

Role of the Phagocyte in Host-Parasite Interactions

XXIV. Aldehyde Generation by the Myeloperoxidase- H₂O₂-Chloride Antimicrobial System: a Possible In Vivo Mechanism of Action

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Myeloperoxidase (MPO), H₂O₂, and chloride ions in the presence of bacteria form aldehydes and are bactericidal. The use of heat-inactivated MPO prevented both killing and aldehyde generation. Decarboxylation and deamination of carboxyl and amino group substrates arising from the bacterial surface may participate in the reaction which yields aldehydes. Bacterial contact was essential for killing. Decarboxylation and bactericidal activities were noted when physiological concentrations of chloride were used. When MPO was replaced with horseradish peroxidase (HPO) in the chloride medium, decarboxylation and bactericidal activities were no longer noted. In contrast, iodide functioned in the antimicrobial system with either MPO or HPO. The iodide concentrations required were at least sixfold greater than circulating blood iodide levels. Moreover, decarboxylation did not occur in the presence of iodide with either enzyme. Thus, both halides function in the MPO-H₂O₂ system but by different mechanisms. It is likely that in vivo under most conditions chloride is the functional halide and that generation of aldehydes is the mechanism responsible for the antimicrobial activity of the MPO-H₂O₂-chloride system.

It has recently been shown that myeloperoxidase (MPO), H₂O₂, and halide ions act in concert to kill bacteria (6, 7, 11), fungi (9), and viruses (1). The precise mechanism by which this system functions has not, as yet, been satisfactorily explained. A suggestion has been made that, when iodide is the participating halide, the function of MPO and H₂O₂ is to convert iodide by peroxidation to a stronger antimicrobial agent (8). However, the functioning of this system in vivo is questionable, because the in vitro concentrations of iodide required under most conditions are not attainable in vivo (12). On the other hand, since chloride functions in this system at physiological concentrations, it well may be the participating halide in vivo. Therefore, a study of the mechanism of action of the MPO-H₂O₂-chloride system would be of extreme interest.

The ability of peroxidases to oxidize a variety of different substrates is well known (18). Recently, it was reported that MPO, isolated from leukocytes of patients with chronic granulocytic leukemia, catalyzed the decarboxylation and deamination of several amino acids to aldehydes (26).

This reaction is, as expected, H₂O₂-dependent and is stimulated by chloride ions. Aldehydes are antimicrobial (24), and substrates suitable for aldehyde formation, for example, amino and fatty acids, are present in abundance in bacteria and phagocytes.

Recently, we suggested that the aldehydes formed from the peroxidative decarboxylation and deamination reaction are responsible for the antimicrobial action of the MPO-H₂O₂-chloride system (5, 22). In this report, we present direct evidence that the MPO-H₂O₂-chloride system in the presence of bacteria forms measurable quantities of aldehydes. It is shown that killing occurs within a short period of exposure and that direct bacterial contact with MPO is mandatory for bactericidal action. Further, amino acids added to this bactericidal system inhibit bactericidal activity, possibly by competing with the substrates present in the bacteria or MPO-containing granules, or in both. Finally, data are presented which suggest that chloride and iodide participate in the antimicrobial system but by different mechanisms.

MATERIALS AND METHODS

All chemicals used were of reagent grade. Alcohol dehydrogenase (ADH), 250 units/mg, and reduced nicotinamide adenine dinucleotide (NADH) were obtained from Sigma Chemical Co., St. Louis, Mo. Before use, ADH was dialyzed against cold water to remove $(\text{NH}_4)_2\text{SO}_4 \cdot \text{H}_2\text{O}_2$, 30%, was obtained from Merck & Co., Inc., Rahway, N.J. Horseradish peroxidase (HPO), type VI, 295 Purpurogallin units/mg, and RZ 3.00, was purchased from Sigma Chemical Co. L-Alanine- I - ^{14}C was obtained from New England Nuclear Corp., Boston, Mass.

