N-terminal stretch Arg[2], Arg[3], Arg[4] and Arg[5] of human lactoferrin is essential for binding to heparin, bacterial lipopolysaccharide, human lysozyme and DNA

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Human lactoferrin (hLF), a protein involved in host defence against infection and excessive inflammation, interacts with heparin, the lipid A moiety of bacterial lipopolysaccharide, human lysozyme (hLZ) and DNA. To determine which region of the molecule is important in these interactions, solid-phase ligand binding assays were performed with hLF from human milk (natural hLF) and N-terminally deleted hLF variants. Iron-saturated and natural hLF bound equally well to heparin, lipid A, hLZ and DNA. Natural hLF lacking the first two N-terminal amino acids (Gly^1-Arg^2) showed reactivities of one-half, two-thirds, one-third and one-third towards heparin, lipid A, hLZ and DNA respectively compared with N-terminally intact hLF. A lack of the first three residues (Gly^1-Arg^2-Arg^3) decreased binding to the same ligands to one-eighth, one-quarter, one-twentieth and one-seventeenth respectively. No binding occurred with a mutant lacking the first five residues (Gly^1-Arg^2-Arg^3-Arg^4-Arg^5). An anti-hLF monoclonal antibody (E11) that reacts to an N-lobe epitope including Arg^5 completely blocked hLF–ligand interaction. These results show that the N-terminal stretch of four consecutive arginine residues, Arg^2-Arg^3-Arg^4-Arg^5, has a decisive role in the interaction of hLF with heparin, lipid A, hLZ and DNA. The role of limited N-terminal proteolysis of hLF in its anti-infective and anti-inflammatory properties is discussed.

INTRODUCTION

Lactoferrin (LF) is a metal-binding glycoprotein of molecular mass 77 kDa [1] found in milk, tears, saliva and bronchial, intestinal, vaginal and other secretions. It is also present in the secondary granules of neutrophils [2]. On the basis of the many reports of antimicrobial and anti-inflammatory activity *in vitro*, LF is thought to be involved in host defence against infection and severe inflammation, most notably at mucosal surfaces [2].

The amino acid sequence of LF has been determined by protein [3] and DNA sequencing [4,5]. Human LF (hLF) consists of a polypeptide chain of 692 amino acid residues folded into two globular lobes [1]. Each lobe in turn folds into α-helix and β-sheet arrays to form two domains (I and II) connected by a hinge region creating a deep iron-binding cleft within each lobe. Each cleft binds a single ferric ion while simultaneously incorporating a suitable anion [1]. However, some of the biological activities of hLF do not arise from the binding of iron but from its capacity to bind to other molecules. For example, interaction of hLF with heparin [6] results in the neutralization of the anticoagulant activity of this molecule [7], possibly acting to localize an infectious process through encapsulation with fibrin. Interaction of hLF with bacterial outer membrane components such as lipopolysaccharide (LPS) [8] and porins [9] is presumably important in the antimicrobial activity of hLF. Binding of hLF to the lipid A portion of LPS [10] inhibits the priming of neutrophils for enhanced formyl-Met-Leu-Phe-triggered superoxide release [11] and might also account for the decreased production of cytokines after challenge with LPS [12]. Direct intermolecular interaction between hLF and human lysozyme (hLZ) [13] might contribute to the synergy between the antibacterial actions of these two proteins [14]. The interaction with DNA of hLF translocated to the nucleus might alter the transcriptional activity of certain genes and underlie some of the immunomodulatory effects of hLF [15].

The N-terminal portion of hLF contains two potential heparin-binding sites, Arg^2-Arg^3-Arg^4-Arg^5 and Arg^{28}-Lys^{29}-Val^{30}-Arg^{31}. Mann and colleagues [6], studying the interaction of hLF with glycosaminoglycans (GAGs), found that these two basic clusters act synergistically in the binding of hLF to GAG. These authors also concluded that Arg^4 and Arg^5 were crucial for hLF–heparin interaction. Wu et al. [16] postulated that binding of hLF to heparin was predominantly mediated through the second basic cluster, Arg^{28}-Lys^{29}-Val^{30}-Arg^{31}. Here we present evidence that each consecutive arginine residue of the first basic cluster (Arg^2-Arg^3-Arg^4-Arg^5) contributes to the interaction of hLF with physiologically relevant ligands, including heparin, lipid A, hLZ and DNA.

