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# Killing of *Actinobacillus actinomycetemcomitans* by Human Lactoferrin

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***Actinobacillus actinomycetemcomitans*** **is a fastidious, facultative gram-negative rod associated with endocarditis, certain forms of periodontal disease, and other focal infections. Human neutrophils have demonstrated bactericidal activity against *A. actinomycetemcomitans*, and much of the oxygen-dependent killing has been attributed to the myeloperoxidase-$H_2O_2$-halide system. However, the contribution of other neutrophil components to killing activity is obscure. Lactoferrin, an iron-binding glycoprotein, is a major constituent of neutrophil-specific granules and is also found in mucosal secretions. In this report, we show that human lactoferrin is bactericidal for *A. actinomycetemcomitans*. Killing activity required an unsaturated (iron- and anion-free) molecule that produced a 2-log decrease in viability within 120 min at 37°C at a concentration of 1.9 μM. Besides exhibiting concentration dependence, killing kinetics were affected by minor variations in temperature and pH. Magnesium, a divalent cation thought to stabilize lipopolysaccharide interactions on the surface of gram-negative organisms, enhanced lactoferrin killing of *A. actinomycetemcomitans*, while other cations, such as potassium and calcium, had no effect. Our data suggest that lactoferrin contributes to killing of *A. actinomycetemcomitans* by human neutrophils and that it may also play a significant role in innate secretory defense against this potential periodontopathogen.**

*Actinobacillus actinomycetemcomitans*, a small capnophilic, aerotolerant gram-negative coccobacillus is considered a minor component of the normal oral microflora of humans and may be recovered from dental plaque (38). Although apparently an innocuous parasite in most instances, it has been linked as an etiologic agent in several forms of human disease, including periodontitis, endocarditis, and severe abscess formation. Supporting this disease potential are several virulence factors, including a potent leukotoxin, a lymphocyte suppression factor, and several histiolytic and proteolytic enzymes. Association with disease is well documented in localized juvenile periodontitis (LJP) (30, 38), a rapidly progressive form of periodontal disease which affects between 0.1 and 2.3% of young people 10 to 19 years of age in the United States, with even higher prevalence among certain minorities (28). Recent work suggests that periodontitis affecting older adults may be linked to *A. actinomycetemcomitans* infection as well, especially in recurrent or refractory cases (11).

A number of host defense mechanisms present in saliva and gingival (crevicular) fluid may normally act to control oral colonization by *A. actinomycetemcomitans* and prevent local or more disseminated tissue pathology. Polymorphonuclear neutrophils (PMN) migrate to crevicular sites in response to microbial plaque and are essential for resolution of gingival and periodontal infections. Lysozyme, a cationic protein found in saliva and human phagocytes, is capable of lysing *A. actinomycetemcomitans* in vitro, an effect which is enhanced in the presence of EDTA (17). Myeloperoxidase isolated from the azurophilic granules of human neutrophils has exhibited bactericidal activity against *A. actinomycetemcomitans* as a part of the myeloperoxidase-$H_2O_2$-halide system (23). However, *A. actinomycetemcomitans* was shown to be relatively resistant to the effects of $H_2O_2$ alone (24). Lactoferrin (LF), an iron-binding glycoprotein contained in the specific granules of neutrophils and produced by acinar epithelial cells, can inhibit the growth of numerous microorganisms through simple iron deprivation (31, 36). In addition, bactericidal activity has been demonstrated that is clearly distinct from the bacteriostasis of iron deprivation, since the latter effect can be overcome by exogenous iron while the former cannot (4, 5). Since LF is a major protein component of neutrophil granules and is present in saliva and gingival fluid, its interaction with periodontopathogenic organisms may be an important contributor to host defense against periodontal disease. The present study examines the in vitro action of human LF on *A. actinomycetemcomitans*.

(This research was conducted by John R. Kalmar in partial fulfillment of the requirements for the Ph.D. degree from Emory University, Atlanta, Ga., 1988.)

