Lactoferrin and transferrin damage of the Gram-negative outer membrane is modulated by Ca^{2+} and Mg^{2+}

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Lactoferrin and transferrin have antimicrobial activity against selected Gram-negative bacteria, but the mechanism of action has not been defined. We studied the ability of lactoferrin and transferrin to damage the Gram-negative outer membrane. Lipopolysaccharide release by the proteins could be blocked by concurrent addition of Ca^{2+} and Mg^{2+} . Addition of Ca^{2+} also blocked the ability of lactoferrin to increase the susceptibility of *Escherichia coli* to rifampicin. Transferrin, but not lactoferrin, increased susceptibility of Gram-negative bacteria to deoxycholate, with reversal of sensitivity occurring with exposure to Ca^{2+} or Mg^{2+} . In transmission electron microscopy studies polymyxin B caused finger-like membrane projections, but no morphological alterations were seen in cells exposed to EDTA, lactoferrin or transferrin. These data provide further evidence that lactoferrin and transferrin act as membrane-active agents with the effects modulated by Ca^{2+} and Mg^{2+} .

Introduction

Lactoferrin and transferrin are iron-binding proteins found in essentially all human secretions. The highest level of transferrin is in serum, and that of lactoferrin within polymorphonuclear leucocytes and in mucosal fluids (Masson *et al.*, 1966, 1969). They have protean effects on micro-organisms; variably acting as growth factors, inducing iron uptake systems, and for selected bacteria producing bacteriostasis (Bullen, 1981; Bullen *et al.*, 1978; Griffiths, 1975; Norrod & Williams, 1978). It has been suggested that this last effect contributes to antibacterial host defence, and that the mechanism of bacteriostasis is sequestration of environmental iron causing nutritional deprivation of susceptible organisms (Finkelstein *et al.*, 1983).

However, a variety of studies have indicated additional antimicrobial effects of the proteins not readily explainable by this hypothesis. The antimicrobial activity of lactoferrin for Gram-negative organisms can be increased by addition of immunoglobulins (Boesman-Finkelstein & Finkelstein, 1985; Rainard, 1986; Spik *et al.*, 1976; Stephens *et al.*, 1980). While this could be due

to further iron deprivation through anti-siderophore antibodies, enhancing antibodies have been found to be directed against lipopolysaccharide (LPS) O-antigens (Finkelstein *et al.*, 1983; Stephens *et al.*, 1980). Similarly, transferrin contributes to antibody- and complement-mediated serum antibacterial activity towards several Gram-negative species, although the mechanism of action is not known (Bullen *et al.*, 1978; Griffiths, 1975; Norrod & Williams, 1978; Fitzgerald & Rogers, 1980). Finally, other work indicates that lactoferrin may directly kill selected bacterial strains through an undefined mechanism (Arnold *et al.*, 1977, 1982; Bortner *et al.*, 1986; Kalmar & Arnold, 1988).

Recent work now suggests that lactoferrin and transferrin can directly damage the outer membrane of Gram-negative bacteria in a manner similar to the synthetic chelator EDTA (Ellison *et al.*, 1988). Gram-negative bacteria have an outer membrane composed of an asymmetric lipid bilayer with large LPS molecules restricted to the outer leaflet (Leive, 1974; Nikaido & Vaara, 1985). The LPS molecules are anionic and are stabilized within the membrane by associated cations, primarily Ca^{2+} and Mg^{2+} (Coughlin *et al.*, 1983). By binding membrane-stabilizing cations, EDTA releases LPS from the outer membrane, concurrently increasing membrane permeability to hydrophobic molecules and sensitizing bacteria to membrane-active agents (Leive,

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1974; Nikaido & Vaara, 1985). As the transferrin proteins can bind numerous metal ions in addition to iron (Bullen *et al.*, 1978), it was theorized that lactoferrin and transferrin could have effects on the Gram-negative outer membrane comparable to EDTA. Lactoferrin, and to a lesser extent, transferrin, were shown to release LPS from Gram-negative bacteria at physiological concentrations and pH (Ellison *et al.*, 1988). Moreover, the proteins also sensitized bacteria to the hydrophobic antibiotic rifampicin (Ellison *et al.*, 1988). To further define the mechanism of membrane damage by these iron-binding proteins, the impact of the membrane-stabilizing cations Ca^{2+} and Mg^{2+} on their effects has now been studied.

Methods

Lactoferrin and transferrin. Human lactoferrin purified from milk was purchased commercially (Sigma or Calbiochem) as was human transferrin purified from serum (Sigma). It was known that the lactoferrin from one of the commercial sources had not been exposed to any known chelators during its purification (personal communication, Calbiochem).

