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Killing of *Actinobacillus actinomycetemcomitans* by the Human Neutrophil Myeloperoxidase-Hydrogen Peroxide-Chloride System

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Actinobacillus actinomycetemcomitans **is a facultative gram-negative coccobacillus associated with periodontal disease and nonoral infections. This organism is resistant to serum bactericidal mechanisms but is nevertheless killed by human neutrophils under aerobic and anaerobic conditions. Most of the killing attributable to oxidative mechanisms is inhibited by sodium cyanide, which suggests that the myeloperoxidase-hydrogen peroxide-chloride (MPO-H_2O_2-Cl^-) system may be a key factor in the oxidative killing process. In this report, we examine whether the isolated MPO-H_2O_2-Cl^- system is bactericidal against *A. actinomycetemcomitans*. We found that three major chromatographic forms of MPO were capable of killing *A. actinomycetemcomitans* at sublethal concentrations of H_2O_2 and that both catalase-positive and catalase-negative strains of this organism were sensitive to killing by the MPO-H_2O_2-Cl^- system. We conclude that the isolated MPO-H_2O_2-Cl^- system is bactericidal for *A. actinomycetemcomitans* independent of other neutrophil granule constituents and may be an important component of the oxygen-dependent bactericidal activity of the neutrophil with respect to this periodontopathic organism.**

Actinobacillus actinomycetemcomitans is a capnophilic, gram-negative coccobacillus, closely related to the oral haemophili (13). It causes severe infections in humans including actinomycoses, subacute bacterial endocarditis, and abscesses (27). It also has a strong association with a severe form of periodontitis occurring in adolescents known as localized juvenile periodontitis (18, 19, 27) and with recurrent or refractory periodontal disease in adults (L. Bragd, M. Wilkström, and J. Slots, Proc. Am. Assoc. Dent. Res. abstr. 538, 1985). *A. actinomycetemcomitans* is resistant to both nonimmune and immune complement-mediated serum bactericidal mechanisms (22; M. E. Wilson, unpublished data). The neutrophil appears to play an important role in host defense against periodontopathic bacteria (3, 4, 6, 23). Neutrophils kill *A. actinomycetemcomitans* by both oxidative and nonoxidative mechanisms, and most oxidative killing appears to be sensitive to inhibition by cyanide (7). The latter observation indicates that oxidative killing may be dependent upon the myeloperoxidase-hydrogen peroxide-chloride (MPO-H_2O_2-Cl^-) system (5, 7).

The MPO-$H_2O_2Cl^-$ system appears capable of exerting antimicrobial activity directly by interaction with a number of bacterial surface components which results in the destruction of the bacterial cell envelope (15, 17, 21). However, it has been reported that the antimicrobial activity of this system is potentiated by elastase, a primary granule-associated neutral protease which is considered part of the nonoxidative killing arm of neutrophils (11). MPO is also involved in the oxidative autoactivation of latent collagenase by neutrophils (24). Thus, one consideration is that the MPO-H_2O_2-Cl^- system may exert antimicrobial activity alone, or it may depend upon or act in concert with other neutrophil granule constituents to potentiate or mediate the killing process.

Another consideration is that there appear to be three major forms (MPO I, II, and III) of human neutrophil MPO, which can be resolved by cation-exchange chromatography (12). These three forms localize within distinct subpopulations of azurophilic granules and also are found in human promyelocytic leukemia cell lines (12, 26). It has been hypothesized that each form may serve different functions (12). Recently, we have developed methods for isolating the three forms in a highly purified state (8). The purpose of this study was to assess the capacity of the isolated MPO-H_2O_2-Cl^- system to kill *A. actinomycetemcomitans* in the absence of other neutrophil cytoplasmic granule constituents, and to determine whether the MPO forms differ in their abilities to exert such bactericidal activity.

MATERIALS AND METHODS

Bacteria. The bacterial strains used in this study include *A. actinomycetemcomitans* Y4, ATCC 29523, SUNYaB 67, and 650, which have been characterized elsewhere (9, 10, 28). The acatalatic variant strain 650 was kindly provided by Anne Tanner of the Forsyth Dental Center, Boston, Mass. Bacteria were grown at 37°C for 1 day under aerobic conditions in 5% CO_2 on chocolate agar consisting of 5% hemolyzed defibrinated horse blood (Crane Laboratories, Geneva, N.Y.), 5 μg of equine hemin III (Sigma Chemical Co., St. Louis, Mo.) per ml, 0.001% menadione (Sigma), 0.1% yeast extract (Difco Laboratories, Detroit, Mich.), and 1% IsoVitaleX (BBL Microbiology Systems, Cockeysville, Md.) in Trypticase soy agar (BBL).

