

Lactoferrin–lipopolysaccharide interaction: involvement of the 28–34 loop region of human lactoferrin in the high-affinity binding to *Escherichia coli* 055B5 lipopolysaccharide

Elisabeth ELASS-ROCHARD,* Anca ROSEANU,† Dominique LEGRAND,* Mihaela TRIF,† Valérie SALMON,* Cecilia MOTAS,† Jean MONTREUIL* and Geneviève SPIK*‡

*Laboratoire de Chimie Biologique, Unité Mixte de Recherche du CNRS no. 111, Université des Sciences et Technologies de Lille, 59655 Villeneuve d'Ascq Cedex, France and †Institut de Biochimie de l'Académie Roumaine, Splaiul Independentei 296, 77.700 Bucarest 17, Rumania

The ability of lactoferrin (Lf), an iron-binding glycoprotein that is also called lactotransferrin, to bind lipopolysaccharide (LPS) may be relevant to some of its biological properties. A knowledge of the LPS-binding site on Lf may help to explain the mechanism of its involvement in host defence. Our report reveals the presence of two *Escherichia coli* 055B5 LPS-binding sites on human Lf (hLf): a high-affinity binding site (K_d 3.6 ± 1 nM) and a low-affinity binding site (K_d 390 ± 20 nM). Bovine Lf (bLf), which shares about 70% amino acid sequence identity with hLf, exhibits the same behaviour towards LPS. Like hLf, bLf also contains a low- and a high-affinity LPS-binding site. The K_d value (4.5 ± 2 nM) corresponding to the high-affinity binding site is similar to that obtained for hLf. Different LPS-binding sites for human serum transferrin have been suggested, as this protein, which is known to bind bacterial endotoxin, produced only 12%

inhibition of hLf–LPS interaction. Binding and competitive binding experiments performed with the N-tryptic fragment (residues 4–283), the C-tryptic fragment (residues 284–692) and the N2-glycopeptide (residues 91–255) isolated from hLf have demonstrated that the high-affinity binding site is located in the N-terminal domain I of hLf, and the low-affinity binding site is present in the C-terminal lobe. The inhibition of hLf–LPS interaction by a synthetic octadecapeptide corresponding to residues 20–37 of hLf and lactoferrin B (residues 17–41), a proteolytic fragment from bLf, revealed the importance of the 28–34 loop region of hLf and the homologous region of bLf for LPS binding. Direct evidence that this amino acid sequence is involved in the high-affinity binding to LPS was demonstrated by assays carried out with EGS-loop hLf, a recombinant hLf mutated at residues 28–34.

INTRODUCTION

Lactoferrin (Lf), also called lactotransferrin [1,2], is an iron-binding glycoprotein present in most biological fluids of mammals [2,3] and released from neutrophil granules during inflammatory responses [4]. Various biological functions of human Lf (hLf) and bovine Lf (bLf) have been demonstrated in host defence, especially in immunological response [5,6] and antibacterial activity (for reviews see refs. [7,8]). *In vitro* studies on a wide range of Gram-negative micro-organisms suggested two reasons for the antimicrobial property of Lf: (i) the capacity of the protein to induce an iron-deficient environment [9] and (ii) its ability to interact with several components present at the surface of the bacterial membrane [10,11]. With respect to the second reason, it has been shown that hLf can bind to lipopolysaccharide (LPS) [11], a major component of the outer membrane of Gram-negative bacteria. In agreement with these findings, Appelmek et al. [12] found high-affinity binding of hLf to the lipid A region of *Escherichia coli* LPS.

hLf–LPS interactions could also have relevance to the role of Lf in the inflammatory process. Indeed, hLf interacts with specific receptors present on mononuclear cells and regulates cytokine release [13–15]. This biological activity is inhibited in the presence of LPS suggesting that hLf loses the ability to bind to its receptor after hLf–LPS complex-formation [14].

A knowledge of the hLf structure has enabled its binding sites for the lymphocytic receptor to be identified [16–19]. Sequence analyses and crystallographic studies [20–22] have established that the hLf polypeptide chain is folded into two homologous

lobes, one N-terminal (residues 1–333), the other C-terminal (residues 345–692), each being organized into two domains NI, NII and CI, CII respectively. The primary structure of hLf shows about 70% sequence identity with that of bLf and 59% identity with that of human serum transferrin [23]. Three main regions located in the N-terminal domain I of hLf are involved in the hLf receptor-binding site: residues 4–6, 28–34 and 38–45 [19]. Comparison of the primary and tertiary structures of these regions with the homologous sequences of the C-terminal lobe of hLf and the N- and C-terminal lobes of serum transferrin revealed that residues 28–34 and residues 4–6 possess structural features specific to the N-terminal moiety of hLf. The loop region (residues 28–34) is also present in lactoferrin H (residues 1–47) and lactoferrin B (residues 17–41), two peptides derived from partial proteolytic hydrolysis of hLf and bLf respectively [24,25]. Both exhibit more potent antibacterial properties than the native proteins. It has been reported that the antibacterial sequences are precisely located in a loop region corresponding to residues 20–37 of hLf and 19–36 of bLf [24].

Therefore the region containing amino acid residues 28–34 could play an important role not only in hLf binding to its specific receptor but also in its antimicrobial activity. As hLf binds to LPS, the interaction of amino acid sequence 28–34 of the protein with bacterial endotoxin could be suggested.

