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LACTOFERRIN: A GENERAL REVIEW

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ABSTRACT

Lactoferrin is a 703-amino acid glycoprotein originally isolated from milk. Plasma lactoferrin is predominantly neutrophil derived but indications are that it may also be produced by other cells. Lactoferrin in body fluids is found in the iron-free form, the monoferric form and in the diferric form. Three isoforms of lactoferrin have been isolated, ie two with RNase activity (lactoferrin-β and lactoferrin-γ) and one without RNase activity (lactoferrin-α). Receptors for lactoferrin can be found on intestinal tissue, monocytes/macrophages, neutrophils, lymphocytes, platelets, and on certain bacteria. A wide spectrum of functions are ascribed to lactoferrin. These range from a role in the control of iron availability to immune modulation. More research is necessary however to obtain clarity with regard to the exact mechanism of action of lactoferrin.

Key words: lactoferrin, lactotransferrin, iron-binding protein, immunomodulation

The name lactoferrin is derived from its past classification as a major iron-binding protein in milk. Lactoferrin, also referred to as lactotransferrin, was first identified in 1939 in bovine milk,[1] and in 1960 it was isolated from human milk by Johannson.[2] Subsequently it has also been shown to be a major iron-binding protein of other exocrine secretions such as bile, pancreatic juice and small intestinal secretions, and has been localized in a host of other tissues, both in man and in other mammals.[3] The size and structure of lactoferrin is closely related to that of another group of iron-binding proteins, the tranferrins, and lactoferrin is considered by many to be a member of the transferrin family.[4] Plasma lactoferrin is currently considered to be predominantly neutrophil derived but indications are that it may also be produced by other cells. In the past it was traditionally seen as a mere bacteriostatic iron-transporting protein of milk, but this view is being challenged by recent research findings.

Structure and properties

The controversies surrounding lactoferrin function are probably the result of misconceptions and ignorance about its structure. The complete amino acid sequence of human lactoferrin has been determined and found to contain 703-amino acid residues.[4] Hololactoferrin consists of a single polypeptide chain folded into two globular lobes, each with one iron binding site.[5] Iron binding to lactoferrin occurs concomitantly with the bonding of two bicarbonate anions, a process essential for the ligation of iron to lactoferrin.[6] There is a notable degree of internal homology between the two lobes (residues 1-338 and 339-703, respectively), which demonstrates 125 (or 37%) identical amino acid residues in the corresponding portions.[4] This has led to a theory of gene duplication, proposed to have occurred some 500 million years ago when the original 40 kDa molecule duplicated, forming the two domains and thus giving rise to a family of proteins with molecular masses in the range of 80 kDa (Table 1).[7] Lactoferrin is suggested to be the youngest of the transferrins.

Lactoferrin is a basic glycoprotein with an isoelectric point of 8.7.[8,9] Human milk lactoferrin has two poly-N-acetyl-lactosaminic glycans

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Table 1. Molecular weight of human milk lactoferrin as determined using various methods.

	Molecular weight (daltons)	*References*
Milk (apo form; by electrophoresis)	76,800	(5)
Milk (apo form; by sedimentation)	75,000	(5)
Milk (apo form; theoretical)	76,400	(5)
Milk (from amino acid sequence)	82,400	(4)
Milk (dry weight determination)	78,000	(18)
Milk (holo form; by sedimentation)	82,600	(7)

that contain N-acetylneuraminic acid (sialic acid), fucose and galactose.[4,7] These sugars have been found to bind to asparagine residues 137 and 478, one located in the C- and the other in the N-terminal zone.[4,9] The primary structure of human polymorphonuclear neutrophil (PMN) lactoferrin glycans is identical to that of the major glycans from human milk lactoferrin. The two glycans, attached to lactoferrin through N-glycoside linkages, are nonetheless structurally heterogeneous and differ from those of other transferrins.[4,9] The precise role of these glycans has not been established, and their removal is said to have no apparent effect on lactoferrin functions and properties, such as receptor binding.[6,10] However, this assumption has been contested by isolated studies in which a role in receptor binding was implicated.[11]

Lactoferrin is remarkably resistant to proteolytic degradation by trypsin and trypsin-like enzymes, rendering it at least partially resistant to digestion in the gut.[6] This property, postulated to be glycan-dependent, facilitates neonatal absorption of lactoferrin from maternal milk. It is of interest that the iron-saturated form (ie hololactoferrin) is more resistant to proteolysis than the apoform.[12] Lactoferrin not only binds iron but copper, zinc, manganese, gallium,[13-15] and possibly vanadium as well.[16] The degree of lactoferrin iron saturation in plasma is unknown.[17]

Similarities between lactoferrin and other transferrins, like transferrin and ovotransferrin, are pronounced. The same polypeptide folding pattern is found in all members of the transferrin family.[7,9] Lactoferrin, like transferrin, is an iron transporter and as such exists in both the hololactoferrin (iron-saturated) and apolactoferrin (iron-depleted) form. The molecular mass of transferrin (apo-form: 75-76.6 kDa; holo-form: 73.8-86 kDa) lies within the reported range for lactoferrin (apo-form: 75-76.4 kDa; holo-form 82.6 kDa).[5] The amino acid compositions of lactoferrin and transferrin were found to be closely related,[5,19] with 59% and 49% homology between the two corresponding domains of the respective molecules.[4] The secondary structures, including their disulphide linkages,[18] as well as the tertiary ones[7] are notably similar. These findings have led to speculation that the two molecules may share the same phylogenic origin.[5,19] Lactoferrin, however, differs from transferrin in its immunologic or antigenic properties, carbohydrate composition, water solubility, isoelectric point, and the localization of its iron binding and glycosylation sites.[3,4,7,20] Lactoferrin and transferrin have, as previously mentioned, comparable molecular masses with similar C-terminal and N-terminal iron-binding domains, consisting of β-sheets as well as α-helices.[9] The inter-lobe connecting peptide is however helical in lactoferrin, while in transferrin it is irregular. The binding site for each lobe, which houses the Fe^{3+} and CO_3^{2-} ions, lies deep within the interdomain cleft. The iron binding sites in the N- and C-lobes are similar: three anionic ligands, 2 tyrosine and 1 aspartic amino acids, with a fourth neutral histidine amino acid that matches the plus three charge on the metal ion, forming a hydrogen bonding network.[9] The role of the carbonate anion is proposed to be twofold: (a) it neutralizes positive charges which might otherwise repel the cation, and (b) it partially prepares the metal binding site on the apo-protein by adding two more potential ligands.[9]

