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Antibacterial Activity of Lactoferrin and a Pepsin-Derived Lactoferrin Peptide Fragment

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Although the antimicrobial activity of lactoferrin has been well described, its mechanism of action has been poorly characterized. Recent work has indicated that in addition to binding iron, human lactoferrin damages the outer membrane of gram-negative bacteria. In this study, we determined whether bovine lactoferrin and a pepsin-derived bovine lactoferrin peptide (lactoferricin) fragment have similar activities. We found that both 20 μ M bovine lactoferrin and 20 μ M lactoferricin release intrinsically labeled [³H]lipopolysaccharide ([³H]LPS) from three bacterial strains, *Escherichia coli* CL99 1-2, *Salmonella typhimurium* SL696, and *Salmonella montevideo* SL5222. Under most conditions, more LPS is released by the peptide fragment than by whole bovine lactoferrin. In the presence of either lactoferrin or lactoferricin there is increased killing of *E. coli* CL99 1-2 by lysozyme. Like human lactoferrin, bovine lactoferrin and lactoferricin have the ability to bind to free intrinsically labeled [³H]LPS molecules. In addition to these effects, whereas bovine lactoferrin was at most bacteriostatic, lactoferricin demonstrated consistent bactericidal activity against gram-negative bacteria. This bactericidal effect is modulated by the cations Ca²⁺, Mg²⁺, and Fe³⁺ but is independent of the osmolarity of the medium. Transmission electron microscopy of bacterial cells exposed to lactoferricin show the immediate development of electron-dense "membrane blisters." These experiments offer evidence that bovine lactoferrin and lactoferricin damage the outer membrane of gram-negative bacteria. Moreover, the peptide fragment lactoferricin has direct bactericidal activity. As lactoferrin is exposed to proteolytic factors in vivo which could cleave the lactoferricin fragment, the effects of this peptide are of both mechanistic and physiologic relevance.

Lactoferrin is an iron-binding glycoprotein present in milk, tears, saliva, vaginal secretions, semen, bronchoalveolar lavage fluid, and specific granules of polymorphonuclear leukocytes (PMNs) (10, 13, 39). Biological properties ascribed to this protein include the regulation of absorption of iron and other metals in the gastrointestinal tract, modulation of both the production of PMNs and the growth of animal cells, and finally antimicrobial activity against bacteria and yeasts (34, 40, 45). Initially, the antimicrobial effect of lactoferrin was considered to be a function of its ability to chelate iron, with the protein inhibiting microbial growth through nutritional deprivation of iron (21). However, several investigators have suggested that lactoferrin has other effects against microorganisms. Work by Arnold and associates (2, 3, 7, 8, 30) has suggested that lactoferrin is capable of a direct bacterial effect on strains of *Streptococcus mutans*, *Vibrio cholerae*, *Escherichia coli*, *Actinobacillus actinomycetemcomitans*, and *Legionella pneumophila*. Additionally, several research groups have found that the antimicrobial activity of lactoferrin against *E. coli* strains is enhanced by concurrent exposure of the bacteria to immunoglobulin G or secretory immunoglobulin A (44, 48, 49). More recently, we have found that human lactoferrin can directly damage the outer membrane of gram-negative bacteria (16-19). Lactoferrin causes the release of lipopolysaccharide (LPS) molecules from the membrane and enhances bacterial susceptibility to hydrophobic antibiotics and human lysozyme. These effects on the outer membrane of

gram-negative bacteria appear to be related to a direct interaction of lactoferrin with the bacterial cell (16).

Work with bovine lactoferrin has found that the antimicrobial activity of an enzymatic hydrolysate generated by digestion with porcine pepsin is stronger than that of the whole protein against an *E. coli* O111 isolate (51). The bacteriostatic activity is associated with low-molecular-weight peptide fragments, and an active lactoferrin peptide fragment has been purified by reverse-phase high performance liquid chromatography (5). Sequence analysis indicates that this peptide fragment is 25 amino acids long (Phe-Lys-Cys-Arg-Arg-Trp-Gln-Trp-Arg-Met-Lys-Lys-Leu-Gly-Ala-Pro-Ser-Ile-Thr-Cys-Val-Arg-Arg-Ala-Phe) and has exact homology with an amino-terminal segment of the whole lactoferrin sequence, as reported by Pierce et al. (43) and by Goodman and Schanbacher (26). The segment of the N terminus involved is distinctly separate from the two iron-binding regions of the protein. It contains five arginine and three lysine residues, making it strongly cationic, and lacks detectable carbohydrate. A search of the NBRF-PIR databank found that it has strong homology with an N-terminal region of mouse lactoferrin, but not with other cationic antimicrobial proteins. In this report, we have investigated the effects of both whole bovine lactoferrin and its peptide fragment, lactoferricin, on the gram-negative bacterial outer membrane and have further characterized the antimicrobial activity of lactoferricin.

MATERIALS AND METHODS

Lactoferrin and lactoferricin. Bovine milk lactoferrin was prepared from fresh skim milk by the method described by Law and Reiter (32), and purity was ascertained by sodium

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dodecyl sulfate-polyacrylamide gel electrophoresis (51). The bovine lactoferrin peptide lactoferricin was prepared by the method of Bellamy et al. (5). Human milk lactoferrin (Sigma Chemical Company [St. Louis, Mo.] or Calbiochem Corporation [La Jolla, Calif.]) and human placental lysozyme (Calbiochem) were purchased commercially.

