Killing of Gram-negative Bacteria by Lactoferrin and Lysozyme

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Abstract

Although lactoferrin has antimicrobial activity, its mechanism of action is not fully defined. Recently we have shown that the protein alters the Gram-negative outer membrane. As this membrane protects Gram-negative cells from lysozyme, we have studied whether lactoferrin's membrane effect could enhance the antibacterial activity of lysozyme. We have found that while each protein alone is bacteriostatic, together they can be bactericidal for strains of *V. cholerae, S. typhimurium,* and *E. coli.* The bactericidal effect is dose dependent, blocked by iron saturation of lactoferrin, and inhibited by high calcium levels, although lactoferrin does not chelate calcium. Using differing media, the effect of lactoferrin and lysozyme can be partially or completely inhibited; the degree of inhibition correlating with media osmolarity. Transmission electron microscopy shows that *E. coli* cells exposed to lactoferrin and lysozyme at 40 mOsm become enlarged and hypodense, suggesting killing through osmotic damage. Dialysis chamber studies indicate that bacterial killing requires direct contact with lactoferrin, and work with purified LPS suggests that this relates to direct LPS-binding by the protein. As lactoferrin and lysozyme are present together in high levels in mucosal secretions and neutrophil granules, it is probable that their interaction contributes to host defense. (*J. Clin. Invest.* 1991. 88:1080–1091.) Key words: *Escherichia coli* • *Salmonella typhimurium* • *Vibrio cholerae* • lipopolysaccharide • transferrin • iron

Introduction

The proteins lysozyme and lactoferrin are major components of the specific granules of human polymorphonuclear leukocytes and are also found in high concentration in human mucosal secretions, including those of the eye, the oropharynx, the breast, the respiratory tract, and the vagina (1–3). Several lines of evidence suggest that their localization at these sites is related to roles in the human host defense system. First, during the inflammatory response both are actively secreted by the polymorphonuclear leukocyte into the external environment as this cell releases the contents of its specific (secondary) granules (4). Second, each of the proteins has been found to have independent antimicrobial activity (5–7).

Human lysozyme is a 1,4-β-*N*-acetylmuramidase that enzymatically degrades a glycosidic linkage of bacterial peptidoglycan (8). Proteins with comparable activity have been identified in essentially all vertebrates suggesting that this is a highly conserved enzyme. Acting alone human lysozyme lyses and kills several Gram-positive microorganisms by damaging their surface-exposed peptidoglycan. In contrast, most Gram-negative organisms are resistant to killing by the protein. In these organisms an outer membrane shields the peptidoglycan murein sacculus from the external environment (9, 10). As lysozyme cannot easily penetrate the outer membrane, the organisms are resistant to its effects and the protein has routinely been considered to have at most a secondary function in the host defense against these pathogens (11).

Lactoferrin was first recognized as a high-affinity iron chelator in human milk, but in the 1960s was found to have bacteriostatic properties (2, 12). Because these effects on bacteria could be markedly reduced or eliminated by iron saturation of the protein, it was hypothesized that lactoferrin acts to deprive microorganisms of the essential nutrient iron (13). Whereas subsequent work has continued to confirm this theory, several studies have indicated that lactoferrin may be directly bactericidal and that it can also markedly enhance the activity of secretory IgA (sIgA) (14–17). These observations suggest that lactoferrin has additional mechanisms of action towards bacteria beyond those related to iron deprivation.

Recent work in our laboratory has confirmed such activity. We have found that lactoferrin, and the related iron chelator transferrin, can damage the outer membrane of enteric Gram-negative bacilli (18–20). To varying degrees the two proteins cause release of LPS molecules from the bacterial cell, sensitize organisms to antibiotics such as rifampin and cefotaxime, and enhance susceptibility to the detergent deoxycholate. This constellation of properties is similar to those of a number of different agents that permeabilize the Gram-negative outer membrane, including metal chelators such as EDTA and polycationic compounds such as polymyxin B nonapeptide, poly-*l*-lysine, the defensins, and the bactericidal permeability increasing protein of neutrophils (BPI) (21–24). In that these agents can enhance the susceptibility of Gram-negative organisms to lysozyme by increasing penetration of the protein through the outer membrane, we have undertaken studies to determine if lactoferrin and transferrin could have comparable effects. Additionally, as lactoferrin and lysozyme almost always coexist in vivo, demonstration of an interaction is of both physiological and mechanistic relevance.

Methods

Lactoferrin, transferrin, and lysozyme. Human milk lactoferrin was purchased commercially (Sigma Chemical Co., St. Louis, MO, or Calbiochem-Behring Corp., La Jolla, CA), as was human serum trans-

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ferrin (Sigma Chemical Co.) and human placental lysozyme (Calbiochem-Behring Corp.). Iron-saturated lactoferrin was obtained both commercially and prepared according to the method of West and Sparling (25). For selected experiments further purification of the commercial lysozyme was performed on an FPLC system (Pharmacia Fine Chemicals, Piscataway, NJ) using a Mono S column (Pharmacia Fine Chemicals) and 0.01 M, pH 7.0, imidazole buffers (buffer A was imidazole alone, and buffer B imidazole with 1 M NaCl) in a series of linear gradients with the following parameters: flow rate 1 ml/min, 0–5 min 0% buffer B, 8 min 17% buffer B, 55 min 22% buffer B, 60 min 100% buffer B. Eluted fractions were tested for lysozyme lytic activity on *Micrococcus luteus* agar plates (26). Immunological identity was confirmed by double immunodiffusion using goat anti-human lysozyme (Kallestad, Austin, TX) and subsequent SDS-PAGE. Material from two runs was pooled, concentrated, and rechromatographed under identical conditions. This material was concentrated before use in time-kill assays.

Analytic procedures. Protein concentrations were determined either by the biuret or the bicinchoninic acid–copper sulfate techniques (27, 28). The levels of iron in individual samples were measured using flameless atomic absorption spectrophotometry (model 5000 spectrophotometer [Perkin-Elmer Corp., Norwalk, CT] equipped with a model 500 programmer and an AS-1 auto sampler) in the laboratory of Dr. Allen Alfrey (Department of Veterans Affairs Medical Center [DVAMC], Denver, CO). The osmolarity of solutions was determined by freezing point depression (Laboratory Wide-Range Model 3WII Osmometer; Advanced Instruments, Needham Heights, MA). Determination of concentrations of sodium, potassium, chloride, bicarbonate, calcium, and magnesium were performed in the Clinical Chemistry Laboratory of the Denver DVAMC. Analysis of carbohydrate fermentation by bacteria was performed using modified chromogenic assays in a microtiter system (MicroScan, West Sacramento, CA) (29). Measurement of LPS concentration was performed using a quantitative, kinetic, chromogenic limulus amebocyte lysate assay (Whittaker M. A. Bioproducts, Walkersville, MD) with a kinetic ELISA reader and software system (Molecular Devices, Menlo Park, CA) using purified *E. coli* O55 B5 endotoxin as a standard. Discontinuous SDS-PAGE analysis of proteins was performed in a modified Laemmeli system using 1.5-mm slab gels with 4.5% stacking and 10% resolving sections, and subsequent analysis by silver stain (30–32). The methods of Perez-Perez and Blaser were used for SDS-PAGE of bacterial LPS (33). Briefly, pelleted cells were resuspended in 200 μl solubilizing buffer and the supernatants lyophilized and resuspended in 100 μl solubilizing buffer. All samples were treated with proteinase K for 1 h at 60°C and boiled for 10 min. Discontinuous SDS-PAGE using the Laemmeli system was performed with 4.5% stacking and 15% resolving gels and analysis by silver stain (34).

