Production and characterization of recombinant human lactoferrin

Veen, H.A. van

Chapter 3

Characterization of monoclonal antibodies against human lactoferrin

Patrick H.C. van Berkel [1], Harrie A. van Veen *, Marlieke E.J. Geerts, Jan H. Nuijens

Pharming, Archimedesweg 4, 2333 CN, PO Box 451, 2300 AL Leiden, The Netherlands

Received 23 November 2001; received in revised form 30 April 2002; accepted 2 May 2002

Abstract

The iron-binding glycoprotein human lactoferrin (hLF) is involved in the host defense against infection and is a modulator of inflammatory reactions. We generated monoclonal antibodies (mAbs) to hLF as tools to assist both structure–function studies and the development of recombinant human lactoferrin for applications in human health care. Binding experiments with ten distinct anti-hLF mAbs to tryptic and recombinant hLF fragments in ELISA and/or on immunoblots revealed that five mAbs bound to conformational epitopes residing in the N-lobe (residues 1 to 334), whereas the other five bound to C-lobe conformational epitopes (residues 335 to 692). None of the mAbs bound to hLF denatured upon reduction. Monoclonal antibody E11 appeared to bind to the arginine-rich N-terminus of hLF, which is the binding site for heparin, bacterial lipopolysaccharide, human lysozyme, DNA and receptors. The dissociation constant of the distinct mAbs for hLF ranged from 0.5 to 18 nM, without differences in affinity for unsaturated or iron-saturated hLF, indicating that the conformational changes subject to incorporation of iron do not seem to affect the exposure and/or conformation of the antibody epitopes. The mAbs did not bind to human transferrin, a protein closely related to hLF in size, primary amino acid sequence and structure. Two C-lobe specific mAbs, E2 and E8, cross-reacted with bovine and/or porcine lactoferrin, indicating that human, bovine and porcine lactoferrin share antigenic determinants. This panel of mAbs will be used to develop quantitative and qualitative immunoassays for hLF and to delineate which regions of hLF are relevant to its anti-infective and anti-inflammatory properties.

Keywords: Monoclonal antibodies; Human lactoferrin; Structure; Function

1. Introduction

Lactoferrin is a glycoprotein of Mr 77 000, which belongs to the transferrin family (Crichton, 1990). Members of this family of iron-transporting proteins are widely distributed in the body fluids of vertebrates and invertebrates. Lactoferrin is present in milk and mucosal secretions as well as in the secondary granules of neutrophils. Human lactoferrin (hLF) consists of a polypeptide chain of 692 amino acids (Metz-Boutigue et al., 1984; Rey et al., 1990), folded into two globular lobes (Anderson et al., 1989), designated the N- and C-

Abbreviations: bLF, bovine lactoferrin; hLF, human lactoferrin; hTF, human transferrin; K_d, dissociation constant; mAb, monoclonal antibody; pLF, porcine lactoferrin; Lfc, lactoferricin H, peptide comprising hLF residues Gly^1 to Ile^{47}.

* Corresponding author. Tel.: +31-71-5247190; fax: +31-71-5247494.

E-mail address: h.veen@pharming.com (H.A. van Veen).

[1] Current address: Crucell Holland BV, Archimedesweg 4, Leiden, The Netherlands.

lobe. These lobes share an amino acid homology of about 40%, suggesting that lactoferrin has evolved by gene duplication from an ancestral gene half its size (Crichton, 1990). There is substantial homology between distinct lactoferrin species. For example, bovine lactoferrin (bLF) shows 69% and 64% amino acid identity with human and murine lactoferrin, respectively (Pierce et al., 1991), whereas the amino acid sequence of porcine lactoferrin (pLF) shows 73%, 71% and 62% homology with bovine, human and murine lactoferrin (Lydon et al., 1992). The two lobes of lactoferrin are connected by a three-turn α-helix. Each lobe folds into α-helix and β-sheet arrays to form two domains per lobe. The domains, connected by a hinge region, create a deep iron-binding cleft within each lobe. One aspartate, two tyrosines, one histidine and a bicarbonate ion are involved in the binding of iron within this cleft (Anderson et al., 1989).

Based on the many reports of its antimicrobial and other properties, lactoferrin is thought to represent a component of the innate host defense (Nuijens et al., 1996). Its growth inhibiting activity towards microorganisms likely occurs via sequestration of free iron (Reiter et al., 1975). The bactericidal activity of lactoferrin towards certain bacteria (Arnold et al., 1977) is mediated through destabilization of the bacterial cell wall after binding to cell wall components, such as bacterial lipopolysaccharide (Ellison and Giehl, 1991; Nibbering et al., 2001). Less clear is the mechanism of the many in vitro anti-inflammatory properties of lactoferrin such as the inhibition of complement activation (Kijlstra and Jeurissen, 1982) and the inhibition of cytokine production (Crouch et al., 1992).

We generated monoclonal antibodies (mAbs) to hLF to serve as tools in studies of the regions of hLF that are involved in its antibacterial and anti-inflammatory activities. Here, we describe the generation, properties and provisional epitope mapping using proteolytic and recombinant hLF fragments of 10 distinct mAbs against hLF.

2. Materials and methods

2.1. Reagents

Human transferrin (hTF), bovine pancreatic trypsin (type III-S) and soybean trypsin inhibitor (SBTI) were purchased from Sigma (St. Louis, MO, USA). Peroxidase labelled and unlabelled goat anti-mouse immunoglobulin, normal goat serum and recombinant IL-6 were from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (CLB, Amsterdam, The Netherlands). Freund's adjuvant was from Cappel (Organon Teknika, Turnhout, Belgium). Polysorp immunoplates were from Nunc (Roskilde, Denmark). All cell culture reagents were from Gibco (Paisley, UK). ^{125}I was from Amersham Biosciences (Bergrand, The Netherlands) and Iodo-beads were from Perbio (Tattenhall, UK).

2.2. Purification of human, bovine and porcine lactoferrin

Human lactoferrin was isolated from fresh human milk, as previously described (van Berkel et al., 1995) and is further designated as 'natural hLF'. Bovine and porcine lactoferrin (bLF and pLF, respectively) were isolated from milk by cationic exchange chromatography using essentially the same procedure as for the isolation of natural hLF. Porcine lactoferrin and bLF were eluted from Mono S at 0.5 and 0.8 M NaCl, respectively (Nuijens and van Veen, 1998). Saturation of natural hLF with iron was performed as described (van Berkel et al., 1995). Saturation was complete as assessed by absorbance measurements of hLF in solution and upon non-reducing SDS-PAGE of non-boiled samples (Nuijens et al., 1997).

2.3. Cell culture

Sp2/0-Ag14 myeloma cells [ATCC CRL 1583] were cultured in Iscove's Modified Dulbecco's Medium supplemented with 5% fetal calf serum (FCS), 50 U/ml penicillin, 50 μg/ml streptomycin and 0.0003% (v/v) β-mercaptoethanol. Hybridomas were cultured in the same medium further supplemented with 500 U/ml IL-6 and 0.1 mM hypoxanthine. CHO/DHFR$^-$ cells [ATCC CRL 9096] were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% FCS, 50 U/ml penicillin, 50 μg/ml streptomycin, 0.1 mM hypoxanthine and 10 μM thymidine. Cell cultures were maintained at 37 °C in a humidified CO_2/air (1:19) incubator.

