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Oxidative Inactivation of *Actinobacillus actinomycetemcomitans* Leukotoxin by the Neutrophil Myeloperoxidase System

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The leukotoxin of *Actinobacillus actinomycetemcomitans* has been implicated in the pathogenesis of inflammatory periodontal disease. We examined a potential mechanism for detoxification of this microbial product by the neutrophil myeloperoxidase system. Exposure to myeloperoxidase, H_2O_2, and a halide resulted in marked inactivation of leukotoxin, an effect which required each component of the myeloperoxidase system. Toxin inactivation was blocked by agents which inhibit heme enzymes (azide, cyanide) or degrade H_2O_2 (catalase). Reagent H_2O_2 could be replaced by the peroxide-generating enzyme system glucose oxidase plus glucose. The latter system, in fact, was more potent than reagent H_2O_2 in terms of the capacity to inactivate high concentrations of toxin. Toxin inactivation was complete within 1 to 2 min at 37°C. These observations suggest a possible role for oxidative inactivation of leukotoxin by secretory products of neutrophils.

The pathogenicity of many microorganisms is related to the formation of toxins directed at host tissues (7, 19, 26). Even though the detoxification of such agents appears to be a critical feature of host defenses, the means for accomplishing this in the nonimmune host lacking neutralizing antibody have not been well characterized. Based on early studies suggesting a potential role for polymorphonuclear leukocyte (PMN) products in the inactivation of diphtheria and tetanus toxins (1, 2), we have recently established mechanisms for the oxidative inactivation of microbial toxins by the myeloperoxidase (MPO) system of the PMN (27). For the cytotoxin of *Clostridium difficile* (27) and the hemolytic toxin of *Streptococcus pneumoniae* (R. A. Clark, J. Immunol., in press), a combination of MPO, H_2O_2, and a halide proved to be a potent detoxification system.

In this report, we define the ability of the MPO-H_2O_2-halide system of the PMN to inactivate the leukotoxin of *Actinobacillus actinomycetemcomitans*. Interest in this toxin derives from several lines of evidence. *A. actinomycetemcomitans* can be isolated in high numbers from subgingival plaque samples from patients with juvenile periodontitis (34). The majority of *A. actinomycetemcomitans* strains are able to selectively destroy isolated human PMN (5); by contrast, other subgingival plaque isolates do not appear to possess this property (28). Since PMN probably constitute a critical defense system in the gingival crevice, their destruction by *A. actinomycetemcomitans* leukotoxin may represent a mechanism of microbial virulence in the development of juvenile periodontitis.

MATERIALS AND METHODS

Special materials. MPO was purified from canine neutrophils by the method of Agner (3). Enzyme activity was assayed daily by the *o*-dianisidine method (Worthington Enzyme Manual, p. 43; Worthington Biochemical Corp., Freehold, N.J., 1972) and expressed in international units, with 1 IU defined as the amount causing the utilization of 1 μmol of substrate per min at 25°C (21). H_2O_2 (30% solution), sodium azide, sodium cyanide, and other routine chemicals were from Fisher Scientific Co., Fairlawn, N.J. Catalase (beef liver; 87,000 U/ml), from Worthington Biochemical Corp., was dialyzed overnight against distilled water before use. Glucose oxidase (type V from *Aspergillus niger*; 200 U/mg) was obtained from Sigma Chemical Co., St. Louis, Mo.; concentrations expressed in units (1 U oxidizes 1 μmol of substrate per min at 25°C) were based on the specifications of the supplier. Also, the rate of H_2O_2 formation from oxidation of glucose was determined under the conditions used by measurement of oxygen consumption with a Clark oxygen electrode and oxygen monitor (Yellow Springs Instrument Co., Yellow Springs, Ohio) (10).

