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Damage of the Outer Membrane of Enteric Gram-Negative Bacteria by Lactoferrin and Transferrin

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Many studies have shown that lactoferrin and transferrin have antimicrobial activity against gram-negative bacteria, but a mechanism of action has not been defined. We hypothesized that the iron-binding proteins could affect the gram-negative outer membrane in a manner similar to that of the chelator EDTA. The ability of lactoferrin and transferrin to release radiolabeled lipopolysaccharide (LPS) from a UDP-galactose epimerase–deficient *Escherichia coli* mutant and from wild-type *Salmonella typhimurium* strains was tested. Initial studies in barbital-acetate buffer showed that EDTA and lactoferrin cause significant release of LPS from all three strains. Further studies found that LPS release was blocked by iron saturation of lactoferrin, occurred between pH 6 and 7.5, was comparable for bacterial concentrations from 10^4 to 10^7 CFU/ml, and increased with increasing lactoferrin concentrations. Studies using Hanks balanced salt solution lacking calcium and magnesium showed that transferrin also could cause LPS release. Additionally, both lactoferrin and transferrin increased the antibacterial effect of a subinhibitory concentration of rifampin, a drug excluded by the bacterial outer membrane. This work demonstrates that these iron-binding proteins damage the gram-negative outer membrane and alter bacterial outer membrane permeability.

The host defense systems active at mucosal surfaces are poorly characterized, even though most bacterial infections develop through extension of disease from these sites. Mucosal secretions have an antibacterial armamentarium very different from that of serum (28, 41). While there is less complement and relatively little immunoglobulin G (IgG) and IgM, there are high concentrations of secretory IgA (sIgA), lysozyme, and the iron-binding protein lactoferrin. Lactoferrin is found at low levels in serum but has been measured at concentrations of up to 9 to 14 mg/ml in mucosal secretions (28, 29, 41, 50, 51). Of further interest, this protein is also present at high concentrations within the specific granules of polymorphonuclear leukocytes (30, 41, 51).

Both in vivo and in vitro studies indicate that lactoferrin has antibacterial activity (12). A large number of independent investigators have found that lactoferrin has a bacteriostatic effect on a diverse spectrum of gram-negative bacteria and that this effect is blocked if the protein is saturated with iron (7, 15, 21, 35, 41, 42, 46, 48). The major hypothesis advanced for this activity has been that lactoferrin produces an iron-deficient environment that limits bacterial growth.

Several lines of evidence have suggested that lactoferrin may have effects on gram-negative bacteria in addition to those resulting from nutritional deprivation of iron. First, several investigators have found that the antimicrobial activity of lactoferrin against bacteria is enhanced with concurrent exposure of the organisms to IgG or sIgA (9, 39, 45–47). It has been suggested that this interaction develops because antibodies directed against bacterial siderophores suppress bacterial iron metabolism more than does lactoferrin alone. However, this hypothesis has not yet been confirmed, and a precise explanation for the combined effect has not been offered (17).

Second, although it is controversial, work by Arnold and associates has suggested that lactoferrin has direct bactericidal activity against several bacteria, including strains of *Escherichia coli*, *Vibrio cholerae*, *Streptococcus mutans*, *Streptococcus pneumoniae*, and *Legionella pneumophila* (2–5, 10). As in the bacteriostasis produced by lactoferrin, this effect is inhibited by iron saturation of the protein. The effect has not been reproduced by exposure of bacteria to an iron-poor environment alone (5). Also, the activity against *L. pneumophila* is inhibited by the addition of magnesium (10). Immunofluorescence studies have demonstrated that lactoferrin appears to be cell associated (3); on this basis, these investigators have postulated that membrane destabilization may contribute to the bactericidal effect of lactoferrin (10).

As for lactoferrin, several studies have indicated that the serum iron-binding protein transferrin also has antibacterial activity (12, 14). The antibody- and complement-dependent bactericidal activity of serum against *E. coli*, *Neisseria gonorrhoeae*, and *Pasteurella septica* can be blocked by saturation of the serum iron-binding capacity (13, 20, 36). As these assays are performed in the presence of fresh serum that contains partially saturated transferrin, it suggests that this protein contributes to the serum-mediated killing of gram-negative bacteria. Moreover, similar to some of the inconsistencies observed with lactoferrin, a mechanism by which iron deprivation could alter the efficiency of complement-mediated antibacterial activity has not been defined.

In a different field, researchers studying the structure of gram-negative bacteria have shown that synthetic metal chelators have significant antibacterial activity against enteric gram-negative bacilli (26, 34). EDTA releases up to 50% of the lipopolysaccharide (LPS) from the bacterial outer membrane, increases the permeability of the membrane to hydrophobic molecules, and sensitizes the bacteria to the effects of complement and lysozyme (34). These and other studies have contributed to a model of the gram-negative outer membrane as an asymmetric lipid bilayer with large LPS molecules restricted to the outer leaflet and nonpolar

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phospholipids on the inner membrane. While hydrophobic LPS-LPS or LPS-protein interactions contribute to maintenance of outer leaflet integrity, the presence of divalent cations within the membrane appears to be critical to stabilizing the strong negative charges of the core oligosaccharide chain of the LPS molecules (16). By binding these membrane-associated cations, EDTA releases LPS molecules from the outer leaflet. Moreover, if the released LPS is replaced by nonpolar phospholipids from the inner leaflet, the membrane becomes more permeable to hydrophobic agents (such as rifampin) that are normally excluded by the hydrophilic LPS barrier (26, 34).

