INFECTION AND IMMUNITY, May 1987, p. 1029–1036
0019-9567/87/051029-08$02.00/0

Vol. 55, No. 5

Role of High-Avidity Binding of Human Neutrophil Myeloperoxidase in the Killing of *Actinobacillus actinomycetemcomitans*

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The binding of the neutrophil enzyme myeloperoxidase (MPO) to microbial surfaces is believed to be the first step in its microbicidal activity. The MPO-H_2O_2-Cl^- system is responsible for most oxidative killing of *Actinobacillus actinomycetemcomitans* by human neutrophils. There appear to be three forms of MPO (MPO I, II, and III), all of which can kill this organism in the presence of H_2O_2 and chloride. In this study, we characterized the binding of native human neutrophil MPO to *A. actinomycetemcomitans* by an elution procedure dependent on the cationic detergent cetyltrimethylammonium bromide. Binding of native MPO was rapid and reached apparent equilibrium within 1 min. A proportion of binding under equilibrium conditions was saturable and highly avid, with a capacity of 4,500 sites per cell and a dissociation constant of 7.9×10^{-10} M. At equal protein concentrations, more MPO III bound than MPO II, and more MPO II bound than MPO I. The high-avidity interaction was inhibitable with yeast mannan and with the serotype-defining mannan of *A. actinomycetemcomitans*. Binding was also partially reversible with yeast mannan. MPO bound to the high-avidity sites did not oxidize guaiacol but oxidized chloride, as detected by the chlorination of taurine. MPO bound to the high-avidity sites was incapable of killing *A. actinomycetemcomitans* alone in the presence of H_2O_2 and Cl^-, but potentiated killing when sufficient additional MPO was provided. The killing of *A. actinomycetemcomitans* by the MPO-H_2O_2-Cl^- system was inhibited by yeast mannan and a serotype-defining mannan of *A. actinomycetemcomitans*. We conclude that high-avidity binding of MPO to the surface of *A. actinomycetemcomitans* is a mannan-specific interaction and that MPO bound to the high-avidity sites is essential but not alone sufficient to kill *A. actinomycetemcomitans*.

Actinobacillus actinomycetemcomitans is a facultative, gram-negative coccobacillus indigenous to dental plaque which can cause severe, occasionally fatal infections, such as endocarditis, brain abscesses, and coinfections in actinomycoses (8, 43). *A. actinomycetemcomitans* has been associated with localized juvenile periodontitis, a rapidly progressing periodontal disease of young adults characterized by massive increases of *A. actinomycetemcomitans* in dental plaque and subsequent bacterial invasion of gingival tissues. Such actinobacillary periodontal infections do not resolve with dental scaling and root planing alone (27, 31). It has been found that *A. actinomycetemcomitans* possesses a number of potential virulence factors, including a biologically active lipopolysaccharide; the release of membrane vesicles; the ability to inhibit fibroblast proliferation; collagenolytic activity; the production of leukotoxin, epitheliotoxin, immune suppressor factors, and capsular mannans; and resistance to serum bactericidal activity (11, 26, 30, 33, 35, 44).

The neutrophilic polymorphonuclear leukocyte appears to represent an important component of host defense against *A. actinomycetemcomitans*. Patients with localized juvenile periodontitis often exhibit defective neutrophil function, in which both phagocytosis and chemotaxis are reported to be impaired (5, 7). Although serum alone does not kill *A. actinomycetemcomitans*, neutrophils in the presence of serum are bactericidal (17). Most of the killing of *A. actinomycetemcomitans* by neutrophils under normoxic conditions proceeds via oxygen-dependent, cyanide-sensitive pathways (17). This suggests that the myeloperoxidase (MPO; EC 1.11.1.7: donor:hydrogen peroxide oxidoreductase)-H_2O_2-chloride antimicrobial system is a primary mechanism involved in the oxidative killing of *A. actinomycetemcomitans* by neutrophils (17). Although MPO may function to activate potentially bactericidal proteases or inactivate their inhibitors (15, 38) and interact synergistically with other neutrophil granule constituents (21), it has been found that the isolated MPO-H_2O_2-Cl^- system is highly bactericidal against *A. actinomycetemcomitans* (19).

MPO is involved in the killing of a number of microorganisms, including viruses, parasites, bacteria, and fungi (6, 13). In certain instances, including the interaction of MPO with enteric bacteria and *Candida albicans*, it has been proposed that the killing activity of MPO is dependent on the binding of MPO to the surface of the microorganism. Electron microscopic examination of neutrophil phagolysosomes reveals that much of the intraphagolysosomal MPO is adsorbed to the surface of the ingested microorganism (12). The physical separation of bacteria and MPO by a dialysis membrane blocks killing despite the presence of H_2O_2 and Cl^- (23). Moreover, microorganisms preadsorbed with MPO are killed more efficiently in the presence of H_2O_2 and Cl^- than organisms to which all three components are added simultaneously (28, 40). Finally, the binding of MPO to *C. albicans* is inhibitable by soluble mannans derived from either *C. albicans* or *Saccharomyces cerevisiae* (39, 41), and

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these fungal mannans also inhibit the killing of *C. albicans* by the MPO-H_2O_2-Cl^- system despite their inability to inhibit the oxidation of guaiacol by MPO (40). A potentially significant observation relevant to oral host-parasite interactions is that MPO appears to adsorb to oral facultative gram-negative bacteria such as *Leptothrix buccalis* and *Capnocytophaga* spp. (34). Together, these observations indicate that the binding of MPO to bacterial surfaces may be important in promoting the cidal activity of the MPO-H_2O_2-Cl^- system against a number of microorganisms, including those indigenous to the oral cavity.

MPO exhibits several properties which relate to its ability to bind to the surfaces of microorganisms. First, it is a glycoprotein possessing mannose and glucosamine which is bound by lectins such as concanavalin A (1, 16). Second, it is highly cationic and binds to cation-exchange resins at pH 4.7 or 8.0 (18, 24, 42). Third, MPO binds to yeast mannans, presumably by charge interaction with phosphate groups (39, 40). Fourth, there appear to be at least three forms of MPO (MPO I, II, and III), which differ in net charge, hydrophobicity, and molecular weight (24), and these forms exhibit differential binding properties to cation-exchange resins and concanavalin A-Sepharose (18, 24, 42).