Toxin. *A. actinomycetemcomitans* JP2 was isolated from a subgingival plaque sample of an 8-year-old male with advanced juvenile periodontitis (32). Based on serotyping and biotyping, the organism was classified as belonging to serotype B (34). The strain were grown at 37°C for 24 h in fluid thioglycolate medium containing 0.4% sodium bicarbonate. Harvested organisms were washed with phosphate-buffered saline (pH 7.4), sonicated in distilled water, and clarified by centrifugation (100,000 × *g*, 4°C, 60 min). The resulting supernatant was dialyzed for 18 h against distilled water, lyophilized, and stored at −20°C. The sonicate contained 480 μg of protein per mg (dry weight) of cells as determined with the Bio-Rad assay kit (Bio-Rad Laboratories, Richmond, Calif.), with bovine plasma albumin as the standard. Small amounts of the preparation were reconstituted in distilled water (250 μg [dry weight]/ml) and stored in portions at −80°C.

Toxin assay. The ability of *A. actinomycetemcomitans* toxin to lyse human PMN was quantitated by a modification of the method of Tsai et al. (32). PMN were purified from heparin-anticoagulated normal human blood by sequential Hypaque-Ficoll density centrifugation, sedimentation in dextran, and hypotonic lysis of contaminating erythrocytes (8, 13). The resulting preparation of 95 to 98% pure granulocytes was labeled with $Na_2{}^{51}CrO_4$ (200 to 500 μCi of Cr per μg; New England Nuclear Corp., Boston, Mass.) as described previously (16), washed, and suspended in Hanks balanced salt solution (HBSS) at 5×10^6 PMN/ml. A 50-μl

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TABLE 1. Toxin inactivation by the MPO system[a]

Change to complete system	Mean % ^{51}Cr release ± SE (no. of expts)	
	MPO-H_2O_2-Cl^-	MPO-GO-G-Cl^-
None	−2.2 ± 1.0 (12)	−2.4 ± 1.0 (12)
MPO omitted	82.8 ± 6.5 (5)	83.9 ± 9.9 (5)
MPO heated	77.2 ± 5.4 (4)	75.3 ± 6.7 (4)
H_2O_2 omitted	81.3 ± 10.1 (5)	—[b]
GO omitted	—	73.1 ± 9.3 (4)
G omitted	—	71.6 ± 11.5 (4)
Cl^- omitted	75.0 ± 4.6 (5)	76.7 ± 7.1 (5)
Cl^- replaced by I^-	9.8 ± 9.5 (5)	4.6 ± 3.5 (4)
Azide added	57.1 ± 8.5 (7)	61.6 ± 8.2 (7)
Cyanide added	61.0 ± 5.9 (9)	58.8 ± 8.0 (8)
Catalase added	56.2 ± 8.9 (8)	46.0 ± 6.7 (6)
Heated catalase added	−4.5 ± 2.8 (4)	−0.9 ± 0.7 (2)

[a] Reaction mixtures contained 5 μg of toxin in 500 μl of 0.1 M sodium–sodium phosphate buffer, pH 7.0, and the supplements indicated, as follows: MPO, 8 mU; H_2O_2, 10 μM; glucose oxidase (GO), 11 mU; glucose (G), 10 mM; NaCl, 0.1 M; NaI, 0.1 mM; NaN_3, 0.1 mM; NaCN, 0.1 mM; and catalase, 435 U. When NaCl was present the concentration of phosphate buffer was decreased to 36 mM to maintain isotonicity. Where indicated, MPO or catalase was heated at 100°C for 15 min. After incubation at 37°C for 30 min, catalase (435 U) was added to all samples, and duplicate 100-μl portions of each sample were tested for toxin activity. With no supplements (toxin alone), ^{51}Cr release was 75.1 ± 4.4% (13 experiments). Values for the complete system were significantly different ($P < 0.001$) from those for toxin alone and for all omissions or additions except heated catalase.

[b] —, Not applicable.

portion of this cell suspension (2.5×10^5 PMN) was mixed with 25 μl of a 1:10 dilution in HBSS of human AB serum, a portion of the test sample (usually 100 μl of an isotonic solution), and sufficient HBSS to give a total volume of 200 μl. These mixtures were incubated in 96-well microtiter plates at 37°C and 5% CO_2–95% air for 40 min. The plates were centrifuged at 800 × *g* at 4°C for 10 min; a 100-μl portion of supernatant was removed from each well and counted for ^{51}Cr in a Packard Autogamma spectrometer (Packard Instrument Co., Downers Grove, Ill.). Parallel wells containing no sample (minimum release standard) or 0.1% Triton X-100 (maximum release standard) were run, and the leukotoxic activity was calculated as percent ^{51}Cr release as follows: % ^{51}Cr release = [(sample cpm − minimum cpm)/(maximum cpm − minimum cpm)] × 100.