The transferrin proteins from different species have been shown to bind a wide spectrum of metal ions in addition to iron, including copper, chromium, cobalt, cadmium, manganese, nickel, indium, gallium, aluminum, scandium, and zinc (14). Although the spectrum of metal binding has not been fully characterized for human lactoferrin and transferrin, we hypothesized that these proteins could be broadly active as chelators and could have an effect on the gram-negative outer membrane similar to that of EDTA. We report that, in a manner comparable to that of this synthetic chelator, lactoferrin causes LPS release from enteric gram-negative bacteria and that both iron-binding proteins alter the permeability of the gram-negative outer membrane.

MATERIALS AND METHODS

Lactoferrin, transferrin, and antiserum preparations. Human lactoferrin purified from milk was purchased commercially (Sigma Chemical Co., St. Louis, Mo., or Calbiochem-Behring, La Jolla, Calif.), as was human transferrin purified from serum (Sigma). The following antiserum preparations were purchased commercially and used to test the purity of lactoferrin: rabbit anti-human lactoferrin (Organon Teknika, Malvern, Pa.), goat anti-human transferrin (Organon Teknika), goat anti-human lysozyme (Kallestad Laboratories, Inc., Austin, Tex.), and goat anti-human sIgA (Organon Teknika).

Measurements of protein. Protein concentrations were determined by the biuret method of Mokrasch and McGilvery (33).

Measurements of iron. The levels of iron in individual samples were measured by using flameless atomic absorption (Perkin-Elmer 5000 spectrophotometer [Norwalk, Conn.] equipped with a 500 programmer and an AS-1 auto sampler) in the laboratory of Allen Alfrey (Veterans Administration Medical Center, Denver, Colo.).

Double immunodiffusion. Double immunodiffusion was carried out at room temperature for 18 h in 0.6% agarose in phosphate-buffered saline, pH 7.0.

SDS-PAGE of lactoferrin. Discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of lactoferrin was performed in a modified Laemmli gel system on 1.5-mm slab gels with 4.5% stacking and 10% resolving gels (8, 25). SDS-PAGE gels were analyzed by either modified silver staining or Western blotting (immunoblotting) by the methods of Oakley et al. and Towbin et al., respectively (37, 54). The anti-human lactoferrin serum was diluted 1:500, and the other sera were diluted 1:100. Horseradish peroxidase conjugated to *Staphylococcus aureus* protein A (Amersham International plc, Amersham, United Kingdom) was used at a dilution of 1:2,000 to identify bound antibody.

Preparation of saturated and apolactoferrin and of transferrin. Solutions of apoproteins were prepared by the method of Mazurier and Spik (31), and saturated preparations were made by the method of West and Sparling (59).

Growth of bacteria. A mutant UDP-galactose epimerase–deficient *E. coli* strain (CL99-2) was obtained from Keith A. Joiner (National Institutes of Health, Bethesda, Md.). This is a smooth, serum-resistant strain that produces LPS containing galactose within the O-polysaccharide repeating subunits. However, it is incapable of manufacturing galactose and, unless provided with exogenous galactose, cannot produce a complete LPS molecule (24). When the strain is grown in the presence of [^{3}H]galactose, the radiolabel is almost exclusively incorporated into the LPS O-polysaccharide (19). The mutant strain is not encapsulated and has been shown to have more LPS in its outer membrane than does the parent strain (18). For studies, the strain was grown in WMS broth with slight modifications (52). The medium was prepared as a solution of 0.12 M Tris, 0.02 M KCl, 0.02 M NH_4Cl, 3 mM Na_2SO_4, 1 mM $MgCl_2$, 2×10^{-4} M $CaCl_2$, 2×10^{-6} M $ZnCl_2$, 2×10^{-3} M K_2HPO_4 supplemented with 0.5% glutamate, 1.0 g of Casamino Acids (Difco Laboratories, Detroit, Mich.) per liter, and 0.25% glycerol with or without 0.03 mM galactose as carbon sources.

Two wild-type, smooth *Salmonella typhimurium* strains, SL696 and SH4247, which also incorporate a large percentage of exogenous galactose into LPS, were obtained from Ilkka M. Helander (National Public Health Institute, Helsinki, Finland) (23, 27, 61). These strains were initially grown in Luria broth supplemented with 2 mM CaCl before they were tested for sensitivity to lactoferrin- or transferrin-mediated LPS release (23).