To check this hypothesis, we have studied the binding of hLf and bLf to *E. coli* 055B5 LPS. As human serum transferrin binds to LPS [26], its ability to inhibit hLf–LPS interaction was also investigated. The domain of the hLf molecule implicated in the endotoxin recognition was specified by binding and competitive

binding assays performed with different hLf fragments: N- (residues 4–283) and C- (residues 284–692) fragments, N2-glycopeptide (residues 91–255) [27,28], a chemically synthesized octadecapeptide corresponding to residues 20–37 of hLf and lactoferricin B. Further, the hLf amino acid sequence responsible for LPS binding was more precisely defined by binding assays using EGS-loop recombinant hLf (EGS-loop rhLf), a mutated protein in which residues 28–34 of the N-terminal lobe were replaced by the homologous loop of the C-terminal lobe of hLf.

MATERIALS AND METHODS

Materials

Lf was purified from pooled human lactoserum by ion-exchange chromatography as previously described [29]. bLf was kindly provided by Biopole (Brussels, Belgium). Homogeneity of the proteins was checked by SDS/PAGE [30]. Iron saturation of Lfs was carried out as described elsewhere [31]. Diferric hLf was submitted to mild tryptic hydrolysis to isolate the 30 kDa monoferric N-tryptic fragment (residues 4–283) and the 50 kDa monoferric C-tryptic fragment (residues 284–692) [27,28]. The 20 kDa N2-glycopeptide (residues 91–255), which corresponds to the N-terminal domain II of hLf described by Anderson et al. [22], was prepared from the tryptic hydrolysate of the N-tryptic fragment [28]. These fragments were further purified by gel-filtration chromatography on Bio-Gel P-60 for the N- and C-tryptic fragments and Bio-Gel P-30 for the N2-glycopeptide [27,28]. An octadecapeptide corresponding to the amino acid sequence 20–37 of hLf was chemically synthesized by Dr. A. Tartar (Institut Pasteur, Lille, France). Lactoferricin B, a homologous peptide of hLf present in the N-terminal lobe of bLf (residues 17–41) was a gift from Morinaga Milk Industry Co. (Tokyo, Japan). Lf samples were passed through a Detoxi-Gel column (Pierce Chemicals Co., Rockford, IL, U.S.A.) before use. LPS contamination of Lfs, tryptic fragments and buffer solutions was less than 50 pg of endotoxin/mg of protein as estimated by *Limulus* amoebocyte lysate assays (QCL1000; BioWhittaker, Walkersville, MD, U.S.A.).

Dulbecco's PBS, pH 7.3, without Ca^{2+} and Mg^{2+} , BSA recommended for ELISA experiments and *E. coli* 055B5 LPS were purchased from Sigma Chemicals (St. Louis, MO, U.S.A.). Iron-free human serum transferrin was from Behring (Marburg, Germany). PD10 prepacked Sephadex G-25 M columns were from Pharmacia-LKB Biotechnology (Uppsala, Sweden). Pyrogen-free water was used to prepare all buffer solutions.

Expression and purification of recombinant hLf and EGS-loop hLf

A full-length 2.3 kb cDNA coding for hLf was obtained from a human mammary-gland cDNA library (Clontech, Palo Alto, CA, U.S.A.) [32]. rhLf was expressed in BHK cells and purified as previously described [32]. The EGS-loop rhLf was obtained by site-directed mutagenesis of the cDNA coding sequence of Lf using the Sculptor *in vitro* mutagenesis system kit (Amersham International, Amersham, Bucks., U.K.). This mutated protein corresponds to hLf in which the sequence $^{28}\text{RKVRGPP}^{34}$ was replaced by the sequence $^{365}\text{EGS}^{367}$, located in the C-terminal lobe counterpart (Figure 1). A mutagenic oligonucleotide, 5'-TGG CAA AGG AAT ATG GAAGGT ICT GTC AGC TGC ATA AAG-3', was synthesized by Eurogentec (Seraing, Belgium) and used to replace nucleotides 433–451 (numbered as described by Rey et al. [21]) by the sequence GAAGGTT. The template for the mutagenesis was the phage M13-mp11 containing a 310 bp *EcoRI*–*AccI* fragment (nucleotides 295–606 of the coding sequence) obtained from the Lf cDNA cloned into

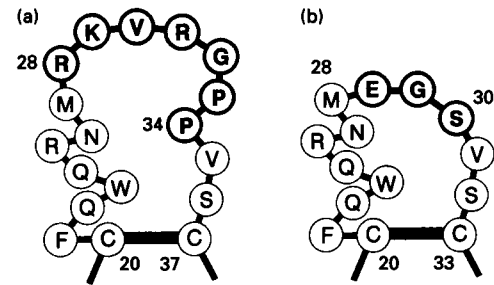


Figure 1 Schematic representation of the loop covering residues 28–34 of hLf

(a) The amino acid sequence of the 20–37 region of hLf N-terminal lobe; (b) residues 20–33 of the rhLf mutant (EGS-loop rhLf) where residues 28–34 of hLf are replaced by residues EGS. Bold letters indicate mutated amino acid residues.

pBluescript SK [32]. The presence of the mutated codons was confirmed by DNA sequence analysis [33] and the mutated *EcoRI*–*AccI* fragment was then ligated back into pBluescript with the 3' complementary part of the full-length cDNA of hLf. The resulting plasmid was then digested with *XbaI* and *HindIII* and the *XbaI*–*HindIII* fragment, made blunt-ended using the Klenow fragment of *E. coli* DNA polymerase I, was subcloned into the *SmaI* site of the expression vector pNUT, generously provided by Dr. R. Palmiter (University of Washington, Seattle, WA, U.S.A.) [34]. Transfection, expression in BHK cells and purification of the recombinant mutated protein were performed as previously described [32]. Both non-modified rhLf and EGS-loop rhLf exhibited only one 80 kDa single protein band similar to that of human milk Lf, as checked by SDS/PAGE (results not shown).