Bacterial strains. Vibrio cholerae 3083 was obtained from Dr. Mary Boesman-Finkelstein (University of Missouri, Columbia, MO); the *O*111 B4 UDP galactose epimerase deficient *Escherichia coli* CL99-2 strain from Dr. Keith A. Joiner (National Institutes of Health, Bethesda, MD); and *Salmonella typhimurium* strain SL696 from Dr. Ilkka M. Helander (National Public Health Institute, Helsinki, Finland) (16, 35–38). *E. coli* 5448 is a human gastrointestinal isolate from the University of Colorado Health Sciences Center Clinical Microbiology Laboratory (19). SDS-PAGE analysis of bacterial LPS demonstrated that all four strains had a "smooth" wild-type LPS phenotype (data not shown).

Time-kill studies. Peptone media were obtained commercially (Difco, Detroit MI) and 1% wt/vol solutions prepared. WMS, Davis and Luria broth were prepared according to published recipes (18, 39–41). Bacteria were grown to stationary phase, the cells centrifuged and washed. A 5×10^5 CFU bacterial inoculum was added to 500 μl of media with or without transferrin (2.0 mg/ml), lactoferrin (2.0 mg/ml), lysozyme (0.5 mg/ml), or EDTA (8×10^{-5} M). The mixtures were incubated at 37°C, aliquots removed, serially diluted, and plated overnight at 37°C on soybean casein digest agar (Sigma Chemical Co.). For studies with *V. cholerae* 3083 the strain was grown to stationary phase in proteose peptone 3 with 0.3 mM $CaCl_2$ and then tested in proteose peptone 3. For all other strains, bacterial cells were grown and tested in the same media. For osmolarity studies, 1% bacto-peptone was supplemented with myo-inositol (Sigma Chemical Co.). For selected experiments *E. coli* 5448 cells were grown for 24 h in 1% bacto-peptone with 0.05 mg/ml lysozyme, washed, and then incubated with lactoferrin and lysozyme as above. Dialysis cell studies were performed with 1% bacto-peptone in 1 ml equilibrium dialysis chambers with 6,000 D exclusion regenerated-cellulose membranes (Bel-Art, Pequannock, NJ).

Transmission electron microscopy (TEM). 5×10^8 CFU inocula of *E. coli* 5448 were added to 500 μl of 1% bacto-peptone with or without lactoferrin (2.0 mg/ml) or repurified lysozyme (0.005 mg/ml). The mixtures were incubated at 37°C for 24 h and the bacterial cells pelleted. The pellets were resuspended in 2% glutaraldehyde in 0.1 M cacodylic acid, pH 7.4, for 1 h at 4°C. Samples were postfixed in buffered 1% osmium tetroxide, dehydrated through a graded series of ethanols, and embedded in poly/bed 812-araldite (Mollenhauer medium; Polysciences, Inc., Warrington, PA). For electron microscopy, 70-nm thin sections were obtained with diamond knives and stained routinely with aqueous solutions of uranyl acetate and lead citrate. Sections were examined with a Philips CM-12 transmission electron microscope at 60 kV.

Equilibrium dialysis studies. Binding of calcium was studied by adding 750 μl of a solution of $^{45}CaCl_2$ in pH 7.4 HBSS lacking calcium and magnesium (HBSS-CM) (Whittaker M. A. Bioproducts) to one side of acrylic equilibrium dialysis cell chambers with 6,000 D exclusion regenerated-cellulose membranes. To the opposite side of the dialysis chambers were added solutions of either 2 mg/ml of BSA (Sigma Chemical Co.), transferrin, lactoferrin, apo- or iron-saturated lactoferrin, or 260 μl of 20% Chelex 100 (Bio-Rad Laboratories, Inc., Richmond, CA) in HBSS-CM. The cells were allowed to equilibrate at room temperature and serial aliquots sampled over 96 h.

LPS binding studies. To study the ability of the iron-binding proteins to bind LPS; lactoferrin, transferrin, BSA, poly-*l*-lysine (Sigma Chemical Co.), and soybean trypsin inhibitor (Sigma Chemical Co.) were coupled to cyanogen bromide-activated Sepharose 4B beads (Pharmacia Fine Chemicals) at a concentration of 1×10^{-7} mol/ml gel (molecular weight range of poly-*l*-lysine is 1,000–4,000, and was estimated at 2,000). After protein coupling the beads were blocked in pH 8.0 Tris buffer, and stored in pH 7.2, 0.03 M barbital-acetate, 0.116 M NaCl buffer (BABS) with 0.02% thimerosol. To control for nonspecific binding, Sepharose beads were also prepared that were not reacted with protein, but instead simply blocked with Tris.

Tritium-labeled LPS was prepared by growing *E. coli* CL99-2 in modified WMS broth supplemented with D-[6-^{3}H] galactose (Amersham Corp., Arlington Heights, IL), and extracting LPS either by washing the cells in pH 8.0 barbital-acetate buffer or by the phenol-water method of Westphal (18, 42). When this strain is grown in the presence of [^{3}H]-galactose, the radiolabel is almost exclusively incorporated into the LPS *O*-polysaccharide (35). For experiments requiring high LPS concentrations the [^{3}H]-LPS was supplemented with similarly prepared unlabeled LPS.

Binding of the LPS was determined by incubating 0.1 ml of the protein-Sepharose or Tris-Sepharose beads with varying concentrations of [^{3}H]-LPS in pH 7.2 BABS for 5 min with gentle shaking. The beads were then pelleted by centrifugation, washed twice with BABS, and the beads and pooled BABS wash material counted by liquid scintillation.

Results

Interaction of lactoferrin and lysozyme. Initial studies were performed addressing the effects of lactoferrin and lysozyme against *V. cholerae* 3083 based on a preliminary observation of Boesman-Finkelstein and Finkelstein that this strain could be killed by human milk lactoferrin and lysozyme (16). We found that over 24 h this strain consistently demonstrated over a 1,000-fold increase in CFU/ml when grown either in proteose peptone 3 broth alone or with 0.5 mg/ml human lysozyme