Aldehyde estimation. The reaction mixture contained 300 μmoles of phosphate buffer (pH 6.5), 0.3 μmole of NADH, and sample of the bactericidal reaction mixture. Twelve units of ADH were added last to start the reaction. Final volume was made to 3.0 ml with distilled water. The temperature of the spectrophotometric assay was maintained at 15 C. Total change in NADH absorbancy at 340 nm was recorded. The aldehydes present were reduced to the corresponding alcohols by ADH in the presence of NADH. An optical density change of 0.21 was considered to be equivalent to 0.1 μmole of aldehyde (15).

Bactericidal system. Granules containing MPO were isolated from freshly prepared guinea pig polymorphonuclear (PMN) leukocytes as described previously (22). The peroxidase activities of the granules and of HPO were determined by guaiacol oxidation (14). *Escherichia coli* was grown overnight (10), harvested in the logarithmic growth phase, washed, and suspended in Krebs-Ringer phosphate buffer (KRPB), pH 5.5. The reaction mixture for bactericidal activity contained granules having 0.016 guaiacol unit of MPO, 0.4 μmole of H_2O_2 , and 8×10^4 *E. coli* cells, unless otherwise indicated. The volume was made to 8.0 ml with KRPB (pH 5.5). The chloride concentration in KRPB is sufficient to satisfy the halide requirements of our bactericidal system. After 5 min of incubation at 37 C, viable bacterial cell counts were made. A sample was assayed for aldehydes as described above. For inactivation of MPO, granules were heated for 30 min in a boiling-water bath.

Bacteria were physically separated from direct contact with MPO by placing 2×10^4 *E. coli* cells and 0.1 μmole of H_2O_2 in 2.0 ml of KRPB (pH 5.5) in a dialysis bag. The bag was then placed in 6.0 ml of KRPB (pH 5.5) containing 0.012 guaiacol unit of MPO and 0.3 μmole of H_2O_2 . The system was incubated for 30 min at 37 C. Alternately, MPO was placed inside the bag and the bacteria were placed outside. H_2O_2 and chloride ions were uniformly distributed both inside and outside the dialysis bag.

To study the effect of L-alanine or glycine on the bactericidal activity, the amino acids were added to the bactericidal system described below: *E. coli*, 2×10^4 cells; MPO, 0.003 guaiacol unit; H_2O_2 , 0.1 μmole ; and glycine, 10^{-3} to 10^{-7} M or L-alanine, 2×10^{-2} to 1×10^{-7} M, final concentration. The total volume was made to 2.0 ml with KRPB (pH 5.5). After incubation for 30 min at 37 C, samples were plated for viable bacterial cell counts.

Decarboxylation of L-alanine- I - ^{14}C was studied in Warburg flasks. The reaction mixture contained either

0.24 guaiacol unit of HPO or 0.03 guaiacol unit of MPO, 0.3 μmole of KI or 300 μmoles of NaCl, 5.4 μmoles of L-alanine- I - ^{14}C (specific activity, 0.055 $\mu\text{Ci}/\mu\text{mole}$), 0.3 μmole of H_2O_2 , and 0.10 M phosphate buffer (halide-free), pH 5.5, to a final volume of 3.0 ml. After 30 min of incubation at 37 C, 0.2 ml of 30% trichloroacetic acid was tipped from the side arm, and incubation was continued for an additional 10 min. $^{14}\text{CO}_2$ was collected in 20% KOH in the center well, and a 0.05-ml sample was counted in 15 ml of an aqueous phosphor (13) in a Packard Tri-Carb liquid scintillation spectrometer equipped with an external standard.

RESULTS

Aldehyde production by the bactericidal system was measured after 5 min of incubation. It may be seen in Table 1 that measurable quantities of aldehydes were detectable in the complete bactericidal system, i.e., *E. coli*, MPO, H_2O_2 , and chloride. The amount detectable was low but was significantly above that observed in any of the controls. The number of viable bacteria remaining after the 5-min incubation period was also determined. The complete system killed 99.99% of the bacteria present. H_2O_2 or MPO alone did not have any significant bactericidal activity. When heat-inactivated MPO was substituted for MPO in the complete system, a complete loss of bactericidal activity was noted. Aldehyde levels were similarly reduced to control levels (Table 1).