## MATERIALS AND METHODS

**Preparation of LF.** LF was purified from pooled human colostrum by heparin affinity chromatography as previously described (9). Fidelity of final preparations was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 10% acrylamide gels stained by silver-Coomassie blue and by transimmunoblot and immunoelectrophoresis. Iron-free LF (apo-LF) was prepared by dialysis against an acetic acid-sodium acetate buffer containing 40 mM EDTA and 0.2 M sodium phosphate (pH 4.0) (21). Iron-saturated LF was prepared by dialysis against 0.03 mM $FeCl_3$–0.1 M $NaHCO_3$–0.1 M sodium citrate. Both preparations were then exhaustively dialyzed against deionized, distilled water, and aliquots were stored at −20°C in sterile, polypropylene tubes (Baxter Health Care). Protein concentration was determined by $A_{280}$ (extinction coefficients: apo-LF, 11.0, iron-saturated LF, 13.8) and Bio-Rad protein assay using human transferrin (Sigma Chemical Co.) as the standard.

**Bacteria.** A clinical isolate (designated A7154) of *A. actinomycetemcomitans* from an active case of LJP was kindly provided by V. R. Dowell, Jr., Centers for Disease Control, Atlanta, Ga. Strain 627, generously provided by Anne Tanner, Forsyth Dental Center, Boston, Mass., and ATCC

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25923 were also tested. The bacteria were maintained on tryptic soy agar supplemented with 5% sterile horse serum (TSA-HS) at 37°C in 85% $N_2$–10% $H_2$–5% $CO_2$. Lyophilized stock cultures were maintained at −70°C and subcultured on TSA-HS or in Schaedler broth when required.

**LF killing assay.** A 0.05-ml sample of a stationary-phase (48-h) culture of *A. actinomycetemcomitans* in Schaedler broth was added to 8 ml of fresh Schaedler broth and grown to mid-exponential phase (optical density at 660 nm, between 0.15 and 0.18) under anaerobic conditions with constant stirring. Clumps were disrupted by mild sonication (Branson model 200 sonifier), and the organisms were harvested by centrifugation (10,000 × *g*, 10 min). Following two washes with 0.15 M NaCl, the cells were suspended to a final concentration of approximately $5 \times 10^7$ CFU per ml in 0.15 M NaCl and maintained at room temperature and atmosphere. Specified amounts of LF were added to 0.1 ml of bacteria suspension, and the final volume was brought to 1.0 ml with simple broth (SB). For controls, the volume of LF solution was replaced by deionized, sterile $H_2O$. SB consisted of 0.85% NaCl (wt/vol), 0.20% $NH_4Cl$ (wt/vol), 1.00% glucose (wt/vol), and 0.10% (wt/vol) yeast extract (Difco Laboratories). Unless otherwise specified, the pH of SB was adjusted to 5.5 with 0.01 N HCl or 0.01 NaOH. Following filter sterilization, pH values for all solutions were reconfirmed before each experiment. For experiments with magnesium, $MgCl_2$ was dissolved at 10× concentration in SB and pH was adjusted prior to filtration. A colorimetric assay for magnesium (procedure no. 595; Sigma) in SB alone gave results below detectable (0.02 mM) levels. Unless noted otherwise, incubations were carried out at 37°C in dry-heat temperature blocks (Fisher Scientific Co.) controlled to ±0.1°C. Samples were withdrawn at specified intervals, diluted in deionized, sterile $H_2O$, and plated in duplicate on Schaedler agar by using the Spiral Plater System (Spiral Systems, Inc.). Plates were incubated at 37°C in 85% $N_2$–10% $H_2$–5% $CO_2$ for 48 h, and CFU were quantitated with a laser-actuated bacteria colony counter (Spiral Systems, Inc.). The theoretical limit of detection for all experiments was 2.4 log units.

**Statistics.** A minimum of three repetitions were performed for each experiment. For all figures, each point represents the mean of at least two determinations performed in duplicate. Error bars were omitted for figure clarity; however, the standard error for all points was less than 10% of the mean. Results were analyzed by one-way analysis of variance and the Fisher least significant difference method at the 5% level of significance as described by Koopmans (19).