2.4. Production and purification of anti-hLF monoclonal antibodies

BALB/c mice were immunized by intraperitoneal injection with 50 µg natural hLF and/or iron-saturated hLF in complete Freund's adjuvant and were boosted every 2 weeks with the same amount of antigen in incomplete adjuvant. Four days after the fourth injection, spleen cells were fused with Sp2/0-Ag14 myeloma cells. Fusion and hybridoma selection were done as previously described (Fazekas de St Groth and Scheidegger, 1980) except that dimethylsulfoxide was omitted from the polyethylene glycol solution and IL-6 at 500 U/ml was used during culture to promote cell proliferation. Culture supernatants were screened for the presence of specific anti-hLF antibodies by RIA and ELISA as described below. Hybridomas producing hLF-specific antibodies were cloned by repeated limiting dilution. Immunoglobulin-enriched fractions of culture supernatant were prepared by precipitation with ammonium sulphate. Precipitates were redissolved and dialyzed against 10 mM sodium phosphate, 0.15 M NaCl, pH 7.5 (PBS). Monoclonal antibodies were purified using the Repligen antibody purification kit (Cambridge, MA, USA). SDS-PAGE of 10 µg of these antibody preparations showed that they were at least 99% pure. The antibody concentration was determined by absorbance measurements at 280 nm, assuming an absorbance coefficient of 1.4 for a 0.1% (w/v) solution. Isotype determination of mAbs was done with the Mouse mAb Isotyping kit of Hycult Biotechnology BV (Uden, The Netherlands).

2.5. Hybridoma screening

2.5.1. RIA

Goat anti-mouse immunoglobulin antibody (30 µg) coupled to 1.5 mg Sepharose 4B in 250 µl PBS, 0.1% Tween-20, 0.05% sodium azide was added to 50 µl hybridoma supernatant and 50 µl ^{125}I-hLF and/or ^{125}I-Fe-hLF (approximately 35 000 cpm in PBS, 0.1% Tween-20, 1% bovine serum albumin (van Berkel et al., 1995)). After incubation for 6 h, the Sepharose was washed and Sepharose-bound radioactivity was measured using a gamma-counter (LKB Bromma, Uppsala, Sweden).

2.5.2. ELISA

PBS containing 1 µg/ml purified natural hLF and/or iron-saturated hLF was incubated for 16 h at 20 °C in Polysorp plates. The plates were then washed with PBS, 0.02% Tween-20 and incubated for 3 h with hybridoma supernatant diluted two-fold in PTG (PBS, 0.02% (w/v) Tween-20, 0.2% (w/v) gelatin). After washing, PTG containing 3.5 µg/ml peroxidase labelled goat anti-mouse immunoglobulin and 1% (v/v) normal goat serum was added to each well. Plates were incubated for another hour, washed and substrate solution (0.01% (w/v) 3,3′,5,5′-tetramethylbenzidine, 0.003% (v/v) H_2O_2 in 0.11 M sodium acetate, pH 5.5) was added. Substrate conversion was stopped by the addition of 2 M H_2SO_4 and the absorbance of converted substrate was measured at 450 nm with a SLT 340 ATCC microplate reader (SLT-labinstruments, Salzburg, Austria). All incubations were done in 100 µl volumes.

2.6. Expression and purification of the recombinant N- and C-lobe of hLF

A cDNA, encoding the N-lobe of hLF (residues Gly^{1} to Lys^{334}), was generated from pCMV/hLF (van Berkel et al., 1996) by mutagenesis via PCR. The 5′ primer 5′ GTCAAGCTTGAGGTGTGG-CAGGCTTGA 3′ was complementary to the 5′ sequence of the hybrid splice acceptor site (van Berkel et al., 1995). With the 3′ primer, 5′ GCTCTAGA-**TCATTA**TTTCTTCAAGTTCTGGATGGCAGT 3′, a *Xba*1 restriction site (underlined) and the complement of two translational stop codons (boldface) were introduced. Using PCR, the 1.15-kb fragment was amplified. This PCR-mixture was used for TA-cloning into pCR/II (InVitrogen, San Diego, CA, USA). After isolation of the plasmid, the 1.15-kb fragment was excised by *Hind*III/*Xba*1 and ligated into the expression vector pRc/CMV. The resulting plasmid was designated pCMV/rN.

The expression vector pCMV/rC encoding the connecting three-turn α-helix and the C-lobe of hLF (residues Ser^{335} to Lys^{692}) was produced with the Transformer™ site-directed mutagenesis kit as described (van Berkel et al., 1996). The entire N-lobe was deleted with the mutagenic primer 5′ CTG-TTGCTCTTGCC**AGTGAGGAGGAAGTGG** 3′, which corresponded to the 14 last nucleotides of the

bovine αS_1 casein signal sequence (van Berkel et al., 1995; underlined) fused to nucleotides 1354 to 1369 of the hLF cDNA (boldface; van Berkel et al., 1996). After mutagenesis, the nature of the deletion was confirmed by dideoxy sequencing.

Human 293(S) [ATCC CRL 1573] cells constitutively expressing pCMV/rN or pCMV/rC were generated as described (van Berkel et al., 1996). Expression of the recombinant lobes was measured with the hLF-specific ELISA (van Berkel et al., 1996). The recombinant N-lobe was purified from the supernatant of 293(S) cells expressing pCMV/rN using S Sepharose (van Berkel et al., 1995). The supernatant of 293(S) cells expressing pCMV/rC was batch-wise incubated for 16 h with C-lobe specific anti-hLF monoclonal E19 (van Berkel et al., 1997) coupled to CNBr-activated Sepharose 4B. This was poured into a column and washed with PBS containing 1.0 M NaCl to remove a-specifically absorbed protein. Recombinant C-lobe was eluted with 0.1 M glycine, 0.15 M NaCl, pH 2.5. Eluted fractions of 500 µl were immediately neutralized in 25 µl 1.5 M Tris–HCl, pH 8.7.

2.7. Expression of lactoferricin H

The expression vector pCMV/lfc encoding lactoferricin H (residues Gly^1 to Ile^{47}; Bellamy et al., 1992) was generated from pCMV/hLF by PCR as described above for the recombinant N-lobe, except that 3′ primer 5′ TCATTAGATACACTGGATGGGGGAGTCTCT 3′ was used. pCMV/lfc was transfected to CHO/DHFR$^-$ as described (van Berkel et al., 1996). After transfection, 800 µg/ml Geneticin was added to the culture medium to select for pCMV/lfc containing cells. Recombinant lactoferricin H was isolated from culture supernatant using S Sepharose as described for natural hLF (van Berkel et al., 1995).