Crystallographic studies have shown conformational changes upon iron-binding in both lactoferrin and transferrin.[9] Iron-binding affinities and characteristics of the individual lobes have been well studied for transferrin,[21] but less is known about these characteristics for lactoferrin. Transferrin can exist in any of four molecular forms:[9,22,23] apotransferrin, monoferric

transferrin, either in the A- or B-form, and diferric transferrin. As the degree of iron saturation increases the apparent molecular mass of transferrin decreases, implying that as iron binds to transferrin the binding areas must induce a conformational change that leads to a closed iron-binding domain. Separation of three lactoferrin forms has also been successfully performed using high-performance liquid chromatography, but absolute certainty about the existence of four iron-binding forms of lactoferrin has not as yet been achieved, since differentiation between possible A- and B-forms of monoferric lactoferrin by electrophoresis has not been carried out.[24]

It has long been recognized that apotransferrin and iron-saturated transferrin differ in their reactivities to specific antisera on crossed immuno-electrophoresis.[25] These findings have severe implications for determinations in which the antibodies used were raised against only one of the forms of the transferrins. The conformational change in lactoferrin that occurs when it binds iron, and its implication in lactoferrin level determination is also emphasized by findings that hololactoferrin has an altered plant lectin binding capability with respect to the apoform.[26] This gives additional substance to findings that certain forms of lactoferrin have a higher affinity for lactoferrin receptors than others.[13] The specific receptor affinity of lactoferrin and transferrin could perhaps also be ascribed to the difference between lactoferrin and transferrin inter-domain interactions.[9] The molecule exhibits a pronounced tendency to polymerize *in vitro* as well as *in vivo* at concentrations as low as 10^{-10} M.[27,28] This may possibly further contribute to the wide range of reported serum lactoferrin levels.

Lactoferrin is known to exist in various isoforms.[8] Three such isoforms, two with RNase activity (termed lactoferrin-β and lactoferrin-γ) and one without RNase activity (termed lactoferrin-α), have been isolated; all three are present in both human breast milk and in granulocytes.[8,29] These isoforms share the same physical, chemical and antigenic characteristics, but differ in their functional properties. The iron-independent isoforms with RNase activity do not exhibit functional iron-binding, while the iron-binding isoform has no RNase activity.[8] These findings may partially explain the reported diversity in functions attributed to lactoferrin.

Lactoferrin levels in plasma

Lactoferrin is present in plasma in relatively low concentrations, with substantially higher levels being found in colostrum, human breast milk, and seminal plasma. Markedly higher levels occur in cord blood, tears, and vaginal mucus (Tables 2-4). The reported differences are probably attributable to factors such as (a) analytical methods, (b) the type of anticoagulant used, (c) variations in lactoferrin iron saturation, (d) the reported spontaneous *in vivo* as well as *in vitro* polymerization,[27,28] and (e) the time interval between venipuncture and analysis or storage.

Plasma lactoferrin is predominantly neutrophil derived.[6] Its presence in specific granules is often used to identify these granules. However, recent findings have shown that lactoferrin is also found in other granules, probably tertiary ones, albeit in lower concentrations.[30] Plasma lactoferrin concentrations may or may not correlate with the neutrophil count,[31-33] depending on the magnitude of degranulation and perhaps the contribution of other organs, such as bone marrow, endometrium and placenta, to the plasma content of lactoferrin.[34-36] A summary of other lactoferrin-containing tissues has been provided elsewhere.[19]

Several authors reported higher lactoferrin levels in males than in females;[17,37-39] one reported similar levels, but a greater standard deviations for females,[17] and yet another reported higher levels in females than males.[40] In view of the higher granulocyte lactoferrin content found in men by Freeman *et al.*,[41] one cannot dismiss the higher plasma levels in males reported by the majority of workers as a mere degranulation difference.

Lactoferrin plasma levels change during pregnancy. The changes in maternal plasma lactoferrin levels manifest as a progressive rise in concentration, with stabilization at week 29 of pregnancy.[38] Several factors may contribute to this:

Table 2. Reported human blood lactoferrin levels.