## RESULTS

**Killing of purified human LF by *A. actinomycetemcomitans*.** Although the ultimate intraphagolysosomal concentration of LF in PMN is unknown, it likely exceeds the concentration found in human saliva (0.05 to 0.09 mg/ml; 0.6 to 1.2 μM) and gingival plaque fluid (0.2 to 0.85 mg/ml; 2.5 to 11 μM) (14, 27). Treatment of each *A. actinomycetemcomitans* strain with 1.9 μM apo-LF resulted in the loss of recoverable CFU over time compared with treatment of controls. The loss in CFU versus controls for each strain was as follows ($\log_{10}$ of LF-treated bacteria [$6.3 \times 10^6$ CFU/ml] per ml minus CFU of controls ± the standard error of the means of three experiments performed in duplicate): strain 29523, −1.88 ± 0.07; strain 627, −1.94 ± 0.12; strain A7154, −1.72 ± 0.04. Since sensitivity to killing by LF and other parameters examined below were equivalent among the various strains, we chose for clarity to focus this report on results obtained with A7154. Also, clinical access to the LJP patient source of this strain was relevant to future work discussed later. Killing of *A. actinomycetemcomitans* A7154 was concentration dependent and could be detected with as little as 0.5 μM LF (Fig. 1). With 1.9 μM LF, this killing achieved significance at the 5% level in 40 min. Note that a 3-log reduction in CFU (99.9%) seen in 180 min when 1.9 μM LF was used required only 80 min with 3.8 μM LF. As demonstrated with other microorganisms, saturation of the LF molecule with iron and coordinate anion abolished bactericidal activity (3). Viability of *A. actinomycetemcomitans* incubated with 3.8 μM saturated LF was equivalent to that of controls. Even at the highest concentration of saturated LF tested (10 μM; data not shown), no loss of CFU was detected.

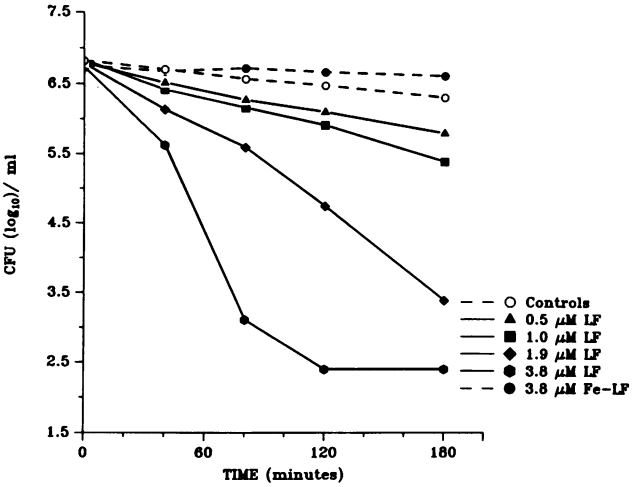


FIG. 1. Bactericidal activity of human LF against *A. actinomycetemcomitans* A7154. The effect of LF concentration and prior iron-anion saturation of LF (Fe-LF) on killing is shown. Each point represents the mean of CFU from duplicate reaction mixtures plated twice on Schaedler agar. The standard error for all points was less than 10% of the mean (error bars omitted for clarity). The theoretical detection limit was 2.4 log units.

**Factors that influence killing. (i) Temperature.** Suspensions of *A. actinomycetemcomitans* with and without 1.9 μM apo-LF were incubated in preequilibrated dry-heat temperature blocks (Fisher) at 35, 36, 37, 38, and 39°C ± 0.1°C. Minor temperature differences substantially altered the kinetics of killing by LF (Fig. 2). While bactericidal activities at 36 and 37°C were equivalent, significantly more (nearly 1 log) *A. actinomycetemcomitans* were killed in 120 min at 38°C. Killing was virtually complete (99.99%) in 80 min at 39°C. Higher temperatures resulted in loss of control CFU and were not tested. Compared with activity at 36 or 37°C, bactericidal activity of LF at 35°C was significantly decreased by 80 min. No differences in viability were detected in 180 min between LF-treated and control suspensions held at room temperature (22°C).