Analytical procedures. Protein concentrations were determined by the biuret method of Mokrasch & McGilvery (1956). The levels of calcium, magnesium or iron in individual samples were measured using flameless atomic absorption spectrophotometry (Perkin-Elmer 5000 spectrophotometer equipped with a model 500 programmer and an AS-1 auto sampler) in the laboratory of Dr A. Alfrey (Department of Veterans Affairs Medical Center, Denver, CO, USA).

Bacteria and culture conditions. UDP-galactose epimerase deficient strains *E. coli* CL99-2 and *Salmonella montevideo* SL5222 were obtained from Dr K. A. Joiner (National Institutes of Health, Bethesda, MD, USA). *E. coli* CL99-2 is a 'smooth', O111 serotype, serum-resistant strain that incorporates galactose into the O-polysaccharide repeating LPS subunits but is incapable of synthesizing galactose. Unless provided with exogenous galactose, this organism cannot produce a complete LPS molecule (Joiner *et al.*, 1984). When the strain is grown in the presence of [^3H]galactose, the radiolabel is almost exclusively incorporated into the LPS O-polysaccharide (Goldman & Leive, 1980). *S. montevideo* SL5222 lacks both UDP-galactose epimerase and phosphomannose isomerase, both of which affect LPS biosynthesis (Joiner *et al.*, 1986). Only cells grown in the presence of both mannose and galactose can synthesize a complete LPS structure. Galactose is incorporated solely in the core oligosaccharide while four mannose residues are incorporated into each O-antigen subunit. In the present studies, *E. coli* CL99-2 was grown in a defined galactose-deficient medium (modified WMS broth) as described previously (Ellison *et al.*, 1988), and *S. montevideo* SL5222 was grown in Luria broth. For selected experiments the media were supplemented with defined concentrations of MgCl_2 , CaCl_2 or ferric chloride.

A wild-type 'smooth' *Salmonella typhimurium* strain, SL696, that also incorporates a large percentage of exogenous galactose into LPS was obtained from Dr I. M. Helander (National Public Health Institute, Helsinki, Finland) (Hukari *et al.*, 1986). This strain was initially grown in Luria broth supplemented with 2 mM- CaCl_2 before testing for sensitivity to lactoferrin or transferrin-mediated LPS release (Hukari *et al.*, 1986). For selected experiments the medium was supplemented with defined concentrations of MgCl_2 , CaCl_2 or ferric chloride.

E. coli 11744 has a non-encapsulated 'smooth' LPS phenotype and is a human blood isolate from the University of Colorado, Health Sciences Center Clinical Microbiology Laboratory blood culture collection. Clinical isolates of *E. coli* (including a serotype O26 strain), *Serratia marcescens*, *Enterobacter cloacae*, *Proteus mirabilis* and *Pseudomonas aeruginosa* were obtained from Dr W.-L. Wang, Denver Department of Veterans Affairs, Medical Center Clinical Microbiology Laboratory.

LPS release assay. Release of LPS from bacteria was determined using the assay described previously (Ellison *et al.*, 1988). Briefly, the strain to be tested was grown for 4 h at 37 °C in 1 ml of defined medium supplemented with 0.1 mM unlabelled galactose and 4–15 μCi D-[6- ^3H]galactose. The cells were then centrifuged, washed and suspended in Hanks' balanced salt solution lacking calcium and magnesium (HBSS-CM) (Sigma). Duplicate 1.0 ml release assays were prepared containing approximately 5×10^7 c.f.u. [^3H]galactose-labelled bacteria, buffer and varying concentrations of test materials in polypropylene tubes. The pH was maintained between 7 and 7.5. After addition of bacteria, 0 min samples were immediately agitated, centrifuged and the supernatant and pellet fractions counted for 5 min for β -emissions 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 as follows:

$$\text{Percentage release} = \frac{[30 \text{ min sample supernatant c.p.m.}/(30 \text{ min sample supernatant c.p.m.} + 30 \text{ min sample pellet c.p.m.}) \times 100] - [0 \text{ min buffer supernatant c.p.m.}/(0 \text{ min supernatant c.p.m.} + 0 \text{ min pellet c.p.m.}) \times 100]}{100}$$

For studies of the ability of calcium, iron or magnesium to block LPS release, each experiment compared a series of samples incubated in HBSS-CM with samples incubated with a selected single supplemental concentration of CaCl_2 , ferric chloride or MgCl_2 . For example, for 3 d parallel LPS release assays were done with lactoferrin and transferrin in HBSS-CM and in HBSS-CM supplemented with 1.3×10^{-3} M- Ca^{2+} . The Ca^{2+} concentrations were chosen to represent 0, 6.25, 12, 25, 50 and 100% of the calcium in HBSS, and the Mg^{2+} concentrations to represent 0, 50, and 100% of the magnesium in HBSS. The iron concentrations were chosen to provide approximately 100% and 200% of the iron necessary to saturate lactoferrin and transferrin.