2.8. Determination of dissociation constant

The antibody–antigen interaction constant, K_d, was determined according to Müller (1983). Briefly, Medium Binding plates (Costar, Cambridge, MA, USA) were incubated for 16 h at 20 °C with PBS containing 1–2 µg/ml of purified mAb. Plates were washed with PBS, 0.02% Tween-20 and ^{125}I-hLF (approximately 2 ng per well; 35,000 cpm) was added in the presence or absence of competitor. All incubations were done in 100 µl volumes. After 4 h at 20 °C, the plates were washed, the wells were snapped apart, placed into tubes and radioactivity was measured. The K_d was calculated as described by Müller (1983) using the equation:

$$K_d = ([I] - [T]) \times (1 - 1.5b + 0.5b^2)M$$

Where b is the fraction of the tracer (^{125}I-hLF) bound by the antibody in the absence of competitor, T is the total tracer concentration and I the competitor concentration at 50% inhibition of the tracer antibody binding.

2.9. Mapping of the anti-hLF antibody epitopes by ELISA and immunoblotting

Localization of the anti-hLF mAb binding sites to the N- and C-lobes was performed by ELISA as previously described (van Berkel et al., 1997). Briefly, Polysorp plates were coated with 100 µl PBS containing 1 µg/ml purified mAb. Plates were washed and incubated with serial dilutions of natural hLF or culture medium of 293(S) cells secreting either the recombinant N- or C-lobe. After washing, bound hLF was measured by adding peroxidase conjugated bovine anti-hLF. Non-reducing and reducing SDS-PAGE and immunoblotting were performed as previously described (van Berkel et al., 1995, 1996; Nuijens et al., 1997).

2.10. Partial trypsin digestion of human lactoferrin

Partial trypsin digestion of natural hLF was done in 50 mM Tris–HCl, 2 mM $CaCl_2$, pH 8.0 at 37 °C for 4 h with an enzyme to substrate ratio of 1:1. In preliminary experiments, we determined that after 4 h of digestion approximately 50% of the molecules had been cleaved into major cleavage products of Mr 39,000 and 51,000 (Legrand et al., 1986; Hutchens et al., 1991; van Berkel et al., 1995).

3. Results

3.1. Generation and characterization of anti-hLF monoclonal antibodies

Mice were immunized with natural hLF and/or iron-saturated hLF and the serum immune responses

were monitored by RIA and ELISA. Spleen cells were fused with Sp2/0 myeloma cells, resulting in 890 proliferating hybridomas. Anti-hLF producing hybridomas were selected as described in Materials and methods. After subcloning, 10 hybridomas showed stable anti-hLF production (Table 1). Heavy and light chain subclass determination showed that all mAbs were of the IgG1 type with a Kappa-light chain, except E10 (IgG2a, Table 1).

A RIA using radiolabeled hLF as a tracer was used to determine the dissociation constant (K_d) of purified anti-hLF mAbs for natural hLF (3% saturated) and fully iron-saturated hLF. The K_d of the various mAbs for natural hLF ranged from 0.5 to 18 nM (Table 1). The affinities of E2 and E8 for fluid-phase radiolabeled hLF appeared to be too low to yield significant binding of the tracer, which precluded the assessment of possible differences in affinity for natural and iron-saturated hLF. All other mAbs had about the same K_d for natural and iron-saturated hLF. This indicates that the exposure and/or conformation of these epitopes are not affected by the binding of iron.

3.2. Specificity of anti-human lactoferrin monoclonal antibodies

Human transferrin (hTF), bLF or pLF were immobilized onto microtiter plates and incubated with serial dilutions of purified mAbs, to determine their specificity. None of the mAbs bound to hTF (not shown). Monoclonal antibody E2 bound to hLF and bLF but not to pLF, whereas E8 bound to all three lactoferrin species (Fig. 1). None of the other mAbs bound to bLF or pLF (Fig. 1 shows the representative results with E3). This indicates that human, bovine and porcine lactoferrin share at least one antigenic determinant, namely the one recognized by E8. Furthermore, hLF and bLF share the E2 epitope.

Table 1
Characterization of anti-human lactoferrin monoclonal antibodies

	Subclass	K_d (nM)		ELISA mapping	
		Natural hLF	Iron-saturated hLF	N-lobe	C-lobe
E1	IgG1, κ[a]	18[b]	18	–[c]	–
E2	IgG1, κ	–[d]	–	–	+
E3	IgG1, κ	3	3	+	–
E8	IgG1, κ	–[d]	–	–	+
E10	IgG2a, κ	1	0.5	–	+
E11	IgG1, κ	4	3	+	–
E13	IgG1, κ	3	3	–	+
E17	IgG1, κ	3	3	+	–
E19	IgG1, κ	3	4	–	+
E31	IgG1, κ	4	4	+	–

[a] κ indicates a κ light-chain.
[b] Dissociation constants (K_d) are given as the mean of two duplicate experiments.
[c] No binding in ELISA.
[d] No binding of ^{125}I-hLF.

3.3. Epitope localization by antibody binding to fluid-phase recombinant hLF fragments

To assign the antibody epitopes to the N- or C-lobe, we added serial dilutions of culture supernatants containing either the recombinant N- or C-lobe to mAbs immobilized onto microtiter plates. Natural hLF was used as a positive control. Fig. 2 shows the dose–response curves with E13 and E17. Similar experiments were done with the other mAbs (Table 1). Four mAbs reacted with the N-lobe, but not with the C-lobe, whereas five mAbs bound to the C-lobe alone. Monoclonal antibody E1 bound to neither the recombinant N- nor the C-lobe.

3.4. Epitope localization by immunoblotting

Fig. 3 shows the result of immunoblotting with the distinct mAbs on purified natural hLF (Fig. 3A), recombinant N- (Fig. 3B) and C-lobe (Fig. 3C) as well as trypsinized hLF (Fig. 3D). The positive control, lane P, shows the binding of HRPO-labelled polyclonal bovine anti-hLF antibodies (van Berkel et al., 1995), whereas lane C shows the lack of binding of a control mAb against C1 esterase. All mAbs bound to non-reduced hLF (Fig. 3A), but not to reduced hLF (not shown), which indicates that the mAbs bind to conformational epitopes, which are apparently destroyed upon reduction of the protein.