Molecular modelling

The crystallographic data of diferric hLf and bLf were kindly provided by Professor E. N. Baker (Massey University, Palmerston North, New Zealand). Molecular modelling was performed on an Evans and Sutherland PS 350 graphic station and a Vax 6320 host computer using the Sybyl 5.3 molecular modelling package (Sybyl, 1988) (Professor G. Vergoten, CRESIMM, Lille, France). The crystallographic data for rabbit serum transferrin used to compare the three-dimensional structures of hLf and serum transferrin were provided by Professor P. F. Lindley (CCL Daresbury Laboratory, Warrington, Lancs., U.K.).

Radiolabelling of proteins

Iron-saturated hLf and bLf, rhLf and mutated EGS-loop rhLf were labelled in 10 mM PBS, pH 7.4, with 0.2 mCi of Na^{125}I (ICN Biomedical, Orsay, France) in the presence of Iodo-Gen as a catalyst, as previously described [16]. Excess reagent was removed by gel filtration through a Sephadex G-25 PD10 column. Radiolabelling of N-tryptic fragment, C-tryptic fragment and N2-glycopeptide was performed under the same experimental conditions as used for native Lfs. Radioactivity of samples was measured with a compugamma LKB Wallac (Turku, Finland) γ radiocounter, and specific radioactivity was determined.

Binding assays to LPS

Binding experiments to endotoxin were carried out with radio-labelled hLf, bLf and tryptic fragments derived from hLf.

LPS was immobilized as previously described [35]. Briefly, LPS suspensions were sonicated, diluted to $5 \mu\text{g}/\text{ml}$ in PBS and transferred ($200 \mu\text{l}$) to radioimmunoassay Maxisorp tubes (Nunc, Kamstrup, Denmark). After overnight incubation at 4°C , tubes were washed twice with PBS, and saturated with $200 \mu\text{l}$ of 0.1% BSA/ 0.01% Tween in PBS, for 3 h at 37°C . The solution was discarded and tubes were washed three times. Radiolabelled Lfs or tryptic fragments ($200 \mu\text{l}$; concentration ranging from 10 to 500 nM) were added directly to LPS-coated tubes and further incubated overnight at 4°C . After three washes with PBS, the radioactivity was measured. A control of binding of radiolabelled protein to LPS-free tubes was carried out. Non-specific binding was estimated for each protein concentration by adding a 100-fold molar excess of unlabelled protein in the presence of LPS. Specific binding was defined as the difference between total and non-specific binding of proteins to LPS. The non-specific binding of Lfs and tryptic fragments represented between 18 and 30% of total binding. Similar binding experiments were performed with rhLf and EGS-loop rhLf. The dissociation constants (K_d) were determined by Scatchard-plot analysis [36] using the Enzfitter program software 1.05 (BioSoft).

Competitive binding assays

Inhibition of ^{125}I -hLf binding to LPS by unlabelled hLf, bLf and human serum transferrin was assessed in the presence of a 10–100-fold molar excess of each unlabelled protein. Radioimmunoassay tubes were coated with $5 \mu\text{g}/\text{ml}$ LPS and incubated overnight at 4°C . The wells were blocked with PBS/ 0.1% BSA, washed and incubated with $2 \mu\text{g}/\text{ml}$ ^{125}I -hLf overnight at 4°C in the presence of increasing concentrations of unlabelled proteins. After three washes with PBS, the radioactivity was measured. A control of binding of radiolabelled protein to LPS-free tubes was carried out. Similar competitive experiments were performed between $2 \mu\text{g}/\text{ml}$ ^{125}I -hLf and a 10–100-fold molar excess of unlabelled N- and C-tryptic fragments, N2-glycopeptide, synthetic octadecapeptide, lactoferricin B, rhLf and EGS-loop rhLf.

RESULTS

Interactions of hLf and bLf with LPS

Specific binding of hLf to immobilized *E. coli* 055B5 LPS was found to be concentration-dependent and saturable at about 450 nM (Figure 2). Scatchard-plot analysis revealed the presence of two binding sites: (i) a high-affinity binding site with a K_d of 3.6 ± 1 nM; (ii) a low-affinity binding site with a K_d of 390 ± 20 nM (Table 1).

As reported for hLf, bLf also exhibits concentration-dependent and saturable binding to LPS (Figure 2). According to Scatchard analysis, two binding sites were found for bLf: (i) a high-affinity binding site with a K_d of 4.5 ± 2 nM, similar to the corresponding site of hLf; (ii) a low-affinity binding site with a lower K_d (576 ± 30 nM) (Table 1).

The specificity of the ^{125}I -hLf binding to LPS was checked in competitive studies with increasing concentrations of unlabelled hLf, bLf or human serum transferrin. As shown in Figure 3, the interaction of hLf with LPS was $76 \pm 4\%$ inhibited by a 100-fold molar excess of unlabelled hLf, suggesting specific binding. At the same concentration, bLf inhibited the binding of ^{125}I -hLf to LPS with an effectiveness similar to that of unlabelled human protein, $69 \pm 3\%$. We have also investigated whether human serum transferrin, a protein closely related in structure to hLf, could inhibit these interactions. Our results revealed that human serum transferrin did not prevent LPS binding to hLf, no more than $12 \pm 3\%$ inhibition being detected (Figure 3). These