LPS release studies. The abilities of bovine lactoferrin and lactoferricin to release LPS was tested by previously described methods with three different bacterial strains (*E. coli* CL99 1-2, *Salmonella montevideo* SL5222, and *Salmonella typhimurium* SL696) (16, 18). Briefly, the LPS of each of these bacteria was intrinsically radiolabeled through the incorporation of tritiated galactose into the carbohydrate component of the LPS molecule (6, 17, 18, 25, 28, 29, 56). The strain to be tested was grown at 37°C in 1 ml of defined medium (WMS broth, Davis minimal medium, Luria broth, or Luria broth with calcium [16, 18]) supplemented with 0.1 mM unlabeled galactose and 4 to 15 μ Ci of D-[6-³H]galactose to reach a concentration of $\approx 5 \times 10^8$ CFU. The cells were then centrifuged, washed, and suspended in Hanks' balanced salt solution lacking calcium and magnesium (HBSS-CM) (Sigma). Duplicate 1.0-ml samples containing approximately 5×10^7 CFU of [³H]galactose-labeled bacteria, buffer, and various concentrations of test materials in polypropylene tubes at pH 7 to 7.5 were prepared. After the addition of bacteria, 0-min samples were immediately agitated and centrifuged, and the beta emissions from the supernatant and pellet fractions were counted. The 30-min samples were incubated at 37°C and then similarly agitated, centrifuged, and counted.

The percentage of radiolabel released at 30 min was determined as follows: percent release = $[(30\text{-min sample supernatant cpm}/(30\text{-min sample supernatant cpm} + 30\text{-min sample pellet cpm})) \times 100 - (0\text{-min buffer supernatant cpm}/(0\text{-min buffer supernatant cpm} + 0\text{-min buffer pellet cpm})) \times 100]$, where cpm is the counts per minute.

Time-kill studies. Bacto Peptone medium was obtained commercially (Difco, Detroit, Mich.), and 1% (wt/vol) solutions were prepared. *E. coli* CL99 1-2 cells were grown to stationary phase, centrifuged, and washed. A bacterial inoculum was added to 500 μ l of medium (Bacto Peptone) with or without lactoferrin, bovine lactoferrin, human lactoferrin, or human lysozyme. The mixtures were then incubated at 37°C, and aliquots were removed, serially diluted, and plated on tryptic soy agar (BBL) to determine bacterial colony counts. For data analysis, if no viable bacteria were observed at the lowest dilution, the bacterial count was recorded as 1 CFU at that dilution. For example, if the lowest dilution without bacterial growth for a given experiment was 1:10², the bacterial CFU was considered to be 10².

LPS binding studies. We studied the ability of the proteins to bind LPS using our previously described assay (16). Bovine lactoferrin, lactoferricin, bovine serum albumin (BSA) and poly-L-lysine (Sigma Chemical Co.) were coupled to cyanogen bromide-activated Sepharose 4B beads (Pharmacia Fine Chemicals) at a concentration of 100 nM/ml of gel. After protein coupling, the beads were blocked in Tris buffer (pH 8.0) and stored in 0.03 M barbital-acetate-0.116 M NaCl buffer (pH 7.2) (BABS) with 0.02% thimerosal. To control for nonspecific binding, Sepharose beads that were not reacted with protein but instead simply blocked with Tris were also prepared.

Tritium-labeled LPS was prepared by growing *E. coli* CL99 1-2 in modified WMS broth supplemented with D-[6-³H]galactose, and LPS was extracted either by washing the cells in barbital-acetate buffer (pH 8.0) or by the phenol-

water method of Westphal and Jann (17, 55). When this strain is grown in the presence of [³H]galactose, the radiolabel is almost exclusively incorporated into the O-specific side chain of LPS (25). For experiments requiring high LPS concentrations, [³H]LPS was supplemented with similarly prepared unlabeled LPS.

Binding of the LPS was determined by incubating 0.1-ml portions of the protein-Sepharose or Tris-Sepharose beads with various concentrations of [³H]LPS in BABS (pH 7.2) for 1 h with occasional gentle shaking. The beads were then pelleted by centrifugation, washed twice with BABS, and the beads and pooled BABS wash material was subjected to liquid scintillation counting.

MIC and MBC tests. Determination of the MIC and MBC of lactoferricin for bacterial strains was performed in 1% Bacto Peptone medium, using a standard microdilution technique with an inoculum of 2×10^5 CFU/ml (23).

TEM. Inocula (5×10^7 CFU) of *E. coli* CL99 1-2 were added to 1-ml portions of 1% Bacto Peptone with or without 100 μ g of lactoferricin. The mixtures were incubated at 37°C for various time periods, and the bacterial cells were pelleted. The pellets were resuspended in 2% glutaraldehyde in 0.1 M cacodylic buffer (pH 7.3) for 30 min at 4°C and washed twice in 0.1 M cacodylic buffer. The 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., Warrenton, Pa.). For transmission electron microscopy (TEM), thin sections (70 nm thick) 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.

RESULTS

LPS release studies. To study the effects of bovine lactoferrin and lactoferricin on the outer membrane, we determined whether they could release LPS from three bacterial strains, *E. coli* CL99 1-2, *S. typhimurium* SL696, and *S. montevideo* SL5222. In initial studies performed with bacterial cells grown with 2 mM calcium, we found that 18 μ M lactoferricin caused a dramatic release of ³H-labeled LPS from all three bacterial strains (Table 1). In comparison, the approximately the same molar concentration of whole bovine lactoferrin protein caused a lower degree of LPS release from the two *Salmonella* strains. These results are comparable to those from a previous study with human lactoferrin (18) and suggest that both bovine lactoferrin and lactoferricin, the peptide with N-terminal region, can damage the gram-negative bacterial outer membrane.