LPS release assay. The strain to be tested in the LPS release assay was grown to stationary phase in a defined galactose-deficient medium (modified WMS broth), and a portion was then added to 1 ml of WMS broth supplemented with 0.03 mM unlabeled galactose and 4 to 15 μCi of D-[6-^{3}H] galactose (24, 52). The cells were grown for 4 h (mid-log phase) at 37°C, centrifuged, washed, and diluted to an appropriate concentration of CFU per milliliter. Initially, duplicate 1.0-ml release assays were prepared that incorporated [^{3}H]galactose-labeled bacteria, barbital-acetate buffer, and various concentrations of test materials in polypropylene tubes. Subsequent work was done with a total volume of 0.5 ml. After addition of the bacteria to complete the assay mixture, 0 min samples were immediately agitated and centrifuged and the supernatant and pellet were counted for 5 min in a beta counter with appropriate corrections for background counts. The 30 min assays were incubated at 37°C and then similarly agitated, centrifuged, and counted.

The percentage of radiolabel released at 30 min was determined with the following formula: percent release = {[30 min sample supernatant cpm/(30 min sample supernatant cpm + 30 min sample pellet cpm)] × 100} − {[0 min buffer supernatant cpm/(0 min supernatant cpm + 0 min pellet cpm)] × 100}. For studies of LPS release over time, it was technically difficult to perform centrifugations in the time available between the different time points. For these experiments, the assay was modified to separate the released LPS from bacterial cells by vacuum manifold filtration with 0.45- or 0.22-μm-pore-size filters (comparable results were obtained with each filter). The percent of initial LPS released was determined as follows: percent release = (time x min sample filtrate cpm/time 0 min sample filtrate cpm) × 100. Subsequent studies were performed by replacing barbital-acetate buffer with 0.1 M Tris buffer (pH 7.2) and either complete Hanks balanced salt solution (HBSS [pH 7.4]), HBSS lacking calcium and magnesium, or HBSS lacking calcium, magnesium, and bicarbonate (Sigma or Whittaker M.A. Bioproducts, Walkersville, Md.).

Analysis of released LPS. For analysis of released LPS, *E. coli* CL99-2 and *S. typhimurium* SL696 were grown with 10 μCi of [^{14}C]galactose and incubated with lactoferrin or EDTA in the LPS release assay described above in HBSS lacking calcium and magnesium. The methods of Perez-Perez and Blaser were used to analyze the released LPS by SDS-PAGE (38). Briefly, the pelleted cells were suspended in 200 μl of solubilizing buffer, and the supernatants were lyophilized and resuspended in 100 μl of solubilizing buffer. All samples were treated with proteinase K for 1 h at 60°C, boiled for 10 min, and redigested with proteinase K. Discontinuous SDS-PAGE using the Laemmli system was performed with 4.5% stacking and 15% resolving gels prepared with either bisacrylamide or AcrylAide (FMC BioProducts, Rockland, Maine) cross-linkers. The gels were subsequently silver stained, dried, and autoradiographed (22).

Effect of lactoferrin and transferrin on bacterial susceptibility to rifampin. *E. coli* CL99-2 was grown to a density of 10^8 CFU/ml in modified WMS with 0.03 mM galactose, and a bacterial inoculum containing 5×10^5 CFU was added to 1 ml of WMS broth with or without EDTA, lactoferrin, or rifampin. The mixture was incubated at 37°C; portions were removed at 0, 2, and 4 h, and bacterial colony counts were determined by plating on tryptic soy agar (Sigma).

Statistical analysis. Data were analyzed by independent and paired Student's *t* tests by using the ABSTAT software package (Anderson-Bell, Canon City, Colo).

RESULTS

The initial studies were performed with lactoferrin from Sigma. The purity of the lactoferrin was ascertained by SDS-PAGE with a modified silver stain showing a single band of approximately 80,000 Da (Fig. 1A). This was further confirmed by double-immunodiffusion experiments with commercial anti-human lactoferrin, anti-human transferrin, and anti-human sIgA showing a precipitin band only with anti-human lactoferrin (data not shown). Finally, immunoblotting with commercial antisera showed that anti-human lactoferrin identified a major 80,000-Da band and a weak second band of approximately 65,000 Da; anti-human transferrin, anti-human sIgA, and anti-human lysozyme were not reactive (Fig. 1B).

The commercial lactoferrin preparations and apolactoferrin solutions were found to be 3 to 10% saturated, apotransferrin solutions were found to be 0.2% saturated, and saturated lactoferrin solutions were found to be 101 to 102% saturated. Initial studies performed with 10^4 CFU/ml at pH 6.8 with lactoferrin, transferrin, and EDTA found that untreated and apolactoferrin-treated preparations produced significant LPS release, although less than that produced by EDTA (Table 1). In contrast, saturated lactoferrin, transferrin, and "apo-" preparations of transferrin and bovine serum albumin did not release LPS. Parallel incubations indicated that the lactoferrin-mediated LPS release was not associated with cell death; 30 min colony counts were 84 ± 8%, 94 ± 9%, 91 ± 11%, and 103 ± 9% of base line for barbital-acetate buffer, transferrin, EDTA, and lactoferrin, respectively (mean ± standard error of the mean [SEM] of four experiments).