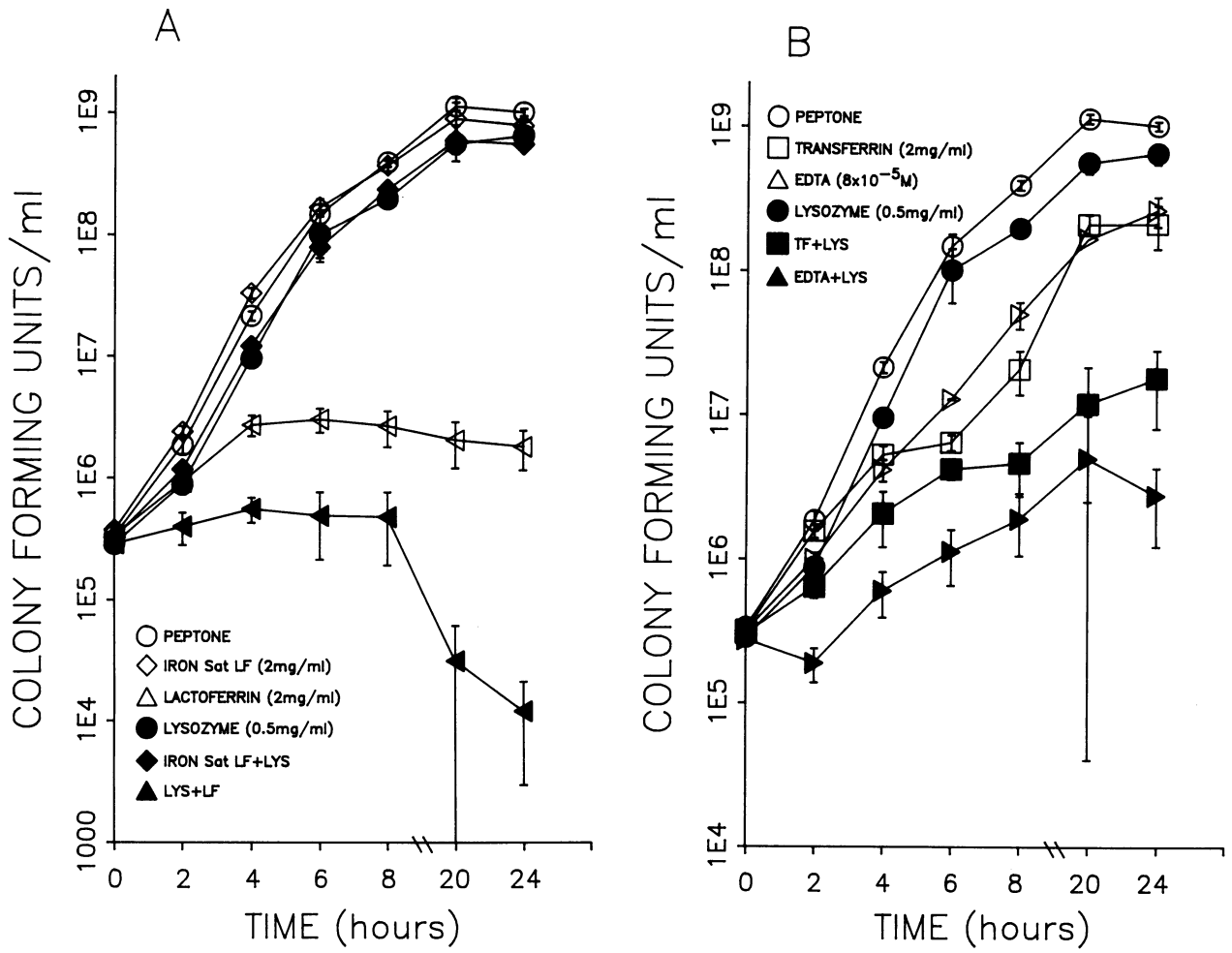

Figure 1. (*A*) Effects of lysozyme (LYS) (0.5 mg/ml) and human apo- and iron-saturated lactoferrin (LF) (2.0 mg/ml) on the growth of *V. cholerae* 3083 in 1% proteose peptone 3 (mean±SEM, seven experiments; 6-, 8-, and 20-h findings represent three, three, and two observations respectively). (*B*) Effects of lysozyme (LYS) (0.5 mg/ml), transferrin (TF) (2.0 mg/ml), and 8×10^{-5} M EDTA on the growth of *V. cholerae* 3083 in 1% proteose peptone 3 (mean±SEM, seven experiments; 6-, 8-, and 20-h findings represent three, three, and two observations, respectively).

(Fig. 1 *A*). In contrast, 2 mg/ml ($\approx 2 \times 10^{-5}$ M) human lactoferrin was bacteriostatic alone, and the combination of lactoferrin and lysozyme was consistently bactericidal. Iron saturation of lactoferrin inhibited both the bacteriostatic and synergistic bactericidal effect of lactoferrin.[1] Parallel studies performed with 2 mg/ml ($\approx 2 \times 10^{-5}$ M) human transferrin and 8×10^{-5} M EDTA indicated that both of these chelators could also enhance the activity of lysozyme, but neither to the degree of lactoferrin (Fig. 1 *B*).

Experiments were then performed to determine if lactoferrin and lysozyme could have similar effects on other Gram-negative organisms. Studies with 1 *S. typhimurium* and 2 *E. coli* strains again found that lactoferrin markedly enhanced the activity of lysozyme (Fig. 2). The strains differed in their susceptibility to the combination of lactoferrin and lysozyme as well as in their susceptibility to lysozyme combined with transferrin or EDTA. Such variability had been noted in prior studies of the effects of lactoferrin, transferrin, and EDTA on LPS release and antibiotic susceptibility of these strains (18, 20).

As *E. coli* 5448 appeared to be the most susceptible strain to the effects of lactoferrin and lysozyme, this isolate was used to define how varying either the concentration of the two proteins or the bacterial inoculum size would affect the interaction. As expected from prior observations on the effects of lactoferrin on LPS release (18), the synergistic activity of the two proteins was found to be dose dependent for lactoferrin (Fig. 3 *A*). Comparable results were found for lysozyme (Fig. 3 *B*). In studies with differing bacterial inocula the activity of lactoferrin and lysozyme was comparable against 5×10^5 and 5×10^6 CFU/ml, but diminished when the bacterial inoculum was increased to 5×10^7 CFU/ml (Fig. 4). To confirm that an impurity in the commercial lysozyme was not contributing to the observed interactions with lactoferrin, transferrin, and EDTA; experiments were repeated with lysozyme that was repurified by ion-exchange chromatography. The purified preparation again showed a synergistic interaction with lactoferrin (data not shown).

Influence of media composition. Studies were then performed to assess the effect of media composition on the lacto-

1. These studies were performed with commercially obtained iron-saturated lactoferrin. Comparable results were subsequently obtained with iron-saturated lactoferrin prepared by the method of West in studies with *E. coli* 5448.

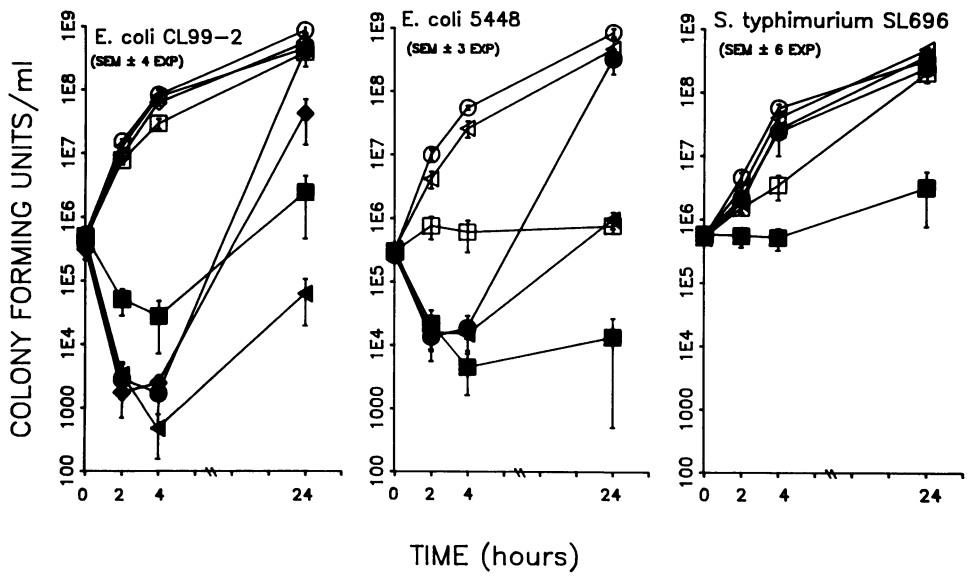

Figure 2. Effects of lysozyme, lactoferrin (2.0 mg/ml), transferrin (2.0 mg/ml), and 8×10^{-5} M EDTA on the growth of *E. coli* and *S. typhimurium* strains. Lysozyme was tested at 0.05 mg/ml for studies with *E. coli* strains and 0.5 mg/ml for *S. typhimurium*. (*Open circles*) Proteose peptone 3; (*open squares*) lactoferrin; (*open triangles*) transferrin; (*open diamonds*) EDTA; (*solid circles*) lysozyme; (*solid squares*) lactoferrin + lysozyme; (*solid triangles*) transferrin + lysozyme; (*solid diamonds*) EDTA + lysozyme.

ferrin-lysozyme interaction. When other media were substituted for proteose peptone 3 in parallel experiments, the activity of lactoferrin and lysozyme towards *E. coli* 5448 changed significantly (Fig. 5). Although the effects of lactoferrin and lysozyme were similar in proteose peptone 3 and proteose peptone, the activity was enhanced in bacto-peptone. In contrast, there was no bactericidal effect noted when the strain was tested in either proteose peptone 2 or in the more defined WMS, Luria and Davis media (Table I). To determine what factors in media composition contributed to the variation in the effects of lactoferrin and lysozyme, the electrolyte composition and osmolarity of each of the media was defined. We noted no apparent relationship between the lactoferrin-lysozyme activity and concentrations of calcium, magnesium, iron, sodium, potassium, or chloride. However, there appeared to be a consistent inverse correlation between the degree of killing and media osmolarity. Lactoferrin-lysozyme activity was absent in media above 60 mOsm.