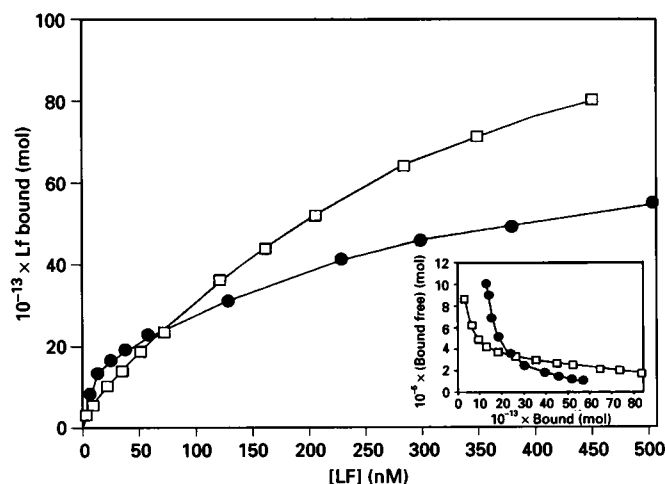


Figure 2 Specific binding of ^{125}I -hLf and ^{125}I -bLf to *E. coli* 055B5 LPS

Immobilized LPS was incubated with various concentrations of ^{125}I -hLf (●) and ^{125}I -bLf (□) at 4°C as described in the Materials and methods section. Non-specific binding was measured in the presence of a 100-fold molar excess of unlabelled Lf and was subtracted from total binding to obtain specific binding. The inset displays data replotted by Scatchard-plot analysis. The results are typical of three separate experiments.

Table 1 Binding parameters of hLf, bLf, rhLf, EGS-loop rhLf and proteolytic fragments derived from hLf to *E. coli* 055B5 LPS

Results are means \pm S.E.M.

Proteins	K_d (nM)	
	High-affinity binding site	Low-affinity binding site
hLf	3.6 ± 1	390 ± 20
bLf	4.5 ± 2	576 ± 30
hLf N-tryptic fragment	13 ± 3	Not measurable
hLf C-tryptic fragment	Not present	580 ± 30
rhLf	7.6 ± 1	650 ± 20
EGS-loop rhLf	Not present	220 ± 14

findings suggest different LPS-binding sites for Lfs and human serum transferrin.

Interactions of tryptic fragments isolated from hLf with LPS

In order to define the region involved in the interaction with LPS, hLf was submitted to partial proteolytic hydrolysis and binding of the resulting fragments to LPS was investigated (Figure 4). At a low concentration, the N-tryptic fragment bound specifically to LPS with high affinity (K_d 13 ± 3 nM), close to that of hLf (Table 1). At a concentration above 150 nM, the binding of the N-tryptic fragment to endotoxin was not saturable and measurable. In contrast, binding of the C-tryptic fragment to LPS was about 2-fold lower than that of native hLf. Only a low-affinity binding site was detected with a K_d of 580 ± 30 nM. Moreover, the binding of N2-glycopeptide to LPS was not saturable and represented only $15 \pm 3\%$ of hLf binding.

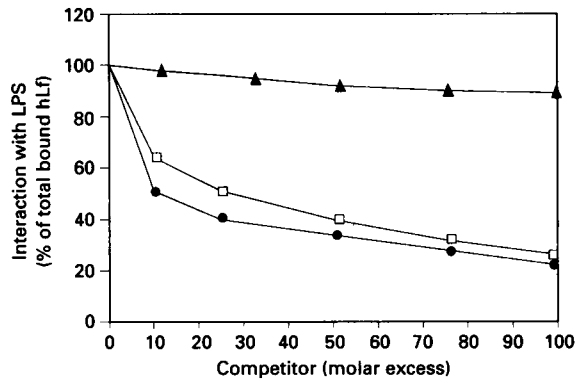


Figure 3 Inhibition of ^{125}I -hLf binding to LPS by unlabelled hLf, bLf and human serum transferrin

Competitive experiments for LPS binding were performed with $2\ \mu\text{g/ml}$ ^{125}I -hLf, in the presence of 10–100-fold molar excess of unlabelled hLf (●), bLf (□) or human serum transferrin (▲) at $4\ ^\circ\text{C}$. The results were calculated from three separate experiments and are expressed as a percentage of total ^{125}I -hLf bound to LPS.

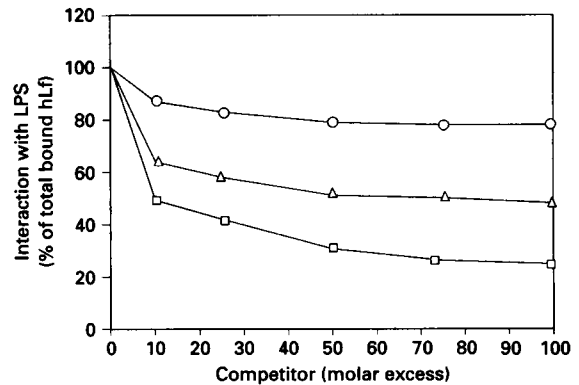


Figure 5 Inhibition of ^{125}I -hLf binding to LPS by unlabelled hLf tryptic fragments

Competitive experiments for LPS binding were carried out, with $2\ \mu\text{g/ml}$ ^{125}I -hLf in the presence of a 10–100-fold molar excess of unlabelled N-tryptic fragment (□), C-tryptic fragment (△) or N2-glycopeptide (○) of hLf at $4\ ^\circ\text{C}$. Results were calculated from three separate experiments. Percentage of total radioactivity bound to LPS was determined.