Efficient bactericidal activity could be demonstrated only if direct contact of bacteria with MPO- H_2O_2 was permitted (Table 2). When MPO

TABLE 1. Estimation of aldehydes in the MPO- H_2O_2 -chloride bactericidal system

Supplements	Organisms/ml ^a	Amt of aldehydes ^b
		nmoles
<i>E. coli</i>	1.2×10^4	6.3 + 2.3
<i>E. coli</i> + MPO.....	1.2×10^4	3.0 ± 1.4
<i>E. coli</i> + H_2O_2	1.3×10^4	1.0 ± 1.0
<i>E. coli</i> + MPO + H_2O_2	$<5.0 \times 10^0$	22.9 ± 5.6
<i>E. coli</i> + heated MPO ^c	1.2×10^4	5.8 ± 1.9
<i>E. coli</i> + heated MPO + H_2O_2	1.2×10^4	5.8 ± 1.9

^a Incubations were carried out for 5 min at 37 C. The bactericidal system contained, where indicated: H_2O_2 , 0.4 μmole ; MPO (guinea pig PMN granules), 0.016 guaiacol unit; 8×10^4 *E. coli* cells; and KRPB (pH 5.5) to 8 ml. Mean of five experiments in each case.

^b Total measurable aldehyde per reaction system. Mean ± SE of the mean for 5 to 15 experiments is given in each case.

^c Heated in a boiling-water bath for 30 min.

TABLE 2. Bactericidal activity of the MPO-H₂O₂-chloride system in the presence and absence of direct bacterial contact with MPO

Conditions	No. of viable organisms per reaction tube
Direct contact ^a	
<i>E. coli</i>	2.0 × 10 ⁴
<i>E. coli</i> + MPO.....	2.1 × 10 ⁴
<i>E. coli</i> + H ₂ O ₂	1.8 × 10 ⁴
<i>E. coli</i> + H ₂ O ₂ + MPO.....	2.2 × 10 ⁴
No direct contact ^b	1.7 × 10 ⁴

^a Bactericidal system containing 0.012 guaiacol unit of MPO, 0.4 μmole of H₂O₂, 2 × 10⁴ *E. coli* cells, and KRPB (pH 5.5) to 8 ml as indicated above.

^b *E. coli* (2 × 10⁴ cells) placed inside a dialysis bag containing 0.1 μmole of H₂O₂ and KRPB (pH 5.5) to a volume of 2 ml. Dialysis bag was placed in 6.0 ml of pH 5.5 KRPB containing 0.012 guaiacol unit of MPO and 0.3 μmole of H₂O₂. The complete reaction system was incubated at 37 C for 30 min. Viability was monitored by sampling the contents in the dialysis bags.

TABLE 3. Effect of amino acids on the bactericidal activity of the MPO-H₂O₂-chloride system

Supplements ^a	Viable-cell count per ml
None.....	7.5 × 10 ⁸
H ₂ O ₂	7.5 × 10 ⁸
MPO.....	7.5 × 10 ⁸
MPO + H ₂ O ₂	<5.0 × 10 ⁰
Glycine.....	7.2 × 10 ⁸
Glycine + H ₂ O ₂	6.8 × 10 ⁸
Glycine + MPO.....	6.5 × 10 ⁸
Glycine + MPO + H ₂ O ₂	6.3 × 10 ⁸
L-Alanine.....	8.3 × 10 ⁸
L-Alanine + H ₂ O ₂	9.3 × 10 ⁸
L-Alanine + MPO.....	7.2 × 10 ⁸
L-Alanine + MPO + H ₂ O ₂	7.4 × 10 ⁸

^a Reaction mixture contained 2 × 10⁴ *E. coli* cells, KRPB (pH 5.5) to a final volume of 2 ml, and the following supplements as indicated above: H₂O₂, 0.1 μmole; MPO, 0.003 guaiacol unit; glycine or L-alanine, 2 μmoles. Incubation was at 37 C for 30 min.

was kept on one side of a dialysis bag, separated from direct contact with bacteria, bactericidal activity dropped very significantly. When the bacteria were not separated from MPO, significant bactericidal activity was readily noted.