MPO. MPO was isolated by using methods previously reported (8). Briefly, the enzyme was solubilized from leukocyte granule preparations by homogenization in a 0.02 M acetate buffer (pH 4.7) containing 0.2 M NaCl, 0.5% cetyltrimethylammonium bromide, 10 μg of phenylmethylsulfonyl fluoride (Sigma) per ml, 10 μg of L-1-tosylamide-2-phenylethylchloromethyl ketone (Sigma) per ml, and 1 mM EDTA. Purification was achieved by gel filtration using Sephadex G-75 and Sephacryl S-200 (Pharmacia) and cation-exchange fast protein liquid chroma-

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TABLE 1. 50% Lethal dose of H_2O_2 required to kill various strains of *A. actinomycetemcomitans* in the presence or absence of MPO[a]

Strain	Serotype[b]	Catalase[c]	H_2O_2 LD_{50} (μM), 1 h	
			−MPO	+MPO
29523	a	+	280	0.7
Y4	b	+	760	0.7
67	c	++	85	0.7
650	c	−	900	0.7

[a] Bacteria (5×10^3/ml) were mixed with 0.075 GU of MPO II per ml in PMS and incubated for 1 h at 37°C. Reaction was terminated, and the suspension (0.05 ml) was spread on chocolate agar plates. The concentration of H_2O_2 correlating with a 50% reduction of bacterial CFU (50% lethal dose; LD_{50}) was recorded.

[b] From Zambon et al. (28). Serotype of strain 650 is inferred from its reactivity with patient serum.

[c] From Miyasaki et al. (10).

tography at pH 4.7 (Pharmacia) (8). The three major forms of MPO isolated by these procedures (MPO forms I, II, and III) exhibited Reinheitzahl values above 0.8. The guaiacol reduction assay of Chance and Maehly (2) was used to determine peroxidase activity. One guaiacol unit (GU) is defined as that amount of enzyme catalyzing a change of 1.0 absorbance unit per min at 470 nm (113 μmol of tetraguaiacol formed per min). MPO forms I, II, and III were dialyzed overnight against 0.14 M NaCl, diluted to 5 GU/ml in 0.14 M NaCl, and sterilized by microfiltration through a 0.2-μm-pore size polysulfone membrane (Gelman, Ann Arbor, Mich.). Samples of 1.0 ml were prepared and stored at −70°C.

MPO-H_2O_2-Cl^- system killing assay. Bacterial cells were removed from chocolate agar plates, dispersed by vortexing, and suspended at 5×10^3 cells per ml in a buffer consisting of 0.01 M sodium phosphate (pH 6.6) containing 0.14 M sodium chloride and 10^{-3} M magnesium chloride (PMS). A sample of MPO was assayed for enzyme activity in triplicate and diluted to the appropriate concentrations in PMS or 0.15 M sodium phosphate (pH 6.6) when appropriate. The molar concentration of stock 30% H_2O_2 (Fisher Chemical Co., Pittsburgh, Pa.) was assessed periodically by absorbance at 230 nm, using the extinction coefficient of 81 cm^{-1} M^{-1}. Fresh dilutions of H_2O_2 in PMS were prepared for each experiment. The bacterial suspension, 100 μl, was mixed with 50 μl of MPO in the wells of sterile microtiter plates and preincubated for 10 min (16). The killing reaction was initiated by adding 50 μl of H_2O_2 of the appropriate concentration. At specified intervals, the reaction was terminated by adding 100 μl of 0.5% tryptone (Difco), 0.5% bovine serum albumin (fraction V; Miles Laboratories, Inc., Naperville, Ill.), and 5×10^{-3} M NaN_3 in 0.14 M NaCl. Sodium azide at 5×10^{-3} M blocks MPO activity but has no effect on bacterial viability (10, 17). CFU were assayed after spreading 50 μl from this mixture onto chocolate agar plates and incubating the plates for 48 h at 37°C under 5% CO_2.

