Production and characterization of recombinant human lactoferrin

Veen, H.A. van

Chapter 1

Introduction

1. General

Lactoferrin (LF) is an iron-binding glycoprotein that belongs to the transferrin family. This family of proteins is widely distributed in vertebrates and invertebrates [1]. Most members of the transferrin family evolved from an ancient gene duplication event, which resulted in a single polypeptide of about Mr 80,000 folded into two homologous lobes [1]. In LF, each lobe can bind a single ferric ion giving the protein a characteristic red color [2]. LF was first reported in 1939 by Sørensen and Sørensen, who separated a "red protein fraction" from cow milk [3]. In 1960, substantial purity of LF was obtained allowing characterization studies [2, 4]. These studies revealed that LF closely relates to transferrin, an abundant serum protein involved in the transport of iron to cells [5]. However, the affinity for iron appeared to be about 300 times higher for LF when compared to transferrin [6] and initial functions ascribed to LF related to this feature i.e. limiting of bacterial growth through iron deprivation [7, 8]. Since then extensive research, both in vitro as well as in vivo, has been performed showing that LF is involved in the innate host defence against infection and severe inflammation, most notably at mucosal surfaces. The diverse functions of LF relate to its binding of iron, binding to a variety of ligands and interactions with specific receptors [9-11].

2. Biosynthesis

Lactoferrin is synthesized by glandular epithelial cells and secreted into milk, tears, saliva, nasal fluids, pancreatic-, bronchial-, gastrointestinal- and reproductive tissue secretions [9]. The concentration of LF in milk varies considerably among species. Human milk has the highest LF concentration (1-6 mg/ml); mouse milk has moderate levels of LF (1-2 mg/ml) and milks from ruminants have relatively low levels of LF (0.01-0.1 mg/ml). Milks from rabbits and rats contain virtually no LF [10]. The LF concentration in lacteal secretions varies also within the lactation phase. In human milk, the LF concentration can be as high as 10 mg/ml in colostrum declining to about 1-2 mg/ml in mature milk. The concentration of bovine LF (bLF) is about 1-2 mg/ml and 0.01-0.1 mg/ml in bovine colostrum and mature milk, respectively [10]. Upon involution of the human and bovine mammary gland, the LF concentration increases to about 50 mg/ml and 20-100 mg/ml, respectively [10]. The concentration of LF in human saliva and tears is about 30 μg/ml and 2 mg/ml, respectively [12, 13].

LF is also released from the secondary granules of activated neutrophils [14]. This process likely accounts for the presence of LF in normal blood plasma at a concentration of about 0.2 μg/ml [15]. In patients with sepsis the levels of LF in plasma are increased to about 1 μg/ml [15]. The level of LF in synovial fluid of patients with non-inflammatory joint diseases is about 0.7 μg/ml; in patients with inflammatory joint diseases the level of LF in synovial fluid is increased to about 4 μg/ml [16].

LF is efficiently removed from the circulation by the liver. Studies in rats showed that about 95% of intravenously administrated LF (0.25 mg/kg body weight) is cleared by the liver within 5 minutes [17].

3. Structure of human lactoferrin

Human LF (hLF) consists of a single polypeptide chain of 692 amino acids [18]. The polypeptide is folded into two globular lobes, designated the N- and C-lobe, connected by an α-helix (Figure 1). Each lobe in turn is folded 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 with high affinity (K ~ 10^{22} M) while simultaneously incorporating a suitable anion such as carbonate or oxalate [19]. The ligands involved in the binding of the ferric ion are the same for both lobes and comprise of two tyrosine residues, one aspartate and one histidine together with two oxygen atoms from the incorporated anion [19].

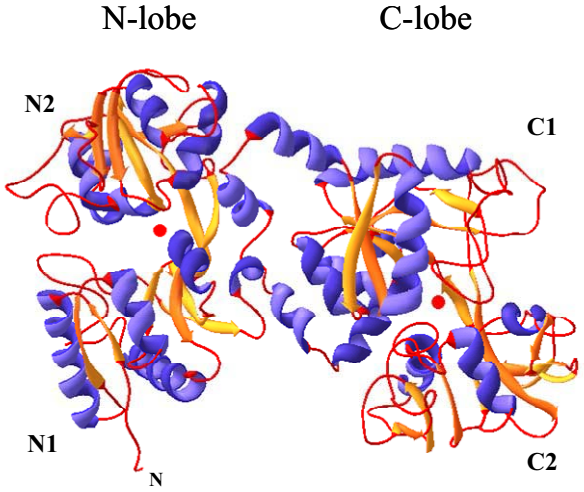

Figure 1 Protein structure of human lactoferrin
The structure of hLF, in its iron-saturated conformation [20], shows the typical bilobal (N- and C-lobe), four domain (N1/N2, C1/C2) folding pattern which is characteristic for proteins of the transferrin family [19]. The α-helices and β-strands are indicated in blue and yellow, respectively. The two iron ions are indicated by red spheres.

Crystallographic studies of hLF have shown that upon binding of iron, domain I of the N- and C-lobe rotates relative to domain II by ~54° and ~20°, respectively, resulting in a more globular, and stable conformation of the entire molecule. This conformational change was also observed upon incorporation of other metals such as manganese, zinc and copper [19].
Whereas some of the biological activities of hLF relate to metal-binding (e.g. limiting bacterial growth through iron sequestration), others are mediated by unique positively charged domains located in the N-terminus i.e. Arg^{2}-Arg^{3}-Arg^{4}-Arg^{5} and Arg^{28}-Lys^{29}-Val^{30}-Arg^{31}, which are juxtaposed to form a cationic cradle [21]. These basic clusters determine the relatively high isoelectric point (pI of 8.7) of hLF [22] and are involved in the binding of hLF to negatively charged ligands such as the lipid A moiety of lipopolysaccharide (LPS) [23], DNA [24], heparin [21], other proteins such as lysozyme [25] as well as cell-surface molecules such as proteoglycans and specific receptors [26, 27]. The release of the positively charged domains from hLF by pepsin action yields lactoferricin (residues Gly^{1} to Ile^{47}), which is a potent bactericidal peptide [28]. Human LF contains three possible N-glycosylation sites, Asn^{138} in the N-lobe and Asn^{479} as well as Asn^{624} in the C-lobe [18], which are utilized in about 94%, 100% and 9% of the molecules, respectively [29]. The glycans of natural hLF are of the sialyl-*N*-acetyllactosaminic type [30].