Blood	*Level*	*MD*	*MC*	*Ref.#*
Blood	0.2-1.5 µg/mL	RIA	EDTA	(49)
	0.05-0.250 µg/mL	LSA	EDTA	(50)
	0.02-0.20 µg/mL	ELISA	–	(51)
	<1.00-3.50 µg/mL	RID	Serum	(52)
	0.13-0.42 µg/mL	RIA	EDTA	(31)
	0.385±0.153 µg/mL	RIA	Serum	(33)
	1.520±0.560 µg/mL	RIA	Heparin	(53)
	0.292±0.110 µg/mL	RIA	Serum	(54)
	0.108±0.059 µg/mL	RIA	EDTA	(54)
	0.888±0.334 µg/mL	RIA	EDTA	(55)
	1.500±1.400 µg/mL	RIA	EDTA	(56)
	0.040-0.100 µg/mL	ELISA	EDTA	(57)
	0.134±0.079 µg/mL	ELISA	EDTA	(58)
	0.307±0.066 µg/mL	ELISA	Serum	(59)
	0.012±0.002 µg/mL	RIA	EDTA	(60)
	0.250-0.750 µg/mL	RIA	EDTA	(61)
	0.540±0.260 µg/mL	ELISA	Serum	(62)
	0.046-0.257 µg/mL	ELISA	EDTA	(63)
	0.168±0.100 µg/mL	ELISA	EDTA	(34)
	0.237±0.155 µg/mL	ELISA	Serum	(34)
♂	0.0978 µg/mL	ELISA	EDTA	(37)
	0.150±0.067 µg/mL	RIA	EDTA	(38)
	0.307±0.141 µg/mL	ELISA	EDTA	(40)
	0.206±0.060 µg/mL	RIA	EDTA	(39)
	1.620±0.077 µg/mL	RIA	EDTA	(17)
♀	0.0847 µg/mL	ELISA	EDTA	(37)
	0.100±0.048 µg/mL	RIA	EDTA	(38)
	0.326±0.127 µg/mL	ELISA	EDTA	(40)
	0.140±0.060 µg/mL	RIA	EDTA	(39)
(pre-menopausal)	0.750±0.036 µg/mL	RIA	EDTA	(17)
(post-menopausal)	1.74±0.10 µg/mL	RIA	EDTA	(17)
Venous plasma	0.122±0.040 µg/mL	EIA	EDTA	(34)
Capillary plasma	0.107±0.073 µg/mL	EIA	EDTA	(34)
Fetal serum	0.05 µg/mL	RIA	–	(36)
Cord Blood	25.8 & 28.0 µg/mL	RIA	EDTA	(17)
(capillary)	0.385±0.113 µg/mL	RIA	EDTA	(34)
	0.02-0.30 µg/mL	ELISA	–	(51)
Infant (7 weeks)	0.267±0.176 µg/mL	RIA	EDTA	(34)
(capillary) (11 weeks)	0.269±0.163 µg/mL	RIA	EDTA	(34)
(15 weeks)	0.176±0.165 µg/mL	RIA	EDTA	(34)

[MD=method of determination; MC=method of collection; RIA = radioimmunoassay; RID = radial immunodiffusion; LSA = luminescence-based sandwich assay; EIA = solid phase enzyme immunoassay; ELISA = enzyme-linked immunosorbant assay]

Table 3. Reported lactoferrin levels in human neutrophils.

Neutrophils	*Level*	*MD*	*Ref.#*
Blood	15 µg/10^6 neutrophils	–	(64)
♂	2.12 µg/10^6 neutrophils	ELISA	(37)
♂	29.2±2.2 µg/10^7 neutrophils	IRA	(41)
♀	1.78 µg/10^6 neutrophils	ELISA	(37)
♀	20.4±2.0 µg/10^7 neutrophils	IRA	(41)
Adults	89.0±7.3 µg/mg cell protein	RE/FA	(65)
Adults	59.6±5.5 µg/10^7 neutrophils	IRA	(66)
Term neonates ♂	12.0±0.6 µg/10^7 neutrophils	IRA	(41)
Term neonates ♀	12.6±0.4 µg/10^7 neutrophils	IRA	(41)
Neonates	30.6±6.1 µg/10^7 neutrophils	IRA	(66)
Newborn	43.2±7.0 µg/mg cell protein	RE/FA	(65)

(MD=method of determination; RIA = radioimmunoassay; ELISA = enzyme-linked immuno sorbent assay; RE/FA = rocket electrophoresis and fluorescent assay).

(a) pregnancy-associated leukocytosis;[42] (b) the reported selective increase in the lactoferrin granular content of neutrophils, while myeloperoxidase content remains the same;[43] (c) a contribution to maternal plasma levels by decidua-derived lactoferrin[36] and, perhaps, (d) a hormonal influence on lactoferrin production by tissues other than the endometrium or decidua, such as breast acini.[44] Indications are that lactoferrin levels may indeed be influenced by endocrine activity. Such a lactoferrin-hormonal link is implicated by (a) the larger standard deviation seen in female serum,[17] (b) the suggested endometrial lactoferrin production during the secretory phase of the menstrual cycle,[35] (c) the increase in plasma levels during pregnancy,[38] (d) in an indirect way, by the correlation between neutrophil count and urinary estradiol levels,[45] (e) the higher postmenstrual than premenstrual levels in vaginal mucus,[46] (f) the decrease in maximal vaginal lactoferrin levels found in women on oral contraceptives,[46] (g) the reported tendency of plasma lactoferrin levels to vary with the menstrual cycle,[47] (h) the differences between male and female levels, (i) the hormonal dependency of prostate lactoferrin concentration,[48] and (j) the higher postmenopausal plasma levels.[17,39] These findings may however be epiphenomenal in nature.