To test the hypothesis that media osmolarity influenced lactoferrin-lysozyme killing, parallel time-kill studies were then performed in 40 mOsm bacto-peptone media that was supplemented with graded concentrations of a metabolically inactive material. Preliminary studies found that *E. coli* 5448 did not ferment inositol, and this sugar was used as an osmotic supplement. It was found that the addition of increasing concentrations of *myo*-inositol to the media did not alter the bacterial growth curve but did affect the bactericidal effects of lactoferrin and lysozyme (Fig. 6). Bacterial killing progressively decreased as the media osmolarity was increased, and was completely absent when the osmolarity was increased to 100 mOsm.

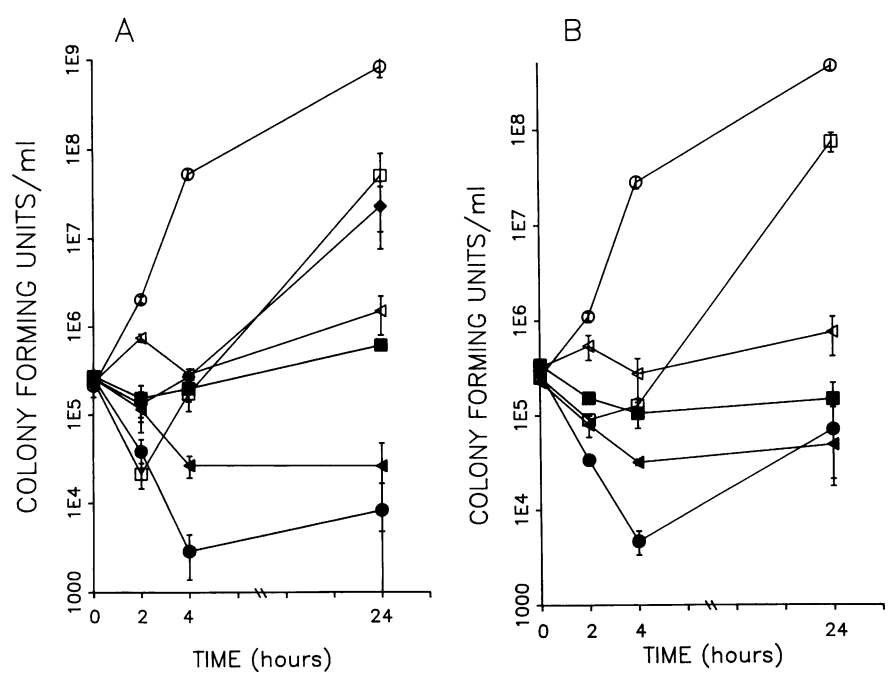

Figure 3. (*A*) Effect of varying lactoferrin concentration on its interaction with lysozyme against *E. coli* 5448 (mean±SEM, three experiments). (*Open circles*) Proteose peptone 3; (*open triangles*) 2 mg/ml lactoferrin; (*open squares*) 0.025 mg/ml lysozyme; (*solid circles*) 4 mg/ml lactoferrin + lysozyme; (*solid triangles*) 2 mg/ml lactoferrin + lysozyme; (*solid squares*) 1 mg/ml lactoferrin + lysozyme; (*solid diamonds*) 0.5 mg/ml lactoferrin + lysozyme. (*B*) Effect of varying lysozyme concentration on its interaction with lactoferrin against *E. coli* 5448 (mean±SEM, four experiments). (*Open circles*) Proteose peptone 3; (*open triangles*) 2 mg/ml lactoferrin; (*open squares*) 0.025 mg/ml lysozyme; (*solid circles*) lactoferrin + 0.05 mg/ml lysozyme; (*solid triangles*) lactoferrin + 0.025 mg/ml lysozyme; (*solid squares*) lactoferrin + 0.0125 mg/ml lysozyme; (*solid diamonds*) lactoferrin + 0.0063 mg/ml lysozyme.

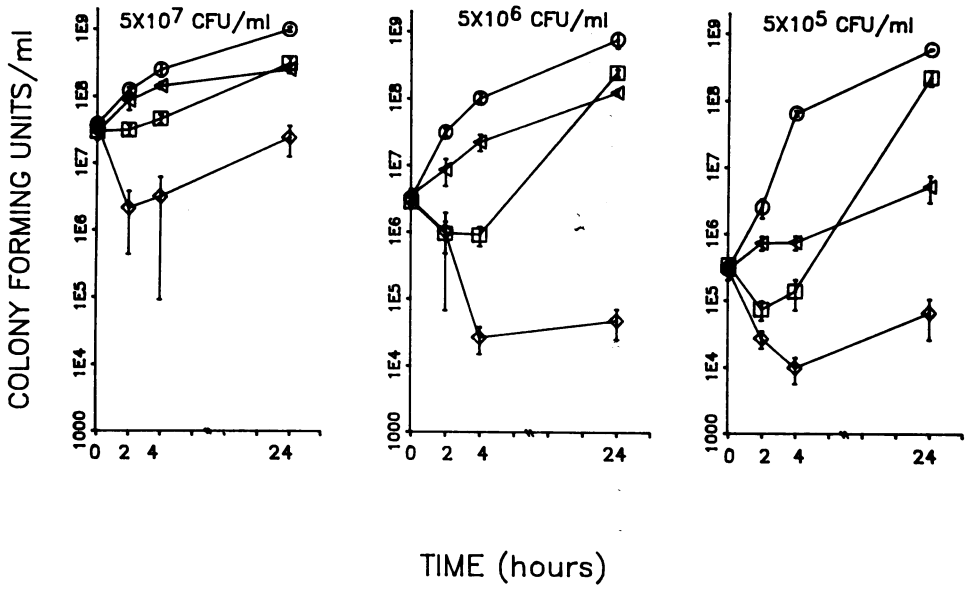

Figure 4. Effect of bacterial inoculum size on the activity of lactoferrin and lysozyme against *E. coli* 5448 (mean±SEM, three experiments). (*Open circles*) Proteose peptone 3; (*open triangles*) 2 mg/ml lactoferrin; (*open squares*) 0.05 mg/ml lysozyme; (*open diamonds*) lactoferrin + lysozyme.

As consistent early killing and subsequent regrowth of *E. coli* 5448 cells was observed with exposure to lysozyme alone, to determine if this represented the emergence of resistant cells, further time-kill studies were performed using cells that had been grown for 24 h in 1% bacto-peptone media with 0.05 mg/ml lysozyme. It was found that these *E. coli* cells grown in the presence of lysozyme remained susceptible to the combined effects of lactoferrin and lysozyme, although in matched studies the concentration of lysozyme needed to be increased fourfold (0.0125–0.05 mg/ml) to achieve a degree of killing comparable with that of cells not previously exposed to lysozyme.