Prior work has indicated that growing bacterial cells in the presence of increasing concentrations of calcium ions increased the percentage of LPS that could be released by EDTA and human lactoferrin (16). Presumably, when the outer membrane is assembled in the presence of high concentrations of cations, increased numbers of cations are incorporated into the membrane to stabilize the anionic charge of the LPS core. The increased amount of cations within the membrane may then make the membrane more susceptible to factors that alter the cation-LPS relationship. Using this hypothesis, we similarly studied the effect of calcium in growth medium on the ability of bovine lactoferrin and lactoferricin to release LPS from *S. typhimurium* SL696 (Table 1). As previously noted with human lactoferrin, the amount of LPS released from the bacterial membrane by bovine lactoferrin significantly increased as the

TABLE 1. Release of LPS from three bacterial strains by bovine lactoferrin and lactoferricin

Bacteria and growth medium	% [³ H]LPS release (mean ± SEM) (n) at 30 min with HBSS-CM		
	Alone	With bovine lactoferrin (2 mg/ml)	With lactoferricin (100 µg/ml)
<i>E. coli</i> CL99 1-2, WMS broth	1.1 ± 1.1 (3)	0.8 ± 0.8 (3)	26.6 ± 3.4 ^a (3)
<i>S. montevideo</i> SL5222, Luria broth + 2 mM calcium	3.4 ± 1.9 (3)	24.6 ± 2.0 ^b (3)	39.8 ± 10.6 ^b (2)
<i>S. typhimurium</i> SL696			
Luria broth	4.5 ± 2.6 (5)	1.5 ± 0.7 (5)	49.5 ± 2.1 ^c (5)
Luria broth + 2 mM calcium	4.6 ± 1.0 (7)	22.8 ± 6.8 ^d (5)	45.5 ± 1.8 ^e (7)
Luria broth + 10 mM calcium	4.9 ± 1.1 (5)	58.0 ± 6.7 ^{f,g} (5)	47.4 ± 0.7 ^f (5)

^a Significantly different from value obtained with HBSS-CM and bovine lactoferrin (*P* < 0.05).
^b Significantly different from value obtained with HBSS-CM alone (*P* < 0.05).
^c Significantly different from value obtained with HBSS-CM and bovine lactoferrin (*P* < 0.0001).
^d Significantly different from value obtained with HBSS-CM (*P* < 0.005).
^e Significantly different from value obtained with HBSS-CM and bovine lactoferrin (*P* < 0.05).
^f Significantly different from value obtained with HBSS-CM (*P* < 0.0001).
^g Significantly different from value obtained with Luria broth and no supplemental calcium (*P* < 0.01).

concentration of calcium ions in the growth medium increased. In contrast, the ability of lactoferricin to release LPS appeared to be independent of the calcium concentration of the growth medium.

With the high degree of radiolabel being released, concurrent experiments were performed to test the effect of bovine lactoferrin and lactoferricin on bacterial viability under these experimental conditions. Although lactoferrin had no effect, lactoferricin caused a greater than 99% decrease in bacterial CFU in HBSS-CM during the 30-min incubation for each of the *Salmonella* strains.

Bacterial susceptibility to human lysozyme. To determine whether the ability of bovine lactoferrin and lactoferricin to release LPS from the bacterial cell also altered the permeability of the outer membrane, we studied the effects of the proteins on bacterial susceptibility to human lysozyme. As previously observed with human lactoferrin, we found that there was increased killing of *E. coli* CL99 1-2 in 1% Bacto Peptone medium containing bovine lactoferrin and human lysozyme (Fig. 1). Similarly, there was also increased killing of bacterial cells that were concurrently exposed to lactoferricin and lysozyme as opposed to either of these compounds alone (Fig. 2). This interaction was dependent on the concentration of lactoferricin, with increasing bacterial killing seen as lactoferricin concentration was increased from 2 to 8 µg/ml (data not shown).

LPS binding studies. Human lactoferrin and polycationic agents not only alter bacterial outer membrane permeability but also directly bind LPS (16, 38). We attempted to ascertain whether bovine lactoferrin and lactoferricin also have this property. In studies with intrinsically radiolabeled LPS, we found that bovine lactoferrin and lactoferricin have similar abilities to bind LPS, and each has a stronger ability to bind LPS than do BSA and poly-L-lysine (Fig. 3). An exact calculation of the number of LPS binding sites and the *K_d* for the interactions with lactoferrin or lactoferricin is not possible both because of the inability to define the molar concentration of LPS (because of size variability) and because of the capacity for free LPS molecules to aggregate in solution.

Bactericidal activity of lactoferricin. As the experiments above indicated that lactoferricin has bactericidal activity in addition to an effect on the gram-negative bacterial outer membrane, further time-kill studies were performed. In experiments with *E. coli* CL99 1-2 in 1% Bacto Peptone medium, we found that lactoferricin exhibited a consistent bactericidal effect (Fig. 4). The activity was proportional to

the concentration of lactoferricin and inversely proportional to the bacterial inoculum. Specifically, lactoferricin at a concentration of 100 µg/ml was highly bactericidal, regardless of the inoculum size. Over a 1-h incubation, there was a greater than 99% reduction in CFU, and subsequent bacterial killing continued through 24-h incubation. A lactoferricin concentration of 10 µg/ml was also bactericidal, but the activity of this lower lactoferricin concentration was inoculum dependent. The lactoferricin concentration of 1.0 µg/ml had no apparent effect on the bacterial cells.

As these studies had been performed with stationary-phase organisms, time-kill curves were also performed with lactoferricin against log-phase bacteria for which a greater bactericidal effect was observed (data not shown). Additionally, because prior work found that a bactericidal effect of lactoferrin against *L. pneumophila* was seen against broth-

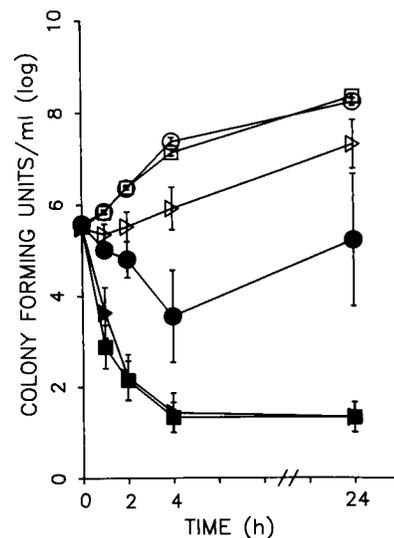


FIG. 1. Effects of human lysozyme (12.5 µg/ml) and human lactoferrin (2 mg/ml) and bovine lactoferrin (2 mg/ml) on the growth of *E. coli* CL99 1-2 in 1% Bacto Peptone alone or supplemented. Each value shown is the mean ± standard error of the mean from three experiments. Symbols: ○, no supplement; □, human lactoferrin; ▽, bovine lactoferrin; ●, human lysozyme; ■, human lactoferrin and human lysozyme; ◼, bovine lactoferrin and human lysozyme.