In this study, we examined the binding of human neutrophil MPO to *A. actinomycetemcomitans* in order to determine its relationship to killing. We describe the nature of the binding interaction between MPO III and *A. actinomycetemcomitans* determined by equilibrium binding analysis. We compared the binding of MPO III with that of MPO I and II. Using mannans from *S. cerevisiae* and *A. actinomycetemcomitans*, we ascertained whether the binding of MPO III to *A. actinomycetemcomitans* was mannan inhibitable. Finally, we characterized the function of cell-bound MPO III in terms of the oxidation of electron donors and the killing of *A. actinomycetemcomitans*.

MATERIALS AND METHODS

Bacteria. *A. actinomycetemcomitans* ATCC 29523 (serotype a) is a catalase-producing organism resistant to the bactericidal effects of H_2O_2 (20). The organism was 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, 0.0001% menadione (Sigma), 0.1% yeast extract (Difco Laboratories, Detroit, Mich.), and 1% IsoVitaleX (BBL Microbiology Systems, Cockeysville, Md.) in Trypticase soy agar (BBL).

MPO. Three forms of MPO were isolated from human neutrophil granules by fast protein liquid chromatography as described previously (18). The purified MPO forms I, II, and III exhibited Reinheitzahl (A_{430}/A_{280} ratio) values above 0.8. The guaiacol oxidation assay was used to determine enzyme activity (4). One guaiacol unit (GU) is defined as the amount of enzyme catalyzing a change of 1.0 absorbance unit per minute at 740 nm (113 nmol of tetraguaiacol formed or 452 nmol of H_2O_2 decomposed per min). The concentration of protein was determined by the dye-binding method (3), and the concentration of MPO by its extinction coefficient at 430 nm of 178 mM^{-1} cm^{-1} (22).

Preparation of serotype mannans. The serotype a antigen from *A. actinomycetemcomitans* SUNYaB 75 (serotype a) was prepared as described in a previous study (44). Antigen-containing fractions were pooled, dialyzed against distilled water, and lyophilized. The serotype a antigen consisted of 85% neutral sugars, of which 72% was mannose.

Binding assay. Bacteria were suspended in a mixture containing 0.1 M sodium phosphate, 0.14 M NaCl, and 0.5 mM $MgCl_2$ (PMS), pH 6.8, and adjusted to an optical density of 0.47 at 540 nm. This provided a final bacterial cell concentration in the binding assay of 0.9×10^9 cells per ml and a total of 10^9 cells per assay tube (using a 1.1-ml reaction volume). Native MPO was diluted in PMS to between 0.34 and 338 μg/ml for saturation studies and to 1.8 μg/ml for examining high-avidity interactions under half-saturation conditions. The final amount of MPO added in saturation studies varied from 0.12 to 135 μg, and the final amount added for studies of the high-avidity interactions under half-saturation conditions was 0.54 μg. The specific activity of MPO ranged from 0.1 to 1.1 GU/μg and is reported when relevant. All binding assays were performed in triplicate. In binding-inhibition studies, the following reagents were preincubated with MPO (0.54 μg) in a volume of 0.4 ml for 5 min at 37°C prior to addition of bacteria (0.7 ml): mannan from *S. cerevisiae* (Sigma), 2.2 mg; lipopolysaccharide (LPS) from *Escherichia coli* serotype O111:B4 (Sigma), 1.1 mg; dextran T40 from *Leuconostoc mesenteroides* (Pharmacia Fine Chemicals, Piscataway, N.J.), 2.2 mg; and α-methyl mannoside (Sigma), glucose (Sigma), galactose (Sigma), lactose (Sigma), and α,α-trehalose (Kodak Laboratory and Specialty Chemicals, Rochester, N.Y.), 2.2 mg. The final concentration of inhibitor in the binding assay was 2 mg/ml, except for LPS, which was 1 mg/ml. Binding of MPO to bacteria was assessed by incubating bacteria and MPO in 1.5-ml polyethylene microfuge tubes for 15 min at 37°C. The reaction was terminated by centrifugation of bacteria and bound MPO for 2 min at $11,600 \times g$. The bacterial pellet was washed twice in PMS, and bound enzyme was eluted with 0.5% cetyltrimethylammonium bromide (CETAB) in 10 mM sodium phosphate, pH 7.0. Following centrifugation to remove the bacteria, eluted MPO was quantified by assessing the enzyme activity in 1.0 ml of the 0.5% CETAB supernatant with the guaiacol oxidation assay (4).

Chloride oxidation assay. The oxidation of chloride was indirectly assessed by analyzing the formation of taurine-chloramine as described by Stelmaszynska and Zgliczynski (32). Briefly, 4 μg of MPO (specific activity, 0.6 GU/μg) or ca. 0.3 μg of MPO bound to 10^9 bacteria was incubated for 10 min at 25°C in the presence of 5 mM taurine (Sigma), 1 mM H_2O_2, and 0.14 M NaCl in 1.1 ml of 20 mM sodium acetate buffer, pH 4.7. The amount of taurine-chloramine formed was estimated spectrophotometrically by the increase in absorbance at 255 nm based on an extinction coefficient of 398 M^{-1} cm^{-1} for chloramine (37).

Bactericidal assay. The bactericidal assay was performed in microtiter plates as described previously (19). In this assay, bacteria (10^4/ml) were incubated in the presence of MPO III (0.075 GU/ml) in PMS (pH 6.5) or PMS supplemented with *S. cerevisiae* mannan, the serotype a mannan from *A. actinomycetemcomitans*, or *L. mesenteroides* dextran T40, 2 mg/ml, for 5 min at 37°C. Triplicate mixtures were incubated for 15 min at 37°C in the presence of H_2O_2, 2 to 20 μM. The bactericidal reaction was terminated by 1:1 dilution in a mixture containing 0.5% tryptone (Difco), 0.14 M NaCl, 0.5% bovine serum albumin (Miles Laboratories, Naperville, Ill.), and 5 mM sodium azide. Bacterial survival was determined by spreading 50 μl of this suspension on tryptic soy agar (Difco) supplemented with 0.1% yeast extract, 5 μg of equine hemin III per ml, and 0.0001% menadione. Agar plates were incubated for 48 h at 37°C in 5% CO_2, and CFU were enumerated.