MATERIALS AND METHODS

Reagents

Mutagenic primers, S-Sepharose and CNBr-activated Sepharose 4B were obtained from Pharmacia Fine chemicals AB (Uppsala, Sweden). Lipid A (from *Salmonella minnesota* Re595), heparin (sodium salt, grade I-A, from pig intestinal mucosa), double-stranded calf thymus DNA, protamine sulphate and polyclonal rabbit anti-hLF antiserum were purchased from Sigma Chemical

Abbreviations used: GAG, glycosaminoglycan; hLF, human lactoferrin; hLF^{-2N}, hLF^{-3N}, $rhLF^{-5N}$, hLF lacking the first two, three or five N-terminal residues respectively; hLZ, human lysozyme; LF, lactoferrin; LPS, lipopolysaccharide; mAb, monoclonal antibody; natural hLF, native hLF from human milk; rhLF, recombinant hLF; RIA, radioimmunoassay.

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Co. (St. Louis, MO, U.S.A.). NHS-LC-Biotin was from Pierce (Rockford, IL, U.S.A.). Streptavidin biotinylated horseradish peroxidase complex and ^{125}I were obtained from Amersham (Little Chalfont, Bucks., U.K.). Maxisorp and Polysorp microtitre plates were from Nunc (Roskilde, Denmark). Universal binding plates were from Costar (Cambridge, MA, U.S.A.). All cell culture reagents were from Gibco (Paisley, Scotland, U.K.). The mouse monoclonal antibody (mAb) isotyping kit was from Hycult Biotechnology (Uden, The Netherlands).

Purification, iron saturation and biotinylation of hLF

hLF was purified from fresh human milk by cation-exchange chromatography on S-Sepharose as described; it is hereafter designated 'natural hLF' [13]. Natural hLF was used in all experiments, unless stated otherwise. Natural hLF was 3.5% saturated with iron; complete saturation of natural hLF with iron was achieved as described [13]. Natural hLF was biotinylated by incubation with a 25-fold molar excess of biotin for 2 h at 20 °C. Unbound biotin was removed by dialysis against 10 mM sodium phosphate/0.15 M NaCl (pH 7.4) (PBS) for 16 h. Biotinylated hLF was stored at 4 °C in PBS containing 0.02% sodium azide.

Production and purification of anti-hLF mAbs

Balb/C mice were hyperimmunized by repeated intraperitoneal injections with 50 μg of natural hLF as described previously [17]; 4 days after the fourth injection, spleen cells were fused with SP2/0-Ag14 [ATCC CRL 1583] myeloma cells. Fusion and hybridoma selection were performed as described [18]. Culture supernatants were initially screened for the presence of specific antibodies by a radioimmunoassay (RIA), in which ^{125}I-hLF was used as an antigen. Hybridomas producing hLF-specific antibodies were cloned by repeated limiting dilution. In all, six different anti-hLF mAbs were obtained. Immunoglobulin subclass determination showed that all mAbs were of the IgG_1 type (determined with the mouse mAb isotyping kit in accordance with the manufacturer's instructions). Immunoglobulin-enriched fractions of culture supernatant were prepared by precipitation with $(NH_4)_2SO_4$ and coupled to Sepharose (20 mg of protein to 1 g of CNBr-activated Sepharose 4B) or used for the purification of anti-hLF mAb with the Repligen antibody purification kit (Cambridge, MA, U.S.A.) as recommended by the manufacturer. SDS/PAGE of 10 μg of purified mAb preparations revealed that they were more than 99% pure. The antibody concentration of purified preparations was determined by absorbance measurement at 280 nm, with an $A_{280}^{0.1\%}$ value of 1.4.

Expression and purification of an N-terminal deleted hLF mutant

The expression vector pCMV/hLF^{-5N}, encoding hLF amino acids Ser^{6} to Lys^{692}, was produced with the Transformer® site-directed mutagenesis kit as described [19]. The mutagenic primer sequence was 5′-CTGTTGCTCTTGCC**AGTGTTCAGTGGTGC**-3′, consisting of the last 14 nucleotides of the bovine αS_1 casein signal sequence (underlined; [20]) fused to nt 365–379 of the hLF cDNA (bold; [4]) to create a deletion from nt 352 to nt 364. After mutagenesis the deletion was confirmed by dideoxy sequencing.

Human 293(S) (ATCC CRL 1573) cells constitutively expressing pCMV/hLF^{-5N} were generated as described [19] and incubated in serum-free Dulbecco's modified Eagle's medium supplemented with 50 i.u./ml penicillin and 50 μg/ml streptomycin for 3 days. S-Sepharose was incubated in batches with the conditioned medium for 4 h, poured into a column and eluted with 20 mM sodium phosphate/0.5 M NaCl (pH 7.5). The S-Sepharose eluate was diluted with 20 mM sodium phosphate, pH 7.5 (buffer A), applied to a Mono S HR 5/5 (Pharmacia) cation-exchange column and eluted with a linear salt gradient of 0–0.5 M NaCl in 60 ml of buffer A at a flow rate of 0.5 ml/min. Whereas natural hLF and recombinant hLF (rhLF) were eluted at 0.7 M NaCl [13], the N-terminally deleted rhLF mutant (designated rhLF^{-5N}) was eluted at 0.33 M NaCl as determined with an hLF-specific ELISA [19].