Studies were performed to determine the optimum conditions for lactoferrin-mediated LPS release. Studies performed from pH 5.5 to 8.0 found that 2×10^{-5} M EDTA caused a similar release of radiolabel at all pH values and lactoferrin for pH values from pH 6.0 to 8.0, although at pH 8.0 the bacterial membrane appeared to be intrinsically unstable (Fig. 2). At pH 7.0, both lactoferrin and EDTA caused comparable LPS release for bacterial concentrations from 10^4 to 10^7 CFU/ml, with the fraction of LPS released differing but being fixed for each (Fig. 3). Increasing lactoferrin concentrations caused increased ^{3}H release over the range of 0.05 to 2 mg/ml, approaching a limit of 16 to 18% ^{3}H release (Fig. 4). The release of LPS over time was evaluated, and both lactoferrin and EDTA were found to cause significant release within 5 min; continued release occurred through 30 min (Fig. 5).

As these studies were performed with a UDP-galactose epimerase–deficient mutant *E. coli* isolate, additional LPS release studies were performed with two smooth, wild-type *S. typhimurium* strains, SL696 and SH4247, that also incorporate a large percentage of exogenous galactose into LPS

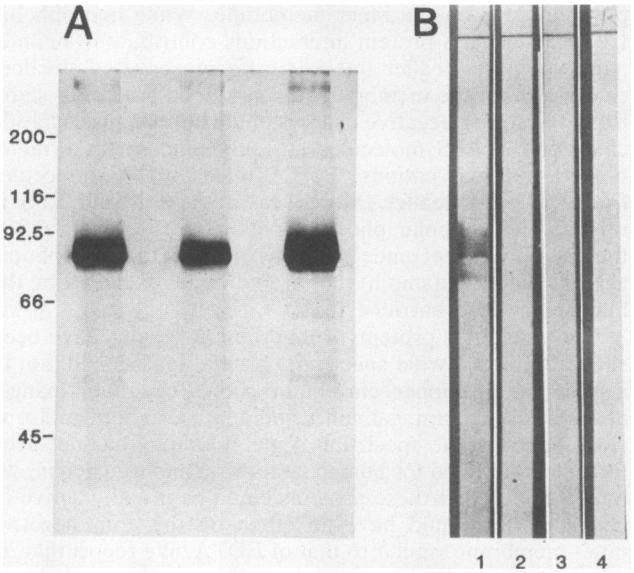

FIG. 1. (A) SDS-PAGE of commercial human milk lactoferrin. Each lane has 1 μg of a different lot of human milk lactoferrin, and the separating gel contained 10% polyacrylamide. The gel was stained with silver by the method of Oakley (13). (B) Immunoblot of goat and rabbit antisera against commercial human milk lactoferrin. Lanes: 1, rabbit anti-human lactoferrin; 2, goat anti-human transferrin; 3, goat anti-human lysozyme; and 4, goat anti-human sIgA. The anti-human lactoferrin serum was diluted 1:500; the other sera were diluted 1:100. Horseradish peroxidase conjugated to *Staphylococcus aureus* protein A was used at a dilution of 1:2,000. Molecular sizes (in daltons) are indicated to the left of panel A.

TABLE 1. Release of ^{3}H from *E. coli* CL99-2 upon exposure to apo- and saturated lactoferrin

Prepn	% ^{3}H released (mean ± SEM of 4 to 9 expts)
Barbital-acetate buffer	2.0 ± 1.3
EDTA, 0.001 M	26.0 ± 3.9[a,b]
Lactoferrin[c], 2 mg (2×10^{-5} M)	8.5 ± 2.1[a,b]
Apolactoferrin, 2 mg	5.9 ± 1.7[b,d]
Saturated lactoferrin, 2 mg	0 ± 1.0
Transferrin, 2 mg	0 ± 1.0
Apotransferrin, 2 mg	2.2 ± 1.7
Apo-bovine serum albumin, 2 mg	1.1 ± 1.3

[a] $P < 0.005$ versus barbital-acetate buffer.
[b] $P < 0.01$ versus saturated lactoferrin.
[c] Obtained from Sigma.
[d] $P < 0.05$ versus barbital-acetate buffer.

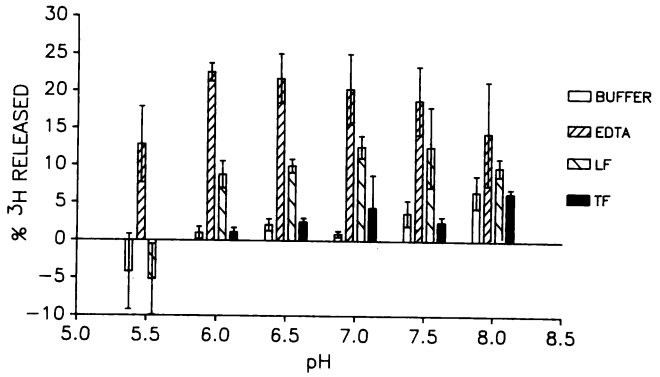

FIG. 2. Effect of pH on the release of LPS from 10^4 *E. coli* CL99-2 CFU upon exposure to 2×10^{-5} M EDTA, lactoferrin (2 mg/ml) (LF), or transferrin (2 mg/ml) (TF) in barbital-acetate buffer (mean ± SEM of three to five experiments).