Exposure of toxin to the MPO system. Toxin (usually 5 μg) was incubated in isotonic phosphate buffer (pH 7.0) alone or with the components of the MPO-H_2O_2-halide system. The total volume of 500 μl in glass tubes (12 by 75 mm) was incubated in a 37°C water bath for 30 min. Catalase (435 U in 5 μl) was added to degrade residual H_2O_2 in order to preclude peroxide-mediated lysis of the ^{51}Cr-PMN in the subsequent assay for toxin activity. Duplicate 100-μl portions of each sample were then tested for leukotoxic activity as described above. Preliminary experiments demonstrated that this amount of toxin (nominally 1 μg) was well within the linear portion of the dose-response curve for leukotoxic activity. In studies of the time course of toxin inactivation, azide (0.1 mM) was added instead of catalase after various periods of incubation and the tubes were immediately placed in an ice bath; this stopped the toxin inactivation reaction more abruptly and was as effective as catalase in preventing an effect of the MPO system components on the ^{51}Cr-PMN.

RESULTS

A. actinomycetemcomitans leukotoxin incubated with buffer alone expressed a mean 75.1% release of ^{51}Cr from labeled human PMN; in contrast, the same amount of toxin exposed to the complete MPO system retained no residual ability to lyse ^{51}Cr-PMN (Table 1). The peroxide component of the MPO system could be provided by either reagent H_2O_2 or the peroxide-generating enzyme system glucose oxidase plus glucose. No leukotoxin inactivation was detected when the MPO was either omitted or inactivated by heating. Omission of H_2O_2 or either glucose oxidase or glucose precluded toxin inactivation. When chloride was omitted, no inactivation of leukotoxin was observed unless an alternative halide, iodide, was added. In both the MPO-H_2O_2-halide and MPO-glucose oxidase-glucose-halide system, toxin inactivation was blocked by the addition of agents which inhibit heme enzymes (azide, cyanide) or degrade H_2O_2 (catalase). Heated catalase had no effect.

In studies not illustrated, we found that the minimum concentration of reagent H_2O_2 required for toxin inactivation in the presence of MPO and chloride was 1 to 2 μM. With the standard glucose oxidase system (Table 1), the rate of H_2O_2 formation was approximately 5 nmol/min. With various amounts of glucose oxidase and standard concentrations of glucose, MPO, and chloride, rates of H_2O_2 formation as low as 0.5 nmol/min were effective in inactivating *A. actinomycetemcomitans* leukotoxin. Kinetic studies demonstrated a rapid rate of leukotoxin inactivation, complete within 1 min in the MPO-H_2O_2-chloride system and within 2 min in the MPO-glucose oxidase-glucose-chloride system (Fig. 1).

The capacity of the MPO system to inactivate leukotoxin was explored further by increasing the amount of toxin in the preincubation from the usual 5 μg to as high as 50 μg and subsequently testing graded amounts of these samples for toxin activity. Thus, a dose response could be established for control and MPO-treated toxin. With low concentrations of toxin in the preincubation (<12.5 μg), no residual activity was detected at the highest testable sample volume (2.5 μg of toxin). As the amount of toxin in the preincubation was increased, progressively more residual activity was detected in samples exposed to the MPO system with reagent H_2O_2. However, when glucose oxidase plus glucose was used as the source of H_2O_2 for the MPO system, toxin activity remained undetectable, even with 50 μg of toxin in the preincubation and a test sample of 10 μg in the ^{51}Cr release assay. Representative data from these analyses, with 25 μg of leukotoxin in the preincubation, are illustrated in Fig. 2.