RESULTS

Binding assay. Earlier studies provided evidence that MPO bound to *Escherichia coli* or *Staphylococcus aureus* does not oxidize guaiacol (25, 28). Hence, to quantify the binding of native MPO by the guaiacol assay, we developed a method of releasing adsorbed enzyme from the microbial surface with CETAB (0.5%, wt/vol), providing a final CETAB concentration in the quaiacol assay of 0.167%. At concentrations below 0.067%, CETAB stimulated enzyme activity, and above 0.167%, some inhibition could be observed; however, between 0.067 and 0.167%, CETAB had no effect on the activity of MPO. The release of adsorbed enzyme from bacteria with 0.5% CETAB was quantitative, and the enzyme released from the bacteria plus the unbound enzyme was found to be equal to the amount of enzyme originally added. No peroxidatic activity could be recovered from untreated bacteria or from the supernatant of bacteria treated with 0.5% CETAB. Enzyme binding to the walls of the polyethylene microfuge tubes was negligible. The relative efficacy of 0.5% CETAB in releasing MPO adsorbed to bacteria was compared with two other procedures, including Nonidet P-40 (NP-40) (Particle Data Laboratories, Elmhurst, Ill.) and 2 M NaCl. Both NP-40 and NaCl inhibited MPO-catalyzed oxidation of guaiacol to some extent, and the values presented are corrected for this inhibition. Elution with NP-40 (0.05 to 2.0%) resulted in the release of 34 to 49% of bound MPO. Elution with 2 M NaCl resulted in the release of 76% of bound MPO. MPO activity could not be eluted with 10 mM sodium phosphate, pH 7.0. Thus, neither NP-40 nor NaCl was as effective as CETAB in releasing MPO from the bacterial surface. Subsequently, 0.5% CETAB was used routinely to elute bound enzyme.

Kinetics of MPO III adsorption. The binding of MPO III to *A. actinomycetemcomitans* ATCC 29523 was rapid, and near maximal binding was achieved within 1 min (Fig. 1). Loss of total MPO activity in the assay system occurred slowly over time and stabilized with 15 min. The loss of activity was proportionally equivalent in both the unbound and bound fractions.

Inhibition of binding. The binding interaction of MPO III with *A. actinomycetemcomitans* ATCC 29523 was specifically inhibitable with *S. cerevisiae* mannan and the serotype a antigen (Table 1). At subsaturating conditions, 94% of the binding of MPO III to *A. actinomycetemcomitans* was inhibited by the yeast mannan and 99% of binding was inhibited by the serotype a antigen at 2 mg/ml. Preincubation of cells with yeast mannan (2 mg/ml) did not inhibit binding of MPO. None of the monosaccharides or disaccharides tested, including glucose, galactose, lactose, α-methyl mannoside, and α,α-trehalose, was capable of inhibiting the binding reaction. Both the LPS from *E. coli* serotype O111:B4 and dextran T40 partially blocked MPO adsorption. The binding of both MPO I and MPO II was inhibited to a similar degree by the yeast mannan.

Specific reversibility of MPO binding. Yeast mannans partially reversed the binding of MPO III to *A. actinomycetemcomitans* ATCC 29523 (Table 2). In comparison to 0.5% CETAB and other reagents such as NaCl and NP-40, the efficiency of the yeast mannan was low, and only 35% of the bound MPO was eluted with mannan.

Saturation of MPO binding. The binding of MPO to *A. actinomycetemcomitans* was found to be saturable (Fig. 2). As the amount of MPO III added to the assay was increased (0.13 to 135 μg added per assay, or 2×10^2 to 2×10^5 molecules per cell), a progressively lower fraction of enzyme bound, producing a curve which exhibited both saturable and nonsaturable components. Scatchard analysis of the saturable binding showed a binding capacity of 4,500 molecules per cell and a dissociation constant (K_D) of 7.9×10^{-10} M. Yeast mannan appeared to depress both saturable and insaturable binding. Nevertheless, the predominant effect appeared to be noncompetitive, as shown by the Scatchard plot, in which the apparent binding capacity of the system was diminished in the presence of yeast mannans by about 83%. All three forms of MPO exhibited similar binding saturation (Table 3), but MPO I was the least efficient and

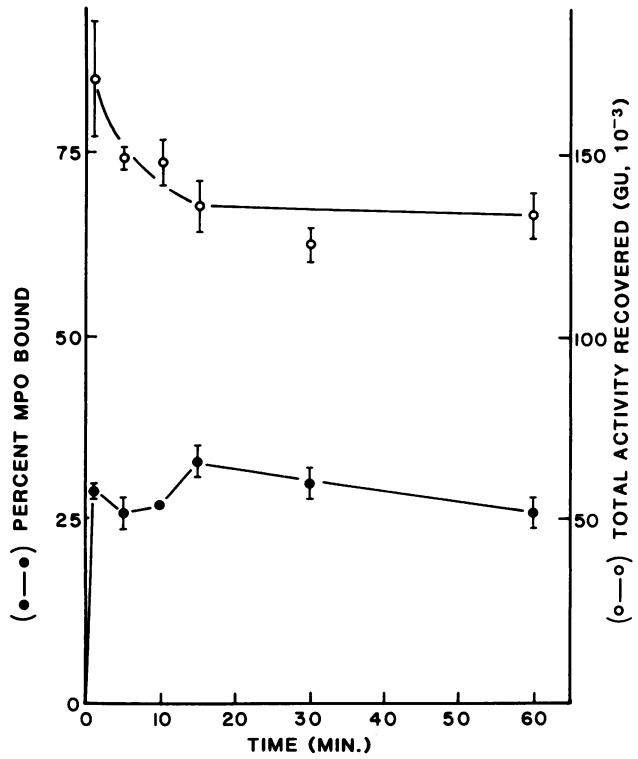

FIG. 1. Kinetics of binding of MPO III (R_z 0.83, 0.54 μg) to *A. actinomycetemcomitans* ATCC 29523 (10^9 cells). The amount of MPO bound was plotted as a function of time. Binding reached maximal values within 1 min. The total MPO activity recovered decreased logarithmically over time for the initial 15 to 30 min. Points represent the mean of three trials, and vertical bars represent the standard deviation.