4. Biological actions of lactoferrin

Extensive research has showed that LF is involved in the innate host defence against infection and severe inflammation. The diverse functions of LF relate to its binding of iron, non-specific binding to a

variety of negatively charged ligands and interactions with specific receptors. Figure 2 provides an overview of biological activities postulated for LF.

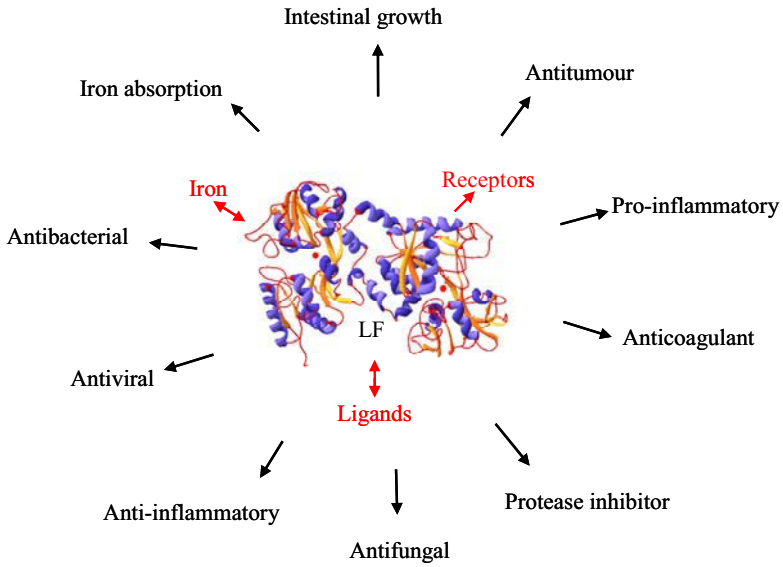

Figure 2 Overview of biological activities postulated for lactoferrin (LF)

One of the first functions ascribed to LF was growth-inhibition of Gram-positive and Gram-negative bacteria by sequestration of environmental iron [7, 8, 31]. In addition, iron deprivation by LF inhibits biofilm formation of *Pseudomonas Aeruginosa* [32]. These antimicrobial activities are reversible as bacterial growth was restored upon the addition of an excess of iron. Some microorganisms such as *Neisseria meningitidis* and *Haemophilus influenzae* can acquire iron from iron-saturated LF through specific receptors for the molecule [33]. Furthermore, LF has been shown to promote the growth of *Bifidobacterium* species [34, 35], the predominant bacteria of the intestinal flora of healthy breast-fed infants.

Besides bacteriostasis, bactericidal activity of LF by destabilization of the cell-wall of Gram-positive and Gram-negative bacteria has been reported [36, 37]. Destabilization of the cell-wall by LF has also been reported for several *Candida* species [38]. The cell-wall destabilization results from the binding of LF to membrane-molecules such as porins [39] and LPS [40, 41]. Furthermore, the binding of LF to membrane molecules, mostly glycosaminoglycans, inhibits cell adhesion and invasion of a large variety of pathogens, including enterovirulent strains of *Escherichia coli* [42, 43] and *Shigella* [44], *Listeria monocytogenes* [45], human cytomegalovirus [46, 47], human herpes simplex virus [46], human immunodeficiency virus [47] and human hepatitis B and C viruses [48, 49]. Besides binding of LF to membrane molecules, LF-mediated proteolysis of molecules involved in the invasion of pathogens (e.g. bacterial invasins) has been reported [50, 51].

The antibacterial, antiviral and antifungal activities of LF have been confirmed by studies in rodents experimentally infected with a variety of pathogens including *Listeria monocytogenes* [52], *Escherichia coli* [53], *Staphylococcus aureus* [54], herpes simplex virus [55], influenza virus [56] and *Candida albicans* [57].

The anti-inflammatory activities of LF include inhibition of hydroxyl-radical ($^{\bullet}$OH) formation by scavenging of iron [58] and inhibition of mast cell tryptase activity by dissociation of the tryptase/heparin complex [59]. As a results of these anti-inflammatory activities LF abolished late phase airway responses (through inhibition of mast cell tryptase activity) in allergic sheep [59] and decreased