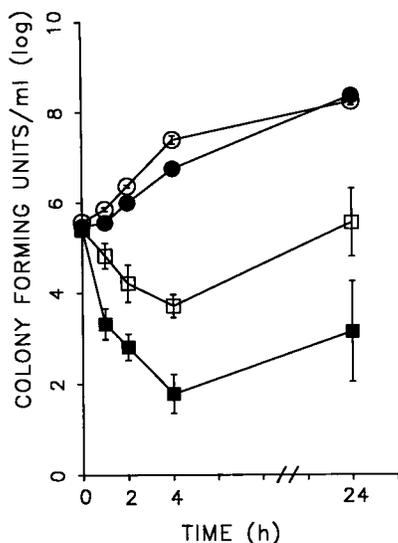


FIG. 2. Effects of human lysozyme (6 $\mu\text{g/ml}$) and lactoferricin (8 $\mu\text{g/ml}$) on the growth of *E. coli* CL99 1-2 in 1% Bacto Peptone alone or supplemented. Each value shown is the mean \pm standard error of the mean from three to five experiments. Symbols: \circ , no supplement; \square , lactoferricin; \bullet , human lysozyme; \blacksquare , lactoferricin and human lysozyme.

grown but not agar-grown cells, we tested the activity of lactoferricin against *E. coli* O111 grown on agar plates (7). In parallel experiments, lactoferricin at a concentration of 100 $\mu\text{g/ml}$ was bactericidal for cells grown under both conditions, but the magnitude of bacterial killing at 24 h was lower for agar-grown cells (decrease in CFU of 3.13 \log_{10} units [mean of three experiments]) than for broth-grown cells (4.69 \log_{10} units).

There appeared to be some bacterial growth at 24 h when high bacterial inocula were exposed to 10 μg of lactoferricin

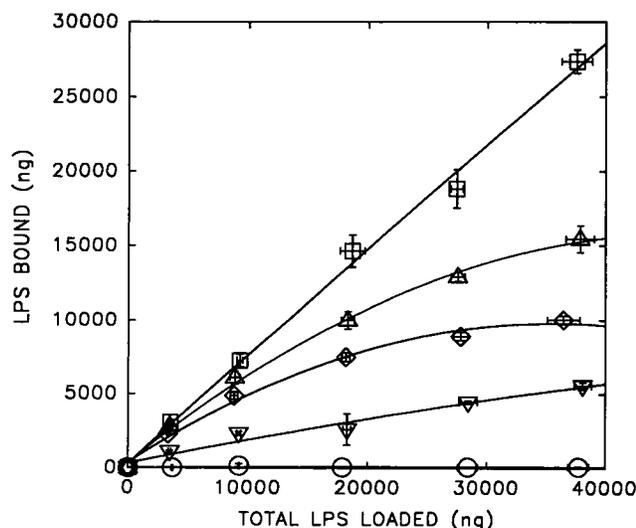


FIG. 3. Binding of *E. coli* CL99 1-2 [^3H]LPS to bovine lactoferrin (\square), lactoferricin (\triangle), poly-L-lysine (\diamond), and BSA (∇) bound to Sepharose and to Tris-blocked Sepharose (\circ). Each value shown is the mean \pm standard error of the mean from three experiments. The binding curves are plotted, using second-order linear regression.

per ml, suggesting that the strain might develop one-step resistance to lactoferricin, as can be seen with selected antibiotics such as rifampin. To test the possibility that such resistance to lactoferricin could occur, bacterial cells surviving 24 h of exposure to 10 μg of lactoferricin per ml at an inoculum size of 10^7 CFU/ml were reexposed to the same lactoferricin concentration in a 10^5 CFU inoculum (Fig. 5). A rapid bactericidal effect for lactoferricin was again observed. These results suggest that the observed variation in activity with inoculum size is not due to the rapid emergence of resistant organisms but instead relates to the ratio of lactoferricin molecules to bacterial cells.

Our prior work with human lactoferrin indicated that lactoferrin and lysozyme could kill gram-negative organisms only under low osmotic conditions (16). To evaluate whether the in vitro killing by lactoferricin was similarly related to the osmolarity of the medium, we tested the activity of the peptide in Bacto Peptone medium supplemented with *myo*-inositol, a sugar not metabolized by *E. coli* CL99 1-2. We found that increasing the osmolarity of the medium up to 288 mosM had no impact on the bactericidal effect (Fig. 6).

The segment of the bovine lactoferrin N-terminus-containing lactoferricin is distinct from the two iron-binding regions of the protein, which suggests that iron would not influence its activity. In testing this hypothesis, we found that 80 μM ferric chloride had no effect on the activity of 18 μM lactoferricin, although it did inhibit the effect of 2 μM lactoferricin, particularly after incubation for 24 h (Fig. 7). In contrast, 80 μM ferric chloride completely inhibited the activity of 20 μM bovine lactoferrin. The fact that the inhibition of lactoferricin occurred at a higher iron-to-protein ratio than the inhibition of lactoferrin suggests that the effect of ferric iron on the activity of lactoferricin is due to a different mechanism than that for bovine lactoferrin.

Similarly, it has been noted that calcium and magnesium can affect the antimicrobial activities of human lactoferrin and several neutrophil-derived cationic proteins, including the bactericidal/permeability-increasing protein (BPI), the defensins, and the bactericins (18, 35, 47, 52). Thus, we tested the effects of increasing calcium and magnesium levels on the antimicrobial activity of lactoferricin (Fig. 8). We found that both cations could inhibit the activity of lactoferricin against *E. coli* CL99 1-2, but the peptide retained a demonstrable effect at calcium and magnesium concentrations of 100 μM .