TABLE 1. Inhibition of binding of MPO to *A. actinomycetemcomitans* ATCC 29523[a]

Inhibitor and concn (mg/ml)	MPO bound (mean ± SD)		% Inhibition of binding
	μg	%	
None	0.30 ± 0.003	56.3 ± 0.6	0
Yeast mannan (2)	0.019 ± 0.003	3.5 ± 0.5	94
Preincubation of bacteria with mannan (2)	0.32 ± 0.018	60.8 ± 3.5	0
LPS (1)	0.22 ± 0.012	41.4 ± 2.3	26
Dextran T40 (2)	0.20 ± 0.006	37.0 ± 1.1	34
Serotype a antigen (2)	0.002 ± 0.001	0.3 ± 0.2	99

[a] Assay: 10^9 bacteria mixed with 0.54 μg of MPO III, R_z 0.83, and inhibitor for 15 min at 37°C. No inhibitory effect was observed in the presence of mono- and disaccharides, including glucose, galactose, lactose, α-methyl mannoside, and α,α-trehalose at 2 mg/ml.

TABLE 2. Reversible binding of MPO III[a]

Eluant	Mean MPO III eluted (mGU) ± SD				% MPO eluted
	Supernatant	1st wash	2nd wash	Elution	
CETAB (control)	66.3 ± 3.1	4.5 ± 0.8	1.6 ± 0.7	56.3 ± 1.3	100
Yeast mannan	63.9 ± 0.9	4.4 ± 0.2	2.0 ± 0.3	19.7 ± 1.9	35

[a] Assay: 10^9 cells of *A. actinomycetemcomitans* ATCC 29523 were mixed with 0.54 μg of MPO III, R_z 0.83, in PMS, incubated for 15 min at 37°C, and washed twice, and bound enzyme was eluted with either 0.5% CETAB or yeast mannan (2 mg/ml).

MPO III was the most efficient in binding to *A. actinomycetemcomitans* ATCC 29523.

Influence of mannans on MPO enzymatic activity. Yeast mannans, the serotype a antigen from *A. actinomycetemcomitans* and dextran from *L. mesenteroides* did not inhibit the enzymatic activity of MPO (Table 4). We confirmed the findings of a previous study demonstrating that mannans do not inhibit guaiacol oxidation (40). However, the relevance of this electron donor in the biologic function of MPO is questionable. Accordingly, we also examined the oxidation of chloride indirectly by spectrophotometric analysis of chloramine generation. Mannans did not inhibit chloride oxidation or the chlorination of taurine.

Oxidation reactions by cell-bound MPO. The enzymatic activity of MPO bound to *A. actinomycetemcomitans* ATCC 29523 was found to be dependent on the electrophile used to assess activity. When the guaiacol assay was used, no enzyme activity was observed when cells with adsorbed MPO were suspended in 10 mM phosphate buffer instead of using the elution procedure with 0.5% CETAB. However, despite the inability to detect enzyme activity with the guaiacol assay, cell-bound MPO oxidized chloride readily, as assessed by the formation of taurine-chloramine by suspensions of bacteria with adsorbed MPO. Cell-bound MPO formed 275 ± 38 nmol of taurine-chloramine, which was equivalent to 18% of the originally added MPO (4 μg), which formed 1,538 ± 60 nmol of taurine-chloramine. This is within the lower range of binding percentages.

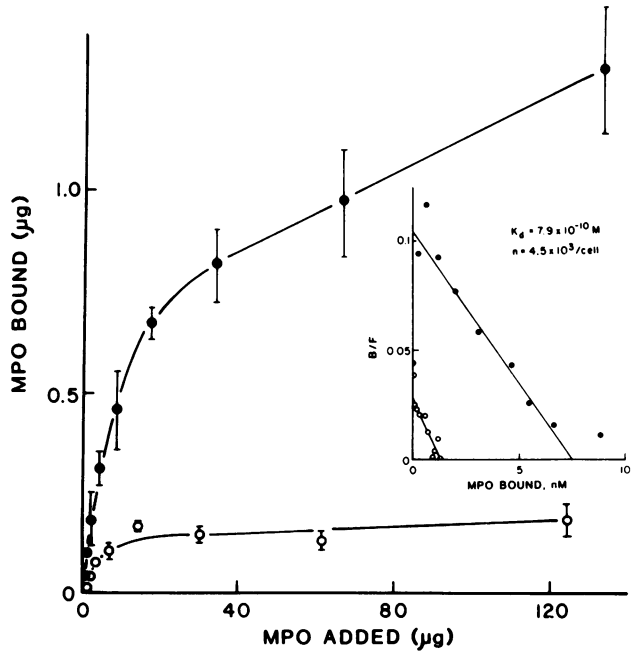

FIG. 2. Binding of MPO to *A. actinomycetemcomitans* ATCC 29523 exhibited saturable and nonsaturable characteristics. Bacteria (10^9 cells) were incubated with 0.13 to 135 μg of MPO III (R_z 0.83, 0.6 GU/μg) for 15 min at 37°C (○). Also shown are bacteria incubated with 0.12 to 124 μg of MPO III (R_z 0.77, 0.4 GU/μg) in the presence of yeast mannan (2 mg/ml) (●). Procedure was modified to include yeast mannan (2 mg/ml) in all wash steps when mannan was used in the binding assay. Points represent the mean of three trials, and vertical bars represent the standard deviation.

TABLE 3. Binding of three forms of MPO to *A. actinomycetemcomitans* ATCC 29523 at saturating and nonsaturating concentrations[a]

Form	Amount (μg)	Binding (mean ± SD)		Molecules bound/bacterium
		μg	%	
MPO I	34	0.9 ± 0.05	2.7 ± 0.2	3,600
	3	0.7 ± 0.04	57.2 ± 1.4	2,800
MPO II	34	1.7 ± 0.10	5.1 ± 0.2	6,800
	3	1.0 ± 0.03	63.6 ± 0.9	4,000
MPO III	34	2.0 ± 0.1	5.9 ± 0.3	8,000
	3	1.1 ± 0.1	66.6 ± 0.7	4,400

[a] 10^9 bacterial cells per assay, 15 min at 37°C. R_z f or MPO I, II, and III was 0.83, 0.82, and 0.83, respectively.

Influence of mannans on MPO-mediated killing. The effect of yeast mannan, dextran, and the serotype a antigen on the killing of *A. actinomycetemcomitans* by the MPO-H_2O_2-Cl^- system was examined (Table 5). All three polysaccharides were observed to exert a protective effect; however, the yeast mannan and the serotype a antigen were far more effective by weight than dextran in preventing the MPO-H_2O_2-Cl^- system from killing *A. actinomycetemcomitans*. Although the molecular weights of the yeast mannan and the serotype a antigen are unknown and probably heterogeneous, they are both probably slightly larger than dextran T40 (2, 44) and therefore more effective on a molar basis as well.