Lane 1 shows that mAb E1 did not bind to the recombinant N-lobe nor the C-lobe (residues 1–334 and 335–692, respectively) confirming the ELISA data (Table 1). The weak binding of E1 to C1^{tryp} (residues 284–692 with one N-linked glycan chain, Mr of 51,000) suggests that residues on the C-terminal part of the hLF N-lobe contribute to the E1 epitope. Lane 2 shows that E2 did not bind to the recombinant

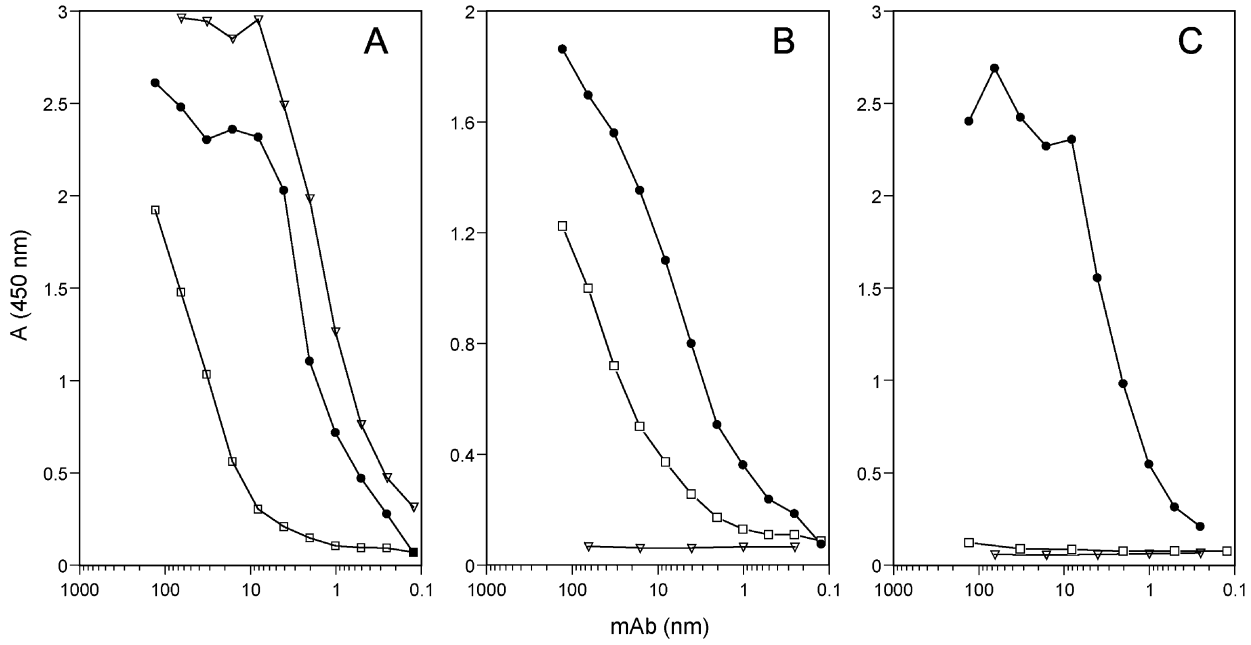

Fig. 1. Binding of mAbs E2, E3 and E8 to human, bovine and porcine lactoferrin. Polysorp plates were coated with 100 µl of 1 µg/ml hLF (A), bLF (B) or pLF (C) in PBS and incubated with serial dilutions of E2 (□), E8 (●) and E3 (▽). Bound mAb was detected by incubation with peroxidase labelled goat anti-mouse immunoglobulin. The A_{450} values of converted substrate are plotted against the amount of mAb added.

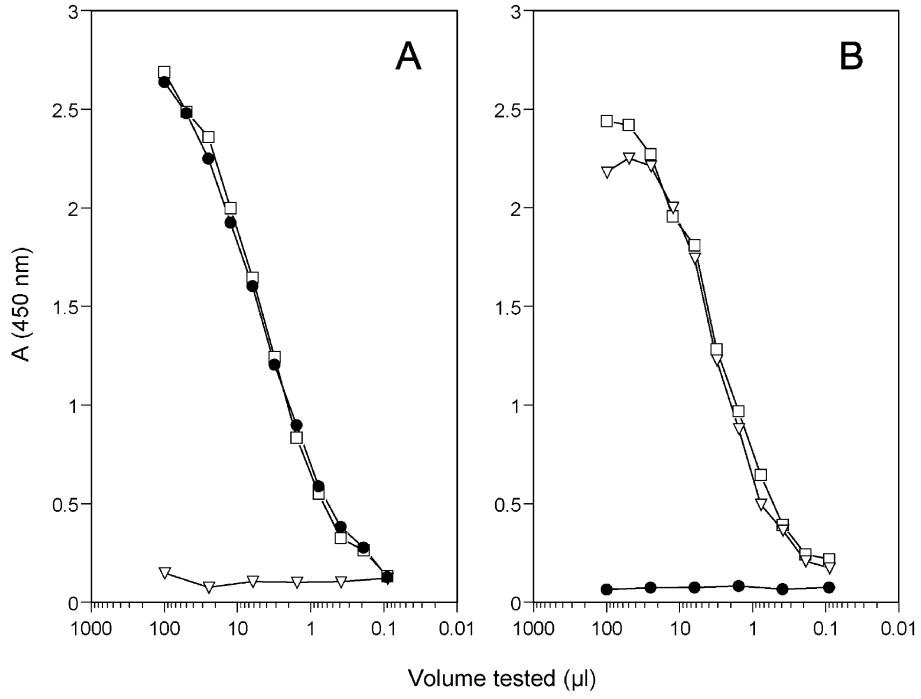

Fig. 2. Binding of mAbs E17 and E13 to the N- and C-lobe. Serial dilutions of natural hLF (100 ng/ml; □) or medium of 293(S) cells secreting the recombinant hLF N-lobe (▽) or C-lobe (●) were incubated with purified mAbs E13 (A) and E17 (B) coated onto microtiter plates. Bound hLF was detected by subsequent incubation with horseradish peroxidase-conjugated anti-hLF. The A_{450} values of converted substrate are plotted against the experimental volume tested.

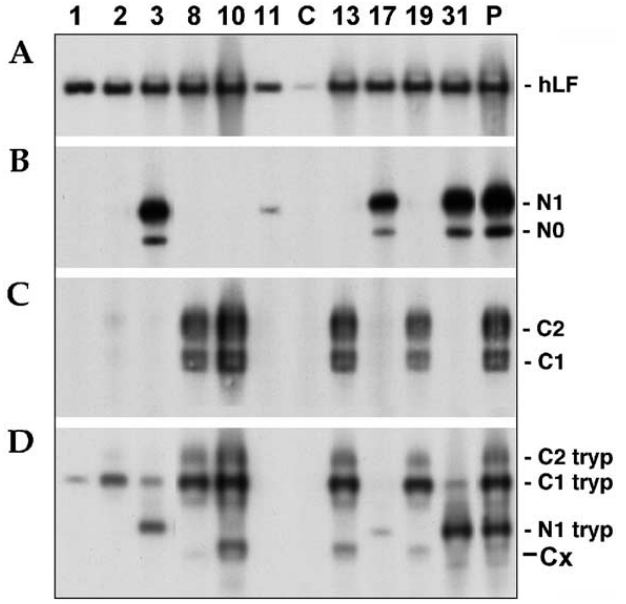

Fig. 3. Localization of the antibody epitopes on human lactoferrin. Non-reducing 12.5% (w/v) SDS-PAGE and immunoblot analysis of anti-hLF mAbs (1 μg/ml) with natural hLF (A), purified recombinant N- or C-lobe (B, C) or trypsinized hLF (D). The antibody used for immunoblotting is indicated above each lane. The recombinant N-lobe band N0 represents unglycosylated N-lobe, whereas N1 represents N-lobe bearing one N-linked glycan. The C-lobe bands C1 and C2 represent C-lobe bearing one and two N-linked glycans, respectively. The tryptic cleavage products of hLF and their sizes are: $N1^{tryp}$ (residues 4 to 283; Mr 39,000), $C1^{tryp}$ (residues 284 to 692; Mr 51,000), $C2^{tryp}$ (same residues as $C1^{tryp}$ with an additional N-linked glycan attached to Asn^{624} (van Berkel et al., 1995); Mr 54,000) and C^{x} (cleavage site unknown; Mr 37,000). Bound mAb was detected by incubation with peroxidase labelled goat anti-mouse immunoglobulin.

