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Review Article

Human lactoferrin: a novel therapeutic with broad spectrum potential

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Abstract

Lactoferrin (Lf), a natural defence iron-binding protein, has been found to possess antibacterial, antimycotic, antiviral, antineoplastic and anti-inflammatory activity. The protein is present in exocrine secretions that are commonly exposed to normal flora: milk, tears, nasal exudate, saliva, bronchial mucus, gastrointestinal fluids, cervico–vaginal mucus and seminal fluid. Additionally, Lf is a major constituent of the secondary specific granules of circulating polymorphonuclear neutrophils (PMNs). The apoprotein is released on degranulation of the PMNs in septic areas. A principal function of Lf is that of scavenging free iron in fluids and inflamed areas so as to suppress free radical-mediated damage and decrease the availability of the metal to invading microbial and neoplastic cells. Mechanisms of action of Lf in addition to iron deprivation are also described. Administration of exogenous human or bovine Lf to hosts with various infected or inflamed sites has resulted in some prophylactic or therapeutic effects. However, an adverse response to the protein might occur if it were to stimulate antibody production or if it were to provide iron to the invading pathogen. The recombinant form of human Lf has become available and development of the product for use in a wide range of medical conditions can now be anticipated.

Introduction

The iron-withholding defence system, possessed by all vertebrate species, serves to scavenge and sequester toxic quantities of the metal. Consequences of over-abundant body iron include catalysis of formation of excessive hydroxyl or ferryl radicals, suppression of various leukocytic defence mechanisms, and stimulation of growth of microbial and neoplastic cell invaders (Weinberg 1993; Kontoghiorghes & Weinberg 1995). A principal component of the iron-withholding defence system is lactoferrin (Lf). The recombinant form of the human protein has become available and development of the product for use in a variety of medical conditions can now be anticipated.

Lf, a 78-kDa glycoprotein, consists of a single chain of 692 amino acids folded into two globular lobes. Each lobe is conjugated to a 3-kDa glycan chain through an N-glycosidic linkage. The lobes each enclose a powerful iron-binding site (active residues are two tyrosines, a histidine and an aspartate). The apo form has an open conformation in which the iron-binding site is near the protein surface. The iron complex is a closed system in which the metal exists below the protein surface and is inaccessible to the surrounding solution (Chung & Raymond 1993).

Lf is structurally similar to transferrin (Tf) with about 44% homology. Similar to Tf, Lf can bind two atoms of iron. For completion of the chelate rings, both Lf and

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Tf require bicarbonate ions. However, the affinity constant of 10^{24} for the iron complex is about 260-fold stronger than that of Tf. Moreover, unlike Tf, Lf avidly retains the metal in acidic environments.

Functions of Lf

Iron-binding in secretions

The two transferrins, Lf and Tf, function in a complementary manner to continuously purge body fluids of non-protein bound free iron. Thus, Tf is responsible for maintaining an environment devoid of free iron in serum, lymph and cerebrospinal fluid. Lf is assigned to exocrine secretions that are commonly exposed to normal flora: tears, nasal exudate, saliva, bronchial mucus, gastrointestinal fluids, cervico–vaginal mucus and seminal fluid.

In addition to its iron-removal function, Tf has an important second function, that is the conveyance of nutritional amounts of the metal to and from cells throughout the body. To accomplish this latter function in humans, Tf normally maintains an iron saturation value of 25–35%. At values above 35%, Tf begins to lose its effectiveness as a scavenger of hazardous iron (Kochan 1973; Weinberg 1974). In serious episodes of infection, the iron saturation value of Tf can be reduced to as low as 5%. This action markedly enhances its ability to withhold the metal from invading pathogens.

Nutritional role?