Isolation of N-terminally truncated natural hLF

hLF variants lacking two (Gly^{1}-Arg^{2}; designated hLF^{-2N}) or three (Gly^{1}-Arg^{2}-Arg^{3}; designated hLF^{-3N}) N-terminal residues were isolated from purified human milk LF from Sigma by Mono S chromatography as described [13]. The hLF^{-3N} and hLF^{-2N} species are eluted from Mono S at 0.5 and 0.6 M NaCl respectively [13].

Solid-phase ligand binding assays

Microtitre plates were coated for 16 h at 20 °C with PBS containing 1 μg/ml lipid A (Polysorp), 10 μg/ml hLZ [Universal binding plates; after being coated, the plates were emptied, exposed to UV in accordance with the manufacturer's instructions and incubated for 30 min with 3% (w/v) BSA in PBS], 25 μg/ml heparin or double-stranded DNA (Maxisorp, precoated with 0.5 mg/ml protamine sulphate in water). After being washed with PBS/0.02% (w/v) Tween-20, plates were incubated with serial dilutions of the test samples in PBS/0.02% (w/v) Tween-20/0.2% gelatin (PTG). After 2 h, plates were washed and incubated for 1 h with 0.4 μg/ml peroxidase-conjugated purified bovine anti-hLF [13] in PTG containing 1% (v/v) normal bovine serum. After another wash, substrate solution [0.01% (w/v) 3,3′,5,5′-tetramethylbenzidine/0.003% (v/v) H_2O_2/0.11 M sodium acetate (pH 5.5)] was added. Substrate conversion was stopped by the addition of 2 M H_2SO_4; A_{450} was read with a SLT 340 ATCC microplate reader (SLT-labinstruments, Salzburg, Austria). All incubations were performed with 100 μl volumes.

To determine the reactivities of distinct hLF variants to the various ligands, serial dilutions of deleted hLF variants as well as N-terminally intact iron-saturated hLF and natural hLF (used as a reference) were tested in parallel in the ligand binding assays as well as the ELISA for hLF, to correct for differences in hLF concentration. The reactivity of an hLF variant towards each of the ligands was expressed as a percentage of the response with natural hLF, which was arbitrarily defined as 100%.

Competitive inhibition of hLF binding to solid-phase ligands

Biotinylated hLF (100 ng) was preincubated for 16 h at 20 °C with a serial dilution of competitor in PTG and added to the microtitre plates coated as described above. After 2 h, plates were washed and then incubated for 30 min with streptavidin biotinylated horseradish peroxidase complex. After another wash, substrate solution was added. Further ELISA procedures were as described above.

Mapping of monoclonal anti-hLF antibody epitopes to the recombinant N-lobe or C-lobe

Polysorp plates were coated for 16 h at 20 °C with PBS containing 1 μg/ml purified mAb. Plates were washed and incubated for 2 h with serial dilutions of natural hLF (100 ng/ml) and conditioned medium of stable 293(S) cell lines secreting either the recombinant N-lobe or the recombinant C-lobe (P. H. C. van Berkel, un-

published work). After a wash, bound hLF was detected with peroxidase conjugated bovine anti-hLF as described.

RIA procedures to compare the binding of distinct hLF variants to anti-hLF–Sepharose

These RIAs were performed as described previously [13]. Briefly, anti-hLF mAbs coupled to Sepharose were incubated with serial dilutions of hLF variants. Bound hLF was detected by subsequent incubation with polyclonal ^{125}I-labelled anti-hLF antibodies. Binding was expressed as a percentage of the total amount of labelled antibodies added.

RESULTS

hLF interacts specifically with heparin, lipid A, hLZ and DNA

Solid-phase ligand binding assays were developed to study the interaction of hLF with heparin, lipid A, hLZ and DNA. Figure 1 shows the binding of natural hLF to each of the ligands immobilized on microtitre plates. No binding of natural hLF was observed when hLF was incubated with uncoated plates or with plates coated with BSA or precoated with protamine sulphate. Incubation of natural hLF in the presence of excess human transferrin, a metal-binding protein closely related to hLF in size and structure, did not affect the binding of hLF to any of the ligands (P. H. C. van Berkel, unpublished work). These results indicate that hLF binds specifically to heparin, lipid A, hLZ and DNA.