Bactericidal activity of bound MPO. In the presence of 20 μM H_2O_2 in PMS buffer, no direct cidal effect of cell-bound MPO was observed after 15 min at 37°C, despite the use of MPO with a specific activity of 1.1 GU/μg and the binding of approximately 3,000 molecules of MPO per bacterial cell

TABLE 4. Influence of mannans and dextran on the oxidation of guaiacol and chloride catalyzed by MPO

Inhibitor (concn)	Guaiacol oxidation[a]		Chloride oxidation[b]	
	Activity (mGU)	Inhibition (%)	Activity (nmol of taurine-chloramine formed)	Inhibition (%)
None	215 ± 9		314 ± 8	
Sodium azide (5 mM)	11 ± 1	95	23 ± 23	93
Yeast mannan (2 mg/ml)	233 ± 3	0	309 ± 12	2
Dextran T40 (2 mg/ml)	203 ± 7	6	331 ± 3	0
Serotype a antigen (2 mg/ml)	ND[c]	ND	350 ± 11	0

[a] Assay of Chance and Maehly (4). MPO III (R_z 0.83), 0.54 μg.
[b] Assay of Stelmaszynska and Zgliczynski (32). MPO III (R_z 0.83), 4 μg.
[c] ND, Not done.

TABLE 5. Inhibition of the bactericidal activity of the MPO-H_2O_2-Cl^- system against *A. actinomycetemcomitans* ATCC 29523 by yeast mannan and the serotype a antigen

Reactants	Bacteria (CFU/plate), mean ± SD	% Survivors
Expt 1[a]		
MPO (no H_2O_2)	319 ± 35	100
MPO, H_2O_2 (20 μM)	0 ± 0	0
MPO, mannan (2 mg/ml), H_2O_2 (20 μM)	232 ± 46	73
MPO, dextran T40 (2 mg/ml), H_2O_2 (20 μM)	77 ± 27	24
Expt 2[b]		
MPO (no H_2O_2)	110 ± 5	100
MPO, H_2O_2 (20 μM)	0	0
MPO, serotype a antigen (2 mg/ml), H_2O_2 (20 μM)	104 ± 14	95

[a] Reaction conditions: MPO III (R_z 0.83), 0.075 GU/ml (specific activity, 0.12 GU/μg); PMS buffer.

[b] Reaction conditions: Same as for experiment 1 except MPO III, R_z 0.81 (specific activity, 1.0 GU/μg).

(67% saturation). However, MPO bound to high-avidity sites decreased the requirement for MPO by about one-half in the bactericidal assay (Fig. 3). Complete killing was achieved in the presence of preadsorbed MPO (3,000 molecules per cell) when the MPO-H_2O_2-Cl^- bactericidal assay enzyme concentration was 0.0188 GU/ml (1.2×10^7 molecules of MPO per cell), whereas twice as much enzyme was required when no MPO was prebound to the high-avidity sites (2.3×10^7 molecules of MPO per cell). Cell-bound MPO also did not kill *A. actinomycetemcomitans* in the presence of 10^{-7} to 10^{-4} M sodium iodide and 20 μM H_2O_2 after 15 min at 37°C. Increasing the concentration of H_2O_2 from 20 to 100 μM did not result in observable killing, nor did increasing the incubation period from 15 min to 1 h.

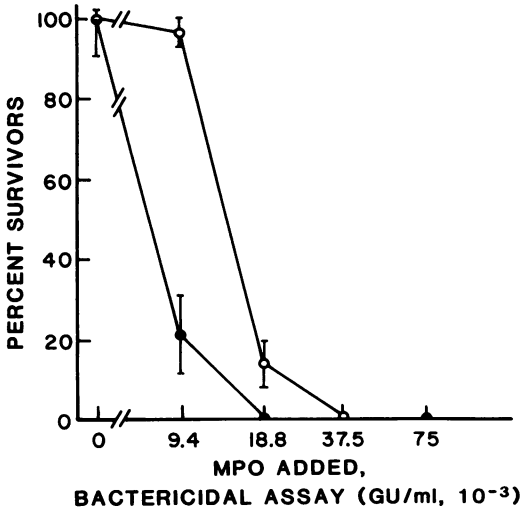

FIG. 3. Augmentation of killing by high-avidity binding of MPO III (R_z 0.83, 1.1 GU/μg) to *A. actinomycetemcomitans* ATCC 29523. Bacteria (10^9 cells) were prebound with MPO III (○) to 67% saturation (3,000 molecules of MPO per cell); alternatively, cells were mock treated (●). Bacteria were washed twice and then diluted to 10^4 cells per ml and mixed with MPO III (R_z 0.83, 0.6 GU/μg) at a concentration of 0 to 0.075 GU/ml (0 to 4.7×10^7 molecules of MPO per cell). In the presence of 2 μM H_2O_2 and 0.14 M NaCl, less MPO was required to kill bacteria when the high-avidity sites were prebound to 67% saturation. Points represent the mean of three trials, and vertical bars represent the standard deviation.

DISCUSSION

A. actinomycetemcomitans is a serum-resistant microorganism implicated as a tissue-invasive pathogen in localized juvenile periodontitis (27, 33). The clearance of *A. actinomycetemcomitans* from host tissues is dependent on neutrophils, which under normoxic conditions, use MPO-dependent processes to kill *A. actinomycetemcomitans* (17). In this study, we explored the relationship between binding of native MPO to *A. actinomycetemcomitans* and killing of *A. actinomycetemcomitans* by the isolated MPO-H_2O_2-Cl^- system. To assess the binding of native enzyme, we devised a method of releasing bound enzyme from the bacterial surface by using the cationic detergent CETAB. This approach circumvented the inhibitory effect that the surface of *A. actinomycetemcomitans* exerts over the oxidation of guaiacol by MPO. This effect has also been observed when MPO is bound to *S. aureus* and *E. coli*, but not to the granules of mast cells (10, 25, 28). The elution of active enzyme showed that the loss of the ability of cell-bound MPO to oxidize guaiacol was not due to irreversible denaturation of the enzyme. Furthermore, bound MPO was capable of oxidizing chloride and enzymatically active at the cell surface. The oxidized chloride was capable of interacting with taurine to form taurine-chloramine. Therefore, our findings as well as the observation of others (25, 28) that MPO bound to bacterial cells cannot oxidize guaiacol suggest that the electron donor-binding site of bound MPO is located in a cell surface domain which is selectively inaccessible to phenolic electrophiles such as guaiacol. Alternatively, bound MPO may undergo a conformational change, rendering it more specific for chloride. Neither of these hypotheses excludes the possibility that cell-bound MPO oxidizes cell-associated structures directly.