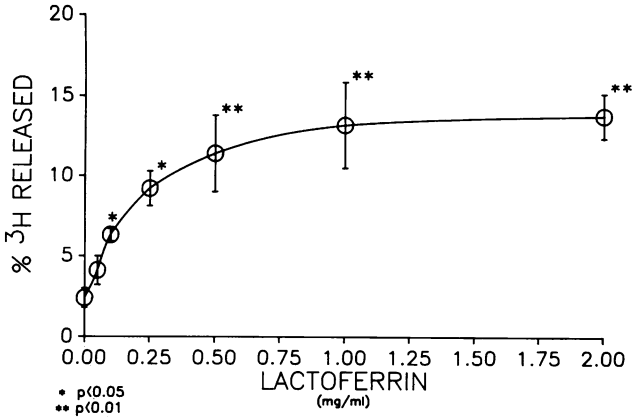

FIG. 4. Release of LPS from 10^7 CFU of *E. coli* CL99-2 per ml in barbital-acetate buffer (pH 7.0) with increasing lactoferrin concentrations (mean ± SEM of three to seven experiments; statistical comparison of each concentration versus preceding lower concentration by paired *t* test).

(23). Lactoferrin and EDTA, but not transferrin, released LPS from both strains, although the amount of LPS released differed for the two isolates (Fig. 6).

In additional studies, we investigated the effects of different buffer systems on the membrane effects of lactoferrin and transferrin (Table 2). The results in a Tris buffer were comparable to those in barbital-acetate. However, in studies with Tris, the buffer was found to release large quantities of LPS by itself and limited the reproducibility of the assay. In complete HBSS, LPS release was not observed with EDTA or with either of the iron-binding proteins. In contrast, lactoferrin and EDTA induced LPS release both in HBSS lacking calcium and magnesium and in HBSS lacking calcium, magnesium, and bicarbonate; significantly greater release was observed in the HBSS containing bicarbonate. In these studies, limited LPS release from the *E. coli* strain was also observed with transferrin exposure in HBSS lacking calcium and magnesium, although the release was less than that observed with lactoferrin.

Studies of LPS release in HBSS lacking calcium and magnesium were repeated by using lactoferrin obtained from a second vendor (Calbiochem). This second lactoferrin preparation also caused LPS release from all three bacterial species (Table 3).

Prior work on EDTA-mediated LPS release showed both that the chelator releases a fixed fraction of LPS and that the retained and released LPS fractions are equally heterogeneous in O-polysaccharide chain length (23, 26). Assuming that the mechanism of lactoferrin is comparable to that of EDTA, it should also release a heterogeneous population of LPS molecules. This hypothesis was confirmed by analyzing the composition of LPS released from *E. coli* CL99-2 and *S. typhimurium* SL696 by using SDS-PAGE. With both strains, lactoferrin and EDTA caused identical release of heterogeneous LPS populations (Fig. 7).

To determine whether the lactoferrin-mediated LPS release is associated with a change in outer membrane permeability, we tested the effect of EDTA, lactoferrin, and transferrin on the susceptibility of *E. coli* CL99-2 to the antibiotic rifampin, which is normally excluded by the outer membrane. EDTA, lactoferrin, and transferrin were all bacteriostatic by themselves, and each had a more than additive effect in increasing bacterial killing due to a subinhibitory concentration of rifampin. The activity of lactoferrin was almost completely eliminated by iron saturation of the protein (Table 4).

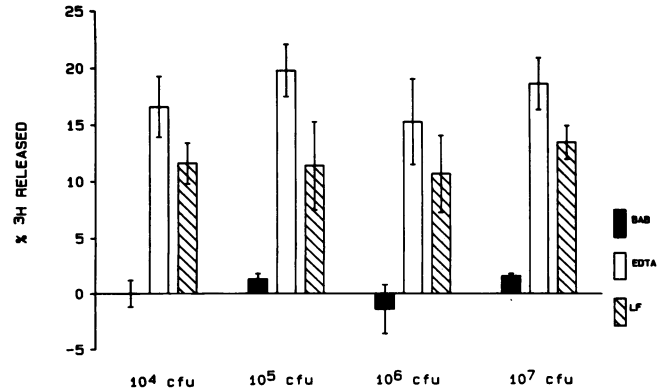

FIG. 3. Release of LPS from *E. coli* CL99-2 by lactoferrin (2 mg/ml) (LF) and 2×10^{-5} M EDTA in barbital-acetate buffer (pH 7.0) (BAB) with increasing bacterial concentrations (mean ± SEM of three to five experiments).