pollen antigen-induced airway inflammation in a murine asthma model by reducing the generation of reactive oxygen species (ROS) such as $^{\bullet}OH$ [60]. The inhibition of ROS formation by LF has also been postulated as mode of action for reducing inflammation in joints of murine arthritis models [61].
Another anti-inflammatory activity of LF is neutralization of LPS, which is a major mediator of inflammatory responses after bacterial infection [62]. LF binds to the lipid A moiety of LPS with high affinity (K_d ~ 4 nM) [23, 63] and competitively inhibits binding of LPS to LPS-binding protein [64]. Furthermore, LF has been shown to inhibit LPS-induced expression of endothelial adhesion molecules through binding to sCD14 and the sCD14-LPS complex [65]. The neutralization of LPS activity by LF has been demonstrated in vivo since LF protected against LPS-induced lethal shock in mice and germ-free piglets [66-68].
LF is involved in the modulation of immune cell activity (recruitment, activation and/or proliferation) of a variety of immune cells such as monocytes/macrophages and natural killer (NK) cells in vitro as well as in vivo [69]. The activation of NK cells contributes to the antitumour activities ascribed to the molecule, which include also modulation of various signaling pathways [9]. The antitumour activities of LF, administrated either orally, intraperitoneally, subcutaneously or intratumorally, has been established for a broad range of tumors experimentally induced in rodents [70-74]. Orally administrated hLF at doses of 1.5 to 9 g/day using a two weeks on, 2 weeks off schedule inhibited growth of refractory solid tumors, especially of non-small cell lung cancer (NSCLC), in humans [75]. In addition, orally administrated hLF has been shown to potentiate conventional chemotherapy in mouse models with established human and mouse tumors [72] and in humans with NSCLC [76].
The modulation of cellular processes by LF is mediated by neutralization of potent stimuli of host immunological responses such as LPS [62] and bacterial unmethylated CpG-containing oligonucleotides [77]. Besides the neutralization of potent inflammatory stimulators, the molecule can modulate cellular processes by binding to receptors and subsequent intracellular signaling pathways [78-81]. Specific receptors for LF have been found on a variety of cells including monocytes [82], lymphocytes [83], liver- [27] and intestinal cells [84]. The presence of a LF-receptor on intestinal cells may explain for the role of LF in iron-absorption in the gut [84]. In addition, LF has been shown to promote the growth of intestinal cells in vitro [85] and in vivo [86] which may explain for the protective effect LF displayed after experimental induced enteropathy in rodents [87, 88] and healthy volunteers [89]. Similarly to protection of intestinal epithelial, prior application of LF suppressed damage of corneal epithelial induced by UV-B [90, 91].
The anticoagulant activities ascribed to LF are related to the binding of glycosaminoglycans such as heparin. LF neutralized heparin activity comparable to platelet factor 4 but was more effective than protamine sulphate [92].

5. Applications of lactoferrin

The diverse biological actions of LF may provide a basis for a large variety of potential nutraceutical as well as topical and systemic applications in human healthcare. The applications may include the prevention and treatment of local or systemic infections and (chronic) inflammations such as occurring in patients with inflammatory bowel diseases, patients receiving high-dose chemotherapy and patients with allergic asthma. Furthermore, LF may be suitable for neutralization of heparin activity after its use as anticoagulant in surgery.
Both hLF and bLF, obtained after fractionation of bovine milk whey, can be used in applications of LF in human health care. However, the use of bLF in human healthcare is limited to oral applications because of its immunogenicity. Furthermore, bLF may be inferior to hLF in applications where interactions with specific receptors are required.

6. Production of recombinant human lactoferrin

The limited availability of human milk and purified hLF has been a major hurdle for (clinical) studies on potential nutraceutical and pharmaceutical applications of hLF. To overcome this limitation, the feasibility of large-scale production of functional recombinant hLF (rhLF) was studied in a variety of expression systems (Table 1). Expression of recombinant hLF (rhLF), at relatively low levels, has been reported for mammalian cells, fungi, yeast, baculovirus-based expression systems and transgenic plants and rabbits. Higher expression levels of rhLF have been reported for *Aspergillus awamori*, transgenic mice, transgenic rice and various transgenic plant cell culture systems (Table 1).

Table 1 Expression of rhLF in various expression systems

Expression system	rhLF expression	Reference
BHK cell culture	~20 µg/ml	[93]
Human 293(S) cell culture	~1 µg/ml	[25]
Aspergillus Oryzae	~25 µg/ml	[94]
Saccharomyces cerevisiae	~2 µg/ml	[95]
Baculovirus/Sf9 cell culture	~15 µg/ml	[96]
Transgenic potato plants	~0.1% of soluble protein	[97]
Transgenic tobacco plants	~0.3% of soluble protein	[98]
Transgenic rabbits	~0.1 mg/ml	[99]
Baculovirus/silkworm larvae	~0.2 mg/ml	[100]
Transgenic tobacco cell culture	~4% of soluble protein	[101]
Transgenic ginseng cell culture	~3% of soluble protein	[102]
Transgenic rice cell culture	~4% of soluble protein	[103]
Aspergillus awamori	~2 mg/ml	[104]
Transgenic mice	~13 mg/ml	[105]
Transgenic rice	~5 g/kg grain	[106]

A disadvantage of most expression systems is that rhLF, in contrast to human-derived hLF, is secreted in its iron-saturated form probably due to the presence of excess of metals during culturing [25, 95]. Such rhLF preparations thus require desaturation (e.g. pH < 3) to obtain biological activities based on the binding of iron. Furthermore, the organism used for expression determines the carbohydrate composition and structures of the glycan chains because glycosylation is species, tissue, cell type and protein-specific [107, 108]. For the parenteral route of administration, the presence of non-human sugar moieties and/or glycan chains may turn them into antigenic determinants [109, 110] and thereby may impair the (immuno) safety of the rhLF containing drug.

Transgenic cows expressing hLF in milk could provide a suitable means to produce large quantities of hLF as one cow can produce annually about 10,000 liters of milk. The costs associated with maintaining transgenic cows are futile as compared to those of large scale mammalian cell-culture based expression systems. In addition, environmental concerns raised for transgenic plants i.e. uncontrolled dissemination of genes to non-transgenic plants don't apply for transgenic cows [111, 112]. Previously, we reported the generation of transgenic cows harbouring mammary gland-specific bovine αS1-casein promoter and hLF cDNA-based expression vectors [113].

7. Outline of thesis

The expression and characterization of rhLF produced in the milk of transgenic cows, bearing the hLF gene under control of the bovine αS1-casein promoter, are described in this thesis.