**(ii) pH.** LF activity against *A. actinomycetemcomitans* was highly dependent on hydrogen ion concentration (Fig. 3). At or above pH 5.8, no significant reduction in CFU was detected with 1.3 μM LF. Below this level, incremental reductions in pH resulted in increasing LF activity. CFU were reduced 78% versus controls in 180 min at pH 5.6, while a 95% reduction was seen at pH 5.5. Moreover, killing was significantly greater in 120 min at pH 5.4 compared with that at pH 5.5. Beginning at pH 5.2 and below, some loss of

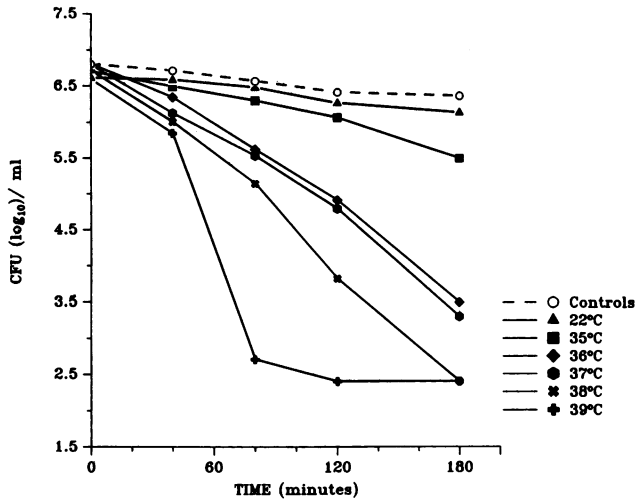


FIG. 2. Temperature dependence of LF activity. LF concentration was 1.9 μM. Controls were incubated at the indicated temperatures without LF, and data represent the mean CFUs of all control mixtures. The limit of detection was 2.4 log units.

control CFU was noted, suggesting toxicity of the acidic environment alone. However, addition of LF under these conditions resulted in rapid and complete (below detectable limits) killing of *A. actinomycetemcomitans* (data not shown).

**(iii) Magnesium.** Magnesium was added as $MgCl_2$ to SB, and the pH was adjusted to 5.5 prior to filtration. While concentrations of $Mg^{2+}$ below 1 mM did not alter LF activity versus controls (data not shown), concentrations between 1 and 10 mM in the presence of 1.3 μM LF progressively reduced recoverable CFU over time versus LF alone (Fig. 4A). Significantly greater killing was seen with both 7 and 10 mM $Mg^{2+}$ in 120 min than with LF alone. Although insignificant at the 5% level, the addition of 1 mM $Mg^{2+}$ to 0.5 μM LF resulted in 57% greater killing than with LF alone in 180 min (Fig. 4B). A significant increase (90%) in killing was seen with the addition of 10 mM magnesium. While 20 mM magnesium further enhanced LF killing activity, bacterial viability was not reduced with that concentration of $Mg^{2+}$ alone. The order of addition of the magnesium and LF to the bacteria did not affect the results. Replacement of $MgCl_2$ by either $CaCl_2$ or KCl at an equivalent molar concentration of cation had no effect on LF activity (Table 1).

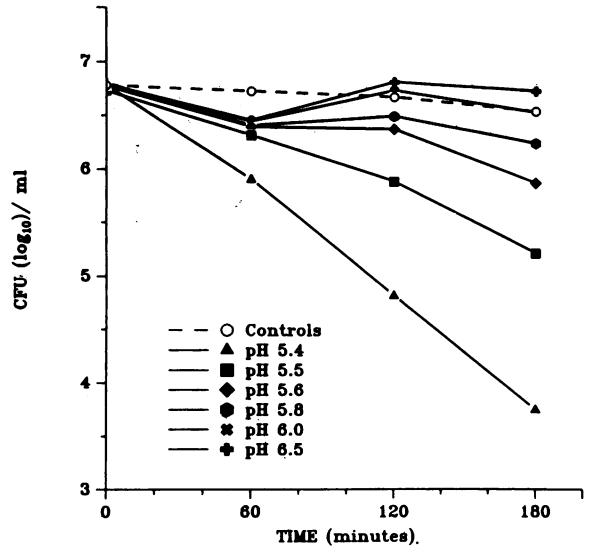


FIG. 3. Effect of pH on LF killing of *A. actinomycetemcomitans*. The LF concentration was 1.3 μM. The data for controls represent means of all reaction mixtures without LF.