Lactoferrin levels in milk, amniotic fluid and neonates

Lactoferrin is present in the milk of all mammalian species investigated to date with the exception of the dog and the rat.[67] Levels in bovine milk are, however, significantly lower

than those in human milk. Masson et al.[67] in fact showed that the levels in human breast milk were the highest among the ten different species investigated. Approximately 30% of the iron in human milk is bound to lactoferrin.[68] It is estimated that only 6-8% of milk lactoferrin is iron saturated, which correlates with the finding of Makino and Nishimura[24] that 95% of milk lactoferrin is in the monoferric and/or apolactoferrin form. Lactoferrin levels in human milk do not appear to be dependent on body iron status, but rather on the general state of maternal nourishment. Lactoferrin is said to be generally lower in malnourished mothers.[69]

Various authors have found colostrum lactoferrin concentration to be significantly higher than that of milk ejected after this period. The levels in milk do not however decline any further upon prolonged lactation.[70] Although no correlation could be shown between gestational age and lactoferrin levels,[71] there is general consensus that the colostrum of preterm deliveries contains significantly higher lactoferrin concentrations than that of full-term deliveries.[71,72] It is unlikely that this could be attributed to either the relatively smaller volume or the higher neutrophil count in preterm colostrum, since there is no difference in protein levels between preterm and full-term colostrum and the difference in neutrophil count is too small to be responsible.[72,73] The initially increased lactoferrin level in preterm colostrum then declines over the colostrum producing period.[73] The finding of markedly higher lactoferrin level in colostrum than in serum despite the substantially lower neutrophil count of colostrum suggests either the production of lactoferrin by mammary tissue, or the active transport of lactoferrin against a concentration gradient.

Lactoferrin levels in amniotic fluid were found to be undetectable before the 20th week of pregnancy.[36] A significant increase is said to occur around week 30, whereafter it remains high until term. Lactoferrin levels in the decidua, amnion and chorion membranes, trophoblast and umbilical cord are shown in Table 4. Indications are that amniotic fluid lactoferrin may be of decidual origin.[36] It is of interest that maternal plasma lactoferrin levels demonstrate a corresponding initial increase and finally stabilize at week 29 of pregnancy.[38] It is therefore plausible that this increase in plasma lactoferrin during pregnancy could be of decidual origin. Amniotic lactoferrin concentrations are, surprisingly, the highest reported levels after those of colostrum, milk, tears and seminal plasma.

Lactoferrin production in the fetus depends on gestational age and was found, by immunohistochemical detection, from 13 weeks onwards.[79] The presence of lactoferrin in fetal salivary glands at a certain level of cytodifferentiation, and the reported decline in salivary gland lactoferrin shortly after birth suggest a contribution to fetal lactoferrin levels by organs other

Table 4. Reported lactoferrin levels in various human secretions and tissues.

Fluid and tissue	*Level*	*MD*	*Ref.#*
Colostral milk	5-7 mg/mL		(67)
(preterm)	6.76±1.50 mg/mL	RIA	(71)
(full-term)	3.10±0.50 mg/mL	RIA	(71)
	6.7±0.7 mg/mL	RIE	(70)
Transitional milk	3.7±0.1 mg/mL	RIE	(70)
Mature breast milk	1-2 mg/mL	RIE	(74)
(human)	1.97-3.20 mg/mL	RIA	(73)
	2.6±0.4 mg/mL	RIE	(70)
Amniotic fluid	2-32 μg/mL	RIA	(36)
Decidua	9-95 μg/g protein	RIA	(36)
Amniotic membrane	2-37 μg/g protein	RIA	(36)
Chorion membrane	2-26 μg/g protein	RIA	(36)
Trophoblast	5-35 μg/g protein	RIA	(36)
Umbilical cord	< 1 μg/g protein	RIA	(36)
Bronchial mucus	35.2±6.5 μg/mL	–	(75)
Tear fluid	2.2 mg/mL	ELISA	(76)
Vaginal mucus			
Postmenstrual	62.9-218 μg/mg protein	–	(46)
Premenstrual	3.8 -11.4 μg/mg protein	–	(46)
♀ on oral contraceptives	≤ 19.8 μg/mg protein	–	(46)
Seminal plasma	1.18±0.74 mg/mL	RID	(77)
Synovial fluid	46.4±35.9 μg/mL	–	(78)

(MD = method of determination; RIA = radioimmunoassay; RID = radial immunodiffusion; ELISA = enzyme-linked immunosorbent assay; RIE = rocket immunoelectrophoresis).

than blood cells.[80] It is also possible that some of the fetal lactoferrin may originate from amniotic fluid, which has significantly higher lactoferrin levels than either fetal or maternal sera. At present it does not appear that lactoferrin can cross the placenta.[81] This view is supported by the demonstrated lack of correlation between maternal and neonatal lactoferrin concentrations.[81]

Plasma lactoferrin levels in the neonate are still controversial. The first reported levels in cord blood were 25.8 and 28.0 μg/mL (n=2), which were at least ten times higher than the values found in adults in the same study.[17] Some authors detected differences of a less significant magnitude between full-term infants (0.385± 0.113 μg/mL) and adults (0.122±0.040 μg/mL), while others could show no difference between neonatal and adult levels.[51,81] Some even reported an inability to detect any lactoferrin in cord blood.[36]

Independent from whether or not plasma lactoferrin levels in the neonate and infant are indeed elevated, these values would appear to stabilize at normal adult levels by the age of 15 weeks.[34] Neonatal plasma lactoferrin levels depend on various factors such as the neutrophil count, neutrophil lactoferrin content, degranulation characteristics, lactoferrin half-life, as well as possible maternal contributions to the fetal plasma lactoferrin pool. Neonatal leukocytosis, which disappears within a week after birth, is well known;[69] however, some controversy exists with regard to granular lactoferrin content, which many report to be decreased,[41,65,66] but which one group of workers found to be comparatively normal.[82]

The general impression with regard to fetal lactoferrin release characteristics would appear to be that of a slight suppression of degranulation, possibly resulting from a subnormal ligand-receptor interaction.[66,82] These findings would, to a degree, correlate with other reports of suboptimal leukocyte activity in the newborn.[83] By the same token less than normal RES activity may prolong lactoferrin half-life. The relatively high neonatal lactoferrin levels with respect to adult values cannot, however, be explained solely by an immature RES.