The effect of addition of glycine or L-alanine to the complete bactericidal system is shown in Table 3. Both of the amino acids significantly inhibited bactericidal activity.

The possible relationship between peroxidase-

catalyzed decarboxylation and bactericidal activity was studied. Two different peroxidases, MPO and HPO, were used. It was noted that the antimicrobial system of MPO, H₂O₂, and chloride ions decarboxylated L-alanine. This system showed significant bactericidal activity. When iodide was the participating halide instead of chloride, decarboxylation did not occur. However, the MPO-H₂O₂-iodide system had bactericidal activity.

When HPO was used, no decarboxylation of L-alanine occurred with either chloride or iodide, and bactericidal activity was noted only when iodide was the participating halide (Table 4). Concentrations of HPO in the range of 0.018 to 10.0 guaiacol units killed 99.99% of the bacteria. Concentrations of HPO 10 times lower or higher than those reported did not decarboxylate L-alanine with either chloride or iodide as the halide (these results are not tabulated).

DISCUSSION

Among the various antimicrobial agents (3, 21, 25) reported to be in the phagocyte, the antimicrobial system of MPO-H₂O₂-halide appears unique in that it can be related to the metabolic activities stimulated during phagocytosis (4, 16, 19, 20), including the stimulation of H₂O₂ production and activation of MPO (14). Further, none of the

TABLE 4. Effect of different halides on peroxidase-mediated decarboxylation and bactericidal activity^a

Supplements ^b	Bacteria killed ^b	Decarboxylation ^c
	%	disintegrations/min
MPO + Cl + H ₂ O ₂	99	24,000
HPO + Cl + H ₂ O ₂	0	120
MPO + I + H ₂ O ₂	99	80
HPO + I + H ₂ O ₂	99	52

^a Incubation was at 37 C for 30 min. Results of the complete system for each set of experiments are presented. In the absence of any of the components, no significant bactericidal or decarboxylation activity was noted.

^b Reaction mixture contained 2 × 10⁴ *E. coli* cells and the following supplements as indicated above: H₂O₂, 0.1 μmole; MPO, 0.003 guaiacol unit, or HPO, 0.018 guaiacol unit; KI, 0.2 μmole, or NaCl, 200 μmoles; and 0.1 M halide-free phosphate buffer (pH 5.5) to a total volume of 2.0 ml.

^c Reaction mixture contained 0.24 guaiacol units of HPO or 0.03 guaiacol units of MPO, 0.3 μmole of KI or 300 μmoles of NaCl, 0.3 μmoles of H₂O₂, L-alanine-1-¹⁴C (specific activity, 0.055 μCi/μmole), and 0.1 M halide-free phosphate buffer (pH 5.5) to 3.0 ml.

three components is individually antimicrobial; yet, collectively they are synergistic (6, 11). These data, along with the fact that physiological concentrations of chloride are present in the host, specifically, within the phagocytic vacuole, suggest that the MPO-H₂O₂-chloride system is operative *in vivo*.

Some recent studies have been particularly helpful in allowing us to postulate a possible mechanism of action for this system. For example, Zgliczynski et al. (26) reported that, in the presence of H₂O₂ and chloride ions, purified MPO isolated from leukocytes collected from patients with chronic granulocytic leukemia decarboxylates and deaminates several amino acids. Reaction products from this system were identified as aldehydes corresponding to the amino acids oxidized. Strauss et al. (22), by using guinea pig PMN granules as the source of MPO, found that decarboxylation and deamination of L-alanine is H₂O₂-mediated and chloride-dependent at pH 5.5. They suggested that products of the above reactions, possibly aldehydes, are the actual bactericidal agents. Jacobs et al. (5) found that taurine, a specific (5, 22) and competitive (26) inhibitor of decarboxylation and deamination (and therefore of aldehyde formation) also inhibits the bactericidal activity of the MPO-H₂O₂-chloride system. These data suggest that the mechanism responsible for the antimicrobial activity of the MPO-H₂O₂-chloride system is its ability to deaminate and decarboxylate appropriate substrates to aldehydes.