In contrast to Tf, Lf is not known to have a normal nutritional function (Sanchez et al 1992). Its ability to retain the metal at mildly low pH values would prevent the protein from quickly releasing iron in acidic endosomes as occurs with Tf. In Tf, interdomain hydrogen bonds are rapidly protonated to trigger opening of the iron cleft with prompt release of the metal (Abdallah & Chahine 2000). In contrast, interdomain hydrogen bonds of Lf do not protonate in mild acidity and iron is retained at pH values > 3.5. Accordingly, Lf fails to provide nutritional iron in hosts. For example, in a study in 2–10-month-old infants, Lf in breast milk was found to suppress, rather than enhance, iron absorption from the diet (Davidsson et al 1994). Nevertheless, in unicellular systems, iron-saturated Lf has been observed to stimulate growth of selected eukaryotes and prokaryotes, for example Fe-Lf enhanced, and apo-Lf inhibited, proliferation of human enterocytes (Caco-2 cells) (Oguchi et al 1995). Similarly, growth of *Legionella pneumophila* was stimulated by Fe-Lf and suppressed by apo-Lf (Byrd & Horwitz 1991). The ability of cells of some bacterial species to bind Fe-Lf and to derive the metal from this sole source of iron is well established (Vogel et al 1997).

Antimicrobial defence

Lf is a major constituent of the secondary specific granules of circulating polymorphonuclear neutrophils (PMNs). The apoprotein is released on degranulation of the leucocytes in septic areas. In such sites, the pH value is lowered by catabolic acids released from metabolically active invading cells as well as from PMNs. With its ability to chelate and retain iron at low pH values, Lf is a useful and probably indispensable defence protein. For disposal of iron-saturated Lf, hepatocytes might serve as a major depository (Brock et al 1994).

Several investigators have noted the joint presence of Lf and lysozyme in milk (Reiter 1983), specific granules of PMNs (Ellison & Giehl 1991), tears (Leitch & Willcox 1998) and tubotympanum mucus (Lim et al 2000). In in-vitro tests with *Escherichia coli*, *Salmonella typhimurium* and *Vibrio cholerae*, each protein alone was bacteriostatic, whereas together they were bactericidal (Ellison & Giehl 1991). In artificial tear fluid, synergy of Lf and lysozyme was observed against *Staphylococcus epidermidis* (Leitch & Willcox 1998).

Some activities of Lf require prior conversion of the apoprotein to the ferrated molecule. In these systems, the mechanism of action would most probably be associated with the oxidant activity of the metal. Intramacrophage killing of *Trypanosoma cruzi* amastigotes and *Listeria monocytogenes* was enhanced by Fe-Lf (Lima & Kierszenbaum 1987), as was suppression of intra-erythrocytic growth of *Plasmodium falciparum* (Fritsch et al 1987). Human Fe-Lf arrested growth of breast carcinoma cells by inhibition of the G1 to S transition of the cell cycle (Damiens et al 1999). Intraperitoneal injection of either iron-saturated or apo-Lf suppressed growth of solid tumours in mice (Bezault et al 1994). In systems in which serum is present, apo-Lf might obtain the requisite iron from transferrin (Fritsch et al 1987).

Examples of other activities of Lf that are not concerned with iron deprivation include enhancement of adherence of PMNs to endothelial cells (Oseas et al 1981) and functions of natural killer cells (Shau et al 1992; Bezault et al 1994; Damiens et al 1998). Lf also modulates the inflammatory process, in part, by preventing endotoxin activation of macrophage cytokine induction by binding to lipid A of lipopolysaccharide (Lee et al 1998; Baveye et al 1999). Administration of Lf before challenge with either endotoxin or bacterial

Table 1 Examples of concentrations of apo-Lf in human body fluids.