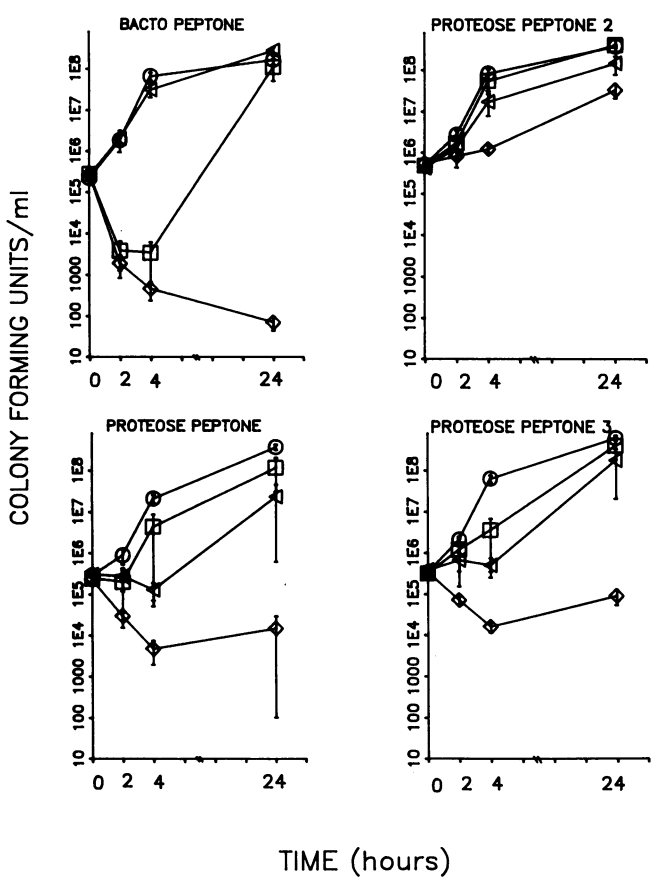

Figure 5. Evaluation of lactoferrin-lysozyme bactericidal activity towards *E. coli* 5448 in differing peptone media (mean±SEM, three experiments). (*Open circles*) Media alone; (*open triangles*) 2 mg/ml lactoferrin; (*open squares*) 0.05 mg/ml lysozyme; (*open diamonds*) lactoferrin + lysozyme.

TEM studies. One model to explain the effect of media osmolarity on bacterial killing by lactoferrin and lysozyme would be that the bactericidal effect is primarily related to degradation of the peptidoglycan matrix. Without the protection of this rigid framework, under low osmotic conditions the bacterial cells would absorb fluid allowing them to balloon and explode. Such a phenomenon would not occur at high osmolarity. To evaluate this possibility, bacterial cells were examined by TEM after 24 h of growth in 40 mOsm bacto-peptone with or without lactoferrin or lysozyme. It was found that the majority of bacterial cells were cylindrical when grown in media alone (Fig. 7 *A*) or in the presence of either lactoferrin (Fig. 7 *B*) or lysozyme (Fig. 7 *C*) individually. In contrast, in keeping with the hypothesis of damage to the peptidoglycan matrix, when cells were exposed to lactoferrin and lysozyme together (Fig. 7 *D*) there was a large amount of cell debris noted and a number of the remaining cells appeared to be quite enlarged, hypodense, and rounded.

Influence of calcium and magnesium concentration. Further work was then initiated to more precisely define how lactoferrin damages the outer membrane and alters bacterial susceptibility to lysozyme. Based on prior work indicating that the calcium and magnesium concentration could significantly influence the ability of lactoferrin and transferrin to either induce LPS release or change bacterial susceptibility to deoxycholate (18, 20), the effects of these cations on lactoferrin-lysozyme synergy towards *E. coli* 5448 were evaluated using concentrations similar to those studied previously. Although the addition of either 1.3 mM $CaCl_2$ or 0.8 mM $MgCl_2$ to bacto-peptone had only minimal effect on media osmolarity (measured osmolarity remaining $\approx$ 40 mOsm), the presence of either cation inhib-

Table I. Analysis of the Bactericidal Activity of Lactoferrin and Lysozyme in Relation to Media Composition

Media studied	Lactoferrin -lysozyme 24 h Log_{10} change	Mg^{+2}	Ca^{+2}	Fe	Na^+	K^+	Cl^-	CO_2	Osmolarity
		mM	*mM*	*ppb*	*mM*	*mM*	*mM*	*mM*	*mOsmol*
Bacto-peptone	−3.7	0.1	0.1	35	10	0.7	12	3	41
Proteose peptone	−2.8	0.2	0.2	114	18	2.8	15	3	54
Proteose peptone 3	−0.7	0.2	0.2	187	18	3.4	12	3	57
Proteose peptone 2	1.7	0.1	0.1	60	23	3.3	16	3	79
WMS II	2	<0.01	0.01	20	37	25	170	3	405
WMS III	2	<0.01	0.01	20	36	24	173	3	444
Supplemented Davis	2	<0.01	0.02	—	14	189	15	3	321
Luria broth	3	<0.01	0.002	550	22	8	15	3	110

Time-kill studies were performed in triplicate in peptone media and singly in others.

ited the bactericidal effect of lactoferrin and lysozyme (Fig. 8 *A*). These studies were extended by evaluating the addition of 8×10^{-5} M or 1.6×10^{-4} M $CaCl_2$ to the bacto-peptone media. With these lower calcium concentrations, there was partial and dose-dependent inhibition of the lactoferrin-lysozyme bactericidal interaction (Fig. 8 *B*).

Calcium chelation studies. A mechanism by which calcium could interfere with the lactoferrin-lysozyme bactericidal effect would be through an inhibition of the ability of lactoferrin to chelate membrane stabilizing calcium. As we were unaware of published data on the ability of lactoferrin (or transferrin) to chelate calcium, equilibrium dialysis studies were performed to directly define $^{45}Ca^{++}$ binding by the proteins. HBSS-CM was chosen as the buffer for these studies as prior work had shown that in this solution the outer membrane effects of lactoferrin and transferrin were both demonstrable and calcium-dependent (20). Whereas the synthetic chelator Chelex caused the expected migration of the isotope across the dialysis chamber, neither lactoferrin or transferrin had any demonstrable effect on distribution of $^{45}Ca^{++}$ within the dialysis chamber; the results with lactoferrin were identical with apo- and iron-saturated preparations (Table II). Thus, these studies indicate that the two proteins do not have any significant ability to chelate calcium under conditions where they have consistent outer membrane effects.

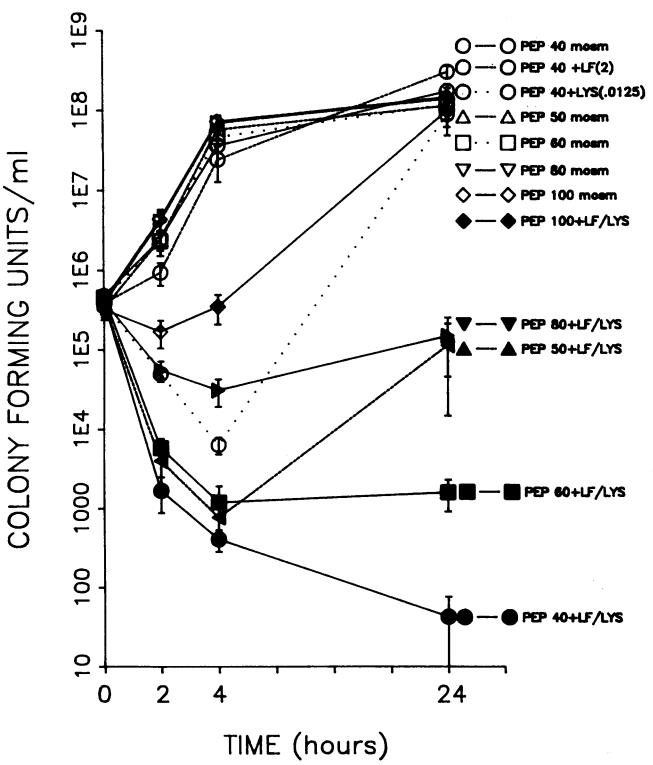

Figure 6. Evaluation of lactoferrin-lysozyme bactericidal activity towards *E. coli* 5448 in bacto-peptone media supplemented with increasing osmotic concentrations of *myo*-inositol (mean±SEM, three experiments).

Dialysis chamber studies. To extend the studies on the mechanism by which lactoferrin enhances bacterial susceptibility to lysozyme, dialysis chamber studies with a 6,000-D exclusion membrane were then performed to define whether direct cell contact was required for lactoferrin's effects (Fig. 9). As expected, lysozyme was not active unless it was in direct bacterial cell contact. The ion exchange resin, Chelex, was found to be more active when in direct cell contact, but also enhanced lysozyme's effects when added to the opposite side of the dialysis chamber (Fig. 9 *B*). In contrast, lactoferrin only enhanced the effects of lysozyme when it was in direct contact with bacterial cells whether tested at concentrations of 1 or 2 mg/ml (Fig. 9 *A*). These observations are more consistent with lactoferrin augmenting lysozyme's effects through a direct interaction with the bacterial outer membrane than with the protein acting through its chelating properties.