The antimicrobial nature of aldehydes has been known for almost a century (24). Yet, to the best of our knowledge no mention that they may function as antimicrobial agents in phagocytes has been made, and quantitative estimation of aldehydes in a bactericidal system has not been reported. The unstable nature of the aldehydes, the microquantities formed, the lack of any specific method for the estimation of most of the aldehydes, and the absence of a need to measure them may all be valid reasons for the paucity of information in this area. As an example of the volatile nature of aldehydes, only a 10% recovery of a 10 mM mixture of different aldehydes was accounted for in our assay. Therefore, the aldehyde values reported in Table 1 are most probably minimal values. In spite of these limitations, the detectable aldehydes in the complete MPO-H₂O₂-chloride bactericidal system are significantly above the control levels. This provides direct evidence that aldehyde formation does occur under our experimental conditions. It must be pointed out that the variability between experiments is high, and in some experiments (6 of 15) the difference in meas-

urable aldehyde between experimental and control was negligible.

Information relative to localization of the substrate source for aldehyde formation is suggested by the finding that in order to get efficient killing the bacteria must be in direct contact with MPO. By physically separating the bacteria from MPO, detectable bactericidal activity was eliminated. This would suggest that the peroxidative activity of MPO is directed principally at the bacteria, possibly the bacterial surface. Free carboxyl and amino groups are associated with the bacterial cell wall (17) and could be available for decarboxylation and deamination by MPO. Cline and Lehrer (2) reported that bacteria can replace amino acids as substrates for D-amino acid oxidases yielding H₂O₂. In addition to amino acids acting as substrates, fatty acids and perhaps other substrates could also give rise to aldehydes. Fatty acids have been shown to be oxidized by peroxidase, yielding aldehydes (23).

Additional support for the concept that bacterial amino acids are involved as substrates for the H₂O₂-mediated decarboxylation by MPO is the fact that the addition of amino acids at concentrations as low as 10⁻⁶ M to the system inhibits bactericidal activity. It would seem that the added amino acids compete or interfere with the substrates already present on the bacteria or phagocyte. As tempting as this suggestion may appear, the possibility that the substrate is originating from granular MPO cannot, as yet, be unequivocally ruled out. Obviously, additional study in this area is needed.

Although in our experiments chloride has been mainly used as the participating halide, iodide has also been studied. However, the concentration of iodide necessary for optimal antimicrobial activity is at least six times higher than circulating iodine blood levels (6, 7, 12). These *in vitro* optimal iodide concentrations may be obtained *in vivo* only at sites of iodine concentrations, e.g., the thyroid and salivary glands. Lehrer suggested that topical iodide treatment of fungal infections may be effective because of the participation of iodide in the MPO-H₂O₂-I system (9). Thus, under certain limited conditions, *in vivo*, iodide may be the participating halide.

It would appear from the above data that the mechanism of action of iodide is different from that of chloride. This is supported by the observation that decarboxylation was not required for bactericidal activity of MPO or HPO when iodide was the halide, whereas in the presence of chloride H₂O₂-dependent decarboxylation was associated with bactericidal activity (Table 4). Lehrer also reported that MPO is fungicidal with chloride or iodide, whereas HPO is fungicidal with iodide but

not with chloride (9). This again would suggest different mechanisms of action for the two halides with peroxidases.

Klebanoff's data showing that under certain conditions H_2O_2 and iodide are bactericidal in the absence of MPO are pertinent (6). He has shown that higher concentrations of iodide and H_2O_2 in the absence of MPO are as bactericidal as lower concentrations of iodide and H_2O_2 in the presence of MPO (6). On the other hand, chloride and H_2O_2 in equivalent and higher concentrations have not been found to be bactericidal in the absence of MPO in our laboratory (*unpublished data*). These observations suggest that when iodide is the participating halide the reaction can go either chemically or enzymatically, whereas with chloride the reaction is enzymatic only. Because chloride functions at physiological concentrations in the MPO- H_2O_2 -halide system, the chloride-mediated aldehyde-generating reaction is the one that is likely to be involved in antimicrobial activity *in vivo*.

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