In Chapter 2 a robust analytical method to assess the identity, purity and N-terminal integrity of hLF preparations is described. The method, employing cation-exchange chromatography on a Mono S column, can discriminate between intact hLF and hLF molecules lacking two or three N-terminal residues, lactoferrins from other species as well as homologous and other whey proteins.
In Chapter 3 the generation and characterization of ten distinct monoclonal antibodies (mAbs) against hLF is described. Localization of the epitopes for these anti-hLF mAbs by using proteolytic hLF fragments and the recombinant hLF lobes revealed that five mAbs could bind to conformational epitopes residing in the N-lobe of hLF, whereas the other five could bind to C-lobe conformational epitopes. One mAb, designated E11, appeared to bind to the arginine-rich N-terminus of hLF. The characterization of the recombinant hLF lobes used for characterization of the anti-hLF mAbs is described in Chapter 4. The recombinant hLF lobes were expressed in human 293(S) cells and the purified lobes were characterized by determining the N-terminal amino acid sequences, the heterogeneity in N-linked glycosylation, the binding of metals like iron and ligands like heparin and LPS. The results confirmed that the major iron-binding associated conformational change and the interaction with lipid A and heparin is determined by the N-lobe of hLF. In addition, the N-linked glycan of the N-lobe is not essential for maintaining the stability of the iron-saturated conformation.
In Chapter 5 the production of rhLF in the milk of transgenic cows is described. Recombinant hLF was expressed at high concentrations in milk (~2.5 g/l) and mainly (> 90%) in its unsaturated form. Comparative characterization studies between rhLF and hLF from human milk revealed identical iron-binding and iron-release properties and, despite differences in N-linked glycosylation, equal effectiveness in various infection models. Crystal structure analysis revealed that the protein structure of iron-saturated rhLF closely matches the structure of iron-saturated hLF from human milk (Chapter 6).
In Chapter 7 two variants of bLF (bLF A and B) are described. These bLF variants differ in utilization of glycosylation-site Asn^{281} and resistance to tryptic proteolysis. In contrast to bLF, N-linked glycosylation is not needed for protection of hLF against tryptic proteolysis. Both recombinant and human milk hLF appeared about 100-fold less susceptible to tryptic proteolysis than bLF (Chapter 7).
The characterization of bovine neutrophil gelatinase-associated lipocalin (bNGAL), which is a potential contaminant of purified LF preparations, is described in Chapter 8. Bovine NGAL was identified based on N-terminal sequence identity with the sequence predicted for the bovine homologue of human neutrophil gelatinase-associated lipocalin (hNGAL), a glycoprotein of Mr 25,000 belonging to the family of lipocalins. A specific ELISA was developed to detect bNGAL in milk and purified LF preparations.
The oral safety of rhLF investigated in Wistar rats is described in Chapter 9. Recombinant hLF was administrated daily, via oral gavage, at doses ranging from 0.2 to 2.0 g/kg body weight/day for 13 consecutive weeks and a large variety of clinical and laboratory safety parameters were monitored. These parameters revealed no treatment-related, toxicologically significant changes on the basis of which the no observed-adverse-effect level (NOAEL) was determined on 2 g/kg body weight/day. The summary and general conclusions of this thesis are provided in Chapter 10.

References

1. Lambert LA, Perri H & Meehan TJ (2005) Evolution of duplications in the transferrin family of proteins, *Comp Biochem Physiol B Biochem Mol Biol* **140**, 11-25.
2. Groves ML (1960) Isolation of a red protein from milk, *J Am Chem Soc* **82**, 3345-3350.
3. Sorensen M & Sorensen SPL (1939) The proteins in whey, *CR Trav Lab Carlsberg* **23**, 55-99.
4. Johanson B (1960) Isolation of an iron-binding red protein from human milk, *Acta Chem Scand* **14**, 510-512.
5. Crichton RR (1990) Proteins of iron storage and transport, *Adv Prot Chem* **40**, 281-363.
6. Aisen P & Leibman A (1972) Lactoferrin and transferrin: a comparative study, *Biochim Biophys Acta* **257**, 314-323.
7. Bullen JJ, Rogers HJ & Leigh L (1972) Iron-binding proteins in milk and resistance to Escherichia coli infection in infants, *Brit Med J* **1**, 69-75.
8. Reiter B, Brock JH & Steel ED (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**, 83-95.
9. Ward PP, Paz E & Conneely OM (2005) Multifunctional roles of lactoferrin: a critical overview, *Cell Mol Life Sci* **62**, 2540-2548.
10. Nuijens JH, van Berkel PHC & Schanbacher FL (1996) Structure and biological actions of lactoferrin, *Journal of Mammary Gland Biology and Neoplasia* **1**, 285-295.
11. Suzuki YA, Lopez V & Lönnerdal B (2005) Mammalian lactoferrin receptors: structure and function, *Cell Mol Life Sci* **62**, 2560-75.
12. Kijlstra A, Jeurissen SH & Koning KM (1983) Lactoferrin levels in normal human tears, *Br J Ophthalmol* **67**, 199-202.
13. Tanida T, Okamoto T, Okamoto A, Wang H, Hamada T, Ueta E & Osaki T (2003) Decreased excretion of antimicrobial proteins and peptides in saliva of patients with oral candidiasis, *J Oral Pathol Med* **32**, 586-594.
14. Baggiolini M, DeDuve, C., Masson, P. L. and Heremans, J. F. (1970) Association of lactoferrin with specific granules in rabbit heterophil leukocytes, *J Exp Med* **131**, 559-570.
15. Nuijens JH, Abbink JJ, Wachtfogel YT, Colman RW, Eerenberg AJ, Dors D, Kamp AJ, Strack van Schijndel RJ, Thijs LG & Hack CE (1992) Plasma elastase alpha 1-antitrypsin and lactoferrin in sepsis: evidence for neutrophils as mediators in fatal sepsis, *J Lab Clin Med* **119**, 159-168.
16. Abbink JJ, Kamp AM, Nieuwenhuys EJ, Nuijens JH, Swaak AJ & Hack CE (1991) Predominant role of neutrophils in the inactivation of alpha 2-macroglobulin in arthritic joints, *Arthritis Rheum* **34**, 1139-1150.
17. Ziere GJ, C vDM, Bijsterbosch MK & van Berkel TJ (1992) Lactoferrin uptake by the rat liver. Characterization of the recognition site and effect of selective modification of arginine residues, *J Biol Chem* **267**, 11229-11235.
18. Rey MW, Woloshuk SL, de Boer HA & Pieper FR (1990) Complete nucleotide sequence of human mammary gland lactoferrin, *Nucleic Acids Res* **18**, 5288.
19. Baker EN & Baker HM (2005) Molecular structure, binding properties and dynamics of lactoferrin, *Cell Mol Life Sci* **62**, 2531-2539.
20. Haridas M, Anderson BF & Baker EN (1995) Structure of human diferric lactoferrin refined at 2.2 Å resolution, *Acta Cryst* **D51**, 629-646.
21. Mann DM, Romm E & Migliorini M (1994) Delineation of the glycosaminoglycan-binding site in the human inflammatory response protein lactoferrin, *J Biol Chem* **269**, 23661-23667.
22. Moguilevsky N, Retegui LA & Masson PL (1985) Comparison of human lactoferrins from milk and neutrophilic leucocytes. Relative molecular mass, isoelectric point, iron-binding properties and uptake by the liver, *Biochem J* **229**, 353-359.