Immunogeneity of lactoferrin from different human sources

The question arises whether any difference exists between lactoferrin from various sources (eg. milk or neutrophils). A study employing double immunodiffusion analysis on human breast milk, colostrum, apolactoferrin and neutrophil lactoferrin did not reveal any obvious disparity among them.[64] The complete DNA sequence of the human mammary lactoferrin gene shows 99.7% agreement with a partial sequence of neutrophil cDNA, and a deduced amino acid homology of 97% to the sequence of human milk lactoferrin.[4] Certain investigators, on the other hand, found a difference in the terminal fucose residues of its glycan chains (which are required for lactoferrin binding to macrophages) between neutrophil- and human milk-derived lactoferrin,[11] while others demonstrated that individual antibodies can be produced which could differentiate between milk and neutrophil lactoferrin.[84] However, it is possible that this observation could be the result of antibody specificity for various iron-saturation forms of lactoferrin. The majority of the existing studies were performed without considering the presence of various isoforms or the degree of lactoferrin iron-saturation.

Sample collection procedure for optimal lactoferrin levels

Correct specimen collection for lactoferrin analyses is of paramount importance. Variations in collection techniques such as the use of heparin instead of EDTA collection tubes are known to give rise to unreliable results.[38] For reliable results it is suggested that (a) EDTA be used as anticoagulant, (b) minimum stasis be applied during venipuncture, (c) separation/centrifugation be performed as soon as possible after blood sampling, or at the latest within 5 hours of blood collection, (d) if separation is delayed, blood be stored at 4°C, and (e) centrifugation be performed preferably at 4°C.[34,50,54,85]

Metabolism of lactoferrin

Lactoferrin is produced in neutrophils and

stored, in the iron depleted state, in the specific granules and possibly in the tertiary granules.[6,86] It appears that the steroid-thyroid receptor superfamily works in concert to modulate lactoferrin gene expression. This supports the hypothesis that lactoferrin levels are hormone dependent. A detailed discussion is perhaps beyond the scope of this writing. Lactoferrin, unlike myeloperoxidase and some other granular products, is not synthesized as a larger precursor and was found to be unphosphorylated.[87] Lactoferrin transfer to its storage granules is dependent on acidification mechanisms and occurs through the medial and transcisternae of the Golgi apparatus.[87] It therefore appears to be processed like proteins destined for secretion. The neutrophil lactoferrin within these granules has two destinations: it can either be secreted into the surrounding tissues or blood,[86] or the granules can fuse with phagosomes.[88] Secretion from polymorphonuclear cells into the circulation is dependent on degranulation factors, which in turn appear to be dependent on the activation of guanylate cyclase, cGMP and proteinkinase C (calcium dependent). This occurs in both aerobic and anaerobic conditions, is unaffected by the presence of hydrogen sulphide and is stimulated by interleukin-8 and surface bound IgG.[88,89] Plasma lactoferrin levels generally increase in iron overload, inflammation, infectious diseases, and during tumor development, demonstrating a multifactorial stimulatory mechanism for lactoferrin release from neutrophils.[90]

Upon release lactoferrin binds metal ions, of which iron has been the most intensively studied. The precise relationship of serum apo- to hololactoferrin has not as yet been determined, because such determinations pose certain experimental difficulties. Lactoferrin removal from circulation appears to occur in one of two ways. First, lactoferrin can be removed from the circulation, as well as from the interstitial spaces, by what would appear to be receptor-mediated endocytosis into phagocytic cells such as macrophages, monocytes and other cells of the RES, with subsequent transfer of the iron to ferritin.[53,86,91] In experiments conducted with rats, the half-life of injected hololactoferrin was prolonged threefold by blocking the RES.[86] Some controversy with regard to the cells involved in this manner of lactoferrin removal still exists.[92] The alternative way of lactoferrin removal would be its direct uptake by the liver through an iron saturation-independent, clathrin-dependent, calcium-dependent process of endocytosis.[93] Kupffer and liver endothelial cells, as well as hepatocytes appear to be involved.[93] The binding sites may perhaps be the same as those for transferrin binding, since lactoferrin was shown to inhibit transferrin uptake by rat hepatocytes.[93]

Bennet and Kokocinski showed that labelled lactoferrin was rapidly cleared from the circulation by the liver and spleen, with all lactoferrin being removed within 7 hours after injection.[55] It is as yet not sure whether lactoferrin, like transferrin, is recycled.[94] Further research is needed to fully understand lactoferrin metabolism in the human adult.

The kidneys appear to play a role in lactoferrin clearance from the circulation since both lactoferrin and lactoferrin fragments were found in the urine of infants.[95] It is of interest to note that the lactoferrin found in breast-fed infants is predominantly of maternal origin.[95] Low molecular fragments of lactoferrin were also reported in stools.[96] Both fecal and urinary elimination of lactoferrin, however, need further investigation because significant controversy still exists.

Lactoferrin receptors

Lactoferrin is a basic protein with a high isoelectric point (8.7), enabling it to undergo nonspecific binding to many target cells or proteins.[9] Some studies with lactoferrin fragments indicate that part of the N-lobe (residues 1-90) is involved in lactoferrin receptor binding.[97] Other studies however found regions in both the C- and N-lobes of human lactoferrin that bind to bacterial lactoferrin receptors.[98] Lactoferrin receptors have been identified in the gastrointestinal tract, on leukocytes and macrophages, platelets, and on bacteria. A summary of these receptors is presented in Table 5.