The binding of hLF to heparin and DNA involves electrostatic interaction, which can be disrupted by increasing the ionic strength [21,22]. Figure 2 shows the effect of the NaCl concentration on the binding of natural hLF [13] to LPS and hLZ. At physiological NaCl concentration (0.15 M), approx. 40% of hLF bound to LPS and hLZ. Decreasing the salt concentration increased hLF binding to LPS and hLZ up to approx. 75% and 55% at 0.013 M NaCl respectively, whereas increasing NaCl above 0.4 M NaCl abolished the binding. These results illustrate the ionic-strength dependence of hLF binding to ligands and suggest that the highly cationic N-terminus of the protein is involved in electrostatic interaction of hLF with the ligands.

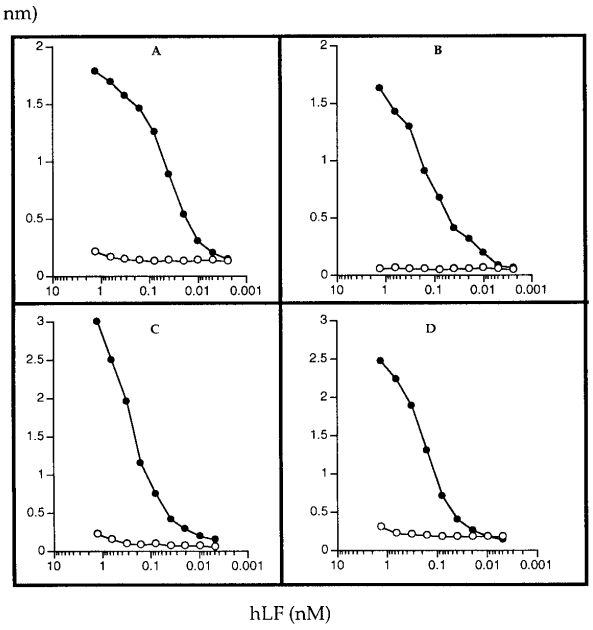

Figure 1 Binding of hLF to solid-phase heparin, lipid A, hLZ and DNA

Serial dilutions of purified natural hLF (●; 100 ng/ml) were incubated with heparin (**A**), lipid A (**B**), hLZ (**C**) or double-stranded DNA (**D**) coated on microtitre plates as described in the Materials and methods section. Bound hLF was detected by subsequent incubation with peroxidase-labelled bovine anti-hLF. Controls (○) were: serial dilutions of natural hLF in protamine sulphate precoated plates without heparin or DNA coating, in plates without lipid A coating, and BSA-blocked plates without hLZ coating. The A_{450} values measured as described are plotted as a function of the hLF concentration in the well.

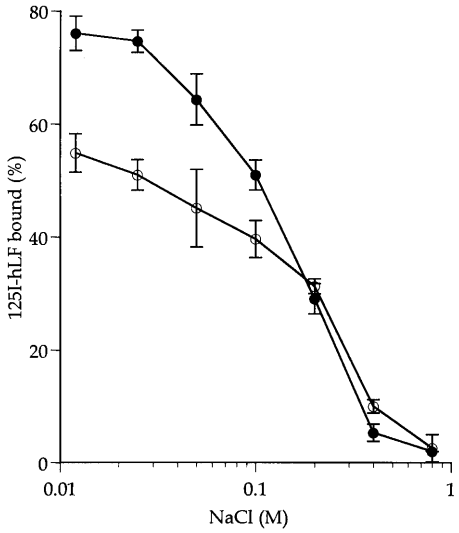

Figure 2 Effect of NaCl on the binding of hLF to LPS and hLZ

Equal amounts of Sepharose, on which LPS from *Salmonella minnesota* Re595 (●) or hLZ (○) had been immobilized [13], were suspended in 10 mM sodium phosphate buffer, pH 7.6, containing 0.02% (w/v) Tween-20 and increasing concentrations of NaCl. Sepharose beads were incubated with ^{125}I-hLF by head-over-head rotation. After 16 h the beads were washed with 10 mM sodium phosphate/0.02% Tween-20 and bound radioactivity was measured. Binding was expressed as a percentage of the total amount of ^{125}I-hLF added. The NaCl concentration of the Sepharose suspension is indicated on the abscissa. The curves represent the means ± S.D. for a representative experiment performed in quintuplicate.

Table 1 Binding of natural hLF, iron-saturated hLF and N-terminally deleted hLF variants to heparin, lipid A, hLZ and DNA

Solid-phase ligand-binding assays were performed as described in the Materials and methods section. The response of natural hLF was arbitrarily defined as 100%. From the parallel dose–response curves the reactivity of an hLF variant towards each of the ligands was expressed as a percentage with reference to natural hLF. Results are given as mean ± S.D. for at least four independent experiments.