RESULTS

Influence of the concentration of substrate H_2O_2 on killing. Although the intraphagolysosomal concentration of H_2O_2 is unknown, it has been postulated that the concentration is not likely to exceed 100 μM (20, 29). Prior studies have revealed that most strains of *A. actinomycetemcomitans* are highly resistant to H_2O_2 at this concentration (9, 10). However, MPO functions optimally below 100 μM H_2O_2 and is most efficient in chlorinating activity at about 20 μM under acidic

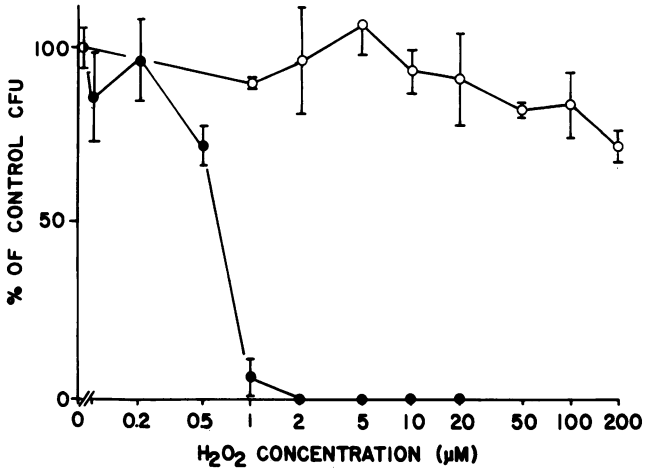

FIG. 1. Killing of *A. actinomycetemcomitans* 650 by the MPO-H_2O_2-Cl^- system as a function of H_2O_2 concentration. Assay was performed at 37°C in PMS (pH 6.6) in the presence (●) or absence (○) of MPO at 0.075 GU/ml. Reaction was terminated in 1 h by a 1:1 dilution in 0.5% tryptone–0.5% bovine serum albumin–3×10^{-3} M NaN_3–0.14 M NaCl. Control CFU per plate was 142 ± 6 in the presence of MPO and 135 ± 13 in the absence of MPO. Points represent the mean of three trials, and vertical bars represent one standard deviation.

(pH 6.6) conditions (29, 30). Hence, we first compared the lethality of the MPO-H_2O_2-Cl^- system with H_2O_2 alone, as a function of H_2O_2 concentration. At 1 h, it was found that the H_2O_2 concentration which resulted in 50% killing (50% lethal dose) of four representative strains of *A. actinomycetemcomitans* ranged between 85 and 900 μM in the absence of MPO. In contrast, inclusion of MPO at 0.075 GU/ml (corresponding to 25 ng of protein per ml), the 50% lethal dose concentration of H_2O_2 for all strains was 0.7 μM (Table 1). The acatalatic variant strain 650 was most resistant to the bactericidal effects of H_2O_2 alone, exhibiting greater than 60% survival after 1 h of exposure to 200 μM H_2O_2. Nevertheless, 2 μM H_2O_2 was sufficient to effect 100% killing of this strain in the presence of MPO (Fig. 1). These data show that sensitivity to the bactericidal effects of the MPO-H_2O_2-Cl^- system is not influenced either by intrinsic bacterial resistance to H_2O_2 or by endogenous bacterial catalase activity. We focused on strain 650 for the remainder of these studies since this strain is acatalatic, resistant to the direct bactericidal effects of H_2O_2, and as sensitive as other strains to the bactericidal effects of the MPO-H_2O_2-Cl^- system.

Requisite for the complete system. To determine whether the MPO-H_2O_2-Cl^- system was responsible for the observed killing, the effect of deleting various components from the

TABLE 2. Role of MPO, H_2O_2, and Cl^- in killing of *A. actinomycetemcomitans* 650

Reaction mixture	CFU/plate[a]	% Killed
Complete system (MPO, H_2O_2, Cl^-)[b]	0 ± 1	99.7 ± 0.6
Without H_2O_2	129 ± 31	0 ± 32
Without MPO	79 ± 6	34 ± 5
Without Cl^-[c]	84 ± 5	21 ± 9

[a] Values represent the mean and standard deviation of triplicate assays.

[b] System consisted of MPO II at 0.075 GU/ml and 2 μM H_2O_2 in PMS buffer. Bacterial viability was assessed after a 15-min incubation at 37°C.

