

Lactoferrin Is a Lipid A-Binding Protein

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Lactoferrin (LF), a cationic 80-kDa protein present in polymorphonuclear leukocytes and in mucosal secretions, is known to have antibacterial effects on gram-negative bacteria, with a concomitant release of lipopolysaccharides (LPS, endotoxin). In addition, LF is known to decrease LPS-induced cytokine release by monocytes and LPS priming of polymorphonuclear leukocytes. Its mechanism of action is incompletely understood. We have now demonstrated by in vitro-binding studies that LF binds directly to isolated lipid A and intact LPS of clinically relevant serotypes of the species which most frequently cause bacteremia (*Escherichia coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*), as well as to lipid A and LPS of mucosal pathogens (among others, *Neisseria meningitidis* and *Haemophilus influenzae*). Binding to LPS was inhibitable by lipid A and polymyxin B but not by KDO (3-deoxy-D-manno-octulosonate), a glycoside residue present in the inner core of LPS. Binding of LF to lipid A was saturable, and an affinity constant of $2 \times 10^9 \text{ M}^{-1}$ was calculated for the LF-lipid A interaction. Our data may explain, in part, the mechanism whereby LF exerts its antibacterial and anti-endotoxic effects. Further studies on the ability of LF to block the detrimental effects of LPS, both in vitro and in vivo, are warranted.

Lactoferrin (LF) is a cationic (calculated pI = 8.5) 80-kDa protein present at high concentrations in the specific granules of polymorphonuclear leukocytes and in mucosal secretions (22). LF has bacteriostatic and bactericidal effects and is thought to contribute to host defense, both systemically and at mucosal surfaces. LF exhibits antibacterial effects on gram-negative bacteria by means of two mechanisms. Firstly, it is an iron-binding protein, which limits the amount of free iron; iron is an essential growth factor for microorganisms (18). Secondly, LF is capable of destabilizing the outer membrane of gram-negative bacteria, which results in liberation of lipopolysaccharides (LPS) (6). One possible mechanism of action is the binding by LF of divalent cations (Ca^{2+} and Mg^{2+}) that neutralize and stabilize the negative charges of LPS (7); this mechanism of action is similar to the destabilizing effect of the metal chelator EDTA on the gram-negative outer membrane. An alternative mechanism for the liberation of LPS is the direct interaction of the cationic LF with the anionic LPS, followed by its withdrawal from the outer membrane (5). Possibly, the negative charges in LPS involved in this interaction are the carboxyl groups on 3-deoxy-D-manno-octulosonate (KDO) present in the inner core and the phosphate residue(s) present in the lipid A part of LPS. This mechanism is similar to that of polymyxin B (PMB), a cationic outer membrane-destabilizing peptide, which binds directly to the lipid A part of LPS (14).

The goal of this study was to investigate systematically, by in vitro-binding studies, the direct interaction of LF with lipid A and LPS of relevant serotypes of gram-negative bacteria that are frequently involved in sepsis and shock, i.e., *Escherichia coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* (4, 9,

12); the interaction with LPS of mucosal pathogens (such as *Neisseria* spp. and *Haemophilus influenzae*) was also studied.

LF was purified from fresh human milk by cation-exchange chromatography on Mono S (HR 5/5; Pharmacia) in 20 mM sodium phosphate buffer (pH 7.5). LF eluted as a single peak at 0.68 M NaCl when a linear 0 to 1 M gradient was used. The sensitivity of the detector was set to 0.01. Gel filtration of purified LF on Superose 12 (HR 10/30; Pharmacia) again showed a single symmetrical peak in absorption and in a nanogram-sensitive radioimmunoassay specific for LF. This excludes the presence of LF polymers. The results of N-terminal protein sequencing showed the presence of a single N terminus of intact LF. Measurement of A_{280} and A_{265} revealed LF to be saturated with iron at about 3.5%. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed pure LF migrating as a closely spaced doublet of protein bands at 80 kDa. LPS contamination of LF was less than 2 U (200 pg) of endotoxin per mg of LF, as estimated by the *Limulus* assay (Kinetic-QCL; BioWhittaker). LF (20 mg/ml in 0.9% NaCl) was stored in portions at -80°C . Portions were thawed, brought to 4°C , and used within 6 days. Affinity-purified polyclonal anti-LF antibodies were prepared from rabbit anti-human LF (Sigma) as described previously (16). Antibodies were kept at -80°C and for daily use were stored at 4°C in the presence of 0.01% merthiolate.