Fluid	Concn (μM)	Underlying condition	Reference
Colostrum	100	Normal	Sanchez et al (1992)
Milk	20	Normal	Hamosh (1998)
	40	Normal	Ford et al (1977)
	60	Normal	Zavaleta et al (1995)
Tears	25	Normal	Hunt et al (1996)
Seminal fluid	1.4	Normal	Buckett et al (1997)
Vaginal fluid	2.0	Just after menses	Cohen et al (1987)
	0.1	Just before menses	Cohen et al (1987)
	< 0.25	Oral contraceptive users	Cohen et al (1987)
Saliva	0.11	Normal adults	Tenovuo et al (1986)
	0.05	Normal children	Smith et al (1981)
	0.25	Children: cystic fibrosis	Smith et al (1981)
Amniotic fluid	0.02	Non-infected	Pacora et al (2000)
	0.04	Infected	Pacora et al (2000)
Cerebrospinal fluid	0.00	Normal children	Maffei et al (1999)
	0.01	Children: aseptic meningitis	Maffei et al (1999)
	0.13	Children: bacterial meningitis	Maffei et al (1999)
Synovial fluid	0.014	Non-inflammatory	Bennett et al (1973)
	0.338	Inflammatory arthritis	Bennett et al (1973)
Serum	0.005	Normal	Kelver et al (1996)
	2.5	Acute sepsis	Vorland (1999)

pathogens can protect against septic shock (Lee et al 1998; Baveye et al 1999).

Lf has been reported to inhibit replication of viruses such as cytomegalo, hanta, hepatitis C, herpes simplex virus, HIV and poliomyelitis, apparently by interfering with attachment of infectious particles to host-cell receptors (Harmsen et al 1995; Marchetti et al 1998; Tanaka et al 1999; Vorland 1999; Murphy et al 2000). Inasmuch as metal-binding causes a conformational change in Lf, the antiviral effect might be expected to vary with the percentage of iron saturation. In replication of human herpes virus in green monkey kidney cells, the ID50 of bovine Lf saturated 10% with iron was 0.36 μM, and at 90% iron was 0.15 μM (Marchetti et al 1998). Iron alone had no antiviral effect.

An antimicrobial function that does not involve iron-trapping has been suggested for specific fragments of Lf. Pepsin digestion of human or bovine apo-Lf yields basic peptide sequences, distinct from the iron-binding regions, which apparently alter cytoplasmic membrane permeability of bacteria, fungi and protozoa. The peptides, termed lactoferricins, range in length from 10–47 amino acid residues (Vorland 1999). Release of the peptides in-vivo might occur upon exposure of Lf to gastric pepsin or to pepsin-like proteases in neutrophilic phagolysosomes.

In in-vitro tests, synthetic peptides corresponding to the first eleven residues of the N terminus of human Lf have been observed to have strong bactericidal (Nibbering et al 2001) and fungicidal (Lupetti et al 2000; Ueta et al 2001) activity. However, the size and tertiary structures of the synthetic peptides may differ considerably from those of natural peptides derived from human Lf (Nibbering et al 2001). Potent antimicrobial peptides are possibly formed from human Lf at specific sites of invasion. Thus, in chemotherapy, human Lf might be more effective than synthetic peptides (Nibbering et al 2001). In a study in mice, cited below, oral human Lf was more active than a synthetic peptide in reducing the level of urinary tract infections (Haversen et al 2000).

Concentration of Lf in body fluids

Examples of the concentrations of apo-Lf in fluids of healthy and infected humans are given in Table 1. The large amount of Lf in human milk suppresses growth of such iron-dependent bacteria in the infant intestine as *Bacteroides*, *Clostridium*, *Escherichia*, *Salmonella* and *Staphylococcus* (Weinberg 2001). Accordingly, the gut of the breast-fed infant, in the absence of supplementary iron, develops a predominantly natural flora of non-pathogenic *Lactobacillus* and *Bifidobacterium*. *Lactobacillus* totally abstains from the use of iron; its enzymes utilize manganese and cobalt in place of iron (Weinberg 1997). Growth of *Lactobacillus* results in a gut pH of 5,

whereas the gut pH of formula-fed infants is 5.9–8.2. Although *Bifidobacterium* requires iron, it has developed a unique ferrous iron-acquisition system that can function at pH 5 and is, to a considerable extent, resistant to iron-withholding by apo-Lf. The fungistatic action of human milk has been shown to depend solely on its content of apo-Lf; the action is abolished by addition of iron (Andersson et al 2000).