23. Appelmelk BJ, An YQ, Geerts M, Thijs BG, de Boer HA, MacLaren DM, de Graaff J & Nuijens JH (1994) Lactoferrin is a lipid A-binding protein, *Infect Immun* **62**, 2628-2632.
24. He J & Furmanski P (1995) Sequence specificity and transcriptional activation in the binding of lactoferrin to DNA, *Nature* **373**, 721-724.
25. van Berkel PHC, Geerts MEJ, van Veen HA, Kooiman PM, Pieper F, de Boer HA & Nuijens JH (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**, 107-114.
26. Legrand D, van Berkel PH, Salmon V, van Veen HA, Slomianny MC, Nuijens JH & 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**, 841-846.
27. Ziere GJ, Bijsterbosch MK & van Berkel TJ (1993) Removal of 14 N-terminal amino acids of lactoferrin enhances its affinity for parenchymal liver cells and potentiates the inhibition of beta-very low density lipoprotein binding, *J Biol Chem* **268**, 27069-27075.
28. Tomita M, Takase M, Wakabayashi H & Bellamy W (1994) Antimicrobial peptides of lactoferrin, *Adv Exp Med Biol* **357**, 209-218.
29. van Berkel PH, van Veen HA, Geerts ME, de Boer HA & Nuijens JH (1996) Heterogeneity in utilization of N-glycosylation sites Asn624 and Asn138 in human lactoferrin: a study with glycosylation-site mutants, *Biochem J* **319**, 117-122.
30. Spik G, Strecker G, Fournet B, Bouquelet S, Montreuil J, Dorland L, Van Halbeek H & Vliegenthart JFG (1982) Primary structure of the glycans from human lactotransferrin, *Eur J Biochem* **121**, 413-419.
31. Weinberg ED (1984) Iron withholding: a defense against infection and neoplasia, *Physiol Rev* **64**, 65-102.
32. Singh PK, Parsek MR, Greenberg EP & Welsh MJ (2002) A component of innate immunity prevents bacterial biofilm development, *Nature* **417**, 552-555.
33. Gray-Owen SD & Schryvers AB (1996) Bacterial transferrin and lactoferrin receptors, *Trends Microbiol* **4**, 185-191.
34. Kim WS, Ohashi M, Tanaka T, Kumura H, Kim GY, Kwon IK, Goh JS & Shimazaki K (2004) Growth-promoting effects of lactoferrin on L. acidophilus and Bifidobacterium spp, *Biometals* **17**, 279-283.
35. Petschow BW & Talbott RD (1991) Response of bifidobacterium species to growth promoters in human and cow milk, *Pediatr Res* **29**, 208-213.
36. Arnold RR, Cole MF & McGhee JR (1977) A bactericidal effect for human lactoferrin, *Science* **197**, 263-265.
37. Arnold RR, Russell JE, Champion WJ, Brewer M & Gauthier JJ (1982) Bactericidal activity of human lactoferrin: differentiation from the stasis of iron deprivation, *Infect Immun* **35**, 792-799.
38. Xu YY, Samaranayake YH, Samaranayake LP & Nikawa H (1999) In vitro susceptibility of Candida species to lactoferrin, *Med Mycol* **37**, 35-41.
39. Sallmann FR, Baveye-Descamps S, Pattus F, Salmon V, Branza N, Spik G & Legrand D (1999) Porins OmpC and PhoE of Escherichia coli as specific cell-surface targets of human lactoferrin. Binding characteristics and biological effects, *J Biol Chem* **274**, 16107-16114.
40. Ellison III RT & Giehl TJ (1991) Killing of gram-negative bacteria by lactoferrin and lysozyme, *J Clin Invest* **88**, 1080-1091.
41. Ellison III RT, Giehl TJ & La FF (1988) Damage of the outer membrane of enteric gram-negative bacteria by lactoferrin and transferrin, *Infect Immun* **56**, 2774-2781.
42. de Araujo AN & Giugliano LG (2001) Lactoferrin and free secretory component of human milk inhibit the adhesion of enteropathogenic Escherichia coli to HeLa cells, *BMC Microbiol* **1**, 25.