Synthetic biphosphoryl *E. coli* lipid A (compound 506) was purchased from Daiichi Fine Chemicals (Tokyo, Japan). Natural biphosphoryl lipid A (lipid A-HAc) and monophosphoryl lipid A (lipid A-HCl) isolated from *E. coli* F515 (Re chemotype) LPS by hydrolysis with acetate buffer and hydrochloric acid, respectively, were gifts from H. Brade. Lipid A from *K. pneumoniae* O1 was provided by R. Zalisz. Lipid A from *P. aeruginosa* 18S was a gift from A. Fomsgaard. Lipid A from *Neisseria meningitidis* was a gift from C.-M. Tsai. LPS from rough mutants of *Salmonella minnesota* R595 (Re chemotype), R4 (Rd2), R7 (Rd1), R5 (Rc), R345 (Rb2), and Ra (R60) were a gift from H. Brade. LPS of *S. minnesota* smooth strain was purchased from Sigma. The sources of other LPS were *S. Cryz*

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(LPS from *E. coli* serotypes O1, O2, O4, O6, O7, O8, O12, O15, O16, O18, O25, and O75 and LPS from *P. aeruginosa* serotypes IT-1, IT-2, IT-3, IT-4, IT-5, IT-6, and IT-7 and Habs 3 and Habs 4), W. Nimmich and R. Zalisz (LPS from *K. pneumoniae* O1, O4, O5, and O2), K. Bryn (*N. meningitidis* LPS), N. Parsons (*Neisseria gonorrhoeae* LPS), L. van Alphen (*H. influenzae* LPS), J. C. Richards (*Branhamella catarrhalis* LPS), A. Moran (*Helicobacter pylori* LPS), and Difco (*Shigella flexneri* LPS). Extraction of rough LPS by the phenol-chloroform-petroleumether method was followed by electro dialysis. Smooth LPS was purified by the phenol-water procedure. LPS and lipid A were stored at 1 mg/ml in water, containing 0.01% merthiolate and 0.1% triethylamine. Synthetic poly- α -(KDO)₂, a copolymerization product of α -allyl-KDO and acrylamide, was prepared by P. Kosma. The copolymer, a 60-kDa polyvalent antigen, contains KDO-KDO disaccharide in an α -2.4 linkage, i.e., the same as that occurring in the natural core (1a). PMB sulfate was obtained from by Pfizer (Brussels, Belgium).

The binding of LF to LPS was investigated by enzyme-linked immunosorbent assay (ELISA). LPS or lipid A was coated for 16 h at room temperature at 1 μ g/ml in 100 μ l of pyrogen-free phosphate-buffered saline (PBS; pH 7.2) onto the surface of polystyrene 96-well plates (Immulon, medium binding capacity; Greiner, Alphen aan den Rijn, The Netherlands). The plates were washed thrice with PBS containing 0.01% Tween and 0.01% merthiolate (PBST). LF was diluted in twofold increments in concentrations ranging from 1 μ g/ml to 1 ng/ml in pyrogen-free PBST (100 μ l) and incubated for 16 h at room temperature. The plates were washed thrice, incubated for 3 h at 37°C in anti-LF (1 μ g/ml in 100 μ l of PBST), washed thrice, incubated for 2 h at 37°C with horseradish-labelled goat antiserum to rabbit immunoglobulin G (heavy and light chains; Nordic, Tilburg, The Netherlands), and diluted 1:1,000 in PBST containing 0.5% goat serum. Then, plates were washed thrice, and color was allowed to develop for 30 min at room temperature in 100 μ l of phosphate-citrate buffer (pH 5.5) containing 1 mg of orthophenylenediamine per ml and 0.015% hydrogen peroxide per ml. The reaction was stopped by the addition of 50 μ l of 1:10-diluted concentrated sulfuric acid, and the plates were read at 492 nm.