The large concentration of apo-Lf in tears, together with lysozyme, efficiently protects ocular tissues from most bacterial pathogens. These two natural (non-immune) proteins permit much lesser reliance on secretory antibody (IgA) for antibacterial defence. Accordingly, possible scarring of delicate ocular tissues as a result of antigen–antibody reactions is minimized.

Note also in Table 1 the remarkable increase in concentration of Lf in serum during severe bacterial infection. The protein is derived from degranulating neutrophils. One million neutrophils have been estimated to contain 3 μg Lf (Bennett et al 1973). Inherited inability to produce specific granules and neutrophilic Lf is associated with recurrent infection and, in untreated persons, death (Breton-Gorius et al 1980). Moreover, the appearance of Lf in increasing amounts in serum is a very early indication of an inflammatory reaction to invasion or trauma. For example, intravenous injection of *E. coli* in piglets caused a rise in serum Lf from 0.01 μM at zero time to 0.1 μM at 1 h and 0.2 μM at 2 h (Gutteberg et al 1988). Similarly, in the initial phase of infection with *Neisseria meningitides*, serum Lf in humans increased at a rate of 0.15 μM h^{-1} (Gutteberg et al 1984).

Administration of exogenous Lf

The considerable range of activities and locations of Lf suggest that the protein might be developed for a variety of prophylactic and therapeutic applications. A number of pilot studies are available that provide information on this possibility. For example, intraperitoneal injection of recombinant human Lf in mice, followed 10 h later by intraperitoneal inoculation of *E. coli*, decreased mortality from 43 to 0% (Ward et al 1995). Topical administration of 1% bovine Lf before inoculation of herpes simplex type 1 on mouse cornea suppressed, but did not eradicate, the infection (Fujihara & Hayashi 1995).

In Table 1, it may be seen that inflamed body joints might be another appropriate site for testing exogenous Lf. Examination of synovial fluid from 25 humans with inflammatory synovitis showed that 30% of the specimens contained free iron. In these samples, concentrations of Lf were significantly lower than in those with no free iron (Guillen et al 1998). Addition of exogenous human apo-Lf to the samples consistently reduced the amount of free iron. In a subsequent study, collagen arthritis was induced in DBA/1 mice and *Staphylococcus aureus* septic arthritis was established in Swiss mice (Guillen et al 2000). In each set, peri-articular injection of human Lf significantly reduced inflammation.

The systemic mechanism of action of orally administered Lf is not well understood. The extent to which available iron in the intestine might modulate the amount of Lf digested or absorbed is unclear. In mice ($n = 24$) inoculated with *E. coli* by bladder instillation and fed 0.5 mg human Lf, serum samples at 24 h contained 0.02–1.1 nM Lf in 11 animals and none in the remaining 13 animals (Haversen et al 2000). At 2 h after feeding Lf, urine specimens contained 0.5–1.0 nM Lf, and at 5 h after feeding contained 0.2–0.4 nM Lf. In this investigation, bovine Lf and synthetic peptide sequence 16–40 were also tested orally. The human Lf-treated group showed the strongest reduction in numbers of kidney and bladder bacteria.

Pre-feeding, or intravenous injection, of human and bovine Lf reduced kidney infections in mice inoculated with *S. aureus* by 40–60% and lowered viable counts 5–12-fold (Bhimani et al 1999). In this study, apo- and holo-Lf were found to be equipotent, but hydrolysed Lf was inactive. Daily feeding of bovine Lf to guinea pigs infected with dermatophytes failed to prevent onset of symptoms during the early phase of infection, but facilitated clinical improvement of skin lesions after the peak of symptoms had occurred (Wakabayashi et al 2000). Development of various tumours in the colon, oesophagus and lungs of rats exposed to chemical carcinogens was partially suppressed by feeding bovine Lf (Ushida et al 1999). Orally administered bovine Lf inhibited angiogenesis in adult rats (Norrby et al 2001). Pre-feeding bovine Lf to germ-free piglets provided significant protection against lethal shock induced by intravenously administered endotoxin (16.7 vs 73.7% mortality; $P < 0.001$) (Lee et al 1998). In a study by Tanaka et al (1999), human patients with hepatitis C were fed bovine Lf for eight weeks. Three of four patients with low pre-treatment levels of viremia experienced a decrease in serum values of HCV-RNA and alanine transaminase. However, no significant changes occurred in seven patients who had high pre-treatment levels of viremia.