To further exclude potential artifacts from unrecognized contaminants, release assays were also done using lactoferrin that was dialysed for 20 h against 0.1 M-sodium perchlorate (Sigma) to dissociate any unrecognized contaminating anions, and then equilibrated with HBSS-CM.

Effect of lactoferrin and transferrin on bacterial susceptibility to rifampicin. *E. coli* strain 11744 was grown to a density of 10^8 c.f.u. ml $^{-1}$ in modified WMS broth with 0.03 mM-galactose. An inoculum of 5×10^5 c.f.u. was added to 1 ml of WMS broth with or without EDTA, lactoferrin, rifampicin or supplemental calcium. Time-kill curves were developed by incubating the mixtures at 37 °C and removing samples at 0, 2 and 4 h. Bacterial colony counts were measured by plating overnight at 37 °C on tryptic soy agar (TSA, Sigma).

Effect of lactoferrin and transferrin on bacterial susceptibility to deoxycholate. The *E. coli* serotype O26 strain was grown in tryptic soy broth, harvested in stationary or early exponential phase of growth, and washed twice in saline (0.9% NaCl). 10^5 c.f.u. inoculum was suspended in 0.9% saline with or without varying concentrations of lactoferrin and transferrin for 30 min at 37 °C. Samples were removed at 0 and 30 min and bacterial colony counts determined by initially plating overnight at 37 °C on blood agar, MacConkey agar, TSA, and TSA with 0.085% (w/v) sodium deoxycholate. Studies of the individual constituents of MacConkey agar used 0.085% sodium deoxycholate, 0.085% (w/v)

cholate, 0.003% (w/v) neutral red (Baker) and 0.0001% (w/v) crystal violet (Matheson, Coleman & Bell). Subsequent experiments were plated only on agar containing deoxycholate (MacConkey or TSA with deoxycholate). Activity was defined by the decrease in c.f.u. remaining at 30 min. To determine the effect of pH, studies were repeated in 0.001 M-barbital acetate buffer at pH 6.5, 7.2 or 8.2.

To determine if transferrin-mediated deoxycholate susceptibility could be reversed, a 10^5 c.f.u. inoculum of *E. coli* cells was first incubated with 1 mg transferrin in 1.0 ml 0.9% saline for 30 min at 37 °C. A 0.1 ml sample was then removed and added to 9.9 ml of varying solutions at 37 °C (water, HBSS, 0.9% saline, 10^{-4} M- CaCl_2 , 10^{-4} M- MgSO_4 , 10^{-5} M-ferric chloride, 10^{-4} M- Na_2HPO_4 , 10^{-4} M- $\text{CaCl}_2/10^{-4}$ M- Na_2HPO_4 and 10^{-4} M- $\text{MgSO}_4/10^{-4}$ M- Na_2HPO_4). Samples were removed immediately after dilution, and 15 and 30 min later. Bacterial colony counts were determined by plating overnight at 37 °C on deoxycholate containing agar. The studies with 0.9% saline were repeated at 4 °C and 37 °C.

Transmission electron microscopy. *E. coli* CL99-2 and *S. typhimurium* SL696 cells were treated as in the LPS release experiments. Cells were grown for 4 h (mid-exponential phase of growth) at 37 °C, centrifuged, washed and diluted. Release assays (1.0 ml) were prepared incorporating 10^9 c.f.u. ml^{-1} in HBSS-CM in buffer alone or in the presence of 2×10^{-5} M-EDTA, 2×10^{-5} M-transferrin, 2×10^{-5} M-lactoferrin or 30 μg polymyxin B ml^{-1} . Samples were incubated at 37 °C for 30 min, agitated and centrifuged. The bacterial pellet was resuspended in 2% (w/v) glutaraldehyde in 0.1 M-sodium phosphate buffer, pH 7.4, for 1 h at 4 °C. Samples were postfixed in buffered 1% (w/v) osmium tetroxide, dehydrated through a graded series of ethanols, and embedded in Poly/bed 812-araldite (Mollenhauer medium, Polysciences). 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.

Statistical analysis. Data analysis was done by independent and paired Student's *t*-tests using the ABSTAT software package (Anderson-Bell, Canon City, CO, USA).