LPS binding studies. Polycationic agents that alter outer membrane permeability appear to directly interact with LPS molecules (10, 24, 43). Several previous studies have indicated that lactoferrin will bind to bacterial cells, although the mechanism of binding may be related to the presence of specific bacterial lactoferrin receptors (44–46). To more carefully elucidate whether lactoferrin might be altering the outer membrane in a manner similar to polycationic agents, we studied whether lactoferrin could directly bind LPS. For these studies equimolar concentrations of human lactoferrin, human transferrin, BSA, poly-*l*-lysine, and soybean trypsin inhibitor (previously shown not to bind LPS [47]) were linked to Tris-blocked Sepharose beads. These protein beads were then incubated with varying

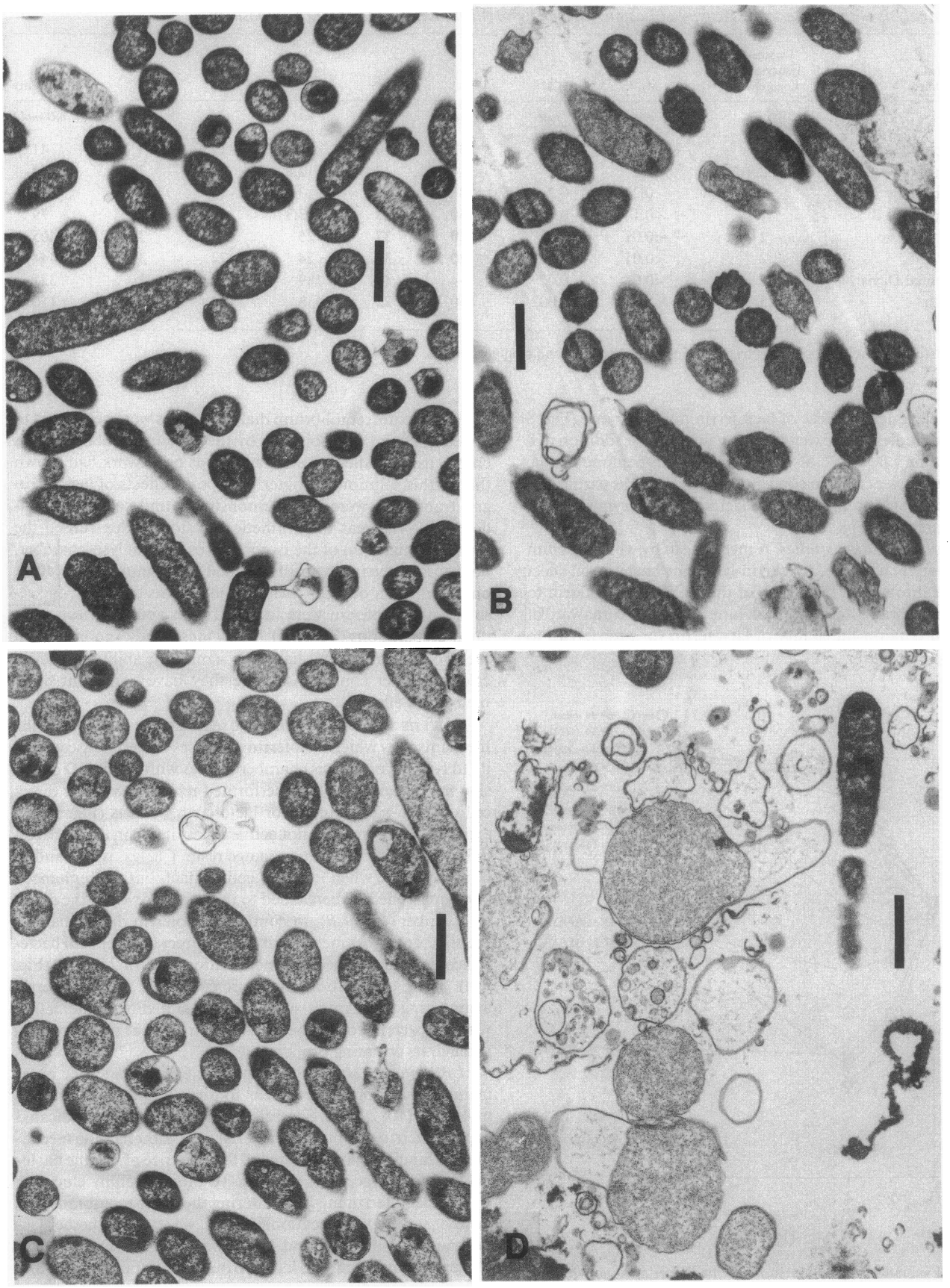

Figure 7. Transmission electron microscopy of *E. coli* 5448 cells incubated for 24 h in (*A*) bacto-peptone, (*B*) bacto-peptone with 2 mg/ml lactoferrin, (*C*) bacto-peptone with 0.005 mg/ml lysozyme, (*D*) bacto-peptone with lactoferrin and lysozyme (markers indicates 1 μm).

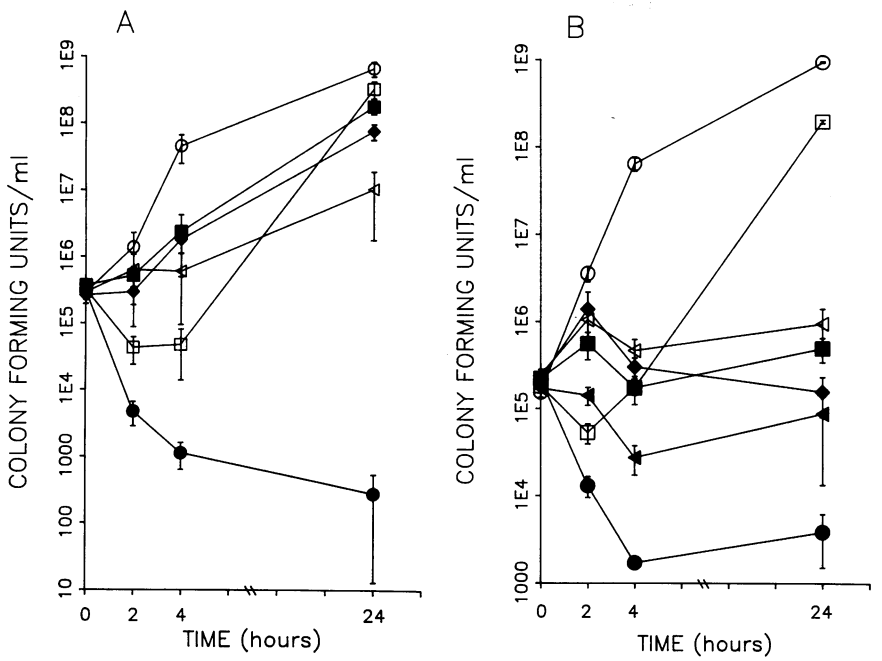

Figure 8. Effect of calcium and magnesium on lactoferrin-lysozyme bactericidal activity towards *E. coli* 5448 in bacto-peptone media (mean±SEM, three experiments). (*A*) (*Open circles*) media alone; (*open triangles*) 2 mg/ml lactoferrin; (*open squares*) 0.05 mg/ml lysozyme; (*solid circles*) lactoferrin + lysozyme in media alone; (*solid squares*) lactoferrin + lysozyme in media supplemented with 1.3×10^{-3} M $CaCl_2$; (*solid diamonds*) lactoferrin + lysozyme in media supplemented with 8×10^{-4} M $MgCl_2$. (*B*) (*Open circles*) media alone; (*open triangles*) 2 mg/ml lactoferrin; (*open squares*) 0.05 mg/ml lysozyme; (*solid circles*) lactoferrin + lysozyme in media alone; (*solid triangles*) lactoferrin + lysozyme in media supplemented with 8×10^{-5} M $CaCl_2$; (*solid squares*) lactoferrin + lysozyme in media supplemented with 1.6×10^{-4} M $CaCl_2$.

concentrations of [^{3}H]-LPS prepared from *E. coli* strain CL99-2 (Fig. 10). The binding curves obtained indicate that lactoferrin and poly-*l*-lysine have a similar ability to bind LPS, and each has a distinctly greater ability to bind LPS than does the other proteins or the Tris-blocked control beads. An exact calculation of the number of LPS binding sites and the K_d for the interactions with lactoferrin and poly-*l*-lysine is limited both because of the inability to define the molar concentration of LPS (due to size variability) and because of the capacity for free LPS molecules to aggregate in solution (48).