Exogenous Lf might be useful as an adjunct in antimicrobial therapy. In in-vitro tests, effective concentrations of diverse antifungal drugs were lowered against

Pneumocystis carinii (Cirioni et al 2000) and *Candida* sp. (Wakabayashi et al 1998; Kuipers et al 1999) by combination with either bovine or human Lf. Similarly, bacteriostatic and bactericidal concentrations of rifampin and doxycycline were lowered against *Pseudomonas aeruginosa* and *Burkholderia cepacia* by combination with recombinant human Lf (Alkawash et al 1999). Of course, only pathogens unable to use Lf as an iron-carrier could be safely attacked in this manner. To date, fungi are not known to extract iron from transferrins such as Lf. However, *Trichomonas* protozoa (Weinberg 1999) and *Helicobacter pylori* bacteria (Dhaenens et al 1997), as well as various members of the bacterial family *Neisseriaceae* (Vogel et al 1997), can acquire iron from human Lf.

Production of recombinant human Lf has been reported in a variety of organisms. These include baby hamster kidney cells (Stowell et al 1991), *Aspergillus nidulans* and *Aspergillus awamori* (Ward et al 1992, 1995), *Saccharomyces cerevisiae* (Liang & Richardson 1993), transgenic dairy animals (Krimpenfort 1993) and transgenic potatoes and tomatoes (Arakawa et al 1999). In the *A. awamori* fermentation, quantities in excess of 25 μM have been obtained. The protein molecules are glycosylated and have excellent metal-binding and antibacterial activity.

Possible hazards of exogenous Lf

A major advantage of human Lf over other chelating drugs is that it is a natural human product and thus should be biocompatible. However, an important potential hazard of therapeutic use of human Lf in human patients is the possible induction of an antibody response. Antibodies to endogenous Lf have been detected in patients with autoimmune diseases such as systemic lupus erythematosus, rheumatoid arthritis and primary sclerosing cholangitis (Skogh & Peen 1993; Afeltra et al 1996). In some patients with rheumatoid arthritis, antibodies to Lf are present in synovial fluid (Guillen et al 1998). Unfortunately, anti-Lf IgG can cause Lf-bound iron to become reactive in the bleomycin assay (Guillen at al 1998).

Another possible hazard of exogenous human Lf is stimulation of growth of specific pathogens. As mentioned above, *Trichomonas vaginalis* obtains iron from human Fe-Lf. The protozoan grows mainly in the Lf-rich environment of human vaginal mucus. Disease symptoms begin or exacerbate during menses at which time the vaginal concentrations of Lf and iron are notably greater than at midcycle. In the male urethra, the illness is self-limited or asymptomatic (seminal fluid contains Lf, but is very low in iron, as is urine). Fortunately, *T. vaginalis* cannot obtain iron from Tf and thus fails to cause systemic infections in either women or men (Weinberg 1999). Were human Lf to be used in treatment of vaginal yeast infections, the patients would first need to be carefully evaluated for freedom from trichomoniasis.