Receptors	*Molecular mass (kDa)*	*Affinity constant (Ka)*	*Specificity*	*Ref.#*
Intestinal	114 (nonreducing) 38 (reducing)	0.3×10^{-6}	+ hololactoferrin + apolactoferrin + deglycosylated lactoferrin + lactoferrin fragments – bovine lactoferrin – transferrin	(6,10)
Monocytes	–	4.5×10^{-9}	+ lactoferrin + transferrin	(99)
Macrophages	–	1.7×10^{-6}	+ lactoferrin	(91)
Neutrophils	–	2.2×10^{-9} 0.6×10^{-9}	+ lactoferrin	(100)
Platelets	–	13.6×10^{-9} 1.23×10^{-9}	+ lactoferrin + transferrin + bovine lactoferrin	(101)
Bacterial: – *Staphylococcus aureus* – *Aeromonas hydrophilia* – *Neisseria meningitides* – *Haemophilus influenzae* – *Shigella flexneri*	–	–	–	(102,103) (74,104) (105,106)
Silent: – *Albumin* – *IGA* – *Casein* – *Secretory component* – *Lysozyme* – *ß–lactoglobulin* – *DNA*	–	–	–	(107,108) (95,109)

Table 5. Lactoferrin receptors identified.

The biological role of lactoferrin

Some controversy still exists as to the exact role and mechanism of action of lactoferrin. It has now been shown that lactoferrin does indeed play a role in the host defense mechanism as well as in iron metabolism. Its role in the host defense mechanism involves much more than that of a mere bacteriostatic agent. Lactoferrin, in addition to its bacteriostatic function, can also exert a bactericidal effect and can curb the proliferation of other microbes such as fungi and viruses. Moreover, it has an extended role in the body's defense mechanism through its immune modulatory actions. The major role of lactoferrin in iron metabolism would appear to be in the control of iron availability. Other mechanisms in which lactoferrin is implicated include a growth regulatory function in normal cells, coagulation, and perhaps cellular adhesion modulation. Lactoferrin is known to have a tendency to bind to a number of other molecules or silent receptors. The functional significance is not clear but certain facts related to these interactions are slowly beginning to emerge.

Role in iron metabolism

Lactoferrin from maternal milk is known to be absorbed in the intact form from the gut of infants.[95] The observation of a higher lactoferrin concentration and a higher iron availability in human than in bovine milk gave rise to the hypothesis that lactoferrin might promote iron absorption in breast-fed infants. This appears to be substantiated by the finding of better iron absorption in breast-fed infants than in newborns on bovine milk-based formulas.[110] Whether lactoferrin does indeed augment iron absorption is, however, still controversial[34] but several reports would seem to support such a possibility, among others:

i) the ability of human enterocytes to extract iron from lactoferrin;[3]
ii) the high lactoferrin uptake by enterocytes;[3]
iii) the correlation of neonatal urinary iron excretion with milk lactoferrin content as well as with breast milk uptake;[67]
iv) the transport of iron across the intestinal brush border by lactoferrin;[13]
v) the accumulation of iron from lactoferrin in brush border membrane vesicles.[13]

Lactoferrin may perhaps affect cellular mechanisms through its influence on iron availability. Iron is known to affect a host of cell functions such as DNA, and to a lesser extent RNA and protein synthesis, the expression of lymphocyte surface markers, immunoglobulin secretion, interleukin-2 receptor expression and many others.[111] Lactoferrin could thus, through its effect on iron availability, indirectly influence a wide spectrum of physiological activities.

Host defense

The role of lactoferrin in the body's defense against micro-organisms is clinically manifested by the recurrent infections seen in patients with an absence of specific granules,[112] and by the altered granulocyte function associated with lactoferrin deficiency.[113] This is experimentally confirmed by research results such as the protective effect shown by lactoferrin in experimental *E. coli* septicemia.[114]

The best known role of lactoferrin in the host defense mechanism is that of a bacteriostatic agent whereby the proliferation of bacteria is inhibited through its iron sequestering properties. Iron withholding as a defence against infection and neoplasia well described by Weinberg.[7,115]

Lactoferrin is known to have a wide spectrum of microbiostatic activities. It is however fairly ineffective against those bacteria which are able to acquire their iron from either lactoferrin or transferrin.[116] It is now known that lactoferrin, in addition to its bacteriostatic action, can also be bactericidal. The bactericidal effect of lactoferricin B, a peptide proteolytically derived from the N-terminal region of lactoferrin, is said to be several times greater than that of lactoferrin. Lactoferricin B was shown to be lethal to a wide spectrum of microbes, and to rapidly inhibit the colony-forming capability of most species tested.[117] The bactericidal effect of lactoferrin is mediated by blistering, i.e. damaging of the outer bacterial membrane, with subsequent alteration of its permeability. The bactericidal membrane damage includes incorporation of lactoferrin into the membrane and subsequent dispersion of lipopolysaccharides (LPS) through a cation (Ca^{++}, Mg^{++} or Fe^{++}) modulated process.[118] The lactoferrin/lactoferricin reactive component of the bacterial membrane is said to be a 38-kDa protein molecule, namely *porin*.[119] Porin is normally shielded by the polysaccharide moiety of LPS, which reduces the anti-microbicidal effect of lactoferrin.