The binding of MPO to *A. actinomycetemcomitans* was rapid and reached maximal proportions within 1 min. Equilibrium binding analysis revealed the presence of both saturable and nonsaturable components in mediating the binding of MPO to bacterial surfaces. The saturable component was found to be highly avid, with a dissociation constant of 7.9×10^{-10} M. Bacteria exhibited a binding capacity of 4.5×10^3 molecules of MPO per bacterial cell via the high-avidity interaction.

The high-avidity binding reaction was specific in that it was almost completely inhibitable with yeast mannan and the serotype a antigen. The structural relationship between the yeast mannan and the serotype a antigen of *A. actinomycetemcomitans* is unknown. Both are known to possess phosphate residues (2, 36, 41). Other polysaccharides such as dextran and LPS inhibited binding slightly. None of the mono- or disaccharides tested inhibited binding of MPO. The ability of mannans to reverse partially the binding of MPO suggested that binding of MPO to *A. actinomycetemcomitans* proceeded in several steps, including a mannan-specific reaction followed by ionic or hydrophobic interactions. This may explain why NP-40, NaCl, and CETAB eluted MPO to various degrees.

The noncompetitive inhibition of MPO III binding by yeast mannan may be explicable on the basis of either allosteric effects of mannan on MPO receptors or irreversible binding of mannan to one of the components of the system. Mannans are most likely associated with MPO rather than cell surface constituents, as evidenced by the

finding that preincubation of bacterial cells with yeast mannans did not inhibit binding of MPO appreciably. The molar ratio of mannan to MPO was not determined, since neither the molecular weight nor the valence of the mannan was known.

The binding of MPO to the high-avidity sites of *A. actinomycetemcomitans* appeared to be necessary but not sufficient to produce bactericidal activity in the presence of H_2O_2 and Cl^-. This conclusion was drawn from three observations. First, bound MPO retained the ability to oxidize chloride, which in turn was capable of chlorinating amines. This finding demonstrated that MPO bound to the high-avidity site was not inactivated as a consequence of the binding interaction. Second, substances, such as yeast mannan, serotype a antigen, and dextran T40, which inhibited the binding of MPO to the high-avidity sites also impeded the killing of *A. actinomycetemcomitans* by the MPO-H_2O_2-Cl^- system. Neither yeast mannan nor dextran appeared to inhibit MPO-mediated oxidation reactions. Hence, the high-avidity interaction was essential for killing to occur. Third, although MPO bound to high-avidity sites did not kill *A. actinomycetemcomitans* in the presence of H_2O_2 (20 to 100 μM), Cl^-, or I^- (incubated for 15 min or 1 h), it decreased the requirement for additional MPO in the 15-min bactericidal assay. The amount of MPO bound to high-avidity sites was negligible compared with the amount of MPO added in the bactericidal assays. It is plausible that the inability to demonstrate that MPO bound to high-avidity sites was lethal in the presence of H_2O_2 and Cl^- was due to the relatively low specific activity of the enzyme. Freshly purified MPO exhibits a specific activity of 3 GU/μg (18). The actual specific activity of MPO in neutrophils is unknown, but it may be higher. Since the high-avidity binding of MPO to *A. actinomycetemcomitans* is saturable, killing mediated by MPO bound to these sites is critically dependent on the ratio of active to inactive molecules. However, if it is assumed that the MPO used in this study had an adequate specific activity, then at least two types of destructive interactions between the MPO-H_2O_2-Cl^- system and the bacterial surface may be required to sustain a kill. Future kinetic analysis or studies with inactive MPO can verify this two-hit hypothesis. Although the killing of *A. actinomycetemcomitans* by the MPO-H_2O_2-Cl^- system is dependent on both H_2O_2 and chloride (19), it is still plausible in a two-hit model that part of the killing process utilizes alternative electron donors.

In other peroxidase-target cell systems, bound enzyme is sufficient to promote cidal effects at sublethal concentrations of H_2O_2. Both *S. aureus* and *toxoplasma gondii* are killed by cell-bound eosinophil peroxidase in the absence of additional peroxidase (14, 25). In these systems halide is required for the cidal activity of the bound enzyme. If it is valid to extrapolate these observations to the interaction of cell-bound MPO with *A. actinomycetemcomitans*, then it is unlikely that the potentiating effects of cell-bound MPO are independent of halide.

It is possible that the potentiating effect that MPO binding to high-avidity sites has on killing is not due to enzymatic activity, as is the case with the bactericidal neutrophil peptides and cationic proteins (9, 21, 29, 36) or the nonbactericidal cell-bound, heat-inactivated, or native elastase (21). Although it is difficult to make comparisons because different bacteria are involved, it would appear that the potentiating effect exerted by bound MPO (3,000 bound molecules per cell) occurs at a much lower density than elastase (1.5×10^6 bound molecules per cell) (21). Similarly, the bactericidal effects of the chymotrypsinlike cationic proteins (5×10^{10} total molecules per cell), the bactericidal- and permeability-increasing protein (10^6 to 10^7 bound molecules per cell), and the defensins (8×10^9 total molecules per cell) occur at far higher concentrations than the potentiating concentration of bound MPO (9, 29, 36). This suggests that the enzymatic activity of bound MPO is very important, since none of the other cationic agents reviewed above exhibits such a low effective density. These considerations also render it extremely unlikely that the potentiating effect observed in this study was due to a contaminant such as elastase. The purity as determined by the R_z value of the MPO preparation was too high and the amount of protein added in the binding phase was insufficient to enable contamination by any one of the above-mentioned substances to be significant.

MPO I, II, and III all exhibited saturable binding characteristics with respect to *A. actinomycetemcomitans*. In this study we observed that, at equal concentrations, MPO III (the most cationic form) bound to a greater extent than MPO II, and MPO II bound to a greater extent than MPO I. Slight differences in specificity or avidity may be responsible for these results. Since the binding of all three forms was inhibited by mannan, dissociation phenomena which do not involve the mannan interaction probably were important. In this case, the strength of a second interaction between the cationic enzyme and the anionic microbial surface probably dictated the observed differences in adsorption.