[c] PMS buffer was replaced with 0.15 M sodium phosphate (pH 6.6).

reaction mixture was assessed. A 15-min incubation with the complete system (0.075 GU of MPO per ml, 2 μM H_2O_2, 0.14 M NaCl, pH 6.6) virtually eliminated all viable bacteria; the deletion of any single component dramatically reduced killing (Table 2). The low levels of bacterial death observed in the absence of MPO have also been observed in buffer alone and were found to be eliminated by the addition of heat-inactivated MPO, boiled rabbit liver powder, cytochrome *c*, or bovine serum albumin. Some death was also observed when chloride (NaCl) was not added, suggesting that the bacteria may provide their own reservoir of electron donors.

Requisite for MPO activity. Enzymatic activity of MPO was inhibited by heme-protein inhibitor such as sodium cyanide (Table 3). Dose-dependent inhibition of MPO enzymatic activity was observed in the concentration range of 5×10^{-4} to 5×10^{-7} M cyanide. Complete loss of viable bacteria was observed when bacteria were exposed to the MPO-H_2O_2-Cl^- system under optimal chlorinating conditions; however, 100% survival was observed when sodium cyanide was incorporated into the reaction mixture at 5×10^{-4} M. Lower concentrations of sodium cyanide were also remarkably inhibitory to the bactericidal activity of the MPO-H_2O_2-Cl^- system, and despite only 29% inhibition of enzyme activity under standardized conditions, 65% inhibition of bactericidal activity was observed. This discrepancy may be due to the lower enzyme and substrate concentrations used in the bactericidal assay. The inactivation of MPO by heating at 100°C for 5 min eliminated both enzymatic activity and the killing of *A. actinomycetemcomitans* 650 in the presence of 2 μM H_2O_2.

Bactericidal activity of three MPO forms. The bactericidal kinetics of highly purified forms MPO I, II, and III were extremely rapid, and virtually all viable bacteria were eradicated within 4 min by the MPO-H_2O_2-Cl^- system at 0.075 GU/ml and 2 μM H_2O_2 (Fig. 2). Under these experimental conditions, all three forms of MPO exhibited comparable kinetics in the killing of *A. actinomycetemcomitans*.

The killing of *A. actinomycetemcomitans* 650 by the MPO-H_2O_2-Cl^- system was highly dependent upon MPO concentration. Although MPO concentrations of 0.075 GU/ml (25 ng of MPO per ml or 0.18 nM MPO) resulted in total killing within minutes, an eightfold dilution resulted in approximately 25% killing in 1 h (Fig. 3).

The three forms of MPO are essentially identical in reaction kinetics with both H_2O_2 and the electron donor, guaiacol (8). Hence, the bactericidal activities of the three forms of MPO were compared at equivalent enzyme activities based upon reactivity with substrate H_2O_2. Large differences in bactericidal activity were neither expected nor observed; however, minor differences were observed at lower MPO activities (0.012 GU/ml) and when incubation periods were shortened to 15 min (Fig. 4). At 0.012 GU/ml,

TABLE 3. Inhibition by sodium cyanide of MPO II activity and killing of *A. actinomycetemcomitans* 650

Sodium cyanide concn (M)	MPO activity[a] (GU/ml)	% Inhibition of MPO activity	Bacterial survival[b] (CFU/plate)	% Inhibition of killing[c]
None	1.42 ± 0.09	0	0 ± 0	0 ± 0
5×10^{-7}	1.01 ± 0.03	29 ± 2.1	53 ± 23	65 ± 28
5×10^{-6}	0.37 ± 0.01	74 ± 0.7	78 ± 16	95 ± 20
5×10^{-5}	0.05 ± 0.00	96 ± 0.0	73 ± 2	89 ± 2
5×10^{-4}	0 ± 0	100 ± 0.0	82 ± 5	100 ± 6
5×10^{-3}	0 ± 0	100 ± 0.0	71 ± 10	87 ± 12

[a] MPO activity determined by the method of Chance and Maehly (2). The assay system included 33 μM H_2O_2 and was performed at pH 7.0. Values are given as GU per milliliter in the initial sample. Activity in the uninhibited reaction mixture was 4.3×10^{-2} GU/ml. Values represent the mean and SD of three trials.

[b] Bactericidal assay was performed in PMS buffer (pH 6.6) plus 20 μM H_2O_2 for 15 min at 37°C. Values represent the mean and SD of three trials.