43. Giugliano LG, Ribeiro ST, Vainstein MH & Ulhoa CJ (1995) Free secretory component and lactoferrin of human milk inhibit the adhesion of enterotoxigenic Escherichia coli, *J Med Microbiol* **42**, 3-9.
44. Willer Eda M, Lima Rde L & Giugliano LG (2004) In vitro adhesion and invasion inhibition of Shigella dysenteriae, Shigella flexneri and Shigella sonnei clinical strains by human milk proteins, *BMC Microbiol* **4**, 18.
45. Antonini G, Catania MR, Greco R, Longhi C, Pisciotta MG, Seganti L & P. V (1997) Anti-invasive activity of bovine lactoferrin towards Listeria monocytogenes, *J Food Protec* **60**, 1-5.
46. Hasegawa K, Motsuchi W, Tanaka S & Dosako S (1994) Inhibition with lactoferrin of in vitro infection with human herpes virus, *Jpn J Med Sci Biol* **47**, 73-85.
47. Harmsen MC, Swart PJ, de Bethune MP, Pauwels R, De Clercq E, The TH & Meijer DK (1995) Antiviral effects of plasma and milk proteins: lactoferrin shows potent activity against both human immunodeficiency virus and human cytomegalovirus replication in vitro, *J Infect Dis* **172**, 380-388.
48. Hara K, Ikeda M, Saito S, Matsumoto S, Numata K, Kato N, Tanaka K & Sekihara H (2002) Lactoferrin inhibits hepatitis B virus infection in cultured human hepatocytes, *Hepatol Res* **24**, 228.
49. Ikeda M, Sugiyama K, Tanaka T, Tanaka K, Sekihara H, Shimotohno K & Kato N (1998) Lactoferrin markedly inhibits hepatitis C virus infection in cultured human hepatocytes, *Biochem Biophys Res Commun* **245**, 549-553.
50. Ochoa TJ, Noguera-Obenza M, Ebel F, Guzman CA, Gomez HF & Cleary TG (2003) Lactoferrin impairs type III secretory system function in enteropathogenic Escherichia coli, *Infect Immun* **71**, 5149-5155.
51. Gomez HF, Ochoa TJ, Carlin LG & Cleary TG (2003) Human lactoferrin impairs virulence of Shigella flexneri, *J Infect Dis* **187**, 87-95.
52. Lee HY, Park JH, Seok SH, Baek MW, Kim DJ, Lee BH, Kang PD, Kim YS & Park JH (2005) Potential antimicrobial effects of human lactoferrin against oral infection with Listeria monocytogenes in mice, *J Med Microbiol* **54**, 1049-1054.
53. Edde L, Hipolito RB, Hwang FF, Headon DR, Shalwitz RA & Sherman MP (2001) Lactoferrin protects neonatal rats from gut-related systemic infection, *Am J Physiol Gastrointest Liver Physiol* **281**, G1140-1150.
54. Bhimani RS, Vendrov Y & Furmanski P (1999) Influence of lactoferrin feeding and injection against systemic staphylococcal infections in mice, *J Appl Microbiol* **86**, 135-44.
55. Wakabayashi H, Kurokawa M, Shin K, Teraguchi S, Tamura Y & Shiraki K (2004) Oral lactoferrin prevents body weight loss and increases cytokine responses during herpes simplex virus type 1 infection of mice, *Biosci Biotechnol Biochem* **68**, 537-44.
56. Shin K, Wakabayashi H, Yamauchi K, Teraguchi S, Tamura Y, Kurokawa M & Shiraki K (2005) Effects of orally administered bovine lactoferrin and lactoperoxidase on influenza virus infection in mice, *J Med Microbiol* **54**, 717-723.
57. Takakura N, Wakabayashi H, Ishibashi H, Teraguchi S, Tamura Y, Yamaguchi H & Abe S (2003) Oral lactoferrin treatment of experimental oral candidiasis in mice, *Antimicrob Agents Chemother* **47**, 2619-2623.
58. Aruoma OI & Halliwell B (1987) Superoxide-dependent and ascorbate-dependent formation of hydroxyl radicals from hydrogen peroxide in the presence of iron. Are lactoferrin and transferrin promoters of hydroxyl-radical generation? *Biochem J* **241**, 273-278.
59. Elrod KC, Moore WR, Abraham WM & Tanaka RD (1997) Lactoferrin, a potent tryptase inhibitor, abolishes late-phase airway responses in allergic sheep, *Am J Respir Crit Care Med* **156**, 375-381.