	Reactivity (%)			
hLF variant	Heparin	Lipid A	hLZ	DNA
Natural	100	100	100	100
Iron-saturated	98 ± 8	96 ± 8	100 ± 11	99 ± 5
hLF^{-2N}	56 ± 4	68 ± 10	38 ± 12	31 ± 2
hLF^{-3N}	13 ± 2	26 ± 3	5 ± 2	6 ± 1
$rhLF^{-5N}$	No binding	No binding	No binding	No binding

Arg^2-Arg^3-Arg^4-Arg^5 stretch of hLF is essential for binding to heparin, lipid A, hLZ and DNA

A comparison of the binding of natural and iron-saturated hLF

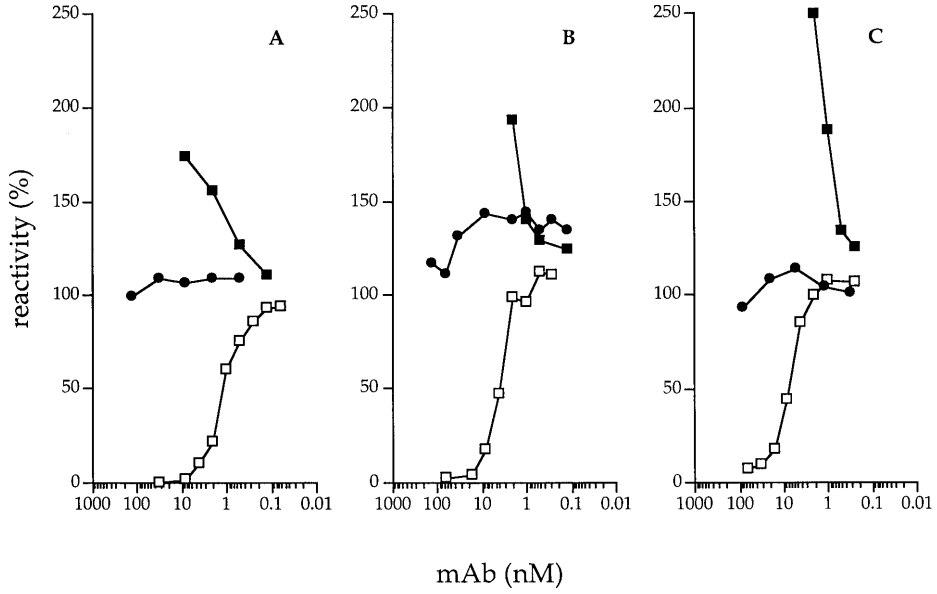

Figure 3 Competitive inhibition by anti-hLF mAb E11 of hLF binding to heparin, lipid A and hLZ

Plates were coated with heparin (**A**), lipid A (**B**) or hLZ (**C**). Biotinylated hLF was preincubated with serial dilutions of anti-hLF mAb E11 (□), anti-hLF mAb E3 (■) or a control mAb directed against human C1-esterase (●) as described in the Materials and methods section. The reactivity of hLF to each ligand in the presence of competitor was expressed as a percentage of the response of hLF without competitor, which was arbitrarily defined as 100%. The mAb concentration in the well (nM) is indicated on the abscissa.

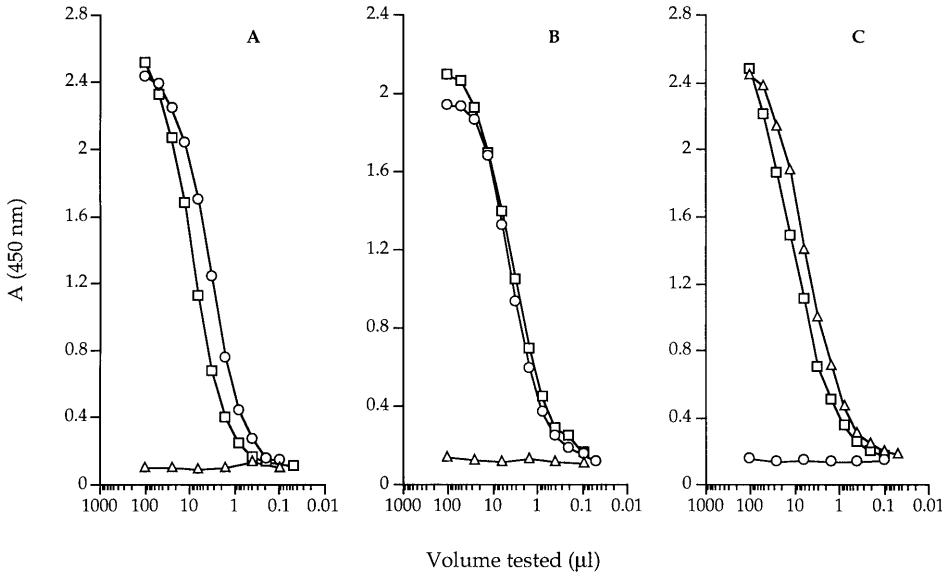

Figure 4 Binding of the recombinant hLF N-lobe and C-lobe and natural hLF to anti-hLF mAbs