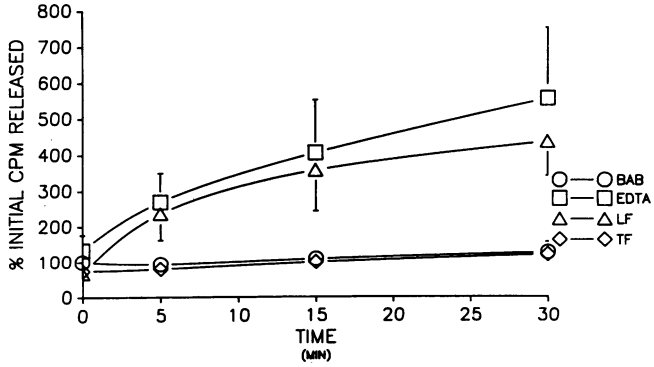

FIG. 5. Release of LPS by lactoferrin (2 mg/ml) (LF), transferrin (2 mg/ml) (TF), and 2×10^{-5} M EDTA from 10^7 CFU/ml in barbital-acetate buffer (BAB; pH 7.0) (mean ± SEM of three experiments; results expressed as percentage of supernatant counts per minute released at 0 min).

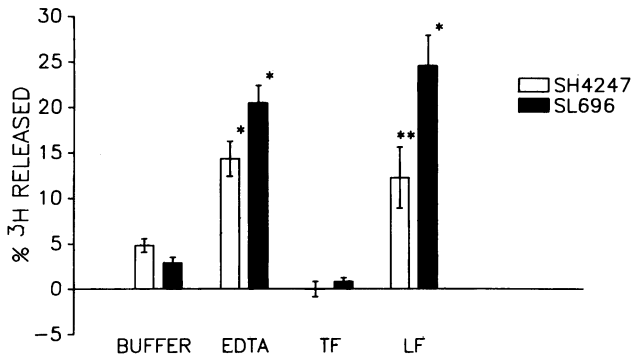

FIG. 6. Release of ^{3}H-labeled LPS from 10^7 CFU of wild-type *S. typhimurium* SL696 and SH4247 by lactoferrin (2 mg/ml) (LF), transferrin (2 mg/ml) (TF), and 2×10^{-5} M EDTA in barbital-acetate buffer (pH 7.0) (mean ± SEM of 10 experiments). *, $P < 0.0005$ versus buffer; **, $P < 0.05$ versus buffer.

DISCUSSION

Gram-negative bacilli have an outer membrane composed of an asymmetric lipid bilayer, with the large LPS molecules restricted to the outer leaflet (26, 34). The LPS molecules are anionic and appear to be stabilized within the membrane by associated cations, including calcium, magnesium, and iron (16). By binding the membrane-stabilizing cations, EDTA releases LPS in the outer membrane (26). Concurrent with the LPS release, the outer membrane becomes more permeable to agents, such as rifampin, that are normally excluded owing to hydrophobicity. The present studies demonstrate that, at a physiologic pH and concentration, lactoferrin releases LPS from enteric gram-negative bacilli. When approximately equimolar concentrations of lactoferrin and EDTA are tested, the respective amounts of LPS released by are comparable and the timing of LPS release is similar. The fraction of LPS released by each compound appears to be fixed for bacterial concentrations from 10^4 to 10^7 CFU/ml. Both lactoferrin and EDTA release heterogeneous populations of LPS molecules. The release of LPS by lactoferrin is inhibited by iron saturation of the protein, an observation consistent with the hypothesis that the membrane effects are dependent on chelating activity and also with previous observations on the bacteriostatic and bactericidal properties of the protein (2–5, 7, 10, 21, 35, 42, 46, 48).

TABLE 2. Release of [^{3}H]LPS from 10^7 *E. coli* CL99-2 or 10^7 *S. typhimurium* SL696 CFU by lactoferrin and transferrin in different buffer systems (four to six experiments)

Buffer system[a]	% [^{3}H]LPS released (mean ± SEM) from:	
	E. coli CL99-2	*S. typhimurium* SL696
Tris	4.9 ± 1.8	10.8 ± 3.5
Tris + EDTA	32.3 ± 13.1[b]	20.4 ± 4.7[c]
Tris + LF	9.8 ± 2.4[d]	58.6 ± 9.7[c]
Tris + TF	−0.3 ± 2.0	7.4 ± 2.8
HBSS	5.6 ± 1.5	2.2 ± 0.4
HBSS + EDTA	7.4 ± 1.8	2.2 ± 0.4
HBSS + LF	3.3 ± 1.8	2.9 ± 0.6
HBSS + TF	4.0 ± 1.2	1.2 ± 0.3
HBSS-Ca,Mg	4.1 ± 0.5	10.0 ± 2.5
HBSS-Ca,Mg + EDTA	22.1 ± 0.9[e]	16.8 ± 1.9[e]
HBSS-Ca,Mg + LF	16.1 ± 0.9[e]	26.7 ± 5.0[e]
HBSS-Ca,Mg + TF	11.7 ± 0.8[e]	11.3 ± 2.0
HBSS-Ca,Mg,HCO_3	2.8 ± 0.4	4.8 ± 0.8
HBSS-Ca,Mg,HCO_3 + EDTA	10.1 ± 1.9[c]	10.4 ± 1.5[c]
HBSS-Ca,Mg,HCO_3 + LF	5.3 ± 1.5	18.4 ± 4.6[c]
HBSS-Ca,Mg,HCO_3 + TF	2.7 ± 0.7	3.9 ± 0.8