Another human pathogen that can specifically derive iron from human Lf is *H. pylori*. This bacterium is the major aetiological agent of chronic gastritis and is a component of the aetiology of gastric ulcers and carcinomas. Cells of this pathogen form a 70-kDa human Lf-binding protein. *H. pylori* also can obtain iron from haeme, but not from human Tf or from bovine or equine Lf or Tf (Dhaenens et al 1997). Thus, bovine Lf can suppress *H. pylori* infection in mice (Dial et al 1998; Wada et al 1999). The singular location of *H. pylori* in human gastric epithelium is apparently a consequence of the availability of human Lf and iron in gastric juice. In a set of 30 *H. pylori* positive and 14 *H. pylori* negative patients with chronic gastritis (Nakao et al 1997), the average level of endogenous Lf in the former was 4.25-fold greater than that in the latter ($P < 0.0007$). In in-vitro susceptibility tests of *H. pylori* to apo-recombinant human Lf, 5 of 13 strains required 10 μM for inhibition, 3 strains required 20 μM, and 5 strains needed > 40 μM (Miehlke et al 1996). No strains were sensitive to low concentrations of the protein. Thus human apo-Lf might not be a successful therapeutic agent for *H. pylori* and could actually intensify the infection. In a recent study, 6 adult humans with *H. pylori* infection were fed 1.25 g and 6 were fed 5 g recombinant human Lf over a 24-h period. The amount of endogenous Lf had not been ascertained. Not surprisingly, none of the 12 subjects cleared the infection. Fortunately, no adverse effects were observed (Opekun et al 1999).

In contrast to *H. pylori*, *Helicobacter felis* does appear to be susceptible to the antibacterial action of human Lf. In mice infected with 3×10^9 viable cells of *H. felis*, recombinant human Lf partially reversed both the infection-induced gastritis and the infection rate (Dial et al 2000). In this system, the efficacy of Lf was comparable with that of amoxicillin as well as with the combination of metronidazole, tetracycline and bismuth subsalicylate.

Perspectives

As recombinant human Lf becomes increasingly available, it may be appropriate to substitute this product for bovine Lf in human studies. However, in some systems, bovine Lf has been observed to be more effective than

human Lf. For example, the ability of *Prevotella nigrescens*, a bacterium associated with dental infections, to adhere to an enamel component (hydroxapatite) was suppressed to a greater extent by bovine than by human Lf (Yasuyuki et al 2001). In a study of the cytopathic effect of HIV-1 on MT4 cells, the IC50 of bovine Lf was 0.5 μM and of human Lf was 1.0 μM (Harmsen et al 1995).

Furthermore, as with any iron chelator, it will be essential to recognize and monitor the iron background of the system under investigation. Effective doses of Lf would be expected to vary with the level of iron available to the protein. The degree to which iron might be required by Lf for the specific activity should also be determined. With the exception of the investigations on synovial fluid (Guillen et al 1998), the pilot studies cited above failed to ascertain tissue or fluid values for endogenous Lf and iron.

In systems in which Lf might to be used as an adjunct to, or a replacement for, antibacterial drugs, consideration should be given to pairing it with lysozyme. However, disease states that would probably not be helped by lysozyme include fungal and viral infections, cancers and sterile inflamed areas.

Sanchez et al (1992) proposed that the "biologic role of Lf is that of a specialized iron-scavenging protein, designed to act particularly under conditions where Tf would be less effective at binding iron due to reduced pH, such as exist in the gastrointestinal tract or inflammatory lesions. By binding iron under these conditions it would render harmless free iron that might otherwise cause free radical-mediated damage to sensitive tissues, reduce absorption of iron in the immediate postnatal period, and decrease its availability to microorganisms". During the past decade, successful research applications of administration of exogenous Lf have tended to validate this proposal. In the coming decade, some of these applications might well be developed into prophylactic or therapeutic products.

Conclusions

Lf, a 78-kDa iron-binding protein, provides antioxidant and antimicrobial activity in secretions of lacrimal and mammary glands and of respiratory, gastrointestinal and genital tracts. It is also released from neutrophils at sites of infection and can scavenge non-protein-bound iron in areas that have lowered and neutral pH values. Recombinant human Lf is becoming available for evaluation of possible prophylactic or therapeutic use in a wide variety of human medical conditions. As a human natural product, it should be efficiently metabolized with no side-effects. However, precautions are needed to avoid antigenic sensitization as well as introduction of the protein into tissues that may be infected with specific protozoa or bacteria that use Lf in their acquisition of host iron.

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