Inhibition experiments. The ability of poly- α -(KDO)₂ and lipid A-HCl of *E. coli* to inhibit the binding of LF to LPS of *P. aeruginosa* IT-1 was investigated. A 100- μ l portion of LF (500 ng/ml) in PBST was mixed with 100 μ l of lipid A-HAc of *E. coli* or poly- α -(KDO)₂, in concentrations ranging from 0.2 ng/ml to 20 μ g/ml in 10-fold increments; the mixture was incubated for 1 h at 37°C. The mixture (100 μ l) was then added to washed plates already coated with LPS, and the ELISA was finished as described above. LF (250 ng/ml) incubated with PBST served as a control representing 100% binding. The ability of PMB to inhibit the interaction between LF and LPS was investigated by two procedures. In the first procedure, PMB (100 μ l), diluted in PBST in concentrations ranging from 0.1 ng/ml to 100 μ g/ml in 10-fold increments, was added to washed plates already coated with LPS of *P. aeruginosa* IT-1 and incubated for 1 h at 37°C. The plates were washed, LF (250 ng/ml) was added, and the ELISA was finished as described above. In the second procedure, the plates were not washed after the incubation of PMB, LF (10 μ l at a concentration of 2.5 μ g/ml) was added to the LPS-coated PMB-containing wells, and the ELISA was done as described previously.

Negative controls in the direct binding studies included wells coated with PBS instead of LPS, and these wells were processed as described above. This control measured the nonspecific sticking of LF to plastic. As another control, LPS-coated

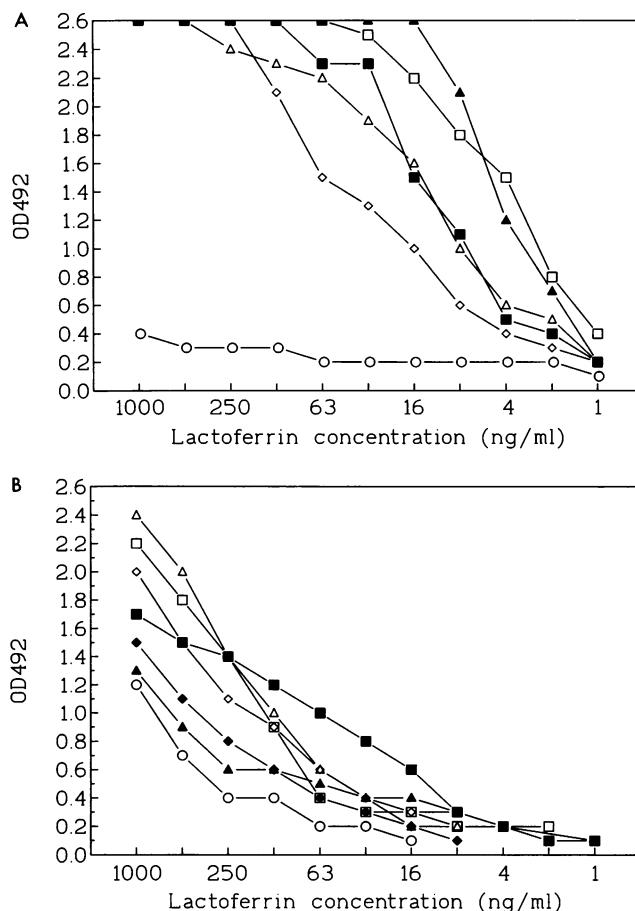


FIG. 1. (A) Binding of LF to various types of lipid A. □, *E. coli*, natural biphosphoryl; ■, *E. coli*, natural monophosphoryl; ◇, *E. coli*, synthetic biphosphoryl; ▲, *K. pneumoniae*; △, *P. aeruginosa*; ○, binding of LF to plastic. (B) Binding of LF to *S. minnesota* with increasing core lengths. □, strain R595 (Re chemotype); ■, R4 (Rd2); △, R7 (Rd1); ▲, R5 (Rc); ◇, R345 (Rb2); ◆, R60 (Ra, complete core); ○, smooth strain. Data are representative results obtained in three independent experiments.

