine/xanthine oxidase (28). Since the influence of superoxide on hypochlorous acid production depends on the hydrogen peroxide concentration (22), these results could reflect differences between the extracellular environment and the phagosome. Also, urate generated from the oxidation of xanthine could mask any stimulatory effect of superoxide in the purified enzyme system (21).

Most of the killing of *S. aureus* that we observed in the absence of myeloperoxidase activity was nonoxidative, but there was a small oxidative component. The mechanism of this slow process has not been elucidated. It may involve direct toxicity of hydrogen peroxide, which accumulates to a greater extent under these conditions (12). However, we (unpublished data) and others have found that higher concentrations of peroxide accumulate in the presence of azide than with myeloperoxidase-deficient cells (39), without any enhancement of killing, suggesting that this is not a very effective mechanism. Another possible killing mechanism is via hydroxyl radicals produced in the iron-catalyzed Haber-Weiss reaction. Prevention of this reaction was the explanation originally put forward to account for the inhibition of killing by superoxide dismutase (18). However, there is now little evidence that neutrophils produce significant numbers of hydroxyl radicals by this mechanism (8). If the Haber-Weiss reaction did occur, it should be enhanced and superoxide dismutase should have more effect when hydrogen peroxide accumulates in the presence of azide. The lack of inhibition by superoxide dismutase under these conditions is further evidence against its playing a significant role.

Myeloperoxidase deficiency and susceptibility to infection. We measured a substantial decrease in killing rate either when myeloperoxidase was inactive (62%) or when the NADPH oxidase was inhibited with DPI (75%). These conditions resemble the situation in myeloperoxidase deficiency and chronic granulomatous disease, respectively. The comparability of these effects might therefore imply that there would not be a major difference in the approach to *S. aureus* in the two clinical conditions. However, individuals with chronic granulomatous disease are severely affected by common pathogens including *S. aureus*, whereas for patients with myeloperoxidase deficiency, there are only occasional reports of increased susceptibility to *Candida* infection and *S. aureus* is not a problem (11). One possible explanation is that myeloperoxidase-deficient neutrophils exhibit an extended oxidative burst (9, 44), so that killing eventually catches up with normal cells. We found no evidence for this subtle difference, even over a longer assay period, when a decrease in killing rate for both normal (12) and myeloperoxidase-deficient cells was apparent. It may be that in vivo, cells other than neutrophils which express the NADPH oxidase but not myeloperoxidase are able to compensate for neutrophil dysfunction and that this explains the differing severity of the two diseases.

Superoxide dismutase and bacterial pathogenicity. Our results with *S. aureus* suggest that bacteria with surface-associated superoxide dismutase would be more resistant to oxidative killing by neutrophils. This may be the case for *Mycobacterium tuberculosis* (32), *Nocardia asteroides* (4), *Helicobacter pylori* (47), and a number of human parasites (17) that all secrete superoxide dismutase. Antibodies to the superoxide dismutase of *N. asteroides* enhanced both bacterial killing by neutrophils (3) and clearance upon inoculation of mice (5). Surface-associated superoxide dismutase on these pathogens could slow killing by phagocytes and thus be a factor in their pathogenicity.

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