It would appear as if lactoferrin can also exhibit fungicidal and perhaps anti-viral effects. The exact antifungal effect is not yet clear, but it is known that monoprotein fractions of *Candida albicans* increases the number of fungi-phagocytosing polymorphonuclear cells and that lactoferrin cannot inhibit this growth in the absence of polymorphonuclear leukocytes.[120] Direct killing and suppression of the colony-forming capability of *Candida albicans* by the N-terminal lactoferrin fragment, lactoferricin B, has also been shown. The mechanism apparently corresponds to that involved in the lactoferricin killing of bacteria and is suppressed in the presence of Ca^{++} and Mg^{++}.[121] Neutrophils were shown to have reduced lactoferrin content during viral infections. This acquired neutrophil lactoferrin deficit is suggested to be instrumental in superimposed postviral bacterial infections.[122] The effect of lactoferrin on viral proliferation *per se* is still controversial.

Lactoferrin, as previously mentioned, appears to play an extended role in the host defense mechanism by modulating other immune processes. Observations that suggest such an immune modulatory role are presented in Table 6.

Lactoferrin and cellular proliferation

A number of studies suggest a role for lactoferrin in cellular proliferation. Such studies include better gastrointestinal development in newborn animals fed maternal milk as compared to newborn animals fed commercial formulas,[104,140] increased thymidine incorporation with lactoferrin supplementation of milk formulas,[141] and *in vitro* augmentation of thymidine incorporation into rat crypt cell DNA by lactoferrin.[142] The dependence of lactoferrin growth stimulatory activity on iron saturation was shown by the fourfold higher DNA synthesis in a mouse embryo cell line under the influ-

Table 6. Host defense/immune modulatory function of lactoferrin.

Modulatory function	*Probable mechanism*	Ref. #
1. Lactoferrin enhances neutrophil accumulation at, and adherence to tissues at the site of injury	• Reduction in the surface charge and thus in the repulsive forces.	27
2. Lactoferrin enhances granulocyte "stickiness" and in so doing promotes cell-to-cell interaction	• Lactoferrin binds to the surface of polymorphonuclear cells and reduces the surface charge.	123
3. The controversy with regard to the role of lactoferrin in free radical production (ie inhibition or augmentation) probably depends on the environmental conditions which cause lactoferrin to be either an iron scavenger or an iron supplier:		
a) In acid environments such as in the phagolysosome, lactoferrin may promote the production of radicals for the intragranulocyte killing of microorganisms.	a) The furnishing of iron by lactoferrin to an oxygen radical-generating system.	124 65
(i) Lactoferrin catalyzes the neutrophilic production of hydroxyl radicals.	(i) Providing iron.	125
b) At normal extracelluar pH values, lactoferrin may inhibit free radical production and in this way perhaps diminish oxidative damage to tissues.	b) Lactoferrin acts as an iron scavenger.	124,126
(i) It inhibits the production of free radicals by stimulated monocytes.	(i) Iron-binding dependent.	126
(ii) Lactoferrin may protect neutrophilic cells from lipid peroxidative damage.	(ii) Iron-binding dependent.	27
(iii) Lactoferrin inhibits lipid peroxidation mechanism.	(iii) Iron-binding dependent, since iron-saturated lactoferrin demonstrated no inhibitory effect	127
4. Lactoferrin, through its growth regulatory function, affects the host defense mechanism:		
a) The effects would appear to be predominantly inhibitory in nature.		
(i) Lactoferrin inhibits mitogen- and alloantigen-induced human lymphocyte proliferation.	(i) Unknown, but would appear to be dependent on its iron chelating properties.	128
(ii) Lactoferrin blocks histamine release from rat mast cells.	(ii) Unknown, but apparently iron saturation dependent.	129
(iii) Lactoferrin inhibits the synthesis of antibodies.	(iii) Unknown.	130
(iv) Lactoferrin helps to control monocyte/macrophage activity.	(iv) Unknown.	131
(v) Lactoferrin has an anticomplement action.	(v) Controversial. Some found a procomplement action.	124
(vi) Lactoferrin augments natural killer cell (NK) cytotoxicity and lymphokine activated killer cell (LAK) cytotoxicity.	(vi) Unknown, but independent of lactoferrin iron saturation or lactoferrin RNase-activity.	83
b) Most of the above mechanisms, shown to be affected by lactoferrin, are generally stimulated by cytokines. The mechanism of action of lactoferrin may thus be through its affect on cytokine activity. A couple of publications would appear to support this possibility.		
(i) Lactoferrin suppresses the secretion of granulocyte-monocyte-colony stimulating factor.	(i) Concentration dependent lactoferrin inhibition of interleukin-1 synthesis (negative feedback).	132,133
(ii) Fifty percent iron-saturated lactoferrin inhibits the release of cytokines, such as tumor necrosis factor, interleukin-1β and interleukin-2, in a dose- and time-dependent way.	(ii) Unknown.	133
(iii) Lactoferrin only affects the release but not the biological activity of the cytokines.	(iii) Unknown.	133
(iv) Lactoferrin, in the presence of lipopolysaccharides, augments the production of interleukin-1β, tumor necrosis factor-α, interleukin-6 and prostaglandins.	(iv) Not known, but independent of iron saturation.	126
5. Lactoferrin may modify the inflammatory response in SLE by binding to DNA.	• Interaction between lactoferrin and DNA prevents the binding of anti-DNA. Lactoferrin is also able to disperse the anti-DNA-DNA bond.	134
6. Lactoferrin was shown to enhance the T-cell autoreactivity associated with Mycobacterium-induced arthritis.	• Cross reactivity between the mycobacterial 65-kDa heat shock protein and lactoferrin.	135
7. Neutrophil lactoferrin augments the antimicrobial capacity of macrophages.	• Macrophages ingest lactoferrin rich granulocytes as their source of lactoferrin and myeloperoxidase.	136
8. Lactoferrin enhances polymorphonuclear cell functioning by increasing their motility and priming them to produce superoxide at a faster rate.	• Unknown, but apparently independent of iron saturation and can be abolished by anti-lactoferrin.	137
9. Lactoferrin potentiates the bactericidal capabilities of bactenectins, a class of arginine-rich antibacterial peptides of bovine neutrophil granules.	• The synergistic action of lactoferrin and bactenectins increases bacterial membrane permeability.	138
10. Lactoferrin is able to substitute for antibodies in order to activate the classical pathway of complement.	• Unknown, but involves the adherence of lactoferrin to the membrane.	124,139