MIC and MBC studies. To further evaluate the spectrum of antimicrobial activity of lactoferricin, we determined the MICs and MBCs of the peptide against a variety of microorganisms in 1% Bacto Peptone medium (Table 2). With 10 Gram-negative strains, the MICs ranged from 1.6 to 5.2 $\mu\text{g/ml}$. For isolates of the family *Enterobacteriaceae*, the MBCs were almost identical to the MICs. In contrast, for two *Pseudomonas aeruginosa* isolates, the peptide had only an inhibitory effect, with the MBC greater than 125 $\mu\text{g/ml}$. For three gram-positive strains and two *Candida albicans* isolates, the MICs and MBCs of lactoferricin were very close and ranged from 0.8 to 13.2 $\mu\text{g/ml}$.

TEM studies. To further characterize the bactericidal effect of lactoferricin, we used TEM to examine *E. coli* CL99 1-2 cells treated with lactoferricin (Fig. 9). We found that bacterial cells exposed to 100 μg of lactoferricin per ml immediately showed an altered cell membrane morphology, with the appearance of membrane "blisters." After 2 h of incubation with 100 μg of lactoferricin per ml, a large amount of cell debris was present, and a number of the remaining cells appear to have a clumping or coagulation of cytoplasmic

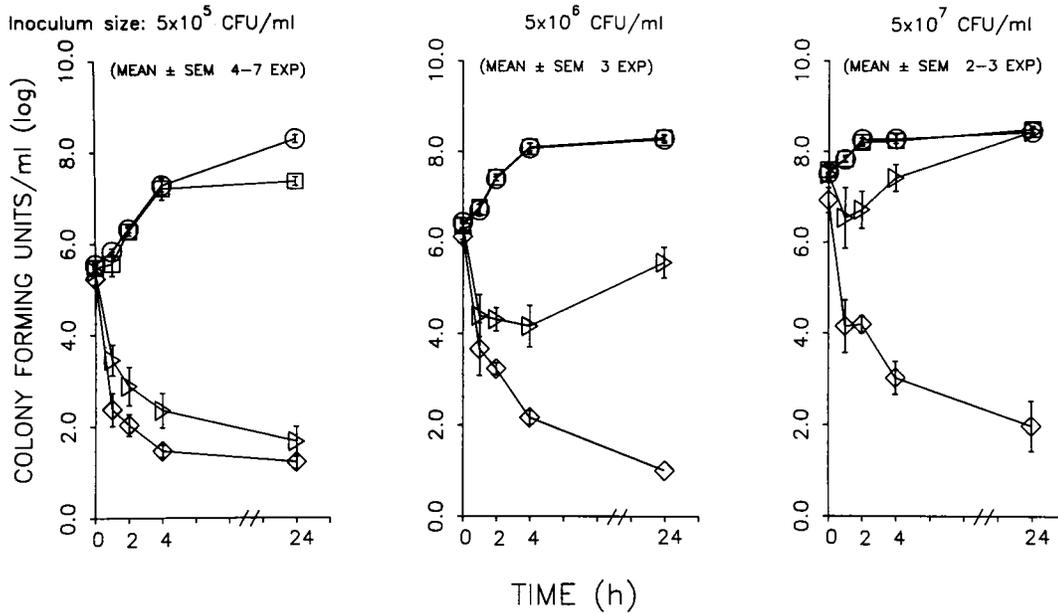


FIG. 4. Effects of the size of bacterial inoculum and the concentration of lactoferrin on the activity of lactoferrin toward *E. coli* CL99 1-2 in 1% Bacto Peptone. Symbols: ○, no lactoferrin; □, 1.0 µg of lactoferrin per ml; ▷, 10 µg of lactoferrin per ml; ◇, 100 µg of lactoferrin per ml. SEM, standard error of the mean; EXP, experiments.

elements, in addition to membrane blistering. These effects are distinctly different from those noted with whole human lactoferrin which has no effect on bacterial morphology by TEM (16).

DISCUSSION

In this work, we have found that bovine lactoferrin alters the structure of the gram-negative bacterial outer membrane.

The protein causes both the release of structural LPS molecules and an increase in killing of bacteria by human lysozyme. In this fashion, it appears to have an effect similar to that of human lactoferrin (16-19). Experiments with human lactoferrin had indicated that its membrane activity was related to an ability to directly interact with the membrane and that the protein binds LPS molecules (16). We have now found that the bovine protein shares this latter property.

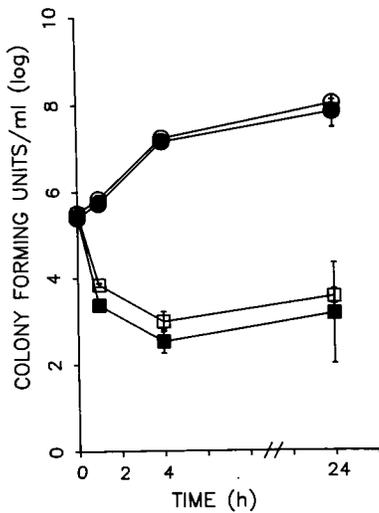


FIG. 5. Evaluation of the bactericidal activity of lactoferrin toward *E. coli* CL99 1-2 cells which survive in the medium (Bacto Peptone) containing lactoferrin. Each value shown is the mean \pm standard error of the mean from two or three experiments. Symbols: ○ and ●, no lactoferrin; □ and ■, 10 µg of lactoferrin per ml; ○ and □, cells which had grown in 1% Bacto Peptone for 24 h; ● and ■, cells which had been inoculated at 10^7 CFU/ml and had survived 24 h in 1% Bacto Peptone containing 10 µg of lactoferrin per ml.