Serial dilutions of natural hLF (100 ng/ml; □) and conditioned medium of 293(S) cells secreting recombinant hLF N-lobe (○) or C-lobe (△) were incubated with purified mAbs E11 (**A**); E3 (**B**) and E19 (**C**) coated on microtitre plates as described in the Materials and methods section. Bound hLF was detected by subsequent incubation with horseradish peroxidase-conjugated anti-hLF.

to heparin, lipid A, hLZ and DNA indicated identical affinities of these molecules for each ligand (Table 1). To delineate the contribution of the four consecutive arginines ('first basic cluster') in the hLF N-terminus in the hLF–ligand interaction, we studied the binding of N-terminally deleted hLF to heparin, lipid A, hLZ and DNA. hLF^{-2N} showed reactivities of one-half, two-thirds, one-third and one-third towards heparin, lipid A, hLZ and DNA respectively, compared with N-terminally intact natural hLF (Table 1). hLF^{-3N} showed one-eighth, one-quarter, one-twentieth and one-seventeenth of the reactivity for heparin, lipid A, hLZ and DNA respectively. No binding of the mutant $rhLF^{-5N}$ was observed. We have shown previously that N-terminally intact rhLF and natural hLF bound to hLZ and LPS with equal affinities [13]. These results indicate that all four

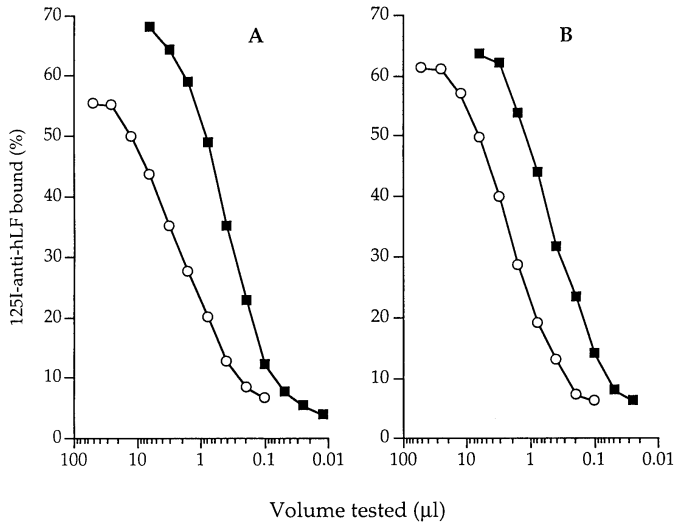

Figure 5 Comparison of the binding of N-terminally deleted and natural hLF to anti-hLF mAbs E11 and E3

Serial dilutions of natural hLF (40 μg/ml; ■) and rhLF^{-5N} (7 μg/ml; ○) were incubated with mAb E11 (**A**) or E3 (**B**) coupled to Sepharose. Bound hLF was detected by subsequent incubation with polyclonal ^{125}I-labelled anti-hLF antibodies. Binding was expressed as a percentage of the total amount of antibodies added.

arginine residues contribute to the interaction of hLF with heparin, lipid A, hLZ and DNA and that the removal of the first basic cluster abrogates the interaction of hLF with these ligands.

Anti-hLF mAb E11 inhibits hLF–ligand interaction

Six different purified anti-hLF mAbs were tested for their ability to inhibit the binding of hLF to immobilized ligands. The results in Figure 3 show that preincubation of hLF with anti-hLF mAb E11 could completely block the interaction of hLF with heparin (Figure 3A), lipid A (Figure 3B) and hLZ (Figure 3C), whereas a control mAb against C1-esterase did not affect hLF binding. The differences in mAb E11 concentration required to completely prevent the binding of hLF to the solid-phase ligands most probably results from differences in the affinities of hLF for each ligand as well as from differences in the amount of immobilized ligand. Surprisingly, all anti-hLF mAbs other than E11 increased the hLF–ligand interaction. Figure 3 shows representative results with mAb E3. The enhancement of hLF binding might be due to the cross-linking of two solid-phase bound hLF molecules by the mAbs with the dimeric complex having a higher affinity for immobilized ligands than monomeric hLF.

Epitope recognized by mAb E11 resides in the N-terminus of hLF

To localize the mAb E11 epitope on hLF, we added serial dilutions of natural hLF and culture supernatant containing either the recombinant N- or C-lobe to E11 immobilized on microtitre plates. Figure 4(A) shows that E11 binds to the recombinant N-lobe. Figures 4(B) and 4(C) show control experiments in which anti-(N-lobe) (E3; Figure 4B) and anti-(C-lobe) (E19; Figure 4C) mAb were used.