[a] HBSS-Ca,Mg, HBSS minus calcium and magnesium (Sigma); HBSS-Ca,Mg,HCO_3, HBSS minus calcium, magnesium, and bicarbonate (Sigma); LF, lactoferrin (2 mg/ml); TF, transferrin (2 mg/ml). EDTA was at 2×10^{-5} M.
[b] $P = 0.053$ versus buffer; all statistical comparisons were performed by paired *t* test.
[c] $P < 0.01$ versus buffer.
[d] $P = 0.075$ versus buffer.
[e] $P < 0.0001$ versus buffer; $P < 0.001$ versus HBSS-Ca,Mg,HCO_3.

TABLE 3. Release of [^{3}H]LPS from 10^7 *E. coli* CL99-2, 10^7 *S. typhimurium* SL696, and 10^7 *S. typhimurium* SH4247 CFU by a second commercial lactoferrin preparation (three experiments)

Prepn[a]	% [^{3}H]LPS released (mean ± SEM) from:		
	E. coli CL99-2	*S. typhimurium* SL696	*S. typhimurium* SH4247
HBSS-Ca,Mg	3.7 ± 0.1	21.8 ± 3.9	7.0 ± 2.9
HBSS-Ca,Mg + EDTA	18.2 ± 0.7[b]	27.1 ± 4.2[b]	14.0 ± 3.8[b]
HBSS-Ca,Mg + LF	9.1 ± 1.4[b]	36.4 ± 7.7[b]	15.1 ± 3.3[b]
HBSS-Ca,Mg + TF	5.8 ± 1.6[c]	15.8 ± 1.8	6.3 ± 1.6

[a] EDTA was at 2×10^{-5} M. LF, Lactoferrin (2 mg/ml); TF, transferrin (2 mg/ml).
[b] $P \leq 0.05$ versus buffer; all statistical comparisons were performed by paired *t* test.
[c] $P = 0.15$ versus buffer.

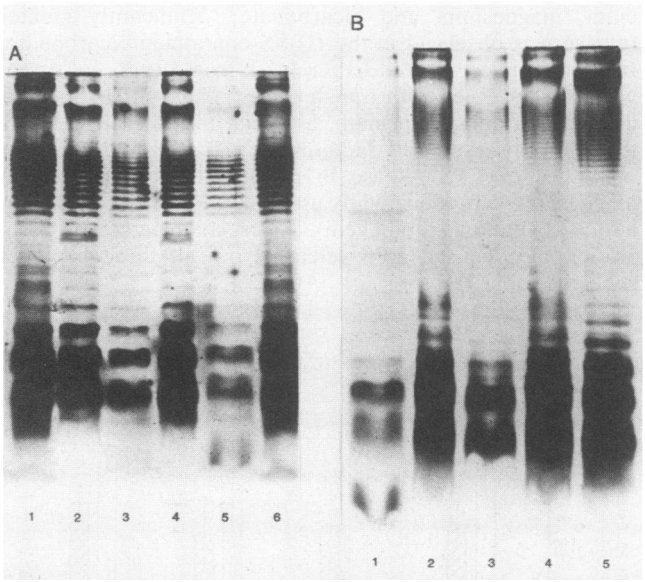

FIG. 7. (A) Silver-stained SDS-PAGE gel of retained and released LPS from *E. coli* CL99-2. Lanes: 1, commercial 0111:B4 LPS (List Biological Laboratories, Campbell, Calif.); 2, cell pellet from EDTA-treated cells; 3, supernatant from EDTA-treated cells; 4, cell pellet from lactoferrin-treated cells; 5, supernatant from lactoferrin-treated cells; 6, commercial 0111:B4 LPS. (B) Silver-stained SDS-PAGE gel of retained and released LPS from *S. typhimurium* SL696. Lanes: 1, supernatant from lactoferrin-treated cells; 2, cell pellet from lactoferrin-treated cells; 3, supernatant from EDTA-treated cells; 4, cell pellet from EDTA-treated cells; 5, whole-cell preparation of *S. typhimurium* SL696.