DISCUSSION

The primary role of the PMN in antimicrobial defense is the ingestion and killing of pathogenic microorganisms by the delivery of toxic agents to the phagocytic vacuole. Among these antimicrobial agents are various reduced forms of oxygen (superoxide anion [O_2^-], hydrogen peroxide [H_2O_2], hydroxyl radical [OH^-]) and the products of the interaction of MPO, H_2O_2, and a halide (4, 11, 20, 22). It has become well established that these agents, including MPO and H_2O_2, are also released outside the cell, where they may perform such biologic functions as killing of extracellular organisms, injury of host cells, and oxidative modification of various humoral mediators (11, 20). For example, the secreted components of the MPO system can inactivate leukoattractants (12, 15, 33), antiproteases (9, 14, 24), leukotrienes (17), iron-binding proteins (R. A. Clark and D. W. Pearson, Clin. Res. **33**:563A, 1985), and bacterial toxins (27). In some instances, specific biochemical reactions such as oxidation of critical thioether moieties have been implicated (11, 15, 24).

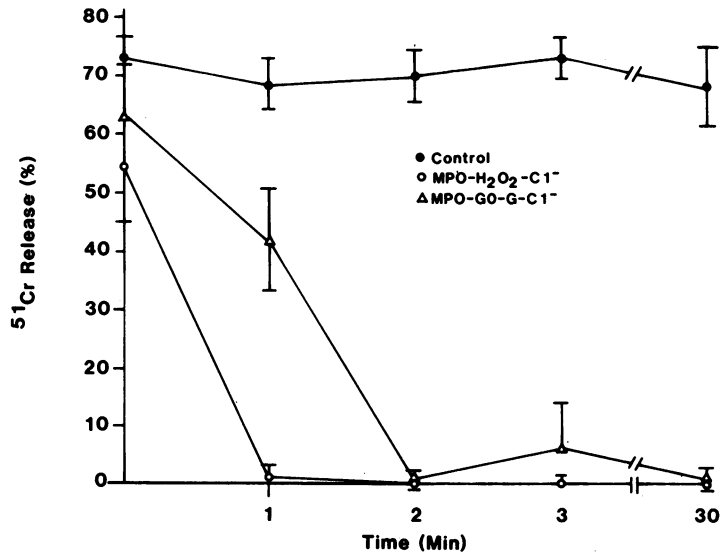

FIG. 1. Time course of leukotoxin inactivation by the MPO systems. The reaction mixtures (Table 1) were for toxin alone (●) and for toxin exposed to the complete MPO system with either reagent H_2O_2 (○) or glucose oxidase plus glucose (△). After the indicated period of incubation at 37°C, 0.1 mM azide (5 μl of a 10 mM stock solution) was added, and the samples were placed in an ice bath to stop the reaction. Portions were then tested for the ability to lyse ^{51}Cr-PMN. Data are mean ± standard error for three to five separate experiments. Abbreviations: Cl^-, halide; GO, glucose oxidase; G, glucose.

Thus, a number of extracellular functions appear to be mediated by oxidants secreted from activated PMN. Our current work shows that inactivation of *A. actinomycetemcomitans* leukotoxin may be one such function. The crude toxin was quickly inactivated by exposure to the cell-free MPO system with either chloride or iodide as the required halide. Reagent H_2O_2 or glucose oxidase plus glucose fulfilled the peroxide requirement. The latter of these displayed a greater capacity for toxin inactivation. Interestingly, the glucose oxidase system with its ongoing formation of H_2O_2 and low steady-state levels provides a closer parallel to the in situ conditions than does bolus H_2O_2. The H_2O_2 flux observed with our standard glucose oxidase system, 5 nmol/min or less, is comparable to that detected from approximately 3×10^6 stimulated human PMN (18). This number of PMN might reasonably be expected in the gingival crevice of patients with periodontal disease (23). Since relatively little is known of the structure of *A. actinomycetemcomitans* leukotoxin, one can only speculate about the biochemical alterations responsible for oxidative inactivation by the MPO system. It will be necessary to consider oxidation of critical methionine, tyrosine, histidine, lysine, and sulfhydryl residues.