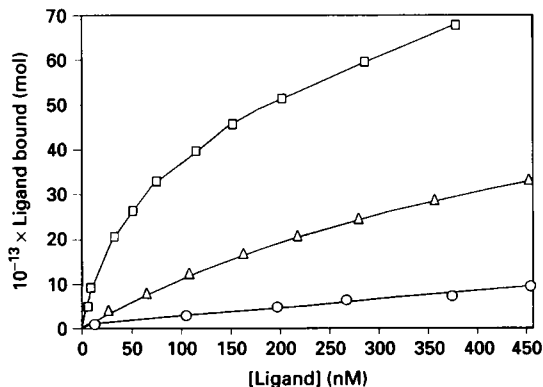


Figure 4 Specific binding of ^{125}I -labelled hLf tryptic fragments to LPS

Equilibrium binding was determined in the presence of various concentrations of radiolabelled N-tryptic fragment (□), C-tryptic fragment (△) or N2-glycopeptide (○) of hLf at $4\ ^\circ\text{C}$, as described in the Materials and methods section. Specific binding was estimated as described in the legend to Figure 1. The results were calculated from three separate experiments.

To investigate whether the two types of LPS-binding sites present in the N- and C-tryptic fragments are similar to that found in the native protein, competitive binding assays between hLf and its proteolytic fragments were also performed (Figure 5). Interaction of hLf with LPS was $75 \pm 3\%$ inhibited by a 100-fold molar excess of N-tryptic fragment whereas inhibition by the C-tryptic fragment was only $50 \pm 3\%$. No significant effect was observed with the N2-glycopeptide, no more than $20 \pm 3\%$ inhibition being measured. These results are in a good agreement with the experiments described above and prove the presence of the high-affinity LPS-binding site in the N-terminal domain I of hLf.

Inhibition of ^{125}I -hLf binding to LPS by the synthetic octadecapeptide and lactoferricin B

Since the N-terminal domain I of hLf seems to be important in the hLf–LPS interaction, we checked whether two well-known antimicrobial peptides located in this region could inhibit hLf–

LPS complex-formation. As illustrated in Figure 6, ^{125}I -hLf binding to LPS was $62 \pm 3\%$ inhibited by the synthetic octadecapeptide corresponding to residues 20–37 of hLf and $82 \pm 3\%$ by lactoferricin B, a homologous peptide of hLf present in the N-terminal lobe of bLf. The binding assays of the two peptides to LPS were not performed because, in the absence of tyrosine residues, their radiolabelling involves the linkage of a chemical reagent to basic amino acids. We have previously reported that for hLf, this type of labelling could modify the capacity of the protein to interact with other molecules [18].

Interaction of native and mutated rhLfs with LPS

To localize the amino acid sequence involved in the interaction with LPS, the $^{28}\text{RKVRGPP}^{34}$ sequence present in the N-terminal lobe of hLf was replaced by the sequence $^{365}\text{EGS}^{367}$ located in the C-terminal lobe of the protein. The EGS loop was obtained by

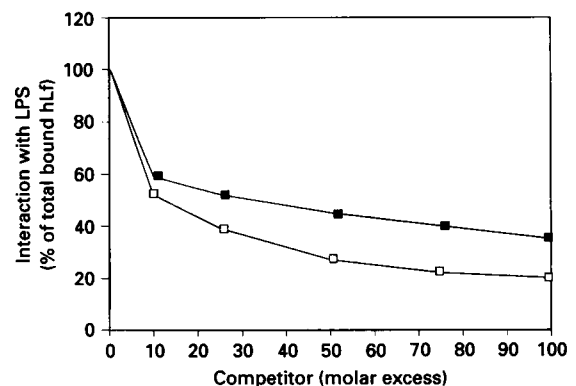


Figure 6 Inhibition of ^{125}I -hLf binding to LPS by unlabelled octadecapeptide and lactoferricin B

^{125}I -hLf ($2\ \mu\text{g/ml}$) was added to immobilized LPS, in the presence of a 10–100-fold molar excess of unlabelled synthetic octadecapeptide covering residues 20–37 of hLf (■) and lactoferricin B, a peptide involving residues 17–41 of bLf (□). Results were calculated from three separate experiments. Percentage of total radioactivity bound to LPS was determined.

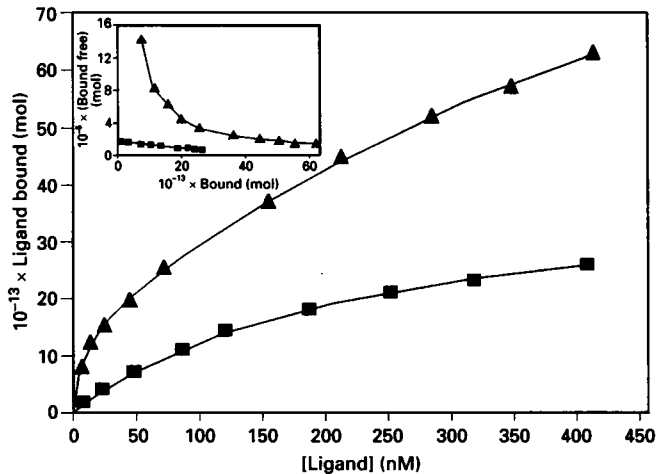


Figure 7 Specific binding of ^{125}I -rhLf and EGS-loop ^{125}I -rhLf to LPS

Equilibrium binding of ^{125}I -rhLf (\blacktriangle) and EGS-loop ^{125}I -rhLf (\blacksquare) was determined at 4 °C, as described in the Materials and methods section. The results are expressed as specific binding to LPS. The results are typical of three separate experiments.