Discussion

The present studies demonstrate that lactoferrin and lysozyme synergistically kill Gram-negative bacteria. This activity is demonstrable against multiple different "smooth" bacteria, is dose dependent and influenced by bacterial inoculum size and basal growth media. The relative activity of lactoferrin and lysozyme appears to correlate with media osmolarity, and can be inhibited by increasing the osmolarity of a permissive media with a metabolically inert material. Morphological analysis of bacterial cells by TEM indicates that the bactericidal effect is associated with the formation of ballooned spheroplasts. These observations appear consistent with lactoferrin acting to increase the penetration of lysozyme into the Gram-negative organism where the enzyme can then disrupt the glycosidic linkages of the rigid murein sacculus. That lactoferrin can enhance lysozyme activity in this fashion is consistent with other observations on the outer membrane effects of lactoferrin. Previous studies have found that the protein causes LPS release and enhances bacterial susceptibility to antibiotics such as rifampin and cefotaxime (18–20).

Enteric Gram-negative bacteria are protected from the external environment by their complex outer membrane structure (9, 10). The membrane has an asymmetric lipid bilayer with negatively charged LPS molecules primarily localized on the outer leaflet and stabilized by the presence of divalent cations. The polysaccharide sidechain of the LPS molecule provides a hydrophilic surface to the bacterial cell, and the saturated fatty acid chains make this membrane relatively rigid as compared with other phospholipid structures (49). The dual hydrophilic/hydrophobic nature of the outer membrane

Table II. Chelation of ^{45}Calcium by Proteins and Chelex 100 Determined by Equilibrium Dialysis

Chelator	Percent migration of ^{45}Ca across dialysis membrane after differing incubation times				
	0 h	2 h	18 h	24 h	96 h
BSA (1 mg/ml)	0.03	24.9±2.3	51.7±0.3	52.3±0.6	47.0±4.8
Human transferrin (1 mg/ml)	0.03	27.9±3.4	48.4±0.4	50.5±0.6	51.3±0.5
Human lactoferrin (1 mg/ml)	0.03	27.3±3.2	48.9±0.1	49.6±0.3	45.9±4.6
Iron saturated human lactoferrin (1 mg/ml)	0.03	27.4±3.3	49.9±1.4	50.6±0.8	52.2±1.9
Chelex resin (20%)	0.03	21.6±1.9	56.6±1.9	74.8±1.8	82.8±15.7

0, 2, and 24 h results are mean±SEM, three experiments; 18 and 96 h results are from two experiments.

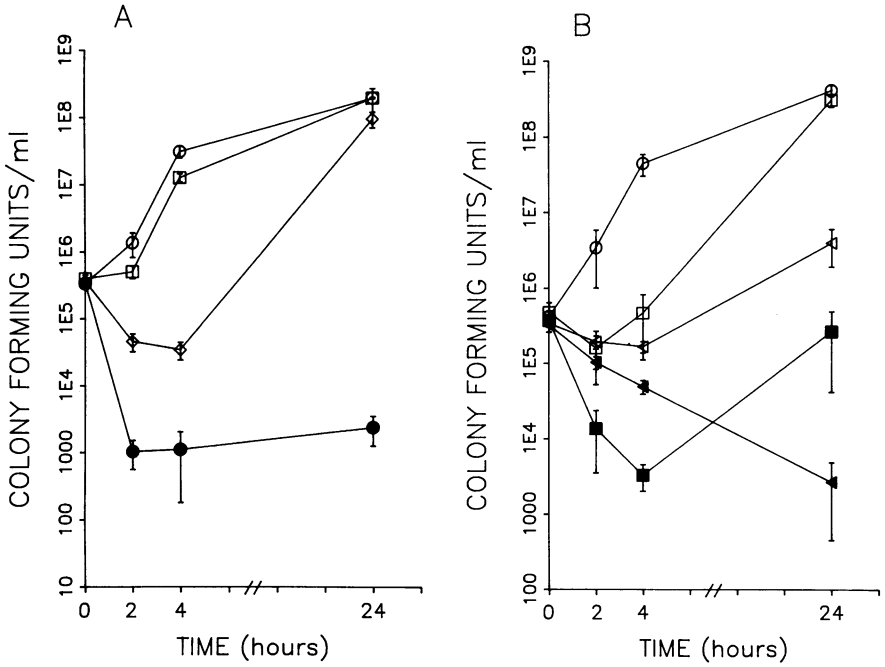

Figure 9. Evaluation of the role of direct cell contact in the ability of lactoferrin or the synthetic chelator Chelex to enhance lysozyme activity against *E. coli* 5448 in dialysis chamber (6,000 D exclusion membrane) (mean±SEM, three experiments). (*A*) (*Open circles*) Bacterial cells and bacto-peptone media alone; (*open squares*) cells and lactoferrin (1 mg/ml) in contact, lysozyme (0.125 mg/ml) on opposite side of membrane; (*open diamonds*) cells and lysozyme in contact, lactoferrin on opposite side of membrane; (*closed circles*) cells, lysozyme, and lactoferrin in contact. (*B*) (*Open circles*) Cells and media alone; (*open squares*) cells and lysozyme (0.125 mg/ml) in contact; (*open triangles*) bacterial cells and Chelex (0.00692 mEq/ml) in contact, lysozyme on opposite side of membrane; (*solid squares*) cells and lysozyme in contact, Chelex on opposite side of membrane; (*solid triangles*) cells, lysozyme, and Chelex in contact.

thereby provides a permeability barrier that limits penetration of both hydrophobic and hydrophilic molecules. In compensation for this barrier, protein porins allow for diffusion of small molecules into the bacterial cell through transmembrane pores, and inducible specific transport systems provide the transport of sugars, vitamin B_{12}, and iron across the membrane (43).

Several groups of agents have been found to damage the Gram-negative outer membrane making the structure more permeable to hydrophobic compounds (10). These include metal chelators such as EDTA and EGTA which remove stabilizing cations and cause release of LPS molecules; and polycations such as polymyxin B nonapeptide, polylysine, and a family of cationic defense proteins of neutrophils which appear to bind to the anionic core components of LPS molecules thereby altering their relationship within the membrane (50). In each instance these agents damage the membrane structure and enhance bacterial susceptibility to hydrophobic substances, such as antibiotics and lysozyme, that have limited ability to penetrate the outer membrane. Thus, the collected observations that lactoferrin causes LPS release and enhances bacterial susceptibility to rifampin and lysozyme strongly suggest that lactoferrin has membrane permeabilizing activity.

Three major mechanisms can be proposed to explain how lactoferrin might alter the permeability barrier of the outer membrane. First, through its metal chelating capacity the protein could remove stabilizing cations in a fashion similar to EDTA (18). Evidence supporting this hypothesis comes from inhibition of the membrane effects by saturation of the protein with iron, and the ability of calcium and magnesium to modulate the activity (20). However, several of the present studies argue strongly against this hypothesis. Specifically, both the inability of lactoferrin to either chelate ^{45}calcium or to enhance lysozyme activity in the absence of direct cell contact are inconsistent with divalent cation chelation being the mechanism of action.