## DISCUSSION

*A. actinomycetemcomitans* was sensitive to killing by LF in vitro at concentrations comparable with those present physiologically in the secretory environment of the oral cavity (14, 27). Besides exhibiting concentration dependence, LF activity against *A. actinomycetemcomitans* required an unsaturated LF molecule. As previously shown with other LF-sensitive organisms, saturated LF (iron plus anion) was not bactericidal (3). Like other members of the transferrin family, specific and high-affinity binding of iron by LF does not occur without suitable anion, usually bicarbonate or carbonate (1, 2). Certain anions, such as EDTA or oxalate, can readily replace bicarbonate, yet others, such as thiocyanate, acetate, or sulfate, are poorly bound in coordinate fashion (1). Readily bound anions have been shown to reduce or block LF killing of *Streptococcus mutans*, while up to 1,000-fold-greater concentrations of lower-affinity anions are required to achieve the same effect (20). Since addition of exogenous iron does not competitively inhibit bacterial killing (including *A. actinomycetemcomitans* [data not shown]), anionic sites on the bacterial surface may be more relevant to LF activity. Experiments to examine the effect of anions on LF killing of *A. actinomycetemcomitans*, however, were compromised by the finding that several anions were independently toxic to the bacteria (data not shown) as previously reported with EDTA (17).

LF activity against *A. actinomycetemcomitans* was strongly influenced by environmental pH. Progressive loss of LF activity with increasing pH may reflect increased protonation of anionic targets or physical alterations in the bacterial surface which may hinder LF binding. In addition, killing may require target binding by the acid-stable iron-anion site of the LF molecule, which would be favored by lower pH (21). Significant conformational changes of the LF molecule that occur with increasing pH may also contribute to the observed reduction in activity (7). Also, as mentioned below for magnesium, LF binding may disrupt bacterial transport mechanisms and render the microorganisms more vulnerable to hydrogen ion toxicity. Salivary pH levels from less than 5.5 to 8.0 have been reported in 12-year-old children (35), and in a study of dental plaque, the pH ranged from 4.3 to 7.3 (37). While more alkaline values (pH 7.0 to 8.0) have been recorded for gingival fluid (8), certain microflora within the periodontal pocket may produce locally acidic conditions that would promote LF killing of *A. actinomycetemcomitans*. This would support the concept that other oral bacteria interfere with *A. actinomycetemcomitans* colonization and growth (16). In addition, intraphagolysosomal acidification following ingestion would be expected to enhance LF killing of *A. actinomycetemcomitans* by host neutrophils (12).

Sensitivity of LF killing to temperature has been previously demonstrated with other microorganisms (6; M. A. Motley and R. Arnold, Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, CCI, p. 11). With gram-negative bacteria, we postulate that increasing fluidity of the outer membrane with higher temperatures may lead to increased exposure of