The potential virulence-promoting properties of mannans have been demonstrated in *C. albicans* (40). In that system, mannans interfere with the release of MPO from neutrophils and the killing of yeast cells by the isolated MPO-H_2O_2-Cl^- system. The serotype capsular antigens of *A. actinomycetemcomitans* all appear to be mannans (manuscript in preparation). In this model system, we have demonstrated that yeast mannans and the serotype a mannan exerted an inhibitory effect on the bactericidal activity of the MPO-H_2O_2-Cl^- system against *A. actinomycetemcomitans* ATCC 29523. Furthermore, the yeast mannan inhibited the binding of MPO to the high-avidity binding site of *A. actinomycetemcomitans* ATCC 29523, suggesting that a component of this interaction is mannan specific.

In conclusion, MPO binds to *A. actinomycetemcomitans* ATCC 29523 in a saturable, mannan-inhibitable manner. MPO is not denatured as a result of binding, and bound MPO III enhances the killing of *A. actinomycetemcomitans* by the MPO-H_2O_2-Cl^- system. The serotype a antigen is capable of inhibiting the binding of MPO III to *A. actinomycetemcomitans* and also of inhibiting the killing of *A. actinomycetemcomitans* by the MPO-H_2O_2-Cl^- system. Thus, serotype antigens may afford protection to *A. actinomycetemcomitans* against the MPO-dependent defense mechanisms of neutrophils.

ACKNOWLEDGMENTS

We gratefully acknowledge the skillful typing of Cathy Drdul and Denise Nicosia. We thank Lawrence A. Tabak for his careful review of this manuscript, Homer S. Reynolds for his help with bacteriology, and Daniel Kosman for his suggestion to examine the chlorinating activity of bound MPO.

This work was funded by Public Health Service grants DE-06757, DE-04898, and DE-08029 from the National Institutes of Health.

LITERATURE CITED

1. **Bakkenist, A. R. J., R. Wever, T. Vulsma, H. Plat, and B. F. vanGelder.** 1978. Isolation procedure and some properties of myeloperoxidase from human leucocytes. Biochim. Biophys.