A second possibility is that lactoferrin could alter membrane permeability through stimulation of bacterial siderophore production. By depleting the environment of available iron, lactoferrin has been found to induce the bacterial production of iron-regulated outer membrane transport proteins (13). The specificity of these transport proteins is still being defined and it is possible that an indiscriminate transport system might allow for the uptake of other molecules aside from iron. Such a mechanism has recently been proposed to explain the enhanced activity of a specific class of catechol-cephalosporins in low iron conditions (51, 52). In considering the present studies, the inhibition of lactoferrin activity by iron saturation would support this hypothesis. Still, it would be expected that this mechanism would not require direct interaction with the bacterial cell, and the inability of lactoferrin to enhance lysozyme activity across a dialysis membrane argues against it.

Another potential mechanism for the membrane effects of lactoferrin is through a direct interaction with the bacterial outer membrane. Such a possibility is supported by the work of Valenti and co-workers correlating the ability of hen ovotransferrin and bovine lactoferrin to bind to bacterial strains with the bacteriostatic effects of the proteins against the strains (44, 53). The dialysis chamber time-kill studies we performed similarly support a direct interaction, and the ability of lactoferrin to bind LPS suggests a mechanism of action. Recently, the crystallographic structure of human lactoferrin has been solved to a resolution of 2.8 Å (54). This analysis indicates that one surface subunit of lactoferrin contains nine arginine and lysine residues in close proximity. This cationic region on the protein surface could function as the binding site for LPS through an interaction with the anionic core structure of the latter molecule. This would allow lactoferrin to act similarly to the well-defined polycationic membrane-active agents where the physical presence of the bound protein then alters normal membrane physiology (21, 24).

Still this hypothesis must also explain how the effects of lactoferrin are altered by changing either the calcium or magnesium concentrations in the assay or the iron saturation of the protein. Lactoferrin is known to form tetramers in the presence

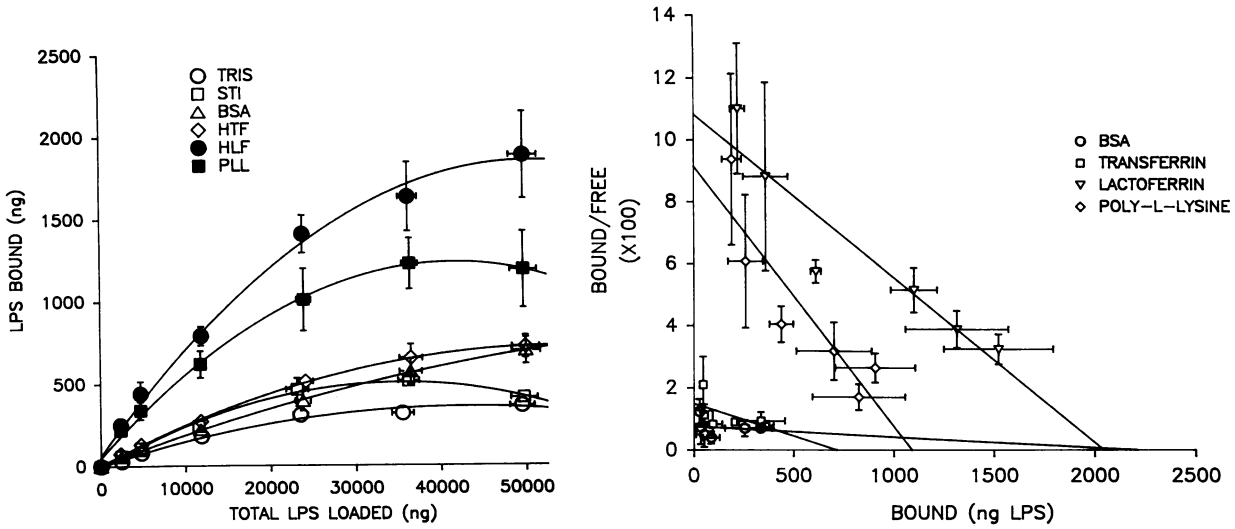

Figure 10. Binding of *E. coli* [^{3}H]-LPS to human lactoferrin (HLF), human transferrin (HTF), poly-*l*-lysine (PLL), soy bean trypsin inhibitor (STI), and bovine serum albumin (BSA) bound to Sepharose, and to Tris-blocked Sepharose (TRIS) (mean±SEM, three experiments). (*A*) The binding curve with second-order linear regression. (*B*) Corresponding Scatchard plot performed by subtracting nonspecific binding to Tris-blocked Sepharose beads; for lactoferrin $r = -0.937$; for poly-*l*-lysine $r = -0.898$.

of calcium, and this may interfere with its biologic activity (55). Additionally, changes in calcium or magnesium concentration in the environment may alter the amount of the cations incorporated into the outer membrane and thereby influence the membrane susceptibility to membrane-active agents (9). Finally, and most importantly, the divalent cations Ca^{2+} and Mg^{2+} have been shown to block the activity of well-characterized polycationic membrane active agents including polymyxin, poly-*l*-lysine, and aminoglycosides (22, 56, 57). Studies with BPI have shown that exposure of bacterial cells to either 40 mM Ca^{2+} or Mg^{2+} blocks the bactericidal activity of the protein against Gram-negative bacteria, apparently by causing the release of protein from the bacterial cell surface (58–60). Thus, the inhibition of lactoferrin's membrane effects by these two divalent cations is consistent with the protein acting as a polycationic agent. In considering the effects of iron saturation of lactoferrin, both electrophoretic and crystallographic studies indicate that iron binding significantly alters the structural conformation of the protein (61, 62). Such a conformational change may then influence an interaction with the bacterial membrane. Clearly each of these hypotheses requires further evaluation.

In these studies lactoferrin was consistently found to be bacteriostatic and not bactericidal. This contrasts with the studies by Arnold and co-workers that describe a direct bactericidal effect of the protein when tested in distilled water (63, 64). However, the observed lactoferrin-lysozyme bactericidal effect appears similar to the results of these investigators. It is conceivable that in the studies of these other investigators, the bactericidal effect ascribed to lactoferrin may have been related in part to inadvertent contamination of the lactoferrin preparations with lysozyme.

The lactoferrin-lysozyme effect must be considered in the context of the natural environment. The in vivo concentrations of lactoferrin and lysozyme are highly heterogeneous. Recent work on the concentration of lactoferrin in mucosal fluids has noted levels ranging from 12 μg/ml in mixed respiratory secretions to 6.7 mg/ml in preterm colostrum (65, 66). Lysozyme varied from 0.77 μg/ml to 0.06 mg/ml in these same fluids. Beyond this variability, the packaging and release of the proteins within granules suggests that there is probably significant microheterogeneity of protein concentrations within the individual secretions. Additionally, precise quantitation of two proteins within the phagolysosome of polymorphonuclear leukocytes has not yet been accomplished. Beyond these considerations on protein concentration, there are also high concentrations of calcium and magnesium in most biological fluids that could limit lactoferrin's membrane activity. However, the in vivo distribution of the cations is also not homogeneous; the calcium concentration in the human macrophage phagolysosome has been found to be < 100 μM (67). This is then one microenvironment where lactoferrin could enhance the activity of lysozyme. Similarly, the osmolarity of most body fluids is higher than that where the combination of lactoferrin and lysozyme has direct bactericidal activity (68). Yet concurrent damage to both the outer membrane and the murein sacculus produced by the two proteins should continue to occur, and should enhance bacterial susceptibility to other host defense systems such as immunoglobulins, the complement cascade, and phagocytic cells. Thus, as lactoferrin and lysozyme have a similar distribution within mucosal secretions and are primary constituents of the specific granules of polymorphonuclear leukocytes (1–4), it is likely that the observed synergistic interaction is relevant to their host defense properties.

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