wells were incubated with PBST substituted for LF, after which the ELISA was done as described above. Direct binding studies were done on two to six separate occasions. Inhibition studies were done in triplicate on two separate occasions. Representative results are presented either in the form of full titration curves or, alternatively, as titers (defined as the smallest amount of LF that still yielded an optical density [OD] of 0.2 above the sum of the results of the two negative controls).

Binding of LF to lipid A of various origins is shown in Fig. 1A. For negative controls, when LF was omitted ODs of 0.1 to 0.2 were obtained. When lipid A (or LPS, see below) was omitted, the ODs were 0.4 for 1,000 ng of LF per ml, 0.3 for 250 ng/ml, and 0.2 for 63 ng/ml (Fig. 1A). LF bound very well to natural mono- and biphosphoryl lipid A of *E. coli* and to biphosphoryl lipid A of *K. pneumoniae* and *P. aeruginosa*; synthetic biphosphoryl lipid A yielded lower ODs. A collection of synthetic lipid A variants would be required to determine the structural elements within lipid A that are important for the interaction between LF and lipid A. Similar studies have been done successfully in the past for anti-lipid A monoclonal antibodies (10) but were not done in this study.

TABLE 1. Binding of LF to LPS of bacteria frequently involved in gram-negative bacteremia^a

Species and serotype	Titer
<i>Escherichia coli</i>	
O1.....	32
O2.....	32
O4.....	1,000
O6.....	1,000
O7.....	1,000
O8.....	125
O12.....	125
O15.....	64
O16.....	16
O18.....	250
O25.....	500
O75.....	125
<i>Pseudomonas aeruginosa</i>	
IT-1.....	64
IT-2.....	250
IT-3.....	32
IT-4.....	125
IT-5.....	32
IT-7.....	250
Habs 3.....	500
Habs 4.....	125
<i>Klebsiella pneumoniae</i>	
O1.....	1,000
O2.....	500
O4.....	125
O5.....	500

^a Binding is expressed as the minimal amount of LF (in nanograms per milliliter) that still yields an OD of 0.2 above the sum of the results for the negative controls. Titers are representative ones obtained from two to six independent experiments.

We next investigated (Fig. 1B) the binding of LF to a series of rough mutants of *S. minnesota* LPS that have increasing core lengths; i.e., LPS of strain R595 (Re chemotype) is the shortest one, consisting of lipid A and KDO only; in R4 (Rd2) and R7(Rd1), LPS contain lipid A, KDO, and one and two heptoses, respectively. Strain R60 expresses the complete Ra core; in the smooth strain LPS, the core is capped with O antigen. The degree of binding to lipid A was higher than to LPS. The difference in binding affinities between R595 LPS and lipid A may be due to steric hindrance of the binding site on lipid A by KDO. By the same token, steric hindrance may be the cause of more inhibition of LF binding to mutant LPS with a complete core (Ra chemotype) than to shorter LPS (Re and Rd chemotypes). However, binding intensity did not follow the exact order of mutant LPS chain lengths. In pilot experiments (data not shown), we found that the interaction between LPS and LF was influenced by the salt concentration; i.e., at an ionic strength below 0.15 M, there was an increased affinity, while 0.8 M NaCl completely abrogated binding. Tween 20, a nonionic detergent did not influence binding. We conclude that the LF-LPS interaction has a predominantly electrostatic nature. Studies investigating the influence of Mg²⁺, Ca²⁺, and pH are in progress.