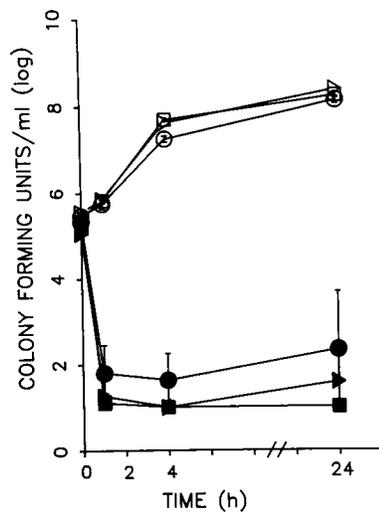


FIG. 6. Evaluation of the bactericidal activity of lactoferrin (10 µg/ml) toward *E. coli* CL99 1-2 in 1% Bacto Peptone medium alone or supplemented with *myo*-inositol and with increasing osmolarities. Each value shown is the mean \pm standard error of the mean from three experiments. Symbols: ○, no inositol or lactoferrin, 54 mosM; □, 100 mM inositol, 148 mosM; ▷, 250 mM inositol, 288 mosM; ●, lactoferrin, 54 mosM; ■, lactoferrin, 148 mosM; ▴, lactoferrin, 288 mosM.

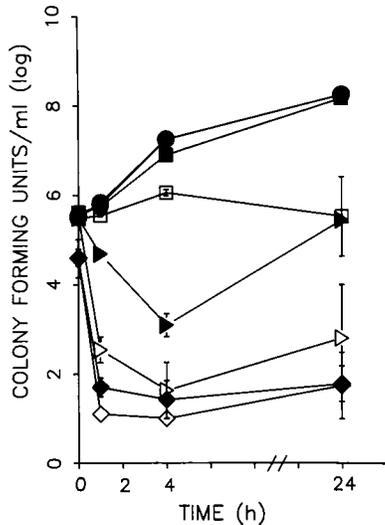


FIG. 7. Evaluation of the bactericidal activity of bovine lactoferrin and lactoferricin toward *E. coli* CL99 1-2 in 1% Bacto Peptone alone or supplemented with ferric chloride. Lactoferrin and lactoferricin were incubated in medium with iron for 1 h at 37°C prior to the addition of bacteria. Each value shown is the mean \pm standard error of the mean from two or three experiments. Symbols: \circ , no supplement; \square , 2 mg of bovine lactoferrin per ml; \triangle , 10 μ g of lactoferrin per ml; \diamond , 100 μ g of lactoferrin per ml; \bullet , 80 μ M Fe^{3+} ; \blacksquare , 2 mg of bovine lactoferrin per ml and 80 μ M Fe^{3+} ; \blacktriangleright , 10 μ g of lactoferrin per ml and 80 μ M Fe^{3+} ; \blacklozenge , 100 μ g of lactoferrin per ml and 80 μ M Fe^{3+} .

The concentration of lactoferrin required to alter the outer membrane is high, indicating that this activity will not occur in all physiologic environments. However, lactoferrin has been found at the following levels in body fluids: 0.5 ± 0.5

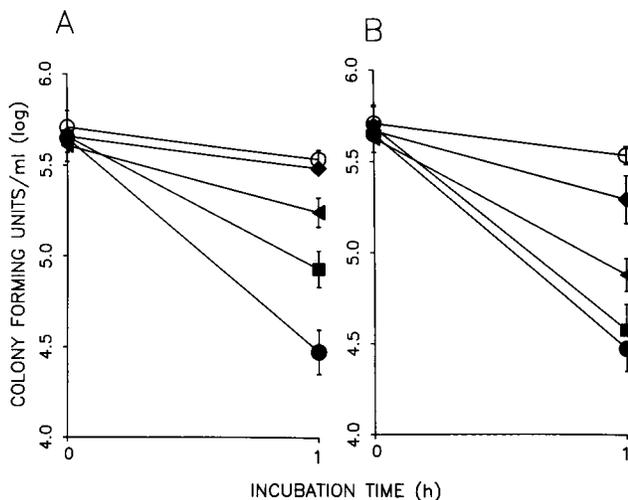


FIG. 8. Evaluation of the bactericidal activity of lactoferricin (100 μ g/ml) toward *E. coli* CL99 1-2 in HBSS-CM alone or supplemented with increasing calcium (A) and magnesium (B) concentrations. Each value shown is the mean \pm standard error of the mean from three experiments. (A) Symbols: \circ , no supplement; \bullet , lactoferricin; \blacksquare , lactoferricin and 100 μ M CaCl_2 ; \blacktriangle , lactoferricin and 200 μ M CaCl_2 ; \blacklozenge , lactoferricin and 1 mM CaCl_2 ; (B) Symbols: \circ , no supplement; \bullet , lactoferricin; \blacksquare , lactoferricin and 100 μ M MgCl_2 ; \blacktriangle , lactoferricin and 200 μ M MgCl_2 ; \blacklozenge , lactoferricin and 1 mM MgCl_2 .

TABLE 2. Antimicrobial activity of bovine lactoferricin against selected bacteria and yeast strains in Bacto Peptone

Bacterial strain or isolate ^a	MIC ^b (μ g/ml)	MBC ^b (μ g/ml)
<i>E. coli</i> CL99 1-2		
Prepn 1	4	8
Prepn 2	13	17
<i>S. typhimurium</i> SL696		
Prepn 1	5	8
Prepn 2	21	21
<i>S. montevideo</i> SL5222		
Prepn 1	3	9
Prepn 2	13	13
<i>S. typhimurium</i> 6749	1.6	3.3
<i>S. typhimurium</i> SH7641	1.6	1.6
<i>E. coli</i> K-12 UB1005	1.6	1.6
<i>E. coli</i> K-12 UB1005 DC-2	1.6	1.6
<i>E. coli</i> ATCC 25922	3.3	3.3
<i>P. aeruginosa</i> ATCC 2783	3.3	>125
<i>P. aeruginosa</i> PAO-1	3.3	>125
<i>Staphylococcus aureus</i> ATCC 29213	6.6	13.2
<i>L. monocytogenes</i> EGD	1.6	3.3
<i>L. monocytogenes</i> 4b (maritime)	6.6	13.2
<i>C. albicans</i> 6372	0.8	0.8
<i>C. albicans</i> 6434	0.8	0.8