N- or C-lobe but did bind to $C1^{tryp}$. This mAb bound to the fluid-phase recombinant C-lobe in the ELISA experiments (Table 1), which suggests that its epitope on the recombinant C-lobe is apparently denatured on SDS-PAGE; hence, we concluded that the conformational epitope for E2 is present on the native and trypsinized C-lobe. Fig. 3 shows that E8, E10, E13 and E19 bound to the C-lobe as well as to $C1^{tryp}$ and $C2^{tryp}$ (residues 284–692 with two N-linked glycan chains, Mr of 54,000). This indicates that they recognize epitopes in the C-lobe. The mAbs E10, E13 and E19 also bound to a tryptic cleavage product of Mr 37,000, designated C^{x}.

The mAbs E3, E11, E17 and E31 recognize N-lobe specific epitopes, present in both fluid-phase (Table 1) and blotted (Fig. 3B) recombinant N-lobe. Binding of E11 to the supposedly denatured N-lobe on the immunoblot was very weak (Fig. 3B). Like E17, E11 did not bind to $N1^{tryp}$ (residues 4–283, Mr of 39,000), suggesting that their epitopes were completely or partially destroyed upon tryptic cleavage of the molecule. Furthermore, two of the N-lobe specific mAbs, E3 and E31, also bound weakly to $C1^{tryp}$ but not to the recombinant C-lobe (Fig. 3C), indicating that C-terminal residues may contribute to the conformational epitope which is mainly formed by N-lobe residues.

3.5. Binding of the mAbs to lactoferricin

We determined whether the mAbs bound to lactoferricin H (residues Gly^{1} to Ile^{47}), a potent bactericidal peptide that is released from hLF by pepsin (Bellamy et al., 1992). It appeared that except for mAb E11, none of the other mAbs bound to lactoferricin H immobilized onto microtiter plates (representative results with E3 are shown in Fig. 4). Thus, E11 binds to a conformational epitope located between amino acids 1 and 47.

3.6. Development of a mAb-based quantitative assay of hLF

A useful application of this panel of mAbs would be in a quantitative sandwich-type immunoassay, e.g. based on the capture of hLF with a high affinity anti-C-lobe antibody and its detection with an anti-N-lobe antibody. An hLF-specific RIA was developed using E19 immobilized onto Sepharose and ^{125}I-E3 (Fig. 5A, open squares). This assay can detect as little as 4 ng of hLF. An ELISA procedure was also developed with a sensitivity of 0.3 ng, which is close to the sensitivity of an hLF-specific ELISA employing rabbit and bovine polyclonal anti-hLF antibodies (0.05 ng; van Berkel et al., 1996). Experiments in which the options of double antibody immunoassay with ^{125}I-E3 and other anti-hLF mAbs coupled to Sepharose were explored, indicated that the binding of E17 to natural hLF prevented the binding of E3, whereas natural hLF bound to E31 could still be detected with E3 (Fig. 5A). Hence, E3 appears to bind closely to the epitope of E17, but not to the epitope of E31. Similar control experiments with polyclonal ^{125}I-anti-hLF instead of ^{125}I-E3 are presented in Fig. 5B.

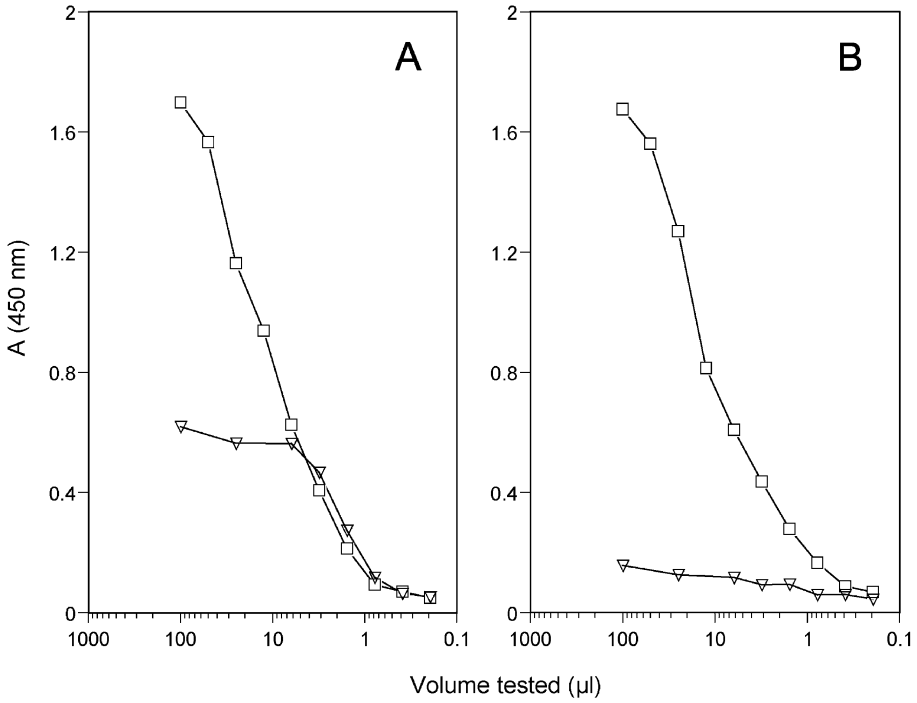

Fig. 4. Binding of mAbs E3 and E11 to recombinant lactoferricin H. Polysorp plates were coated with natural hLF (□) or lactoferricin H (▽) in PBS. After washing, wells were incubated with a serial dilution of 10 µg/ml E11 (A) and E3 (B) in PTG. After another wash, bound mAb was detected by incubation with peroxidase labelled goat anti-mouse immunoglobulin. The A_{450} values of converted substrate are plotted as a function of the volume tested.