Results

LPS release

To define the effects of calcium, iron and magnesium on LPS release due to lactoferrin and transferrin, the effects of the proteins on *E. coli* CL99-2 and *S. typhimurium* SL696 were studied in the presence of graded concentrations of the cations. As expected from previous studies, the degree of LPS release differed slightly between the strains but the results were otherwise similar; only the *S. typhimurium* SL696 data are shown (Table 1). Supplemental iron was able to inhibit LPS release by both of the proteins, with less effect on the activity of EDTA. Release of LPS mediated by lactoferrin and transferrin was similar when studied in HBSS-CM or in HBSS-CM with the addition of 8×10^{-5} M- Ca^{2+} . Comparable to previous work, lactoferrin consistently released more ^3H -labelled LPS than transferrin. As the Ca^{2+} concentration was increased there was graded and parallel inhibition of LPS release due to each of the proteins as well as EDTA. Of note, however, 2×10^{-5} M-lactoferrin caused significant LPS release from both bacterial strains in the presence of 1.6×10^{-4} M- Ca^{2+} , and 2×10^{-5} M-

Table 1. Effect of increasing Ca^{2+} or Mg^{2+} concentration on lactoferrin- and transferrin-mediated release of ^3H -labelled LPS from *S. typhimurium* SL696

The release of ^3H -labelled LPS was determined as described in Methods. Results are means $\pm$ SEM of three experiments. Each concentration of supplemental Ca^{2+} , Fe^{3+} or Mg^{2+} was studied at least in triplicate with a simultaneous unsupplemented control: the respective unsupplemented Ca^{2+} , Fe^{3+} and Mg^{2+} control results represent 16, 6 and 6 assays respectively. The significance of results (versus LPS release in buffer alone) is indicated as follows (all statistics by paired *t*-test): (a) $P < 0.05$; (b) $P < 0.01$; (c) $P < 0.005$; (d) $P \leq 0.0001$.

		LPS release			
Assay buffer supplement	Assay buffer . . .	HBSS-CM buffer	HBSS-CM + EDTA (2×10^{-5} M)	HBSS-CM + lactoferrin (2 mg ml^{-1})*	HBSS-CM + transferrin (2 mg ml^{-1})*
Unsupplemented		7.3 $\pm$ 1	16.3 $\pm$ 1 ^d	18.6 $\pm$ 2 ^d	8.8 $\pm$ 1 ^a
Ca^{2+} 8×10^{-5} M		6.1 $\pm$ 1	14.9 $\pm$ 1 ^d	13.4 $\pm$ 1 ^d	9.6 $\pm$ 1 ^a
1.6 $\times 10^{-4}$ M		6.1 $\pm$ 1	10.5 $\pm$ 1 ^b	10.8 $\pm$ 1 ^c	7.3 $\pm$ 1
3.3 $\times 10^{-4}$ M		3.0 $\pm$ 1	6.2 $\pm$ 2	7.0 $\pm$ 3	4.4 $\pm$ 2
6.5 $\times 10^{-4}$ M		5.2 $\pm$ 1	7.5 $\pm$ 1	8.1 $\pm$ 1	5.0 $\pm$ 0.3
1.3 $\times 10^{-3}$ M		2.2 $\pm$ 0.2	2.1 $\pm$ 0.2	3.1 $\pm$ 0.5	1.5 $\pm$ 0.2
Unsupplemented†		2.7 $\pm$ 1	17.8 $\pm$ 1 ^d	15.7 $\pm$ 1 ^d	8.1 $\pm$ 2 ^b
Fe^{3+} 4×10^{-5} M		2.7 $\pm$ 1	13.1 $\pm$ 2 ^a	9.1 $\pm$ 1 ^c	3.6 $\pm$ 1
8 $\times 10^{-5}$ M		2.6 $\pm$ 2	13.1 $\pm$ 2 ^a	7.4 $\pm$ 2	3.4 $\pm$ 1
Unsupplemented		10.3 $\pm$ 3	19.6 $\pm$ 2 ^d	23.3 $\pm$ 5 ^c	10.9 $\pm$ 2
Mg^{2+} 4×10^{-4} M		3.8 $\pm$ 2	6.5 $\pm$ 3	1.4 $\pm$ 2	0.1 $\pm$ 3
8 $\times 10^{-4}$ M		5.7 $\pm$ 2	9.3 $\pm$ 2 ^c	4.7 $\pm$ 1	4.1 $\pm$ 1

* Molar concentration about 2×10^{-5} .

† $< 4 \times 10^{-8}$ M-iron.

Table 2. Effect of growth in differing cation concentrations on lactoferrin- and transferrin-mediated release of ³H-labelled LPS from two *Salmonella* strains

Values are means ± SEM of three experiments.