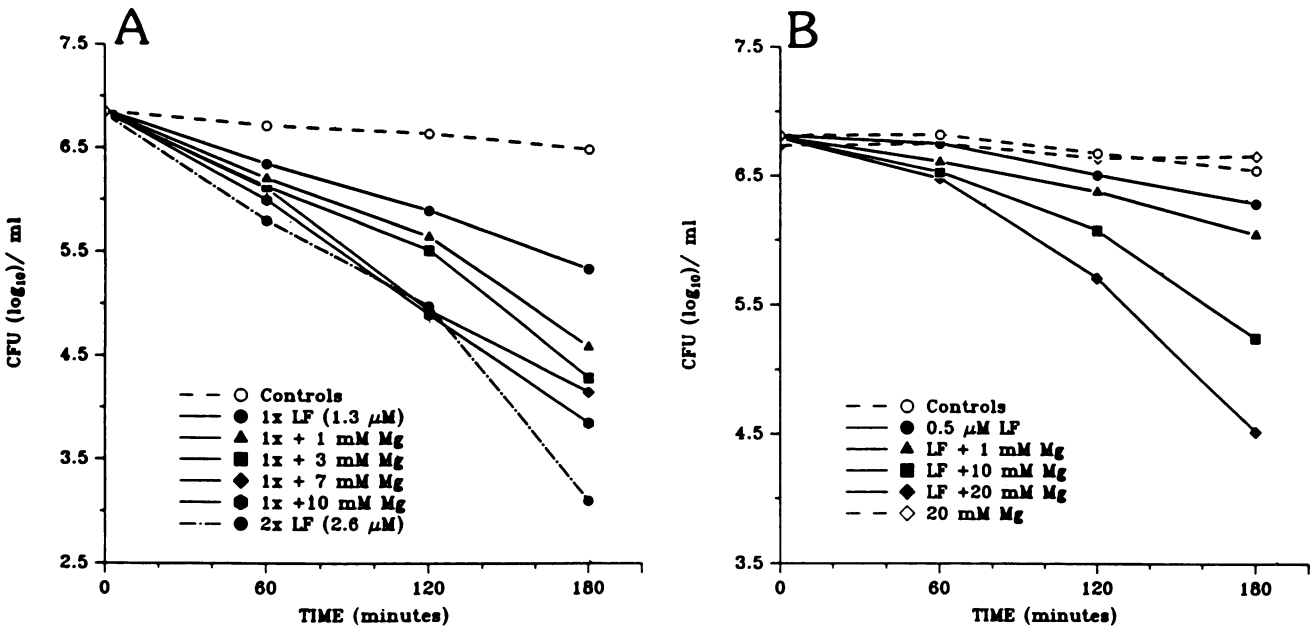


FIG. 4. Effect of magnesium on bactericidal activity of LF. (A) 1×, 1.3 μM LF; 2×, 2.6 μM LF. (B) LF concentration, 0.5 μM. Magnesium was added in the form of $MgCl_2$. The data for controls represent means of all reaction mixtures without LF, except for 20 mM magnesium, which is shown separately.

vulnerable target sites. The finding that LF killing of *A. actinomycetemcomitans* was sensitive to minor changes in temperature is of interest with respect to clinical aspects of periodontal disease. In adult periodontitis, sites of disease activity have been shown to exhibit temperatures 1°C higher than temperatures at inactive sites (R. T. V. Kung and J. M. Goodson, Abstr. Meet. Int. Assoc. Dent. Res. 1986, no. 527, p. 228). This would result in significant enhancement of *A. actinomycetemcomitans* killing by LF at these locations. However, unlike typical adult periodontitis, clinical signs of disease, such as tissue erythema and edema, are not commensurate with the rapidity or severity of alveolar destruction seen in LJP (15). Although the biologic or physiologic basis for this difference is uncertain, it may result in lower ambient temperatures at disease-active sites of LJP patients compared with those of their adult periodontitis counterparts. Our data suggest that this would reduce LF activity and could also impair other temperature-sensitive host defense factors, favoring persistence of *A. actinomycetemcomitans* infection in these individuals.

TABLE 1. Effect of various cations on LF killing of *A. actinomycetemcomitans*

| Treatment | Change in no. of CFU versus control[a] |
|---|---|
| $Mg^{2+}$ | +0.06 ± 0.05 |
| $Ca^{2+}$ | +0.03 ± 0.04 |
| $K^+$ | −0.01 ± 0.11 |
| LF | −0.75 ± 0.06 |
| + $Mg^{2+}$ | −1.46 ± 0.09 |
| + $Ca^{2+}$ | −0.78 ± 0.06 |
| + $K^+$ | −0.74 ± 0.02 |

[a] Data represent CFU ($log_{10}$) of treated bacteria per ml minus CFU of controls. Data are means ± the standard error of the mean of three experiments performed in duplicate. Final cation concentration was 15 mM, and LF concentration was 1.3 μM. Bacteria ($6.2 \times 10^6$/ml) were incubated with cation, LF, or both in SB for 120 min at 37°C and spiral plated in duplicate on Schaedler agar.