^a *E. coli* CL99 1-2, *S. typhimurium* SL696, and *S. montevideo* SL5222 were tested against two separate preparations of lactoferricin purified by two different high-performance liquid chromatographic schema. All other isolates were tested against a single lactoferricin preparation. *E. coli* UB1005 and UB1005 DC-2 are a laboratory parental strain and a polymyxin B-hypersusceptible mutant (45a). *P. aeruginosa* PAO-1 and *L. monocytogenes* EGD are defined laboratory isolates. *L. monocytogenes* 4b (maritime) is a clinical epidemic strain, and the *C. albicans* strains are blood culture isolates from the University of Colorado Health Sciences Center Clinical Microbiology Laboratory.

^b Values shown are the means from two to six experiments.

mg/ml (mean \pm standard deviation) in pooled pulmonary secretions; above 6 mg/ml in preterm colostrum; and above 14 mg/ml in infected parotid fluid (9, 41, 50). Additionally, lactoferrin is released from PMNs in response to cytokine stimulation and in response to gram-negative bacterial infection. As the levels of lactoferrin in plasma during acute sepsis can reach 0.2 mg/ml, it is likely that local concentrations at sites of inflammation will be in the range of milligrams per milliliter (27, 31). Thus, lactoferrin levels similar to those studied may be found in sites of bacterial infection, as well as within the neutrophil phagolysosome and within colostrum milk.

In addition to this activity of whole bovine lactoferrin, we have found that the peptide fragment of the protein, lactoferricin, has very comparable effects on the outer membrane. These observations suggest that at a minimum the amino-terminal domain of the whole protein comprising lactoferricin makes a major contribution to the outer membrane activity of lactoferrin. It is conceivable that this domain is the sole site in lactoferrin contributing to the membrane effects. This hypothesis is consistent with recent X-ray crystallographic observations with human lactoferrin (1). Crystallographic analysis indicates that the region of human lactoferrin approximating the lactoferricin segment of bovine lactoferrin is surface exposed, and thus in a location where it could interact with either free LPS or a bacterial cell.