Supplement to growth medium (Luria broth)	Assay buffer ...	LPS release									
		<i>S. typhimurium</i> SL696					<i>S. montevideo</i> SL5222				
		HBSS-CM buffer	HBSS-CM + EDTA (2 × 10 ⁻⁵ M)	HBSS-CM + lactoferrin (2 mg ml ⁻¹)*	HBSS-CM + transferrin (2 mg ml ⁻¹)*	HBSS-CM buffer	HBSS-CM + EDTA (2 × 10 ⁻⁵ M)	HBSS-CM + lactoferrin (2 mg ml ⁻¹)*	HBSS-CM + transferrin (2 mg ml ⁻¹)*	HBSS-CM + EDTA (2 × 10 ⁻⁵ M)	HBSS-CM + lactoferrin (2 mg ml ⁻¹)*
Ca ²⁺ 2 × 10 ⁻⁴ M		6.8 ± 3	13.5 ± 2	6.7 ± 1	5.0 ± 1	10.6 ± 1	20.8 ± 3	27.7 ± 8	9.0 ± 2	21.1 ± 2	41.6 ± 9
2 × 10 ⁻³ M		15.1 ± 4	18.9 ± 3	24.9 ± 3	11.4 ± 2	15.0 ± 2	21.1 ± 2	41.6 ± 9	12.3 ± 1	22.9 ± 1	48.4 ± 7
1 × 10 ⁻² M		13.9 ± 7	21.7 ± 4	39.1 ± 9	17.0 ± 3	15.5 ± 1	22.9 ± 1	48.4 ± 7	13.0 ± 1	16.6 ± 2	14.1 ± 2
Mg ²⁺ 8 × 10 ⁻⁵ M		2.5 ± 0.1	10.3 ± 0.7	5.7 ± 0.4	4.0 ± 0.1	5.8 ± 2	16.6 ± 2	14.1 ± 2	7.2 ± 1	14.9 ± 1	18.2 ± 4
8 × 10 ⁻⁴ M		6.4 ± 3	12.9 ± 2	9.3 ± 3	6.6 ± 2	6.4 ± 2	14.9 ± 1	21.8 ± 9	8.2 ± 1	14.6 ± 1	21.8 ± 9
8 × 10 ⁻³ M		9.0 ± 3	14.9 ± 2	14.4 ± 2	10.5 ± 2	8.1 ± 2	14.6 ± 1	21.8 ± 9	6.9 ± 1	14.6 ± 1	21.8 ± 9

* Molar concentration about 2 × 10⁻⁵.

transferrin caused LPS release from only the *E. coli* strain at this same Ca^{2+} concentration. Supplemental Mg^{2+} also produced a graded inhibition of LPS release by both proteins from both bacterial strains. Thus, these studies confirm the importance of Ca^{2+} and Mg^{2+} to the membrane effects of lactoferrin and transferrin, and also indicate that LPS release due to the proteins can occur in the presence of Ca^{2+} concentrations that exceed the protein concentration.

Previous work by other investigators has indicated that growing bacteria in the presence of increasing concentrations of Ca^{2+} or Mg^{2+} increases the percentage of LPS released by EDTA, presumably because more cations are incorporated into the outer membrane (Leive, 1974). To further define the mechanism of lactoferrin and transferrin, the effect of media cation concentration on LPS release by these proteins was comparably evaluated. As preliminary studies showed that lactoferrin-mediated LPS release from *S. montevideo* SL5222 was of a greater magnitude than that from *E. coli* CL99-2 (data not shown), this strain and *S. typhimurium* SL696 were used. For both strains, the amount of LPS released from the bacterial membrane increased as the concentration of Ca^{2+} or Mg^{2+} in the growth medium increased (Table 2). However, the increase in LPS release was substantially greater for EDTA and lactoferrin than for transferrin or buffer alone. Growing bacteria in the presence of supplemental iron did not influence LPS release (data not shown). These results are again consistent with the concept that Ca^{2+} and Mg^{2+} are important to the stability of the Gram-negative outer membrane, and that as their concentration within the membrane increases, the membrane becomes more susceptible to damage.

Additional studies also found that lactoferrin-mediated LPS release from *S. typhimurium* SL696 and *S. montevideo* SL5222 was not influenced by pretreatment of lactoferrin with sodium perchlorate indicating that the LPS release was not being mediated by an unrecognized contaminating anion that had chelating activity (data not shown).

Rifampicin susceptibility

To determine whether the Ca^{2+} concentration might influence the previously defined interaction between the iron-binding proteins and rifampicin, time-kill studies were done on a wild type *E. coli* strain (Table 3). Addition of Ca^{2+} had no effect on the growth of the strain in media alone or in the presence of lactoferrin, transferrin or EDTA. However, it significantly decreased the ability of both lactoferrin and EDTA to sensitize this strain to rifampicin, a finding consistent with the observed effect of the cation on LPS release.

Table 3. Effect of Ca^{2+} on the ability of EDTA, lactoferrin and transferrin to enhance the susceptibility of a clinical *E. coli* strain to rifampicin

Values are means $\pm$ SEM of five experiments. The significance of results is indicated as follows (all statistics by paired *t*-test): (a) $P < 0.005$ versus WMS; (b) $P < 0.005$ versus rifampicin; (c) $P < 0.01$ versus experiments without added Ca^{2+} .