[c] Inhibition of killing in comparison to killing in a control mixture plus the standard deviation of the percent of bacterial survival. Control reaction mixture containing all reactants except H_2O_2 yielded 82 ± 10 CFU per plate.

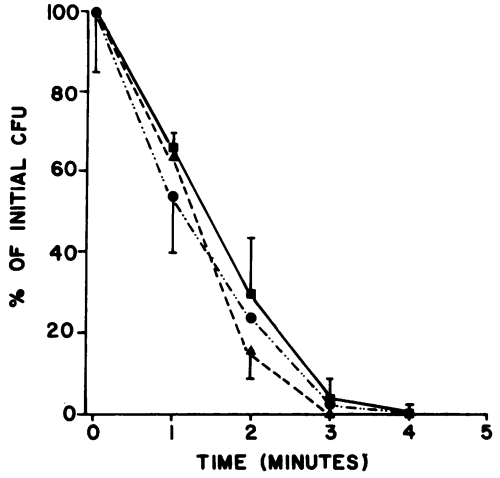

FIG. 2. Kinetics of killing *A. actinomycetemcomitans* 650 by MPO I (●), II (▲), and III (■). Assay was performed in PmS buffer (pH 6.6) and 2 μM H_2O_2 in plastic snap-top tubes. Temperature was maintained at 37°C in a water bath. Reaction was terminated by a 1:1 dilution in 0.5% tryptone–0.5% bovine serum albumin–3×10^{-3} M NaN_3–0.14 M NaCl. Initial CFU values were 63 ± 14, 69 ± 9, and 56 ± 9 for triplicate tubes containing MPO I, II, and III, respectively. Control tubes containing no MPO or no H_2O_2 exhibited 55 ± 8 and 63 ± 15 CFU, respectively, at 5 min. Points represent the mean of three trials, and bars represent one standard deviation.

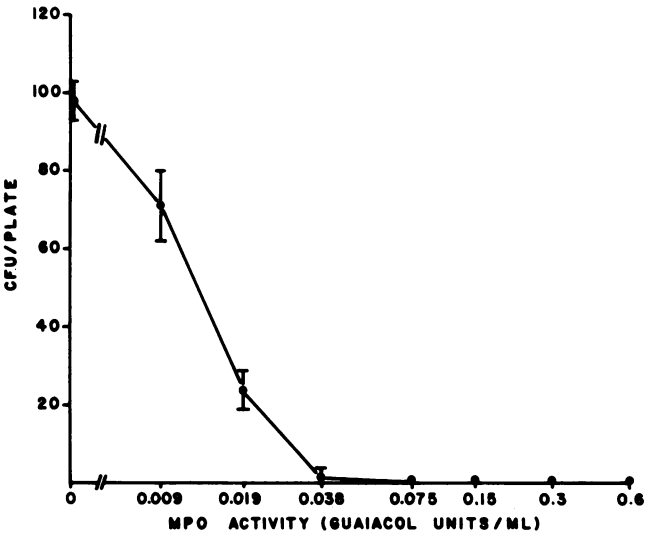

FIG. 3. Killing of *A. actinomycetemcomitans* 650 by the MPO-H_2O_2-Cl^- system as a function of MPO activity. The assays were performed at 37°C in PMS (pH 6.6) in the presence of 2 μM H_2O_2, and the reactions were terminated in 1 h by a 1:1 dilution in 0.5% tryptone–0.5% bovine serum albumin–3×10^{-3} M NaN_3–0.14 M NaCl. Points represent the mean of three trials, and bars represent one standard deviation.