The two N-lobe-specific mAbs E3 and E11 (Figure 4) were coupled to Sepharose and incubated with serial dilutions of natural hLF and rhLF^{-5N}. Dose–response curves of these hLF variants in the RIA with E3-Sepharose showed identical slopes and maximal responses (Figure 5B), suggesting that N-terminally deleted hLF and natural hLF are equally well bound by mAb E3 and detected by the polyclonal antibody. Comparison of the responses in the RIAs with E3 and E11 indicates that the removal of N-terminal residues in rhLF^{-5N} affects the binding of this hLF variant by E11 (as manifested by a decreased plateau and non-parallel curves). These results imply that N-terminal arginine residues are part of the E11 epitope. The observation that the binding of rhLF^{-5N} to mAb E11 was not completely abrogated indicates that the E11 epitope also contains residues C-terminal to Arg^5.

DISCUSSION

In this study we show that a single region, Arg^2-Arg^3-Arg^4-Arg^5, determines the specific electrostatic interaction of hLF with hLZ and polyanions such as heparin, lipid A and DNA. Several studies of the regions in hLF involved in the interaction with polyanions have been published. Mann et al. [6] provided evidence that the heparin-binding site in hLF represents a 'cationic cradle' formed by the juxtaposition of the first (Arg^2-Arg^3-Arg^4-Arg^5) and second (Arg^{28}-Lys^{29}-Val^{30}-Arg^{31}) basic clusters of the hLF N-terminus. Residues Arg^4 and Arg^5 of the cationic cradle were shown to be essential for the binding of hLF to heparin [6]. Wu et al. [16] proposed that a structural motif formed by Arg^5 together with Arg^{25}, Arg^{28}, Arg^{31} and Lys^{29} represents the GAG-binding domain. By side-directed mutagenesis of the second basic cluster, Ellas-Rochard et al. [23] suggested that hLF binding to LPS 055B5 was mediated by the second basic cluster, Arg^{28}-Lys^{29}-Val^{30}-Arg^{31}. However, our results demonstrate unequivocally the essential role of the N-terminal penultimate stretch of four arginine residues in the interaction of hLF with heparin, lipid A, hLZ and DNA. Binding was decreased on the removal of consecutive arginine residues and was abolished with rhLF^{-5N}, a mutant lacking Arg^2 to Arg^5. The latter observation is in line with the conclusion of Mann et al. [6] that Arg^4 and Arg^5 are crucial for the hLF–GAG interaction. However, our results with hLF species lacking one or two N-terminal arginine residues clearly demonstrate that Arg^2 and Arg^3 also contribute to the interaction of hLF with heparin, lipid A, hLZ and DNA. It is noteworthy that residues Arg^4 and Arg^5 are conserved in human, sheep, horse,

bovine and pig LF. Mouse LF lacks a pair of basic residues at these positions [24] and is eluted at 0.3 M NaCl by Mono S chromatography [2], i.e. at virtually the same position as $rhLF^{-5N}$. Mouse LF does not compete for the binding of hLF to ligands (P. H. C. van Berkel, unpublished work).

In addition to the high-affinity binding site (K_d 3.6 nM) in hLF for bacterial LPS, Elass-Rochard et al. [23] also identified a 'low-affinity' LPS-binding site (K_d 390 nM) in hLF, supposedly located on the C-lobe of hLF. This low-affinity binding site was identified by LPS binding studies with the 51 kDa C-terminal tryptic fragment. Obviously, our results provide no clear evidence for the existence of an LPS-binding site on the C-lobe. The mutant $rhLF^{-5N}$ did not bind to any of the solid-phase ligands even when concentrations up to 20 μg/ml were added. Moreover we found no binding of the recombinant C-lobe to LPS and heparin (P. H. C. van Berkel, unpublished work). Apparently the basic cluster Arg^{342}-Arg^{343}-Ala^{344}-Arg^{345} in the C-lobe, which is homologous with the first basic cluster Arg^2-Arg^3-Arg^4-Arg^5 in the N-lobe, is not able to interact with these ligands. This might be due to the three consecutive glutamic residues (Glu^{336}-Glu^{337}-Glu^{338}) preceding Arg^{342}-Arg^{343}-Ala^{344}-Arg^{345} and/or the surface exposure of the positive charge. Minor contamination of the C-terminal tryptic fragment with N-terminal tryptic fragments might explain the apparent binding of the C-terminal tryptic fragment to LPS [23].