Acta **524**:45–54.
2. **Ballou, C. E.** 1974. Some aspects of the structure, immunochemistry, and genetic control of yeast mannans. Adv. Enzymol. **40**:239–270.
3. **Bradford, M. M.** 1976. A rapid sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
4. **Chance, B., and A. C. Maehly.** 1955. Catalases and peroxidases. Methods Enzymol. **2**:764–775.
5. **Cianciola, L. J., R. J. Genco, M. R. Patters, J. McKenna, and C. J. VanOss.** 1977. Defective polymorphonuclear leukocyte function in human periodontal disease. Nature (London) **265**:445–447.
6. **Clark, R. A.** 1983. Extracellular effects of the myeloperoxidase-hydrogen peroxide-halide system, p. 107–146. *In* G. Weissman (ed.), Advances in inflammation research, vol. 5. Raven Press, New York.
7. **Clark, R. A., R. C. Page, and G. Wilde.** 1977. Defective neutrophil chemotaxis in juvenile periodontitis. Infect. Immun. **18**:694–700.
8. **Eng, R. H. K., S. M. Smith, E. J. C. Goldstein, K. T. Miyasaki, S.-E. Quah, and F. Buccini.** 1986. Failure of vancomycin prophylaxis and treatment for *Actinobacillus actinomycetemcomitans* endocarditis. Antimicrob. Agents Chemother. **29**:699–700.
9. **Ganz, T., M. E. Selsted, D. Szklarek, S. S. L. Harwig, K. Daher, D. F. Bainton, and R. I. Lehrer.** 1985. Defensins. Natural peptide antibiotics of human neutrophils. J. Clin. Invest. **76**:1427–1435.
10. **Henderson, W. R., E. C. Jong, and S. J. Klebanoff.** 1980. Binding of eosinophil peroxidase to mast cell granules with retention of peroxidatic activity. J. Immunol. **124**:1383–1388.
11. **Kiley, P., and S. C. Holt.** 1980. Characterization of the lipopolysaccharide from *Actinobacillus actinomycetemcomitans* Y4 and N27. Infect. Immun. **30**:862–873.
12. **Klebanoff, S. J.** 1970. Myeloperoxidase-mediated antimicrobial systems and their role in leukocyte function, p. 89–110. *In* J. Schultz (ed.), Biochemistry of the phagocytic process: localization and the role of myeloperoxidase and the mechanism of the halogenation reaction. North-Holland, Amsterdam.
13. **Klebanoff, S. J., and C. B. Hamon.** 1972. Role of myeloperoxidase-mediated antimicrobial systems in intact leukocytes. J. Reticuloendothel. Soc. **12**:170–196.
14. **Locksley, R. M., C. D. Wilson, and S. J. Klebanoff.** 1982. Role for endogenous and acquired peroxidase in the toxoplasmacidal activity of murine and human mononuclear phagocytes. J. Clin. Invest. **69**:1099–1111.
15. **Matheson, N. R., P. S. Wong, M. Schuyler, and J. Travis.** 1981. Interaction of human α-1-proteinase inhibitor with neutrophil myeloperoxidase. Biochemistry **20**:331–336.
16. **Merrill, D. P.** 1980. Purification of human myeloperoxidase by concanavalin A-Sepharose affinity chromatography. Prep. Biochem. **10**:133–150.
17. **Miyasaki, K. T., M. E. Wilson, A. J. Brunetti, and R. J. Genco.** 1986. Oxidative and nonoxidative killing of *Actinobacillus actinomycetemcomitans* by human neutrophils. Infect. Immun. **53**:154–160.
18. **Miyasaki, K. T., M. E. Wilson, E. Cohen, P. C. Jones, and R. J. Genco.** 1986. Evidence for and partial characterization of three major and three minor chromatographic forms of human neutrophil myeloperoxidase. Arch. Biochem. Biophys. **246**: 751–764.
19. **Miyasaki, K. T., M. E. Wilson, and R. J. Genco.** 1986. Killing of *Actinobacillus actinomycetemcomitans* by the human neutrophil myeloperoxidase-hydrogen peroxide chloride system. Infect. Immun. **53**:161–165.
20. **Miyasaki, K. T., M. E. Wilson, J. J. Zambon, and R. J. Genco.** 1985. Influence of endogenous catalase activity on the sensitivity of the oral bacterium *Actinobacillus actinomycetemcomitans* and the oral haemophili to the bactericidal properties of hydrogen peroxide. Arch. Oral Biol. **30**:843–848.
21. **Odeberg, H., and I. Olsson.** 1976. Microbicidal mechanisms of human granulocytes: synergistic effects of granulocyte elastase and myeloperoxidase or chymotrypsin-like cationic protein. Infect. Immun. **14**:1276–1283.
22. **Olsen, R. L., and C. Little.** 1983. Purification and some properties of myeloperoxidase and eosinophil peroxidase from human blood. Biochem. J. **209**:781–787.
23. **Paul, B. B., A. A. Jacobs, R. R. Strauss, and A. J. Sbarra.** 1970. Role of the phagocyte in host-parasite interactions. XXIV. Aldehyde generation by the myeloperoxidase-H_2O_2-chloride antimicrobial system: a possible in vivo mechanism of action. Infect. Immun. **2**:414–418.
24. **Pember, S. O., R. Shapira, and J. M. Kinkade, Jr.** 1983. Multiple forms of myeloperoxidase from human neutrophilic granulocytes: evidence for differences in compartmentalization, enzymatic activity, and subunit structure. Arch. Biochem. Biophys. **221**:391–403.
25. **Ramsey, P. G., T. Martin, E. Chi, and S. J. Klebanoff.** 1982. Arming of mononuclear phagocytes by eosinophil peroxidase bound to *Staphylococcus aureus*. J. Immunol. **128**:415–420.
26. **Robertson, P. B., M. Lantz, P. T. Marucha, K. S. Kornman, C. L. Trummel, and S. C. Holt.** 1982. Collagenolytic activity associated with *Bacteroides* species and *Actinobacillus actinomycetemcomitans*. J. Periodont. Res. **17**:275–283.
27. **Saglie, F. R., F. A. Carranza, Jr., M. G. Newman, L. Cheng, and K. I. Lewin.** 1982. Identification of tissue invading bacteria in human periodontal disease. J. Periodont. Res. **17**:452–455.
28. **Selvaraj, R. J., J. M. Zgliczynski, B. B. Paul, and A. J. Sbarra.** 1978. Enhanced killing of myeloperoxidase-coated bacteria in the myeloperoxidase-H_2O_2-Cl system. J. Infect. Dis. **137**: 481–485.
29. **Shafer, W. M., L. E. Martin, and J. K. Spitznagel.** 1984. Cationic antimicrobial proteins isolated from human neutrophil granulocytes in the presence of diisopropyl fluorophosphate. Infect. Immun. **45**:29–35.
30. **Shenker, B. J., W. P. McArthur, and C.-C. Tsai.** 1982. Immune suppression induced by *Actinobacillus actinomycetemcomitans*. I. Effects on human peripheral blood lymphocytes responses to mitogens and antigens. J. Immunol. **128**:148–154.
31. **Slots, J., and B. Rosling.** 1983. Suppression of the periodontopathic microflora in localized juvenile periodontitis. J. Clin Periodontol. **10**:465–486.
32. **Stelmaszynska, T., and J. Zgliczynski.** 1974. Myeloperoxidase of human neutrophilic granulocytes as a chlorinating enzyme. Eur. J. Biochem. **45**:305–312.
33. **Sundqvist, G., and E. Johansson.** 1982. Bactericidal effect of pooled human serum on *Bacteroides melaninogenicus*, *Bacteroides asaccharolyticus*, and *Actinobacillus actinomycetemcomitans*. Scand. J. Dent. Res. **90**:29–36.
34. **Tsai, C.-C., B. F. Hammond, P. Baehni, W. P. McArthur, and N. S. Taichman.** 1978. Interaction of inflammatory cells and oral microorganisms. VI. Exocytosis of PMN lysosomes in response to gram-negative plaque bacteria. J. Periodont. Res. **13**:504–512.
35. **Tsai, C.-C., B. J. Shenker, J. M. DiRienzo, D. Malamud, and N. S. Taichman.** 1984. Extraction and isolation of a leukotoxin from *Actinobacillus actinomycetemcomitans* with polymixin B. Infect. Immun. **43**:700–705.
36. **Weiss, J., S. Beckerdite-Quagliata, and P. Elsbach.** 1980. Resistance of gram-negative bacteria to purified bactericidal leukocyte proteins. Relation to binding and bacterial lipopolysaccharide structure. J. Clin. Invest. **65**:619–628.
37. **Weiss, S. J., R. Klein, A. Slivka, and M. Wei.** 1982. Chlorination of taurine by human neutrophils. Evidence for hypochlorous acid generation. J. Clin. Invest. **70**:598–607.
38. **Weiss, S. J., G. Peppin, X. Ortiz, C. Ragsdale, and S. T. Test.** 1985. Oxidative autoactivation of latent collagenase by human neutrophils. Science **227**:747–749.
39. **Wright, C. D., J. U. Bowie, and R. D. Nelson.** 1984. Influence of yeast mannan on release of myeloperoxidase by human neutrophils: determination of structural features of mannan required for formation of myeloperoxidase-mannan-neutrophil complexes. Infect. Immun. **43**:467–471.
40. **Wright, C. D., J. U. Bowie, G. R. Gray, and R. D. Nelson.** 1983.

Candidacidal of activity of myeloperoxidase: mechanisms of inhibitory influence of soluble cell wall mannan. Infect. Immun. **42**:76–80.

41. **Wright, C. D., M. J. Herron, G. R. Gray, B. Holmes, and R. D. Nelson.** 1981. Influence of yeast mannan on human neutrophil function: inhibition of release of myeloperoxidase related to carbohydrate-binding property of the enzyme. Infect. Immun. **32**:731–738.
42. **Yamada, M., M. Mori, and T. Sugimura.** 1981. Purification and characterization of small molecular weight myeloperoxidase from human promyelocytic leukemia HL-60 cells. Biochemistry **20**:766–771.
43. **Zambon, J. J.** 1985. *Actinobacillus actinomycetemcomitans* in human periodontal disease. J. Clin. Periodontol. **12**:1–20.
44. **Zambon, J. J., J. Slots, K. Miyasaki, R. Linzer, R. Cohen, M. Levine, and R. J. Genco.** 1984. Purification and characterization of the serotype c antigen from *Actinobacillus actinomycetemcomitans*. Infect. Immun. **44**:22–27.