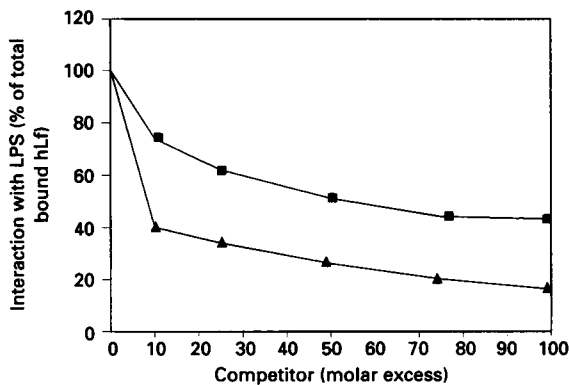


Figure 8 Inhibition of ^{125}I -hLf binding to LPS by unlabelled rhLf and EGS-loop rhLf

Competitive experiments were carried out with 2 $\mu\text{g}/\text{ml}$ ^{125}I -hLf, in the presence of a 10–100-fold molar excess of unlabelled rhLf (\blacktriangle) or EGS-loop rhLf (\blacksquare) at 4 °C. The results were expressed as a percentage of total radioactivity bound to LPS.

in vitro mutagenesis experiments. Binding of native rhLf and EGS-loop rhLf to LPS was then investigated. Like hLf, native rhLf exhibits concentration-dependent and saturable binding to LPS (Figure 7). Scatchard analysis revealed the presence of two binding sites, one of high and one of low affinity, similar to those obtained for hLf; the K_d values were 7.6 ± 1 and 650 ± 20 nM respectively. However, the binding of the EGS-loop rhLf to LPS was about 2-fold less than the native rhLf or hLf and the high-affinity binding site had disappeared. Only one class of low-affinity binding sites (K_d 220 ± 14 nM) was detectable by Scatchard analysis.

In competitive binding experiments, $55 \pm 3\%$ inhibition of ^{125}I -hLf binding to LPS was obtained in the presence of EGS-loop rhLf (Figure 8). In contrast, native rhLf gave a similar inhibition curve to that of unlabelled hLf. These data confirm the results obtained in the binding experiments described above.

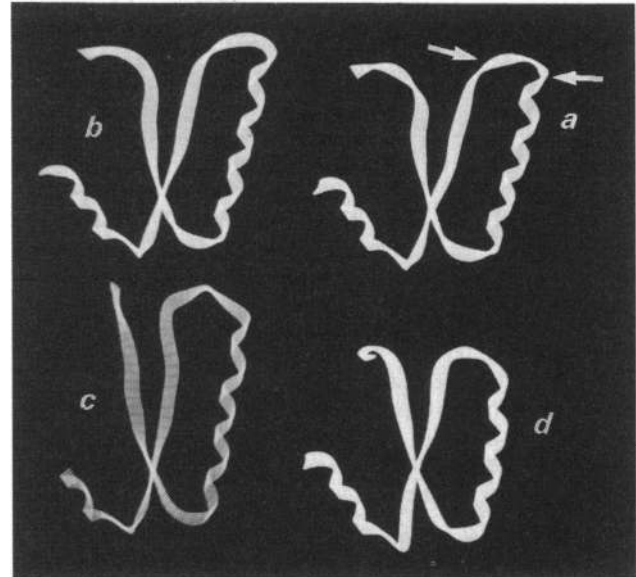


Figure 9 Molecular modelling of the 4–52 amino acid sequence of the N-terminal lobe of hLf (a) and the corresponding regions of the N-terminal lobe of bLf (b), the N-terminal lobe of rabbit serum transferrin (c) and the C-terminal lobe of hLf (d)

Arrowheads in (a) indicate the 28–34 loop region of hLf.

Molecular modelling

We have compared the folding of the N-terminal peptide (residues 4–52) of hLf with the folding of the corresponding regions found in the C-terminal lobe of hLf, N-terminal lobe of bLf and N-terminal lobe of rabbit serum transferrin. The N-terminal lobe of human serum transferrin possesses sequences homologous to that of rabbit protein [37]. However, crystallographic studies on human serum transferrin have not been reported.

As shown in Figure 9, all four peptides have β - α - β - α structures, which differ mainly in the loop equivalent to residues 28–34 of hLf. Very similar folding of this loop can be observed for hLf and bLf, whereas the corresponding loops of rabbit serum transferrin and the C-terminal part of hLf are longer or shorter respectively.

DISCUSSION

Antimicrobial properties of Lf [10,38] and its role in immune defence during inflammation [5,6] have been reported. Some of these activities are modulated, at least in part, by LPS–Lf complex-formation [14]. Recently, it has been demonstrated that inactivation of LPS by neutrophils is due to Lf secreted by neutrophil granules limiting inflammation and avoiding tissue damage by oxygen radicals [39]. However, up to now, no data on the region of Lf recognized by LPS are available. In the present study, two binding sites for *E. coli* 055B5 LPS have been found on hLf: a high-affinity (K_d 3.6 ± 1 nM) site, and a low-affinity (K_d 390 ± 20 nM) site which appears at high protein concentrations. The affinity constant corresponding to the high-affinity site is close to that obtained by Appelmelk et al. [12] for hLf binding to lipid A isolated from *E. coli* LPS (0.5 nM). These authors have not described a low-affinity site, probably because they worked only at very low hLf concentrations. A number of other proteins have also been reported to bind the lipid A region

of LPS. These include bactericidal permeability-increasing protein, a cationic protein present in the granules of polymorphonuclear leucocytes [35], and LPS-binding protein [40], a serum protein. The binding of both proteins is specific and of high affinity with a K_d of 2–5 nM for the former [35] and 1 nM for the latter [40]. In our experiments, as well as in those reported by others [12,35,40], the number of binding sites on different proteins per LPS molecule has not been determined. Indeed, it is well known that the molecular mass of LPS is heterogeneous since the endotoxin forms aggregates of different size. The capacity of ^{125}I -hLf to bind LPS was found to be specific, as 76% inhibition of LPS-Lf interaction was obtained in the presence of a 100-fold molar excess of unlabelled protein.