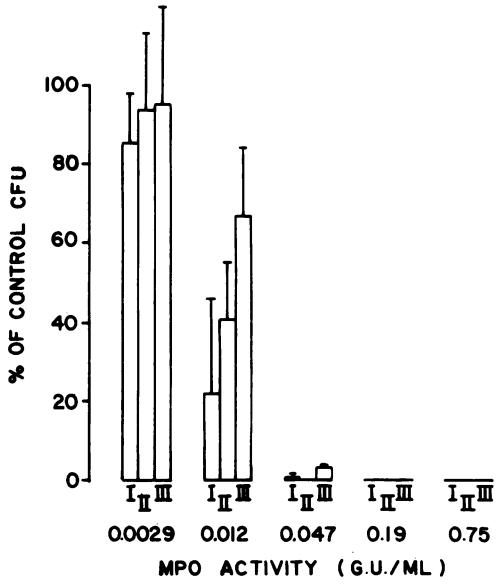

FIG. 4. Differential killing of *A. actinomycetemcomitans* 650 by MPO I, II, and III at different levels of MPO activity. The reaction was performed at 37°C in PMS (pH 6.6) and 2 μM H_2O_2 and terminated after 15 min by 1:1 dilution in 0.5% tryptone–0.5% bovine serum albumin–3 × 10^{-3} M NaN_3–0.14 M NaCl. Control CFU (no H_2O_2) was 156 ± 16. Bars depict the mean and standard deviation of three trials.

MPO I was more efficacious in killing *A. actinomycetemcomitans* 650 than was MPO III. MPO II exhibited intermediate effectiveness.

DISCUSSION

In this report, we examine the killing of the oral pathogen *A. actinomycetemcomitans* by highly purified human neutrophil MPO in a cell-free MPO-H_2O_2-Cl^- system. We found that *A. actinomycetemcomitans* was sensitive to killing by this in vitro system at the pH (1), H_2O_2 level (20, 29), and chloride concentration (30) likely to exist in neutrophil phagolysosomes. In our assay system, 100% killing was achieved at 25 ng of MPO per ml (in the presence of 2 μM H_2O_2), which is equivalent to the amount of MPO found in 3 × 10^3 neutrophils per ml (11). Since the assay system incorporated 5 × 10^3 bacteria per ml, killing was achieved at a ratio of two bacteria per neutrophil enzyme equivalent. The enzyme concentrations used in this study were somewhat lower than those reported for *Enterobacteriaceae* (14, 16, 17) but in accord with previously reported enzyme activities (16), suggesting that laboratory strains of *A. actinomycetemcomitans* are approximately equal to members of the *Enterobacteriaceae* in susceptibility to killing by the MPO-H_2O_2-Cl^- system. However, direct comparisons must be obtained before any conclusion may be drawn, since both bactericidal assay systems and enzyme specific activities may vary from one laboratory to the next.

The requisite for the intact MPO-H_2O_2-Cl^- system was also verified. Omission of any one component resulted in a dramatic decrease in bactericidal activity. Furthermore, the bactericidal activity of the complete system was inhibitable with the heme-enzyme inhibitor sodium cyanide at concentrations found to block the oxidative killing of *A. actinomycetemcomitans* by intact neutrophils (7). The rate of killing of *A. actinomycetemcomitans* by the MPO-H_2O_2-Cl^- system was found to be rapid, which supports the potential importance of this system in the killing process in intact neutrophils under aerobic conditions.

The three forms of MPO all exhibited the capacity to kill *A. actinomycetemcomitans*; however, MPO I was somewhat more effective than MPO II and significantly more effective than MPO III at low concentrations. A key factor involved in the killing of target cells by the MPO-H_2O_2-Cl^- system appears to be the binding of MPO to the target (16, 25). Since MPO I, II, and III exhibit identical k_1 and k_4 values (8), the slight differences observed in killing efficacy may reflect differences in the binding properties of these three forms. If this is true, then it may be hypothesized the MPO I binds more than MPO II and MPO II binds more than MPO III. Another explanation is that MPO I is a larger, more intact enzyme form which is simply less sensitive to either autoinactivation or inactivation by bacterial enzymes. These hypotheses are the subject of ongoing research. Regardless, it appears that MPO I, II, and III may indeed be designed for different functions, but are all capable of killing bacteria if present in adequately high concentrations.

In conclusion, we have demonstrated that the isolated MPO-H_2O_2-Cl^- system is bactericidal against *A. actinomycetemcomitans* in the absence of other neutrophil granule constituents. The supports the hypothesis that oxidative killing of *A. actinomycetemcomitans* by human neutrophils proceeds by an MPO-dependent mechanism (7), especially when these results are compared with previous findings that *A. actinomycetemcomitans* is highly resistant to H_2O_2 toxicity (9, 10) and to superoxide anion-generating systems (unpublished data). Furthermore, these results show that the three MPO forms (I, II, and III) are capable of exerting bactericidal activity in the MPO-H_2O_2-Cl^- system, but MPO I is more effective than MPO II, and MPO II is more effective than MPO III.

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