Iron-free and iron-saturated hLF have been found to possess the same capacity to neutralize heparin coagulant activities [7]. It is shown in Table 1 that natural and iron-saturated hLF bound equally well to heparin, lipid A, hLZ and DNA, suggesting that both hLF species have identical affinities for these ligands. Thus the conformational change that occurs in hLF on the incorporation of iron apparently does not affect the projection of the N-terminal ligand-binding site. Indeed, crystallography has shown that on saturation of hLF with iron, the two domains of the N-lobe rotate as rigid bodies, leaving the relative position of the N-terminus essentially unaffected [25].

mAb E11 specifically inhibited the interaction of hLF with lipid A, heparin and hLZ and was mapped to an epitope including Arg^5 (Figure 5), confirming the importance of the first basic cluster in hLF–ligand interaction. mAb E11 might be a powerful tool for hLF structure–function studies.

It is important that hLF preparations for studies on the structure and biological activity of this protein be subjected to analytical chromatography on Mono S to ensure the protein's N-terminal integrity. We have previously found that many commercially available preparations of human milk LF contain variable amounts of hLF species lacking three or two N-terminal residues that are eluted at 0.5 and 0.6 M NaCl respectively, whereas N-terminally intact hLF is eluted from Mono S at 0.7 M NaCl [13].

Although hLF is very resistant to tryptic proteolysis *in vitro* as assessed by SDS/PAGE analysis [13], we have found that cleavage occurs first and relatively readily at N-terminal arginine residues 2 and 3 [26]. Preparations containing hLF cleaved at its N-terminus will probably display a lower specific activity than intact hLF in those biological assays in which interaction with heparin, LPS, hLZ and DNA is the basis of hLF action. The capacity of cleaved hLF to neutralize heparin's anticoagulant activity and thus enhance thrombin formation and coagulation [7] will probably be lower than that of intact hLF. Cleaved hLF might less effectively prevent infection with CMV if the interference by hLF with virus binding to cell surface GAGs is indeed the basis for its antiviral effect [27]. The ability of cleaved hLF to inhibit LPS-induced priming of neutrophils [11] is likely to be decreased. Similarly, effects on gene transcription through binding to nuclear DNA [15] are less likely to occur with cleaved hLF. The antibacterial activity of cleaved hLF towards some Gram-negative bacteria will be decreased if the binding of hLF to LPS and the resulting effects [14] are an important determinant in the overall antibacterial effect of hLF.

The presence of a ligand in biological systems, either on purpose or as a contaminant, might also affect the biological activity of hLF if the interaction with another ligand is key to its activity or when the activity of the complex overrides the effects of hLF alone. For example, heparin has been shown to block the binding of hLF to *Staphylococcus aureus* [28]. RNA and DNA inhibited the ability of hLF to increase natural killer cell cytotoxicity [29]. We found that preincubation of hLF with lipid A could completely block the binding to heparin (P. H. C. van Berkel, unpublished work). Contamination of hLF with LPS removes the myelosuppressive effect of hLF [30], the suppression of release of monocytic interleukin 1 and tumour necrosis factor and the inhibition of LPS priming of neutrophils for enhanced release of formyl-Met-Leu-Phe-triggered superoxide [11]. Extraction of hLF from milk in batches at 0.4 M NaCl is an effective means of preventing contamination with hLZ and LPS [13].

It is, however, also likely that N-terminally truncated hLF might display a higher specific activity or might perform differently from intact hLF in some other biological systems. The rapid hepatic clearance of hLF from the circulation involves at least two classes of hLF-binding site, i.e. a large number of low-affinity binding sites (presumably cell-associated proteoglycans) and a smaller number of high-affinity binding sites representing the chylomicron remnant receptor and/or the LDL-receptor-related protein [31,32]. Ziere et al. [32] showed that hLF binding to rat hepatocyte chylomicron remnant receptor and subsequent internalization was increased after removal of the first 14 N-terminal residues. With Jurkat human lymphoblastic T-cells we recently observed a decrease in binding of N-terminally deleted hLF to cell-surface-associated sulphated molecules, presumably representing the abundant low-affinity binding sites [26]. This study provided additional evidence that the binding of hLF to a specific receptor involves the second basic cluster of hLF [33]. Limited N-terminal proteolysis of hLF might allow a greater proportion of hLF to bind to this specific receptor, possibly altering the immunomodulatory activity of hLF.

Our understanding of hLF proteolysis *in vivo*, whether simple N-terminal cleavage, generation of fragments such as lactoferricin [34] or complete degradation, is very limited. SDS/PAGE analysis of infant stools revealed cleaved forms of hLF and indicated that approx. 5% survives digestion [35]. Hutchens et al. [36] have shown that approx. 40% of maternal hLF isolated from the urine of preterm infants lacks the first two N-terminal residues. We are currently analysing the fate of hLF in the human gastrointestinal tract by the quantitative and qualitative analysis of hLF in infant stools and in a phase I study on the survival of exogenous hLF in the adult.

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