The binding of LF to LPS of clinically relevant serotypes of the most important gram-negative bacterial species that cause bacteremia and sepsis (4, 9, 12) (*E. coli*, *K. pneumoniae*, and *P. aeruginosa*) is shown in Table 1. Clearly, LF interacts with most of the LPS tested, although with various reactivities. To give some indication of the reproducibility of our results, the reactivities of LF with LPS of *E. coli* O16 on three separate

TABLE 2. Binding of LF to lipid A and LPS of mucosal pathogens^a

Organism	Titer
<i>Neisseria meningitidis</i> (lipid A) ^b	2
<i>Neisseria meningitidis</i> (LPS).....	4
<i>Neisseria gonorrhoeae</i>	125
<i>Haemophilus influenzae</i>	16
<i>Branhamella catarrhalis</i>	125
<i>Shigella flexneri</i>	>1,000
<i>Helicobacter pylori</i>	125

^a See Table 1 for definition of binding and description.
^b The binding of LF to LPS was tested, except for *N. meningitidis* for which lipid A was also used.

occasions were 16, 32, and 16 ng/ml; for O25, the data were 1,000, 500 and 500 ng/ml, respectively. The data for *E. coli* were 125, 250, and 125 ng/ml for serotype O12 and 125, 64, and 64 ng/ml for serotype O15. We did not do formal statistics, but it is clear that LF binds to O16 LPS with greater affinity than to O25, while it is unclear whether such differences exist between O12 and O15. Data on the interactions between LF or lipid A and LPS of mucosal pathogens are shown in Table 2. The ability of lipid-A HAC, KDO, and PMB to inhibit the binding of LF to LPS is shown in Fig. 2. Clearly, lipid A causes inhibition of binding; this shows that the binding site of LF in LPS is located at, or close to, the lipid A part of LPS. KDO did not inhibit binding at concentrations that in the past were shown to be amply sufficient to cause strong inhibition of binding of anti-KDO monoclonal antibodies (1a, 20, 21). Although KDO on its own does not cause inhibition, it is possible that LF does bind to KDO but only when presented in the form of LPS; this situation had been encountered by us in the past with clone 17, a monoclonal antibody that requires the simultaneous presence of KDO and lipid A (covalently linked, i.e., in the form of LPS) for binding, while both isolated lipid A and KDO were without inhibitory effects (20, 21). In summary, lipid A is most likely the major epitope recognized by LF, but we cannot totally exclude contributions by other parts of the LPS molecule. The data presented in Fig. 2 for PMB were obtained by using the second procedure (see above); the first procedure yielded similar results, but more PMB was needed to cause inhibition (data not shown). Our data show that LF

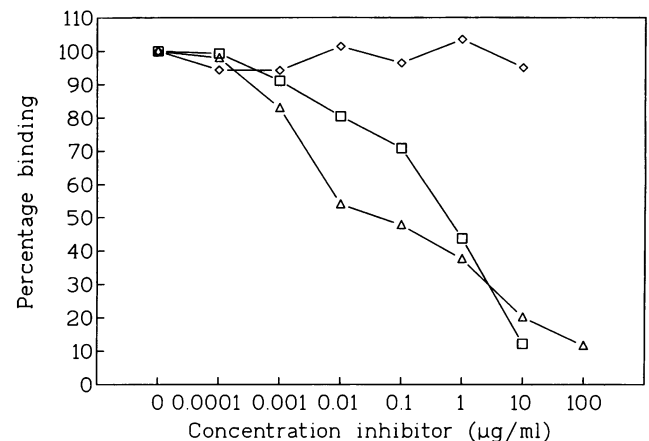


FIG. 2. Inhibitory effects of various compounds on binding of LF to *P. aeruginosa* IT-1 LPS. □, *E. coli* lipid A-HAC; △, PMB; ◇, poly- α -(KDO)₂. Data are representative results obtained as triplicates in two independent experiments.