Supplement to growth medium (modified WMS)	$\log_{10}$ (Change in no. of bacteria after 4 h)	
	$-\text{Ca}^{2+}$	$+1.3 \text{ mM-Ca}^{2+}$
None	2.0 ± 0.1	2.0 ± 0.1
EDTA ($2 \times 10^{-5} \text{ M}$)	1.9 ± 0.2	2.0 ± 0.1
Lactoferrin (2 mg ml^{-1})	1.4 ± 0.1^a	1.3 ± 0.1
Transferrin (2 mg ml^{-1})	1.0 ± 0.1^a	1.1 ± 0.1
Rifampicin ($5 \mu\text{g ml}^{-1}$)	1.3 ± 0.1	1.8 ± 0.1
Rifampicin + EDTA	0.6 ± 0.2	1.7 ± 0.1^c
Rifampicin + lactoferrin	0.0 ± 0.2^b	0.7 ± 0.1^c
Rifampicin + transferrin	0.8 ± 0.1	0.8 ± 0.1

Deoxycholate studies

EDTA and polycationic agents can sensitize Gram-negative bacteria to detergents, and previous studies have demonstrated that other host factors sensitize Gram-negative bacteria to deoxycholate (LaForce & Boose, 1981; Leive, 1974). Thus, experiments were done to investigate whether the iron-binding proteins could sensitize bacteria to this detergent. Lactoferrin- and transferrin-treated serotype O26 *E. coli* were plated on media with or without sodium deoxycholate. Although lactoferrin at concentrations from 0.5 – 2.0 mg ml^{-1} did not affect the bacteria, transferrin at concentrations from 0.1 – 1.0 mg ml^{-1} consistently sensitized this strain to sodium deoxycholate. After a 30 min incubation with $1.0 \text{ mg transferrin ml}^{-1}$, no effect on bacterial colony counts was observed when exposed cells were plated on blood agar or TSA alone. In contrast, a greater than 80% decrease in colony counts was seen when transferrin-treated cells were plated on MacConkey agar or TSA with deoxycholate. By testing isolated components of MacConkey agar individually, it was further confirmed that the factor in the agar that transferrin sensitized bacteria to was deoxycholate; $98.9 \pm 0.8\%$ killing occurred in the presence of deoxycholate and $<10\%$ killing with exposure to crystal violet, neutral red or cholate (mean $\pm$ SEM of three experiments). Transferrin sensitization of cells to deoxycholate was found to be dose dependent, but progressively decreased as the pH was raised from 6.6 to 8.2 (Fig. 1). In addition, saturation of transferrin with iron markedly decreased but did not completely eliminate the antibacterial activity (Fig. 2).

Fig. 1. Influence of pH and transferrin concentration on sensitization of *E. coli* O26 to deoxycholate (expressed as the percentage of initial bacterial inoculum remaining after 30 min incubation with transferrin and subsequent plating on media containing 0.085% sodium deoxycholate; mean of two experiments). □, pH 8.2; ○, pH 7.2; ◇, pH 6.6. Additional experiments with 1.0 mg transferrin ml⁻¹ demonstrated 8 ± 4% (mean ± SEM) bacteria remaining at 30 min at pH 6.6, 49 ± 15% remaining at pH 7.2, and 111 ± 3% remaining at pH 8.2 ($P < 0.05$ for pH 6.6 versus pH 7.2; $P < 0.01$ for pH 7.2 versus pH 8.2; $P < 0.001$ for pH 6.6 versus pH 8.2).

Two other clinical *E. coli* strains as well as single clinical strains of *Serratia marcescens*, *Enterobacter cloacae* and *Pseudomonas aeruginosa* were similarly sensitized to deoxycholate by transferrin. A clinical strain of *Proteus mirabilis* was resistant. Overall, these findings indicate that transferrin alters the Gram-negative outer membrane and thereby increases bacterial susceptibility to deoxycholate.

Fig. 2. Effect of incubating *E. coli* O26 for varying time intervals with saline (○), apotransferrin (△) or iron-saturated transferrin (▲) on subsequent sensitization to deoxycholate (expressed as the percentage of initial bacterial inoculum remaining after incubation with transferrin and subsequent plating on media containing 0.085% sodium deoxycholate; mean ± SEM of 10 experiments).

The outer membrane damage induced by EDTA can be reversed by incubating EDTA-treated cells in media containing a large excess of Ca²⁺ or Mg²⁺ (Leive, 1974). Similar studies were done to evaluate whether transferrin-induced deoxycholate susceptibility could be reversed. This was tested by treating cells with transferrin, then diluting the transferrin concentration 100-fold and holding the exposed cells in solutions containing iron, Ca²⁺, Mg²⁺ or phosphate. Reversal of membrane damage did occur to varying degrees in essentially all solutions, but the greatest bacterial recovery was seen when the cells were held in solutions containing both Ca²⁺ or Mg²⁺ and phosphate (Table 4). To determine if the recovery from transferrin damage was energy dependent further studies compared reversal at different incubation temperatures.