Several cations have been shown to be important in the organization of bacterial outer membranes. In particular, $Mg^{2+}$ seems to stabilize lipopolysaccharide interactions and possibly phospholipid-phospholipid interactions (33). Magnesium concentrations of 0.1 to 5 mM were found to inhibit leakage of periplasmic enzymes from deep rough lipopolysaccharide mutants (13) and from lipoprotein-deficient mutants (26). Deep rough mutant sensitivity to hydrophobic inhibitors was decreased by $Mg^{2+}$ (32), as was the bactericidal action of normal serum on smooth and rough enteric bacteria (25). In previous work, concentrations of $MgCl_2$ up to 100 mM did not block LF killing of *S. mutans*, a gram-positive microorganism (6, 10), while the addition of 8 mM $Mg^{2+}$ completely abolished LF activity against gram-negative *Legionella pneumophila* (10). Similar blocking of LF effects with $Mg^{2+}$ has been detected with other gram-negative bacteria, such as *Escherichia coli* and *Salmonella typhimurium* (manuscript in preparation). Taken together, these data are consistent with the concept of outer membrane lipopolysaccharide stabilization by $Mg^{2+}$ as a protective mechanism against LF bactericidal activity.

The current findings suggest that magnesium may play different or additional roles in membrane organization or metabolic function of *A. actinomycetemcomitans*. While concentrations of $Mg^{2+}$ below 1 mM did not detectably affect bacterial viability with or without LF, higher concentrations in the presence of LF resulted in accelerated loss of CFU versus LF alone. The addition of 7 mM $Mg^{2+}$ to 1.3 μM LF produced killing at 120 min equivalent to twice that with the LF concentration alone. The mere presence of cation was not sufficient to enhance LF activity, since killing was unaffected by the addition of either $Ca^{2+}$ or $K^+$. Biologic relevance of this effect is suggested by magnesium concentrations in plaque fluid ranging from 3.7 to 5.3 mM (34). Intracellular $Mg^{2+}$ concentrations in bacteria are reportedly 20 to 40 mM; however, over 95% is bound, primarily by polynucleotides (29). Although we detected no

toxicity with $Mg^{2+}$ alone up to 50 mM (data not shown), higher concentrations did reduce recoverable CFU. These results are consistent with an LF-induced alteration in normal magnesium-handling mechanisms of *A. actinomycetemcomitans*. LF binding at the bacterial surface may permit normally innocuous extracellular levels of $Mg^{2+}$ to accumulate intracellularly as toxic, free magnesium ions. The addition of magnesium to LF-bacteria mixtures would then be expected to enhance the rate of killing seen with LF alone. Direct immunofluorescence has demonstrated that apo-LF binds to the surface of *S. mutans*, while no binding was detected with the iron-saturated molecule (4). The requirement for LF binding to initiate both bactericidal events and susceptibility to $Mg^{2+}$ was supported by the finding that equivalent concentrations of iron-saturated LF and $Mg^{2+}$ had no effect on bacterial viability (data not shown). Further analysis of the effect of magnesium on *A. actinomycetemcomitans* and related microorganisms such as *Haemophilus* spp. may provide important mechanistic insight into the bactericidal activity of LF.

The association of *A. actinomycetemcomitans* with LJP provides an intriguing model system for host-parasite interaction in human disease. Normal human neutrophils have been shown to kill *A. actinomycetemcomitans* by both oxidative and nonoxidative mechanisms (22). A majority of the oxidative killing was attributed to neutrophil myeloperoxidase; however, the role of other mechanisms was not examined. We have recently confirmed the ability of normal neutrophils to phagocytose and kill opsonized *A. actinomycetemcomitans* (18). It was further observed that PMN from LJP patients phagocytose normally but exhibit impaired killing of *A. actinomycetemcomitans*. Such dysfunction may be due to discrete deficiencies in or modifications of the biochemical armamentarium of the PMN. Alternatively, *A. actinomycetemcomitans* may persist in patient PMN by altering the phagosome-lysosome interaction in these cells. Comparative analysis of granule populations from both patient and control PMN and biofunctional assay of components such as LF may provide information crucial to our understanding of the molecular basis for host susceptibility and development of disease.

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