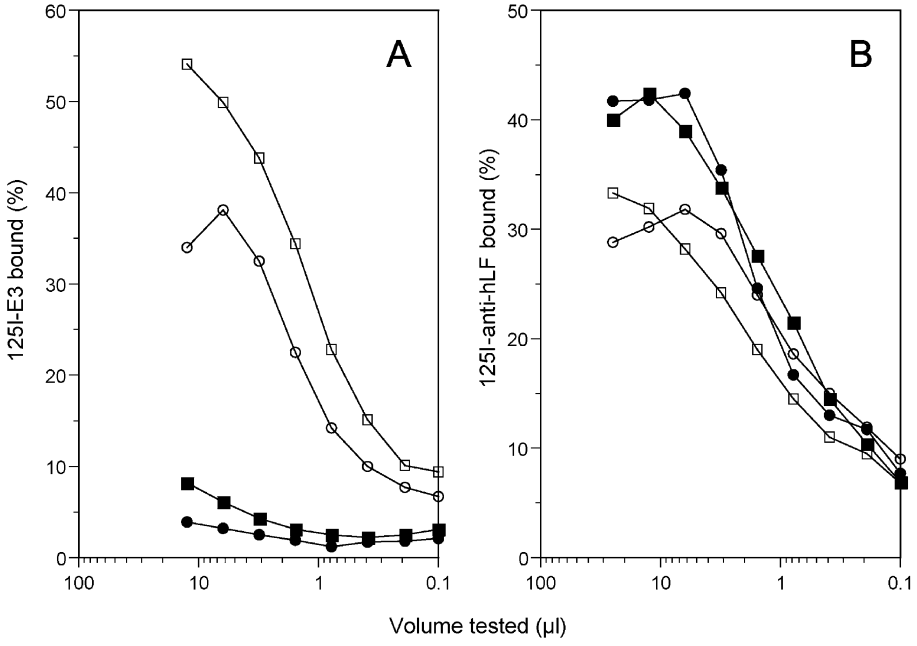

Fig. 5. Development of a mAb-based sandwich-type RIA of hLF. Monoclonal antibodies E3 (■), E17 (●), E31 (○) and E19 (□) were coupled to Sepharose and incubated with a serial dilution of 10 µg/ml natural hLF as previously described (Nuijens et al., 1997). After washing, bound hLF was detected with ^{125}I-E3 (radiolabelled by using Iodo-beads according to the manufacturer's instructions; panel A), or polyclonal rabbit ^{125}I-anti-hLF (panel B). Results are expressed as the percentage binding of the ^{125}I-labelled antibodies added. The volume of the hLF sample tested (µl) is indicated on the abscissa.

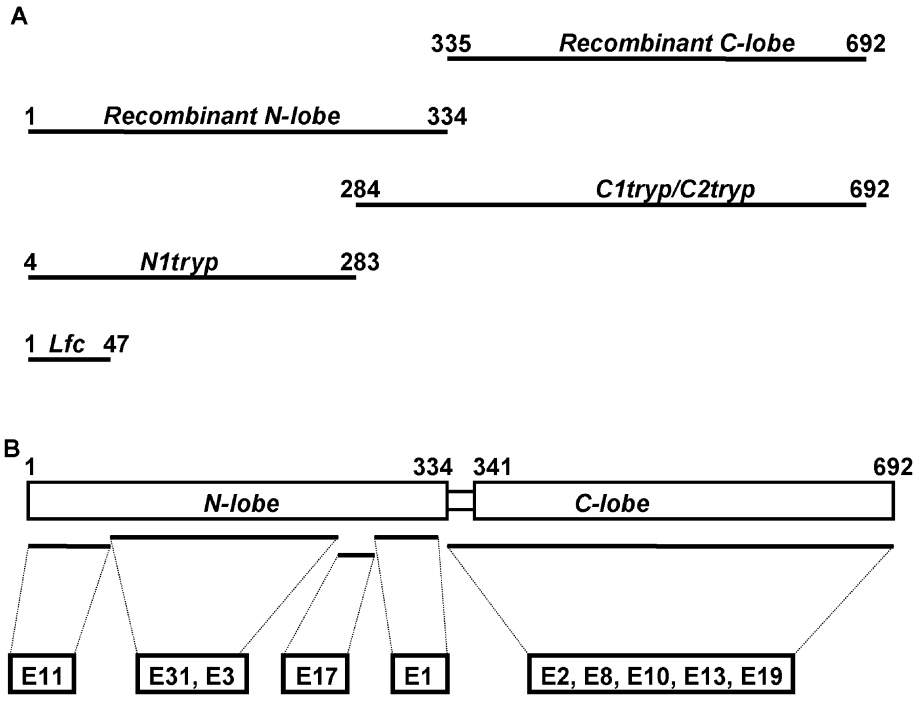

Fig. 6. Localization of the epitopes on hLF. Overview of the recombinant and tryptic hLF-fragments (A) and putative epitope localization (B) of anti-hLF mAbs. The numbers correspond to the amino acid residues of the hLF protein sequence. The region between residues 334 and 341 represents the connecting three-turn α-helix (not drawn to scale).

Fig. 6A gives an overview of the recombinant and tryptic hLF fragments used for the epitope mapping studies. Fig. 6B depicts the putative epitope locations for the anti-hLF mAbs, which are inferred from the results of Table 1 and Figs. 2–5.

4. Discussion

Monoclonal antibodies against hLF appear to be useful tools to identify regions of the molecule that are important for its biological activity (van Berkel et al., 1997). Here, we describe the production of 10 distinct mAbs against hLF recognising conformational epitopes located in both the N- and C-lobe (Fig. 6). Although several reports of anti-hLF antibodies have appeared (Chung et al., 1985; Broxmeyer et al., 1986; Skubitz et al., 1989; Caccavo et al., 1996, 1999), this paper is the first to describe the characterisation of a large panel of mAbs against hLF in more detail.

Human lactoferrin has been shown to display antimicrobial activity in vitro and in vivo (Nuijens et al., 1996). In addition, a variety of in vitro observations suggest that the protein can act in vivo to modulate inflammatory and coagulation responses (Nuijens et al., 1996). The sequestration of free iron plays a central role in at least some of these biological activities. For example, the bacteriostatic activity of hLF is mediated through limiting the availability of environmental iron, a growth requirement for many microbial species (Reiter et al., 1975) and the sequestration of free iron inhibits the iron-catalysed formation of hydroxyl radicals from superoxide and H_2O_2 released from neutrophils (Britigan et al., 1989). Comparative crystallographic studies of unsaturated and iron-saturated hLF revealed that, upon the binding of iron, domain II of the N- and C-lobe rotate 54° and 18°, respectively, with respect to domain I resulting in a more globular closed conformation of the entire molecule (Baker et al., 1996). We expected that the major conformational changes upon iron incorporation might translate to many neo-determinants in the saturated protein. Antibodies specific for unsaturated or saturated hLF would be very useful in assays to quantify the extent to which hLF is saturated with metal ions. Despite specific efforts including immu-

nisation and screening with iron-saturated hLF, we did not succeed in obtaining such an antibody: the exposure and/or conformation of the epitopes for the antibodies described herein appeared not to be affected by the conformational changes upon incorporation of iron as the affinities to each hLF species did not differ (Table 1).