However, in addition to its outer membrane effects, lact-

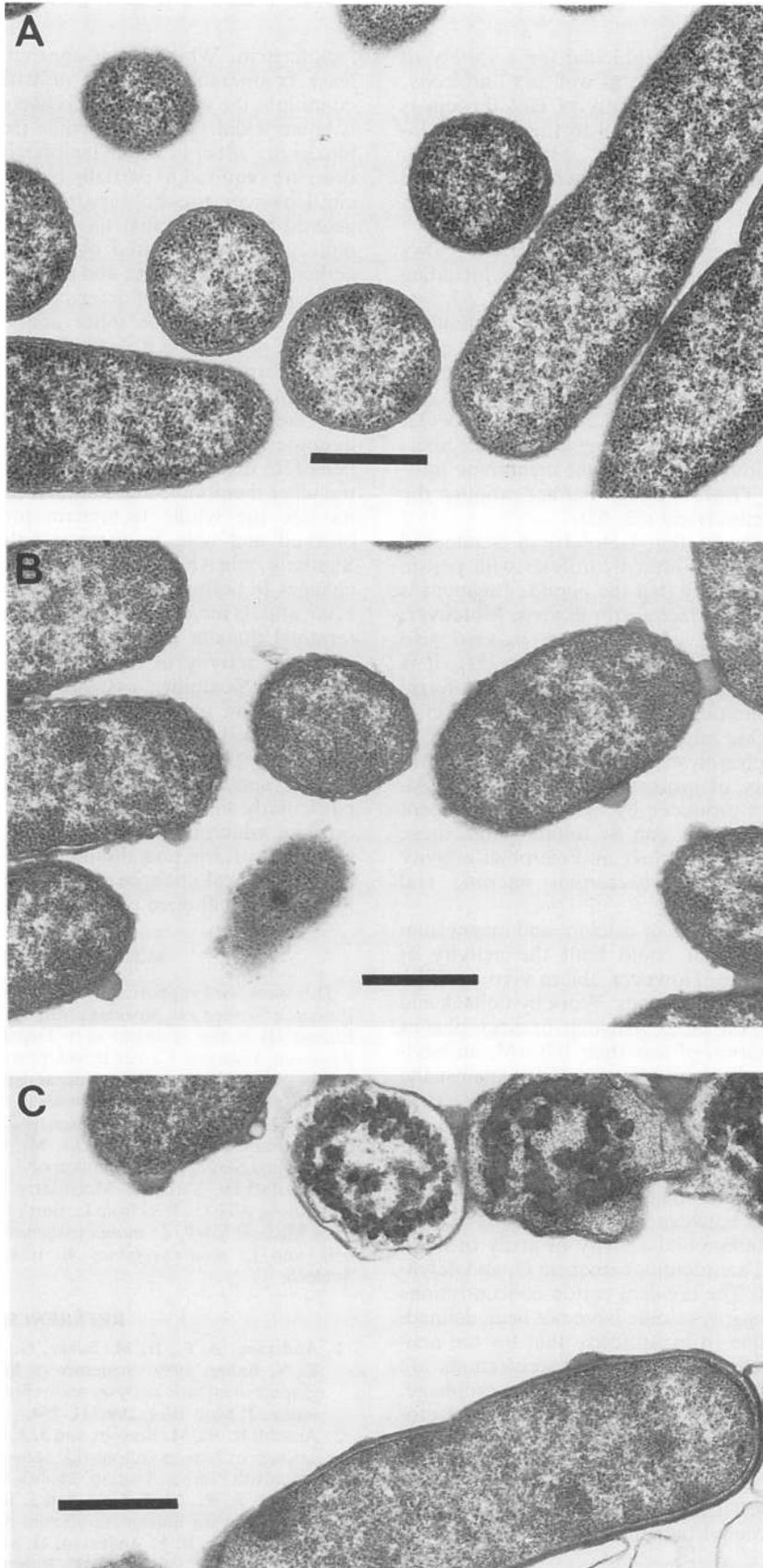


FIG. 9. TEM of *E. coli* CL99 1-2 cells. The cells were incubated for 2 h in 1% Bacto Peptone alone (A), incubated for 0 h in 1% Bacto Peptone with 100 µg of lactoferricin per ml (B), and incubated for 2 h in 1% Bacto Peptone with 100 µg of lactoferricin per ml (C). Bars, 500 nm.

oferricin can also be directly microbicidal for a variety of gram-negative and -positive bacteria, as well as *C. albicans*. For gram-negative bacteria, the activity of lactoferricin is dose dependent, inversely proportional to the bacterial inoculum, and modulated by cations Ca^{2+} , Mg^{2+} , and Fe^{3+} . TEM analysis found that lactoferricin dramatically alters the morphology of both the bacterial cell membrane and cytoplasm.

In all these properties, the peptide lactoferricin shows marked similarities to a variety of PMN proteins including BPI, defensins, and bactericins (22, 24, 33, 37, 47, 53, 54). Although the amino acid sequence of lactoferricin is unique, like these other proteins, it is highly cationic and alters outer membrane permeability (33, 47, 53, 54). With BPI, it shares an ability to bind LPS (38), and with the defensins, it shares an ability to kill gram-negative bacteria and *C. albicans* (22, 35). Additionally, both BPI and the defensins produce alterations in the gram-negative bacterial outer membrane morphology observable by TEM, although neither produce the blister-like effects of lactoferricin (33, 52).

It is important to consider that lactoferricin is released from whole bovine lactoferrin after hydrolysis with pepsin (5), which raises the possibility that the peptide fragment is released from orally ingested lactoferrin in vivo. Moreover, in that an aspartic protease of *Penicillium duponti* also appears to release the peptide from lactoferrin (51), it is possible that lactoferricin will also be freed from lactoferrin in vivo under other conditions when the whole protein is exposed to proteases. One important environment in which this could occur is the phagolysosome of PMNs and macrophages, where a variety of proteases is present (20). Although lactoferrin is not produced by macrophages, recent work suggests that the protein can be found within these cells in vivo and contribute to their antimicrobial activity against *L. pneumophila*, *Mycobacterium microti*, and *Trypanosoma cruzi* (11, 36, 46).

There are high concentrations of calcium and magnesium in most biological fluids that could limit the activity of lactoferrin and lactoferricin. However, the in vivo distribution of the cations is not homogeneous. Work by Pollack and associates indicates that the phagolysosome of macrophages has a calcium concentration of less than 100 μM , an environment where lactoferrin should have activity against the gram-negative bacterial outer membrane and lactoferricin would be bactericidal. Additionally, as noted above, the membrane effects of lactoferrin and lactoferricin appear dependent on a mechanism of action similar to that of the neutrophilic cationic antimicrobial proteins. Both Ca^{2+} and Mg^{2+} (at concentrations between 1 and 10 mM) have been shown to block the antimicrobial activity of many of these proteins, including BPI, azurocidin, cathepsin G, and defensins (12, 35, 37, 42, 53). The divalent cation concentrations within the neutrophil phagolysosome have not been defined. However, it is reasonable to hypothesize that for the neutrophil proteins to function, the cation concentrations are likely to be comparable to those within the macrophage. Thus, this may be another site where lactoferrin and lactoferricin could be active. Quite recently, defensin-like peptides have been isolated from both tracheal tissue (tracheal antimicrobial peptide) and murine small intestine Paneth cells (cryptdins) (14, 15). This result would suggest that there are other in vivo microenvironments where antimicrobial peptides are active.

In spite of the similarities in activity noted above, there are also clear differences between the antimicrobial effects of the whole lactoferrin molecule and its peptide fragment,

lactoferricin. While under appropriate conditions they release comparable amounts of tritiated LPS, under these conditions the whole protein is bacteriostatic and the peptide is bactericidal. Moreover, while iron saturation completely blocks the effect of whole lactoferrin, higher iron concentrations are required to partially block the activity of lactoferricin. However, these observations remain consistent with the general hypothesis that the outer membrane effects of the molecules are mediated by a polycationic mechanism of action. Work by Lehrer and associates (33) suggests that the bactericidal activity of defensins toward *E. coli* relates to an ability to disrupt the inner and outer membrane of these bacteria and that cell death is coincident with inner membrane permeabilization. There is a major size difference between the whole lactoferrin molecule ($\approx 83,000$ Da) and free lactoferricin (3,126 Da) (5, 43). It is reasonable to hypothesize that the peptide fragment might be able to penetrate through the outer membrane to reach and damage the inner membrane and kill the cell. In contrast, because of its size the whole lactoferrin protein may be sterically blocked and able to damage only the outer membrane. Similarly, when lactoferrin becomes iron saturated, there are changes in both the three-dimensional structure of the molecule and its molecular flexibility (4). Thus, while the amino-terminal domain of lactoferrin is not associated with the chelating activity of the molecule, the decrease in overall molecular flexibility associated with iron chelation may decrease the ability of the protein to interact with the bacterial cell. It is also possible that ferric iron could interfere with the activity of lactoferrin and lactoferricin in a manner similar to the divalent cations. Such an effect is particularly likely in relation to the effect of iron on lactoferricin, which is distinct from the iron-binding domain of whole lactoferrin and should therefore not be influenced by conformational changes related to chelation. Each of these hypotheses will need experimental confirmation.

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