60. Kruzel ML, Bacsi A, Choudhury B, Sur S & Boldogh I (2006) Lactoferrin decreases pollen antigen-induced allergic airway inflammation in a murine model of asthma, *Immunology* **119**, 159-166.
61. Guillen C, McInnes IB, Vaughan D, Speekenbrink AB & Brock JH (2000) The effects of local administration of lactoferrin on inflammation in murine autoimmune and infectious arthritis, *Arthritis Rheum* **43**, 2073-2080.
62. Levin J, van Deventer SJH, van der Poll T & Sturk A. (1994) Bacterial endotoxins. Basic science to anti-sepsis strategies, *Progress in Clinical and Biological Research*, **388**, John Wiley & Sons, Inc.
63. Elass-Rochard E, Roseanu A, Legrand D, Trif M, Salmon V, Motas C, Montreuil J & Spik G (1995) Lactoferrin-lipopolysaccharide interaction: involvement of the 28-34 loop region of human lactoferrin in the high-affinity binding to Escherichia coli 055B5 lipopolysaccharide, *Biochem J* **312**, 839-845.
64. Elass-Rochard E, Legrand D, Salmon V, Roseanu A, Trif M, Tobias PS, Mazurier J & Spik G (1998) Lactoferrin inhibits the endotoxin interaction with CD14 by competition with the lipopolysaccharide-binding protein, *Infect Immun* **66**, 486-491.
65. Baveye S, Elass E, Fernig DG, Blanquart C, Mazurier J & Legrand D (2000) Human lactoferrin interacts with soluble CD14 and inhibits expression of endothelial adhesion molecules, E-selectin and ICAM-1, induced by the CD14-lipopolysaccharide complex, *Infect Immun* **68**, 6519-6525.
66. Lee WJ, Farmer JL, Hilty M & Kim YB (1998) The protective effects of lactoferrin feeding against endotoxin lethal shock in germfree piglets, *Infect Immun* **66**, 1421-1426.
67. Kruzel ML, Harari Y, Chen CY & Castro GA (2000) Lactoferrin protects gut mucosal integrity during endotoxemia induced by lipopolysaccharide in mice, *Inflammation* **24**, 33-44.
68. Kruzel ML, Harari Y, Mailman D, Actor JK & Zimecki M (2002) Differential effects of prophylactic, concurrent and therapeutic lactoferrin treatment on LPS-induced inflammatory responses in mice, *Clin Exp Immunol* **130**, 25-31.
69. Legrand D, Elass E, Carpentier M & Mazurier J (2005) Lactoferrin: a modulator of immune and inflammatory responses, *Cell Mol Life Sci* **62**, 2549-2559.
70. Bezault J, Bhimani R, Wiprovnick J & Furmanski P (1994) Human lactoferrin inhibits growth of solid tumors and development of experimental metastases in mice, *Cancer Res* **54**, 2310-2312.
71. Yoo YC, Watanabe S, Watanabe R, Hata K, Shimazaki K & Azuma I (1997) Bovine lactoferrin and lactoferricin, a peptide derived from bovine lactoferrin, inhibit tumor metastasis in mice, *Jpn J Cancer Res* **88**, 184-90.
72. Varadhachary A, Wolf JS, Petrak K, O'Malley BW, Jr., Spadaro M, Curcio C, Forni G & Pericle F (2004) Oral lactoferrin inhibits growth of established tumors and potentiates conventional chemotherapy, *Int J Cancer* **111**, 398-403.
73. Tsuda H, Sekine K, Fujita K & Ligo M (2002) Cancer prevention by bovine lactoferrin and underlying mechanisms--a review of experimental and clinical studies, *Biochem Cell Biol* **80**, 131-136.
74. Wolf JS, Li D, Taylor RJ & O'Malley BW, Jr. (2003) Lactoferrin inhibits growth of malignant tumors of the head and neck, *ORL J Otorhinolaryngol Relat Spec* **65**, 245-249.
75. Hayes TG, Falchook GF, Varadhachary GR, Smith DP, Davis LD, Dhingra HM, Hayes BP & Varadhachary A (2006) Phase I trial of oral talactoferrin alfa in refractory solid tumors, *Invest New Drugs* **24**, 233-240.
76. Petrak K, Varadhachary A, NSCLC Clinical Investigator Group, Yankee E & Wang Y (2005) Double-blind controlled trial of oral Talactoferrin in combination therapy for firts-line non-small cell lung cancer (NSCLC), *7th International Conference on Lactoferrin: Structure, Function and* Applications, Honolulu, Hawaii (October 16-19, 2005), 33.

77. Britigan BE, Lewis TS, Waldschmidt M, McCormick ML & Krieg AM (2001) Lactoferrin binds CpG-containing oligonucleotides and inhibits their immunostimulatory effects on human B cells, *J Immunol* **167**, 2921-2928.
78. Oh SM, Pyo CW, Kim Y & Choi SY (2004) Neutrophil lactoferrin upregulates the human p53 gene through induction of NF-kappaB activation cascade, *Oncogene* **23**, 8282-8291.
79. Oh SM, Hahm DH, Kim IH & Choi SY (2001) Human neutrophil lactoferrin trans-activates the matrix metalloproteinase 1 gene through stress-activated MAPK signaling modules, *J Biol Chem* **276**, 42575-42579.
80. Dhennin-Duthille I, Masson M, Damiens E, Fillebeen C, Spik G & Mazurier J (2000) Lactoferrin upregulates the expression of CD4 antigen through the stimulation of the mitogen-activated protein kinase in the human lymphoblastic T Jurkat cell line, *J Cell Biochem* **79**, 583-593.
81. Haversen L, Ohlsson BG, Hahn-Zoric M, Hanson LA & Mattsby-Baltzer I (2002) Lactoferrin down-regulates the LPS-induced cytokine production in monocytic cells via NF-kappa B, *Cell Immunol* **220**, 83-95.
82. Birgens HS, Hansen NE, Karle H & Kristensen LO (1983) Receptor binding of lactoferrin by human monocytes, *Br J Haematol* **54**, 383-391.
83. Mazurier J, Legrand D, Hu WL, Montreuil J & Spik G (1989) Expression of human lactotransferrin receptors in phytohemagglutinin-stimulated human peripheral blood lymphocytes. Isolation of the receptors by antiligand-affinity chromatography, *Eur J Biochem* **179**, 481-487.
84. Suzuki YA, Shin K & Lönnerdal B (2001) Molecular cloning and functional expression of a human intestinal lactoferrin receptor, *Biochemistry* **40**, 15771-15779.
85. Nichols BL, McKee KS, Henry JF & Putman M (1987) Human lactoferrin stimulates thymidine incorporation into DNA of rat crypt cells, *Pediatr Res* **21**, 563-567.
86. Zhang P, Sawicki V, Lewis A, Hanson L, Nuijens JH & Neville MC (2001) Human lactoferrin in the milk of transgenic mice increases intestinal growth in ten-day-old suckling neonates, *Adv Exp Med Biol* **501**, 107-113.
87. Togawa J, Nagase H, Tanaka K, Inamori M, Nakajima A, Ueno N, Saito T & Sekihara H (2002) Oral administration of lactoferrin reduces colitis in rats via modulation of the immune system and correction of cytokine imbalance, *J Gastroenterol Hepatol* **17**, 1291-1298.
88. Dial EJ, Dohrman AJ, Romero JJ & Lichtenberger LM (2005) Recombinant human lactoferrin prevents NSAID-induced intestinal bleeding in rodents, *J Pharm Pharmacol* **57**, 93-99.
89. Troost FJ, Saris WH & Brummer RJ (2003) Recombinant human lactoferrin ingestion attenuates indomethacin-induced enteropathy in vivo in healthy volunteers, *Eur J Clin Nutr* **57**, 1579-1585.
90. Shimmura S, Suematsu M, Shimoyama M, Tsubota K, Oguchi Y & Ishimura Y (1996) Subthreshold UV radiation-induced peroxide formation in cultured corneal epithelial cells: the protective effects of lactoferrin, *Exp Eye Res* **63**, 519-526.
91. Fujihara T, Nagano T, Endo K, Nakamura M & Nakata K (2000) Lactoferrin protects against UV-B irradiation-induced corneal epithelial damage in rats, *Cornea* **19**, 207-211.
92. Wu HF, Lundblad RL & Church FC (1995) Neutralization of heparin activity by neutrophil lactoferrin, *Blood* **85**, 421-428.
93. Stowell KM, Rado TA, Funk WD & Tweedie JW (1991) Expression of cloned human lactoferrin in baby-hamster kidney cells, *Biochem J* **276**, 349-355.
94. Ward PP, Lo J, Duke M, May GS, Headon DR & Conneely OM (1992) Production of biologically active recombinant human lactoferrin in Aspergillus oryzae, *Bio/Technology* **10**, 784-789.
95. Liang Q & Richardson T (1993) Expression and characterization of human lactoferrin in yeast Saccharomyces cerevisiae, *J Agric Food Chem* **41**, 1800-1807.