The bactericidal and other biological activities of hLF do not depend on the sequestration of free iron, but relate to the binding of hLF to negatively charged molecules such as bacterial lipopolysaccharide, heparin, human lysozyme and DNA (van Berkel et al., 1997). The first basic cluster of hLF, Arg^2–Arg^3–Arg^4–Arg^5, is essential for these interactions (Mann et al., 1994; van Berkel et al., 1997). We have recently shown that E11 binds to an epitope located in the N-lobe and can completely block the interaction of hLF with anionic ligands (van Berkel et al., 1997; El Yazidi-Belkoura et al., 2001). Here we show that E11 binds to lactoferricin (residues 1–47; Fig. 4), but not to $N1^{tryp}$ lacking Gly^1–Arg^2–Arg^3 (Fig. 3). The lack of binding to $N1^{tryp}$ on blots seems to contrast with the previous observation that E11 can bind to fluid-phase $rhLF^{-5N}$, a mutant lacking the first 5 N-terminal residues, although with reduced affinity (van Berkel et al., 1997). However, this apparent discrepancy may reflect the fact that the first cationic cluster contributes to a conformational epitope that is further affected when $N1^{tryp}$ is subjected to additional denaturation by SDS. The latter likely also accounts for the weak reaction of E11 with the recombinant N-lobe on immunoblots (Fig. 3) in comparison with the other anti-N-lobe mAbs having an identical affinity to fluid-phase natural hLF (Table 1). These considerations imply that the N-terminus of hLF not only projects out of the molecule, but indeed may interact with the second cationic cluster to form a cationic cradle (Mann et al., 1994).

Next to the major tryptic cleavage site at Arg^3, the N-lobe of hLF contains another major tryptic cleavage site at Lys^{283} (Legrand et al., 1997). The N-lobe specific mAb E17 bound to natural hLF as well as the recombinant N-lobe (Table 1, Figs. 2 and 3), but only very weakly bound to $N1^{tryp}$, particularly when compared to E3 and E31. This indicates that tryptic cleavage at Lys^{283} affects the epitope for mAb E17, implying that residues at or near Lys^{283} contribute to the conformational epitope for E17.

The mAbs E3 and E31 bind to the N-lobe and $N1^{tryp}$, but not to lactoferricin H, indicating that residues between 48 and 283 contribute to their conformational epitopes. Both E3 and E31 weakly bind to $C1^{tryp}$, whereas they do not bind to the recombinant C-lobe indicating that residues between 284 and 335 may also contribute to their conformational epitope. E3 and E31 bind to different epitopes as was determined by cross-blocking experiments (Fig. 5). Pre-incubation of hLF with E17 inhibited the binding of E3, which suggests that the E3 and E17 epitopes are located near each other (Fig. 5).

E1 likely also binds to the N-lobe. This mAb binds weakly to $C1^{tryp}$ but not to the recombinant C-lobe (Fig. 3) suggesting that its conformational epitope resides between residues 284 and 335, i.e. the N-lobe part of $C1^{tryp}$. At this stage, we do not know why E1 does not bind to the fluid-phase or blotted recombinant N-lobe, although it might be explained by the fact that the conformational epitope for E1 is altered in the recombinant half molecule.

The epitopes of five mAbs, E2, E8, E10, E13 and E19, reside in the C-lobe (Fig. 6). At least three of them, i.e. E2, E8 and E10 have unique features (Table 1, Figs. 1 and 3), hence they likely bind to different epitopes. For more detailed mapping, further research using C-lobe fragments or rhLF mutant molecules is needed. Such effort appears complicated by the C-lobe containing 10 disulphide bridges (Metz-Boutigue et al., 1984) and the fact that reduction abolishes the binding of the mAbs to hLF.

The transferrin family is an important class of iron-transporting proteins widely distributed in the body fluids of vertebrates and invertebrates. The members include lactoferrin, serum transferrin, ovotransferrin and melanotransferrin (Crichton, 1990). These proteins share a high degree of sequence homology and overall structure (Crichton, 1990). For instance, hLF shares 51% amino acid identity with human transferrin. The binding studies with the anti-hLF mAbs suggest that, despite the high degree of sequence homology, considerable differences exist in their surface structure. The issue of common antigenicity between lactoferrin from different mammals has been debated. Some researchers reported that there is no cross-reactivity between hLF and bLF (Wang et al., 1984; Davidson and Lönnerdal, 1986) or hLF and pLF (Kokriakov et al., 1988), while others reported

that hLF, bLF and pLF share antigenic determinants (Masson and Heremans, 1971; Magnuson et al., 1990). Our results clearly demonstrate that at least one epitope is conserved among hLF, bLF and pLF (Fig. 1), namely the C-lobe conformational epitope for mAb E8. The C-lobe epitope for mAb E2 is conserved between hLF and bLF. It is to be noted that sequence alignment of the lactoferrins of different species revealed that the highest homology occurs in the C-lobe, whereas the most pronounced differences are found in domain I of the N-lobe (Pierce et al., 1991; Lydon et al., 1992).

In summary, we have obtained a panel of 10 distinct mAbs against hLF, which will be applied in structure–function relationship studies as well as in quantitative and qualitative immunoassays of hLF, i.e. to develop recombinant hLF from transgenic cattle for applications in human healthcare.

Acknowledgements

We thank Dr. B. Colargol (Erasmus University, Rotterdam, The Netherlands) for stimulating discussions.

References

Anderson, B.F., Baker, H.M., Norris, G.E., Rice, D.W., Baker, E.N., 1989. Structure of human lactoferrin: crystallographic structure analysis and refinement at 2.8 Å resolution. J. Mol. Biol. 209, 711.

Arnold, R.R., Cole, M.F., McGhee, J.R., 1977. A bactericidal effect for human lactoferrin. Science 197, 263.

Baker, E.N., Anderson, B.F., Baker, H.M., Faber, R., Smith, C.A., Sutherland-Smith, A.J., 1996. Structural and Functional Flexibility of Lactoferrin. Humana Press, Totowa, NJ.

Bellamy, W., Takase, M., Yamauchi, K., Wakabayashi, H., Kawase, K., Tomita, M., 1992. Identification of the bactericidal domain of lactoferrin. Biochim. Biophys. Acta 1121, 130.

Britigan, B.E., Hassett, D.J., Rosen, G.M., Hamill, D.R., Cohen, M.S., 1989. Neutrophil degranulation inhibits potential hydroxyl-radical formation. Relative impact of myeloperoxidase and lactoferrin release on hydroxyl-radical production by iron-supplemented neutrophils assessed by spin-trapping techniques. Biochem. J. 264, 447.

Broxmeyer, H.E., Bicknell, D.C., Gillis, S., Harris, E.L., Pelus, L.M., Sledge, G.J., 1986. Lactoferrin: affinity purification from human milk and polymorphonuclear neutrophils using monoclonal antibody (II 2C) to human lactoferrin, development of an immunoradiometric assay using II 2C, and myelopoietic regulation and receptor-binding characteristics. Blood Cells 11, 429.

Caccavo, D., Afeltra, A., Guido, F., Di Monaco, C., Ferri, G.M., Amoroso, A., Vaccaro, F., Bonomo, L., 1996. Two spatially distant epitopes of human lactoferrin. Hybridoma 15, 263.

Caccavo, D., Afeltra, A., Pece, S., Giuliani, G., Freudenberg, M., Galanos, C., Jirillo, E., 1999. Lactoferrin–lipid A–lipopolysaccharide interaction: inhibition by anti-human lactoferrin monoclonal antibody AGM 10.14. Infect. Immun. 67, 4668.

Chung, S., Hayward, C., Brock, D.J., Van, H.V., 1985. A monoclonal antibody-based immunoassay for human lactoferrin. J. Immunol. Methods 84, 135.