SLE: Systemic lupus erythematosus

ence of hololactoferrin than in the same line under the influence of apolactoferrin.[143] A role for lactoferrin as growth stimulatory factor in embryos and neonates is further suggested by the significant enhancement of DNA synthesis in rat neonatal hepatocytes by iron saturated lactoferrin. This mitogenic characteristic of lactoferrin apparently does not apply to adult rat hepatocytes.[144] The effect of lactoferrin on cancerous cells would appear to be inhibitory rather than stimulatory.[145] Some contradictions still exist however.

The exact effect of lactoferrin on myelopoiesis is still being debated. The contrasting views on this subject have previously been referred to as *the lactoferrin controversy*.[28] The reader is referred to a publication in which the controversial points of view are discussed.[28] The majority of research workers are presently of the opinion that lactoferrin acts as a negative feedback regulator of myelopoiesis.[128,133,146,147] The mechanism of action would appear to be through suppression of the release of cytokines such as interleukin-1, tumor necrosis factor and interleukin-2.[133,147] Lactoferrin has been shown to (a) bind to specific receptors on hemopoietic cells, (b) become internalized in such cells, and (c) associate with DNA within the nucleus.[146] Euchromatin has been suggested as the probable functional site for the lactoferrin inhibitory action.[148] Whether lactoferrin can directly influence hemopoietic cell proliferation, or whether its effect is primarily through the regulation of cytokine release must still be confirmed.

Other possible function

Acute phase proteins are defined as proteins whose concentrations in plasma increase by 25% or more following infection or inflammation.[149] Several authors have suggested that lactoferrin be classed as an acute phase protein.

An antithrombotic function has also been ascribed to lactoferrin. The possibility that lactoferrin or lactoferrin-derived substances may influence platelet function is supported by observations such as (a) the presence of lactoferrin receptors on platelet membranes,[101] (b) the inhibition of ADP-treated platelet aggregation,[150] (c) the inhibition of fibrinogen binding to ADP-treated platelets, and (d) the inhibition of platelet aggregation, thromboxane generation, serotonin release and α-granule membrane protein expression.[151]

In addition to its proposed role in the modulation of the host response, lactoferrin may also be involved in immunotolerance. It has been shown to prevent activation of the complement system (confirmed by hemolytic assay).[152] Indications, however, also exist that it may activate the classical complement pathway.[139] The presence of anti-lactoferrin antibodies in certain autoimmune diseases might also imply a role for the molecule in immunotolerance (Table 7).

Possible clinical applications

Since lactoferrin is released in a nonspecific way in response to inflammation, any such event will increase its levels through neutrophil activation and degranulation. The diagnostic application of these levels is similar to that of several different indicators of immune stimulation, such as neopterin and elastase-α1-proteinase inhibitor complex and others, rendering lactoferrin levels relatively nonspecific. A number of clinical applications are nonetheless described in the literature. These are mostly of diagnostic or prognostic predictive value and include plasma lacto-

Table 7. Diseases in which anti-lactoferrin antibodies have been shown to occur, and the frequency with which they occur.

Disease	*Frequency or percentage of anti-lactoferrin antibodies*	*Ref.#*
Crohn's disease	Occasionally, 34%, 8%	(152-154)
Ulcerative colitis	High, 45%, 50%	(152-154)
Primary sclerosing cholangitis	High, 50%	(152,154)
Uncomplicated RA	Occasionally, 10%, 2.4%, 4%, 20%	(152,155,156)
SLE	Occasionally, 20%, 15-20%, 39%	(152,155)
Primary Sjögren's syndrome	Occasionally	(152)
Scleroderma	19%	(155)
Felty's syndrome	50%	(156)

ferrin determination as an index of the total blood neutrophil pool or neutrophil kinetics,[31,53] as a tool in the diagnosis of chronic myeloid leukemia,[54] granulocytic leukemia,[53] chronic calcifying pancreatitis,[108,157] cystic fibrosis,[38,158] septicemia,[50,159] congenital aplasia of the vasa deferentia and seminal vesicles,[77] schizophrenia,[160] joint inflammation and cartilage degradation,[161] psoriasis[89] and rheumatoid arthritis.[162] Lactoferrin antibodies have been demonstrated in patients with Felty's syndrome, and the detection of these antibodies may prove useful in its diagnosis.[156,163] It has further been suggested that β-lactoferrin/RNase and γ-lactoferrin/RNase may be of value in the detection of breast cancer.[164] The weak discriminatory power of changes in total plasma lactoferrin concentration makes it unlikely that the determination of values will ever achieve widespread prognostic or diagnostic application.

Conclusions

A wide spectrum of functions have been ascribed to lactoferrin. This may indicate a relative nonspecificity of function rather than a highly specialized role. It is possible that lactoferrin may exert most of its functions through its effect on iron availability, but this is difficult to explain in the light of our present knowledge about lactoferrin-iron affinity. More insight into the interrelationships and interactions between lactoferrin fragments, isoforms, and the different iron-saturated structures will no doubt go a long way toward providing a better understanding of the mechanism of action of lactoferrin.

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