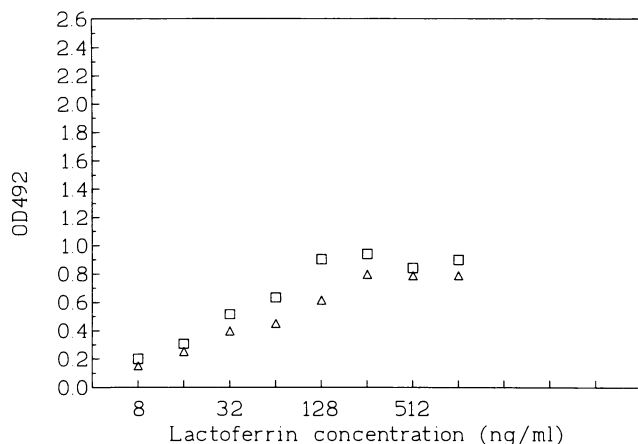


FIG. 3. Saturability of lipid A by LF. Symbols are defined in the legend to Fig. 1A. A coating concentration of 10 ng/ml was used.

and PMB are competing for the same binding site on LPS or to binding sites in close proximity; these findings suggest the possibility that LF may exhibit some of the same effects that PMB has on LPS bioactivity, e.g., inhibition of its ability to induce cytokines (see below). Finally, we investigated whether the binding of LF to lipid A was saturable. These studies were done at coating concentrations of 10 and 100 ng/ml. Results for 10 ng/ml are shown in Fig. 3. It can be seen that binding to biphosphoryl lipid A of *P. aeruginosa* and *E. coli* was indeed saturable, with full saturation occurring at approximately 128 to 250 ng/ml. Assuming that at 10 ng/ml all lipid A is bound to the plate, a stoichiometry at saturation of LF:lipid A of 1:2 can be calculated. When half-saturation (approximately 40 ng/ml) is taken as a reflection of affinity (1), an affinity constant of $2 \times 10^9 \text{ M}^{-1}$ can be calculated. Results for lipid A of *K. pneumoniae* were comparable (data not shown).

Our data demonstrate that LF binds to the lipid A part of LPS of clinically relevant bacterial species. Direct binding to lipid A has not been reported before. As far as we know, previous work on the in vitro binding of LF to LPS has been restricted to two LPSs of *E. coli* (5, 13). It is possible that the direct binding of LF to LPS, followed by withdrawal of LPS from the outer membrane, accounts, in part, for its antibacterial effects, whether in vitro or in vivo, i.e., when bacteria are ingested within phagocytes during the process of oxygen-independent killing or when bacteria are on mucosal surfaces. The decreasing antibactericidal activity of LF on bacteria with increasing core lengths (15) would be in agreement with an involvement of LPS binding in this process.

Our findings may also explain a totally different line of data. LF has very recently been reported to decrease LPS-induced cytokine release by monocytes (3). In addition, LF was capable of blocking LPS priming of human neutrophils for production of superoxide (2). These effects may be due to direct binding of LF to LPS, which decreases its bioactivity, an effect that also occurs when LPS binds to bactericidal/permeability-increasing proteins (8), another protein present in the granules of polymorphonuclear leukocytes. Interestingly, many of the antimicrobial proteins present in polymorphonuclear leukocyte granules bind to LPS (17). Bactericidal/permeability-increasing protein is known to block LPS-induced cytokine secretion in vitro (8) and to cause protection against endotoxic death in vivo (8). Likewise, the cationic, membrane-active oligopeptide PMB binds to the lipid A part of LPS (14), blocks LPS-induced

tumor necrosis factor secretion in vitro (23), and protects in vivo (19). Under the assumption that polymorphonuclear leukocyte-derived LF behaves in a manner similar to that derived from milk, it is possible that the in vivo protective effect of LF on the course of lethal gram-negative infection (24) is at least partially due to its anti-endotoxic effect. In agreement with this possibility is a recent report (11) showing that infusion of LF into mice prior to LPS challenge decreases the concentration of cytokines in the circulation.

In summary, LF binds to the lipid A part of LPS of clinically relevant bacteria, and this may result in bacterial growth reduction and/or killing, as well as in decreased endotoxicity of LPS occurring both in the bloodstream and at mucosal surfaces. Further studies of LPS as an anti-endotoxin agent are therefore warranted.

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