Crichton, R.R., 1990. Proteins of iron storage and transport. Adv. Protein Chem. 40, 281.

Crouch, S.P., Slater, K.J., Fletcher, J., 1992. Regulation of cytokine release from mononuclear cells by the iron-binding protein lactoferrin. Blood 80, 235.

Davidson, L.A., Lönnerdal, B., 1986. Isolation and characterization of rhesus monkey milk lactoferrin. Pediatr. Res. 20, 197.

Ellison III, R.T., Giehl, T.J., 1991. Killing of gram-negative bacteria by lactoferrin and lysozyme. J. Clin. Invest. 88, 1080.

El Yazidi-Belkoura, I., Legrand, D., Nuijens, J., Slomianny, M.C., van Berkel, P., Spik, G., 2001. The binding of lactoferrin to glycosaminoglycans on enterocyte-like HT29-18-C1 cells is mediated through basic residues located in the N-terminus. Biochim. Biophys. Acta 1568, 197.

Fazekas de St Groth, S., Scheidegger, D., 1980. Production of monoclonal antibodies: strategy and tactics. J. Immunol. Methods 35, 1.

Hutchens, T.W., Henry, J.F., Yip, T.T., 1991. Structurally intact (78-kDa) forms of maternal lactoferrin purified from urine of preterm infants fed human milk: identification of a trypsin-like proteolytic cleavage event in vivo that does not result in fragment dissociation. Proc. Natl. Acad. Sci. U. S. A. 88, 2994.

Kijlstra, A., Jeurissen, S.H.M., 1982. Modulation of the classical C3 convertase of complement by tear lactoferrin. Immunology 47, 263.

Kokriakov, V.N., Aleshina, G.M., Slepenkov, S.V., Iakovleva, M.F., Pigarevskii, V.E., 1988. Degree of structural homology of lactoferrins from milk and neutrophilic granulocytes. Biokhimiia 53, 1837.

Legrand, D., Mazurier, J., Aubert, J.P., Loucheux, M.H., Montreuil, J., Spik, G., 1986. Evidence for interactions between the 30 kDa N- and 50 kDa C-terminal tryptic fragments of human lactotransferrin. Biochem. J. 236, 839.

Legrand, D., van Berkel, P.H.C., Salmon, V., van Veen, H.A., Slomianny, M.C., Nuijens, J.H., Spik, G., 1997. The N-terminal Arg2, Arg3 and Arg4 of human lactoferrin interact with sulphated molecules but not with the receptor present on Jurkat human lymphoblastic T-cells. Biochem. J. 327, 841.

Lydon, J.P., O'Malley, B.R., Saucedo, O., Lee, T., Headon, D.R., Conneely, O.M., 1992. Nucleotide and primary amino acid sequence of porcine lactoferrin. Biochim. Biophys. Acta 1132, 97.

Magnuson, J.S., Henry, J.F., Yip, T.T., Hutchens, T.W., 1990. Structural homology of human, bovine, and porcine milk lactoferrins: evidence for shared antigenic determinants. Pediatr. Res. 28, 176.

Mann, D.M., Romm, E., Migliorini, M., 1994. Delineation of the

glycosaminoglycan-binding site in the human inflammatory response protein lactoferrin. J. Biol. Chem. 269, 23661.

Masson, P.L., Heremans, J.F., 1971. Lactoferrin in milk from different species. Comp. Biochem. Physiol., B 39, 119.

Metz-Boutigue, M., Jolles, J., Mazurier, J., Schoentgen, F., Legrand, D., Spik, G., Montreuil, J., Jolles, P., 1984. Human lactotransferrin: amino acid sequence and structural comparisons with other transferrins. Eur. J. Biochem. 145, 659.

Müller, R., 1983. Determination of affinity and specificity of anti-hapten antibodies by competitive radioimmunoassay. Methods Enzymol. 92, 589.

Nibbering, P.H., Ravensbergen, E., Welling, M.M., van Berkel, L.A., van Berkel, P.H.C., Pauwels, E.K.J., Nuijens, J.H., 2001. Human lactoferrin and peptides derived from its N-terminus are highly effective against infections with antibiotic resistant bacteria. Infect. Immun. 69, 1469.

Nuijens, J.H., van Veen, H.A., 1998. Isolation of lactoferrin from milk. US Patent 5849885.

Nuijens, J.H., van Berkel, P.H.C., Schanbacher, F.L., 1996. Structure and biological actions of lactoferrin. J. Mammary Gland Biol. Neoplasia 1, 285.

Nuijens, J.H., van Berkel, P.H., Geerts, M.E., Hartevelt, P.P., de Boer, H.A., van Veen, H.A., Pieper, F.R., 1997. Characterization of recombinant human lactoferrin secreted in milk of transgenic mice. J. Biol. Chem. 272, 8802.

Pierce, A., Colavizza, D., Benaissa, M., Maes, P., Tartar, A., Montreuil, J., Spik, G., 1991. Molecular cloning and sequence analysis of bovine lactotransferrin. Eur. J. Biochem. 196, 177.

Reiter, B., Brock, J.H., Steel, E.D., 1975. Inhibition of *Eschericia coli* by bovine colostrum and post-colostral milk: II. The bacteriostatic effect of lactoferrin on a serum-susceptible and serum-resistant strain of *E. coli*. Immunology 28, 83.

Rey, M.W., Woloshuk, S.L., de Boer, H.A., Pieper, F.R., 1990. Complete nucleotide sequence of human mammary gland lactoferrin. Nucleic Acids Res. 18, 5288.

Skubitz, K.M., Christiansen, N.P., Mendiola, J.R., 1989. Preparation and characterization of monoclonal antibodies to human neutrophil cathepsin G, lactoferrin, eosinophil peroxidase, and eosinophil major basic protein. J. Leukoc. Biol. 46, 109.

van Berkel, P.H.C., Geerts, M.E.J., van Veen, H.A., Kooiman, P.M., Pieper, F., de Boer, H.A., Nuijens, J.H., 1995. Glycosylated and unglycosylated human lactoferrins can both bind iron and have identical affinities towards human lysozyme and bacterial lipopolysaccharide, but differ in their susceptibility towards tryptic proteolysis. Biochem. J. 312, 107.

van Berkel, P.H., van Veen, H.A., Geerts, M.E., de Boer, H.A., Nuijens, J.H., 1996. Heterogeneity in utilization of *N*-glycosylation sites Asn624 and Asn138 in human lactoferrin: a study with glycosylation-site mutants. Biochem. J. 319, 117.

van Berkel, P.H.C., Geerts, M.E.J., van Veen, H.A., Mericskay, M., de Boer, H.A., Nuijens, J.H., 1997. The N-terminal stretch Arg2 Arg3, Arg4 and Arg5 of human lactoferrin is essential for binding to heparin, bacterial lipopolysaccharide, human lysozyme and DNA. Biochem. J. 328, 145.

Wang, C.S., Chan, W.Y., Kloer, H.U., 1984. Comparative studies on the chemical and immunochemical properties of human milk, human pancreatic juice and bovine milk lactoferrin. Comp. Biochem. Physiol., B 78, 575.