An early precedent for MPO-dependent inactivation of bacterial toxins is found in the work of Agner (1, 2), who demonstrated that diphtheria toxin and tetanus toxin lost

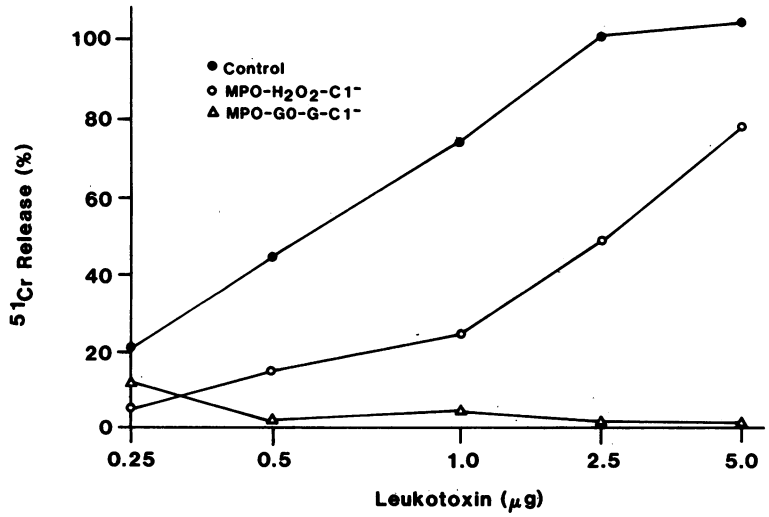

FIG. 2. Dose response for activity of toxin exposed to the MPO systems. The reaction mixtures (Table 1) were for toxin alone (●) and for toxin exposed to the complete MPO system with either reagent H_2O_2 (○) or glucose oxidase plus glucose (△), except that the amount of toxin was increased from 5 to 25 μg. After the preincubation (37°C for 30 min), portions of various volumes containing the nominal amounts of toxin indicated on the abscissa were tested for the ability to lyse ^{51}Cr-PMN. Data are means of duplicates in a single experiment representative of two separate studies. See the legend to Fig. 1 for abbreviations.

their lethal effects on mice during exposure to H_2O_2 and verdoperoxidase (MPO) plus a dialyzable factor. More recently, oxidation of the toxic microbial metabolites aflatoxins by the MPO system was described (25). We have found that secreted MPO and H_2O_2 together with a halide constitute a potent system for inactivating the cytotoxin of *C. difficile* (27) and the hemolytic toxin of *S. pneumoniae* (Clark, in press). The pathophysiologic importance of our present observations on inactivation of *A. actinomycetemcomitans* leukotoxin relates to the role of this microbial product in inflammatory periodontal disease. *A. actinomycetemcomitans* infection is a frequent finding in juvenile periodontitis (34). The pathogenesis of this disorder is still a matter for conjecture, but it is conceivable that the *A. actinomycetemcomitans* leukotoxin may represent a mechanism of microbial virulence in the gingival crevice. If the toxin is capable of destroying PMN in vivo, it may compromise antimicrobial defense in the area. Under these circumstances it would be to the advantage of the host to inactivate this molecule, limiting its biologic potency. We have previously shown that the majority of juvenile periodontitis patients have high titers of neutralizing antibody against this leukotoxin (29, 30). However, these observations were made on subjects with established (long-standing) disease. At some point during the course of infection the host must contend with the toxin without immune protection. It is during this nonimmune period that other systems may be required for detoxification of the leukotoxin. The present studies suggest that PMN MPO may play a role in nonimmune destruction of *A. actinomycetemcomitans* leukotoxin. The leukotoxin is also sensitive to various proteolytic enzymes, including trypsin and elastase (32; N. S. Taichman, unpublished data). PMN exposed to intact *A. actinomycetemcomitans* or isolated leukotoxin discharge granule-derived products (such as MPO and proteases) into the extracellular environment (6, 31). Thus, either cytotoxic or stimulus-dependent release of enzymes from PMN may represent mechanisms for inactivating *A. actinomycetemcomitans* leukotoxin.

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