TABLE 4. Effects of rifampin, EDTA, lactoferrin, and transferrin on growth of *E. coli* CL99-2 (six experiments)

Prepn	Mean log change (mean ± SEM)[a] at:	
	2 h	4 h
WMS media	0.9 ± 0.1	1.7 ± 0.2
EDTA, 2×10^{-5} M	0.6 ± 0.1	1.3 ± 0.1*
Lactoferrin, 2 mg	0.7 ± 0.1	1.1 ± 0.1*
Saturated lactoferrin, 2 mg	1.0 ± 0.0	1.8 ± 0.1
Transferrin, 2 mg	0.7 ± 0.1	0.9 ± 0.1*
Rifampin, 2 μg	0.8 ± 0.1	1.7 ± 0.1
Rifampin + EDTA	−0.2 ± 0.3**	−0.5 ± 0.6***
Rifampin + lactoferrin	0.0 ± 0.3**	−0.2 ± 0.4***
Rifampin + saturated lactoferrin	0.6 ± 0.2	0.9 ± 0.5‡
Rifampin + transferrin	0.0 ± 0.3†	0.2 ± 0.4**

[a] *, $P < 0.002$ versus WMS media; **, $P < 0.0005$ versus rifampin; ***, $P < 0.0001$ versus rifampin; †, $P < 0.01$ versus rifampin; ‡, $P < 0.05$ versus rifampin (all statistics were compared by paired *t* test).

Lactoferrin and EDTA caused LPS release in three different buffer systems with low calcium and magnesium concentrations, but their activity was blocked by the high concentrations of calcium and magnesium found in HBSS (0.00125 M and 0.001 M, respectively). The activity of lactoferrin was enhanced when bicarbonate was present, suggesting that it could act as a companion anion for the chelation of metal ions. This is consistent with the known chelating properties of the metal-binding proteins (57). That exogenous calcium and magnesium ions block the activity of lactoferrin and transferrin suggests that the ability of the proteins to chelate these ions may be important for the observed effects. However, recent work has suggested that lactoferrin may form tetramers in the presence of high concentrations of calcium (6). This could limit the biological activity of the protein and would be an alternative explanation for calcium blocking the effect of this protein. That the concentrations of calcium and magnesium in complete HBSS block LPS release by lactoferrin clearly may limit the physiologic relevance of the observations. To determine the importance of these studies it will be necessary to further define the influence of these two cations on the ability of lactoferrin to release LPS.

Although transferrin altered susceptibility to rifampin, it did not release LPS in all buffer systems, nor did it release as large a percentage of LPS as did lactoferrin. This suggests either that differences in chelating ability are important for the LPS release or that an additional mechanism contributes to the effect of lactoferrin. Although we are unaware of relative studies on the ability of the two proteins to chelate calcium and magnesium, the two proteins do differ in their ability to chelate iron; lactoferrin is the stronger chelator (1, 53). Furthermore, transferrin is anionic at physiologic pH (pI ~ 5.5), while lactoferrin is very cationic (pI ~ 8.5) (32, 60). Because it is cationic, lactoferrin may share a second mechanism of action with a family of polycationic agents. These compounds include polylysine, protamine, the antimicrobial agents polymyxin B and polymyxin B nonapeptide (PMBN), and several cationic proteins isolated from neutrophils (55, 56, 58).

In a manner similar to that of EDTA, the polycationic compounds increase the permeability of the outer membrane to hydrophobic agents, although the compounds have heterogeneous effects and their mechanisms of action are not fully understood. Polymyxin B and PMBN cause both permeability change and LPS release, while a neutrophil bactericidal effect/permeability-increasing protein enhances membrane permeability but does not release LPS. The activity of these polycationic compounds may be the result of an ability to insert into the bacterial outer membrane by binding to the anionic LPS molecules. Differences in binding or membrane insertion could then explain the observed variability in effects (56). As several studies have suggested that lactoferrin can be associated with the bacterial cell membrane, it is reasonable to postulate that lactoferrin may also share a mechanism of action with the polycationic compounds (3, 40).

The demonstration of a more-than-additive interaction between the iron-binding proteins and rifampin is consistent with the proteins altering outer membrane permeability, although other explanations for the effect have not been excluded. Also, the variability between lactoferrin and transferrin in relation to the effects on LPS release and membrane permeability is not unlike the variability noted in the polycationic compounds (56). More-detailed studies will be necessary to clarify the precise mechanisms of outer membrane damage.

If these effects are demonstrable at calcium and magnesium concentrations that exist in vivo, the ability of lactoferrin and transferrin to alter the structure of the outer membranes of gram-negative bacteria may supplement their ability to deprive bacteria of iron. This could contribute to their bacteriostatic (and, in the case of lactoferrin, possibly bactericidal) activities. Perhaps more importantly, the membrane effects of the iron-binding proteins may allow them to amplify other antimicrobial systems. Prior work with EDTA and the polycationic agent PMBN has shown that the compounds can enhance the bactericidal effects of antibiotic agents, the complement system, and lysozyme (11, 26, 34, 43, 44, 50). In 1974, Leive suggested that, except for its toxicity, EDTA might have a valuable role as an antibiotic adjuvant (26). The present work suggests that lactoferrin and transferrin may have comparable effects. A lactoferrin-mediated alteration in outer membrane structure could explain the observed additive effects of lactoferrin and sIgA (9, 39, 45–47). Moreover, there is limited evidence suggesting that lysozyme and lactoferrin may have synergistic activity (9). Such an interaction would be relevant physiologically as the proteins are found at their highest concentrations at the same sites in vivo. Together they may function as a potent bactericidal system against the gram-negative bacteria that are only minimally affected by either protein alone.

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