We have located the hLf binding sites for LPS using different fragments obtained by partial proteolysis of hLf. The high-affinity site was found to be present in the N-terminal fragment (residues 4–283) of hLf (K_d 13 ± 3 nM). It seems that the removal of the first three arginine residues from the N-tryptic fragment during tryptic hydrolysis of hLf [27] does not affect the binding of the fragment to LPS, since similar K_d values for whole native hLf and the N-tryptic fragment were obtained. The C-tryptic fragment possesses only low-affinity binding features and partially inhibits the hLf-LPS interaction. As no significant binding to endotoxin was measured with the N2-glycopeptide (91–255) which corresponds to N-terminal domain II of the protein, it can be assumed that the high-affinity recognition site is located in N-terminal domain I of hLf (residues 4–91 and 256–322). The glycan moiety of the protein is not involved in the binding of hLf to LPS, since domain I is not glycosylated. It has been shown that the lymphocyte-receptor-binding site of hLf is located in the N-terminal part of the molecule, corresponding to residues 4–52 [19]. As reported by Legrand et al. [19], this domain contains three accessible areas, residues 4–6, 28–34 and 38–45, which are specific for the N-terminal moiety of hLf. The 28–34 amino acid sequence is present in the synthetic octadecapeptide, an antimicrobial cationic peptide corresponding to residues 20–37 of hLf which produced 62% inhibition of hLf-LPS interaction. The loop region, which was found to be involved in the receptor-binding site, is somewhat different in the corresponding part of the C-terminal lobe [19]. This fact could explain why the C-tryptic fragment of hLf does not bind to LPS with high affinity.

Direct evidence that residues 28–34 are responsible for LPS binding was obtained by experiments performed with the EGS-loop rhLf, a recombinant hLf in which residues 28–34 of the protein were replaced by the C-terminal loop counterpart. Our results revealed that this mutation on the whole protein leads to suppression of the high-affinity interactions between rhLf and LPS.

As for the C-terminal lobe of hLf, the homologous loop of the N-terminal lobe of serum transferrin exhibits specific structural features which could explain why serum transferrin, despite its ability to bind LPS, did not inhibit hLf-LPS interaction. Our results suggest the existence of different LPS-binding sites on transferrin and hLf, a hypothesis supported by the different biological activities reported for these two proteins [24]. Unlike hLf which possesses bactericidal activity, only a bacteriostatic effect of transferrin has been detected [24]. Nevertheless, the LPS-binding sites on serum transferrin have not been identified and further studies are necessary to elucidate the difference between Lf and serum transferrin in their molecular interactions with LPS.

Scatchard analysis also showed the presence of two LPS-binding sites on bLf, with similar K_d values to those obtained for hLf. Moreover, bLf inhibited hLf-LPS interaction. These results suggest that the same binding sites are present on bLf and hLf.

In fact, considering the high-affinity binding of Lfs to LPS, it can be observed that the loop regions originating from hLf (residues 28–34) and bLf exhibit similar structural features [41]. Lactoferricin B, a proteolytic peptide covering residues 17–41 of bLf, also decreased hLf binding to the endotoxin, but the inhibition was greater than that obtained with the octadecapeptide (82% compared with 62%). This difference could be explained by a greater affinity of lactoferricin B for LPS, in agreement with its more potent activity against Gram-negative bacteria than the peptide derived from hLf [24].

In conclusion, the loop region (28–34) of hLf is essential for the high-affinity binding of LPS. Considering the similar behaviour of hLf and bLf towards LPS, it is reasonable to postulate that the above region is also important for bLf-LPS interaction. Moreover, as hLf has been reported to be a lipid A-binding protein [12], it can be assumed that the loop sequences of hLf and bLf interact directly with the lipid A region of LPS.

Finally, the data on the hLf amino acid sequence involved in interaction with LPS could help to explain the modulation of some biological activities of this glycoprotein in the presence of endotoxin [13,14]. In this context, our results are in agreement with the observation of Miyazawa et al. [14] that hLf loses the capacity to interact with its receptor present on differentiated HL60 cells after the formation of the hLf-LPS complex.

This work was supported by the Université des Sciences et Technologies de Lille I, the Centre National de la Recherche Scientifique (U.M.R. du CNRS no. 111, Director: Professor A. Verbert), by the INSERM contract (no. 930507) and by the Programme International de Coopération Scientifique of the CNRS with Rumania (contract no. 232). We are grateful to Mrs. M. Benaïssa and Mr. J. P. Decottignies for their skilful technical assistance and to Professor A. Tartar (URA no. 1309 du CNRS, Institut Pasteur, Lille) for synthesis of the octadecapeptide. We thank Professor E. N. Baker (Massey University, New Zealand) and Professor P. F. Lindley (CCL, Daresbury Laboratory, Warrington, Lancs., U.K.) for providing the X-ray crystallographic data on hLf, bLf and rabbit serum transferrin. We also thank Professor G. Vergoten (CRESIMM, Lille, France) for molecular modelling studies. We are especially grateful to Dr. J. Mazurier for helpful discussion.