Table 4. Reversal of transferrin sensitization to deoxycholate by incubation of exposed *E. coli* cells in differing salt solutions

Values represent the percentage of initial bacterial inoculum remaining after incubation with transferrin and subsequent plating on media containing 0.085% sodium deoxycholate. The 0 min results indicate the percentage of the initial bacterial inoculum that was present immediately after the 30 min incubation with 1 mg transferrin ml⁻¹. The 15 min and 30 min results indicate the percentage of the initial bacterial inoculum that was present after the bacterial cells were allowed additional time to recover in the respective solution. The values are means ± SEM. Numbers in parentheses are *P* values comparing the percentage of bacteria remaining at 0 min with the percentages at 15 and 30 min; NS, not significant.

Solution	No. of experiments, <i>n</i>	Percentage of bacteria remaining		
		0 min	15 min	30 min
Water	21	8 ± 4	14 ± 3 (<0.05)	14 ± 3 (<0.05)
FeCl ₃ (10 ⁻⁵ M)	6	11 ± 4	19 ± 9 (NS)	21 ± 7 (NS)
CaCl ₂ (10 ⁻⁴ M)	4	6 ± 3	26 ± 8 (NS)	30 ± 6 (<0.05)
MgSO ₄ (10 ⁻⁴ M)	3	6 ± 3	48 ± 6 (<0.05)	51 ± 14 (NS)
Na ₂ HPO ₄ (10 ⁻⁴ M)	11	8 ± 3	20 ± 6 (<0.01)	26 ± 8 (<0.01)
CaCl ₂ (10 ⁻⁴ M) + Na ₂ HPO ₄ (10 ⁻⁴ M)	4	14 ± 5	91 ± 6 (<0.001)	96 ± 6 (<0.001)
MgSO ₄ (10 ⁻⁴ M) + Na ₂ HPO ₄ (10 ⁻⁴ M)	4	12 ± 6	96 ± 11 (<0.001)	102 ± 5 (<0.001)

Fig. 3. Transmission electron micrographs showing the outer membrane of *S. typhimurium* SL696. (a) Incubated for 30 min in HBSS-CM; (b) incubated for 30 min in HBSS-CM containing EDTA (10^{-5} M); (c) incubated for 30 min in HBSS-CM containing transferrin (2 mg ml^{-1}); (d) incubated for 30 min in HBSS-CM containing lactoferrin (2 mg ml^{-1}); and (e) incubated for 30 min in HBSS-CM containing polymyxin B (30 µg ml^{-1}). In (a)–(d) the outer bacterial membrane is relatively uniform without significant surface irregularities. In (e), numerous microvillus-like projections of the bacterial outer membrane are evident. Bars, 100 nm.

Transferrin-exposed *E. coli* could gradually recover when held in 0.9% saline alone at 37°C with the percentage of viable bacteria remaining increasing from $12 \pm 5\%$ immediately after dilution to $51 \pm 3\%$ after 15 min incubation (mean $\pm$ SEM of three experiments). In contrast, recovery was not observed with cells held in saline at 4°C : the percentages of bacteria remaining were $12 \pm 5\%$ immediately after dilution and only $10 \pm 1\%$ after 15 min ($P < 0.01$ compared to the studies at

37°C). These findings were again consistent with the observations made with EDTA, and also suggest that repair of transferrin-induced membrane alteration requires active bacterial metabolism.

Transmission electron microscopy (TEM) of outer membranes

Although EDTA has not been shown to induce membrane alterations detectable by TEM, membrane-active

polycationic agents can produce such effects (Vaara & Vaara, 1983a). Therefore, the effects of lactoferrin and transferrin on the membrane were studied by TEM. Incubating *S. typhimurium* SL696 in HBSS-CM alone (Fig. 3a) or treating the cells with lactoferrin (b), transferrin (c) or EDTA (d) for 30 min had no effect on the ultrastructural features of the outer membrane. Bacteria showed a relatively smooth surface with a well-defined trilaminar outer membrane. Similarly, these agents had no significant ultrastructural effects on the outer membrane of *E. coli* CL99-2 (not shown). In contrast, incubation for 30 min with polymyxin B in HBSS-CM resulted in formation of numerous microvillus-like projections on the outer membrane of both *S. typhimurium* SL696 (Fig. 3e) and of *E. coli* CL99-2 (not shown).