96. Salmon V, Legrand D, Georges B, Slomianny MC, Coddeville B & Spik G (1997) Characterization of human lactoferrin produced in the baculovirus expression system, *Protein Expr Purif* **9**, 203-210.
97. Chong DK & Langridge WH (2000) Expression of full-length bioactive antimicrobial human lactoferrin in potato plants, *Transgenic Res* **9**, 71-78.
98. Salmon V, Legrand D, Slomianny MC, el Yazidi I, Spik G, Gruber V, Bournat P, Olagnier B, Mison D, Theisen M & Merot B (1998) Production of human lactoferrin in transgenic tobacco plants, *Protein Expr Purif* **13**, 127-135.
99. Li L, Shen W, Min L, Dong H, Sun Y & Pan Q (2006) Human lactoferrin transgenic rabbits produced efficiently using dimethylsulfoxide-sperm-mediated gene transfer, *Reprod Fertil Dev* **18**, 689-695.
100. Liu T, Zhang YZ & Wu XF (2005) High level expression of functionally active human lactoferrin in silkworm larvae, *J Biotechnol* **118**, 246-256.
101. Choi SM, Lee OS, Kwon SY, Kwak SS, Yu DY & Lee HS (2003) High expression of a human lactoferrin in transgenic tobacco cell cultures, *Biotechnol Lett* **25**, 213-218.
102. Kwon SY, Jo SH, Lee OS, Choi SM, Kwak SS & Lee HS (2003) Transgenic ginseng cell lines that produce high levels of a human lactoferrin, *Planta Med* **69**, 1005-1008.
103. Suzuki YA, Kelleher SL, Yalda D, Wu L, Huang J, Huang N & Lönnerdal B (2003) Expression, characterization, and biologic activity of recombinant human lactoferrin in rice, *J Pediatr Gastroenterol Nutr* **36**, 190-199.
104. Ward PP, Piddington CS, Cunningham GA, Zhou X, Wyatt RD & Conneely OM (1995) A system for the production of commercial quantities of human lactoferrin: a broad spectrum natural antibiotic, *Bio/Technology* **13**, 498-503.
105. Nuijens JH, van Berkel PH, Geerts ME, Hartevelt PP, de Boer HA, van Veen HA & Pieper FR (1997) Characterization of recombinant human lactoferrin secreted in milk of transgenic mice, *J Biol Chem* **272**, 8802-8807.
106. Nandi S, Suzuki YA, Huang J, Yalda D, Pham P, Wu L, G. B, Huang N & Lönnerdal B (2002) Expression of human lactoferrin in transgenic rice grains for the application in infant formula, *Plant Science* **163**, 713-722.
107. James DC, Freedman RB, Hoare M, Ogonah Ow, Rooney BC, Larionov OA, Dobrovolsky VN, Lagutin OV & Jenkins N (1995) N-glycosylation of recombinant human interferin-gamma produced in different animal expression systems, *Bio/Technology* **13**, 592-596.
108. Opdenakker G, Rudd PM, Ponting CP & Dwek RA (1993) Concepts and principles of glycobiology, *FASEB J* **7**, 1330-1337.
109. Jenkins N & Curling EM (1994) Glycosylation of recombinant proteins: problems and prospects, *Enzyme Microb Technol* **16**, 354-364.
110. van Ree R, Cabanes-Macheteau M, Akkerdaas J, Milazzo JP, Loutelier-Bourhis C, Rayon C, Villalba M, Koppelman S, Aalberse R, Rodriguez R, Faye L & Lerouge P (2000) Beta(1,2)-xylose and alpha(1,3)-fucose residues have a strong contribution in IgE binding to plant glycoallergens, *J Biol Chem* **275**, 11451-11458.
111. Dyck MK, Lacroix D, Pothier F & Sirard MA (2003) Making recombinant proteins in animals--different systems, different applications, *Trends Biotechnol* **21**, 394-399.
112. Houdebine LM (2002) Antibody manufacture in transgenic animals and comparisons with other systems, *Curr Opin Biotechnol* **13**, 625-9.
113. Krimpenfort P, Rademakers A, Eyestone W, van der Schans A, van der Broek S, Kooiman P, Kootwijk E, Platenburg G, Pieper F, Strijker R & de Boer HA (1991) Generation of transgenic dairy cattle using 'in vitro' embryo production, *Bio/Technology* **9**, 844-847.