REFERENCES

- Montreuil, J. and Mullet, S. (1960) *C. R. Acad. Sci. Paris* **250**, 1736–1739
- Montreuil, J., Tonnelat, J. and Mullet, S. (1960) *Biochim. Biophys. Acta* **45**, 413–421
- Masson, P. L. (1970) in *La Lactoferrine, Protéine des Sécrétions Externes et des Leucocytes Neutrophiles* (Masson, P. L., ed.), pp. 93–165, Edition Arscas, Bruxelles
- Masson, P. L., Heremans, J. F. and Schönee, E. (1969) *J. Exp. Med.* **130**, 643–658
- Brock, J. (1986) in *Iron in Immunity, Cancer and Inflammation* (De Souza, M. and Brock, J., eds.), pp. 81–98, John Wiley, Chichester
- Zimecki, M., Mazurier, J., Machnicki, M., Wiczorek, Z., Montreuil, J. and Spik, G. (1991) *Immunol. Lett.* **30**, 377–382
- Spik, G. and Montreuil, J. (1983) *Bull. Eur. Physiopathol. Respir.* **19**, 123–130
- Sanchez, L., Calvo, M. and Brock, J. H. (1992) *Arch. Dis. Child.* **67**, 657–661
- Bullen, J. J. (1981) *Rev. Infect. Dis.* **3**, 1127–1137
- Arnold, R. R., Cole, M. F. and MacGhee, A. G. (1977) *Nature (London)* **197**, 263–265
- Ellison, R. T. and Giehl, T. J. (1991) *J. Clin. Invest.* **88**, 1080–1091
- Appelmek, B. J., An, Y. Q., Geerts, M. et al. (1994) *Infect. Immun.* **62**, 2628–2632
- Crouch, S. P. M., Slater, K. J. and Fletcher, J. (1992) *Blood* **80**, 235–240
- Miyazawa, K., Mantel, C., Lu, L., Morrison, D. C. and Broxmeyer, H. E. (1991) *J. Immunol.* **146**, 723–729
- Birgens, H. S. (1991) *Dan. Med. Bull.* **38**, 244–252
- Mazurier, J., Legrand, D., Hu, W. L., Montreuil, J. and Spik, G. (1989) *Eur. J. Biochem.* **179**, 481–487
- Rochard, E., Legrand, D., Mazurier, J., Montreuil, J. and Spik, G. (1989) *FEBS Lett.* **255**, 201–204
- Legrand, D., Mazurier, J., Maes, P., Rochard, E., Montreuil, J. and Spik, G. (1991) *Biochem. J.* **276**, 733–738
- Legrand, D., Mazurier, J., Ellass, A. et al. (1992) *Biochemistry* **31**, 9243–9251
- Metz-Boutigue, M. H., Jollès, J., Mazurier, J. et al. (1984) *Eur. J. Biochem.* **145**, 659–676
- Rey, M. W., Woloshuk, S. L., De Boer, H. A. and Pieper, F. R. (1990) *Nucleic Acids Res.* **18**, 5288

- 22 Anderson, B. F., Baker, H. M., Dodson, E. J. et al. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 1769–1773
- 23 Pierce, A., Colavizza, D., Benaïssa, M. et al. (1991) *Eur. J. Biochem.* **196**, 177–184
- 24 Bellamy, W., Takase, M., Yamauchi, K., Wakabayashi, H., Kawase, K. and Tomita, M. (1992) *Biochim. Biophys. Acta* **1121**, 130–136
- 25 Jones, E. M., Smart, A., Bloomberg, G., Burgess, L. and Millar, M. R. (1994) *J. Appl. Bacteriol.* **77**, 208–214
- 26 Berger, D. and Berger, H. G. (1990) *Surg. Res. Comm.* **8**, 147–156
- 27 Legrand, D., Mazurier, J., Metz-Boutigue, M. H. et al. (1984) *Biochim. Biophys. Acta* **787**, 90–96
- 28 Legrand, D., Mazurier, J., Aubert, J. P., Loucheux-Lefebvre, M. H., Montreuil, J. and Spik, G. (1986) *Biochem. J.* **236**, 839–844
- 29 Spik, G., Strecker, G., Fournet, B. et al. (1982) *Eur. J. Biochem.* **121**, 413–419
- 30 Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- 31 Mazurier, J. and Spik, G. (1980) *Biochim. Biophys. Acta* **629**, 399–408
- 32 Legrand, D., Salmon, V., Coddeville, B., Benaïssa, M., Plancke, Y. and Spik, G. (1995) *FEBS Lett.* **365**, 57–60
- 33 Sanger, F., Nicklen, S. and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5463–5467
- 34 Palmiter, R. D., Behringer, R. R., Quaife, C. J., Maxwell, F., Maxwell, I. H. and Brinster, R. L. (1987) *Cell* **50**, 435–443
- 35 Gazzano-Santoro, H., Parent, J. B., Grinna, L. et al. (1992) *Infect. Immun.* **60**, 4754–4761
- 36 Scatchard, G. (1949) *Ann. N.Y. Acad. Sci.* **51**, 660–672
- 37 Banfield, D. K., Chow, B. K.C., Funk, W. D. et al. (1991) *Biochim. Biophys. Acta* **1089**, 262–265
- 38 Ellison III, R. T. (1994) *Adv. Exp. Med. Biol.* **357**, 71–90
- 39 Wang, D., Pabst, K. M., Aida, Y. and Pabst, M. J. (1995) *J. Leukoc. Biol.* **57**, 865–874
- 40 Mathison, J. C., Tobias, P. S., Wolfson, E. and Ulevitch, R. J. (1992) *J. Immunol.* **149**, 200–206
- 41 Spik, G., Legrand, D., Leveugle, B. et al. (1993) in *New Perspectives in Infant Nutrition* (Renner, B. and Sawatzki, G., eds.), pp. 77–83, Symposium Antwerp 1992, Georg Thieme Verlag, Stuttgart