Discussion

The results presented show that lactoferrin and transferrin alter the outer membrane of Gram-negative bacteria. Specifically, although differing in activity, these proteins can cause the release of LPS and sensitize bacteria to both a hydrophobic antibiotic and a surface-active detergent. In addition, all of these activities are modulated by Ca^{2+} and Mg^{2+} . These findings are consistent with the studies of C. A. Bortner and co-workers who found that bactericidal effect of lactoferrin against *Legionella pneumophila* is inhibited by the addition of exogenous Mg^{2+} (Bortner *et al.*, 1986).

The amount of LPS that can be released from the outer membrane varies between strains and is influenced by the Ca^{2+} concentration in the growth media. Leive (1974) and other investigators noted similar findings in studies with EDTA, and suggested that the alignment of LPS molecules within the outer membrane may be variable and partially dependent upon the availability of cations at the time of membrane formation. This has led to the hypothesis that for a given bacterial strain, if the outer membrane is assembled in the presence of high concentrations of cations, then increased numbers of cations are incorporated into the membrane and used to stabilize the anionic charge of the LPS core (Leive, 1974). This could then make the membrane more susceptible to factors that alter the cation-LPS relationship such as a chelator. By extension, inter-isolate variation in the incorporation of cations into the outer membrane could contribute to the observed differences between strains in the susceptibility to the chelators.

The ability of bacteria to recover from transferrin-mediated sensitization to deoxycholate indicates that healing of the membrane damage can occur. That

healing does not occur at 4 °C suggests that the process requires active bacterial metabolism. Healing may be related to the incorporation and stabilization of new LPS molecules in the outer membrane, but this hypothesis will require further confirmation. Also requiring further study will be the role of phosphate in this process: it may enhance bacterial metabolism or be incorporated into new LPS molecules.

Since free Ca^{2+} and Mg^{2+} are present in essentially all secretions, definition of the cation concentrations at which the membrane effects of lactoferrin and transferrin occur is important. These studies indicate that these proteins are able to induce at least partial LPS release in the presence of a Ca^{2+} concentration of 1.6×10^{-4} M (a molar concentration eight times greater than that of lactoferrin in the test system). As the concentration of both the iron-binding proteins and Ca^{2+} and Mg^{2+} are heterogeneous *in vivo*, in selected micro-environments ionic conditions may exist where these membrane-active effects of lactoferrin and transferrin can occur. Elegant studies by C. Pollack indicate that the Ca^{2+} concentration within the human macrophage phagolysosome is less than 10^{-4} M, suggesting one micro-environment where lactoferrin could damage the Gram-negative outer membrane under physiological conditions (Bullen, 1981; Pollack *et al.*, 1986).

In these studies, there were consistent differences in the membrane effects of lactoferrin and transferrin, with lactoferrin causing greater LPS release, and transferrin having greater ability to induce deoxycholate sensitivity. Such heterogeneous observations are similar to previous studies with other agents that affect the outer membrane of Gram-negative bacteria. These agents include polylysine, protamine, the antimicrobial agents polymyxin B and polymyxin B nonapeptide (PMBN), and several cationic proteins isolated from neutrophils (Vaara & Vaara, 1983a; Vaara & Viljanen, 1985; Weiss *et al.*, 1984). Similar to EDTA, to varying degrees these polycationic compounds increase the permeability of the Gram-negative outer membrane to hydrophobic agents. The mechanism of action of the compounds appears to be at least partially due to an ability to insert into the bacterial outer membrane by binding to the anionic LPS molecules (Nikaido & Vaara, 1985; Vaara & Viljanen, 1985).

Lactoferrin and transferrin differ significantly in pI with transferrin anionic (pI ~5.5) and lactoferrin cationic (pI ~8.5) at neutral pH (Moguilovsky *et al.*, 1985; White *et al.*, 1978). This difference may contribute to the variation in their membrane effects. In being cationic, lactoferrin may share a mechanism of action with the polycationic agents as well as EDTA. Consistent with this hypothesis are the results of studies indicating that lactoferrin can be associated with the

bacterial cell membrane (Arnold *et al.*, 1977; Redhead *et al.*, 1987).

This work serves to confirm the observation that the iron-binding proteins lactoferrin and transferrin damage the outer membrane of Gram-negative organisms. Such membrane effects could explain several previous observations on the antimicrobial activity of the proteins. Additionally, previous studies with EDTA and the polycationic agents suggest that both of these classes of membrane-permeabilizing compounds sensitize Gram-negative organisms to the effects of lysozyme and complement (Bryan, 1974; Hancock & Wong, 1984; Joiner, 1985; Vaara & Vaara 1983*b*; Vaara *et al.*, 1984). It is reasonable to speculate that in altering the Gram-negative outer membrane, lactoferrin and transferrin may also sensitize bacteria to these other host antibacterial systems and thereby amplify their effects.

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