Lactoferrin: Antimicrobial and Diagnostic Properties

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ABSTRACT

Lactoferrin is the major antimicrobial agent present in human milk, where it has a significant role in the protective effects ascribed to breast-feeding. The mechanisms of the antimicrobial effects of lactoferrin are critically discussed in relation to important bacterial and viral pathogens and the most promising areas for basic and applied research are specifically highlighted. Applications of lactoferrin in the study of the mechanisms of iron uptake by microbial pathogens, as well as in the diagnosis of gastrointestinal inflammation of infectious and autoimmune origin, are addressed. The progress in the use of lactoferrin as an immunomodulating agent are briefly discussed and the observed side effects of lactoferrin administration are singled out for consideration in clinical studies. As with the rest of the components of the innate immune system, the final outcome of the biological activity of lactoferrin is the result of combined effects with other innate and adaptive immune factors. In this regard, the availability of recombinant lactoferrin will allow clinical trials addressing the innocuity and efficacy of single or combined formulations of lactoferrin with other biologically active molecules. Diseases such as AIDS, chronic Hepatitis C, bacterial sepsis, arthritis, and bacterial infections in diabetic subjects, which lack effective treatment and have the potential to be modulated by lactoferrin, may be considered as targets for such trials. Lactoferrin research has reached a stage where a rigurous clinical evaluation of the multiple effects observed *in vitro* would provide a definitive answer to its actual antimicrobial and diagnostic value.

Keywords: antibacterial agents, antiviral agents, endotoxemia, immunomodulation, inflammation, iron, lactoferrin

RESUMEN

Lactoferrina: propiedades antimicrobianas y para el diagnóstico. La lactoferrina es el principal agente antimicrobiano presente en la leche humana y desempeña un papel significativo en los efectos protectores de la lactancia materna. Los mecanismos de los efectos antimicrobianos de la lactoferrina se analizan críticamente en esta revisión, en los ejemplos de patógenos bacterianos y virales importantes, y se señalan en particular las áreas más relevantes para la investigación básica y aplicada. Se describen las aplicaciones de la lactoferrina en el estudio de los mecanismos de captación de hierro por patógenos microbianos, y en el diagnóstico de inflamaciones gastrointestinales de origen infeccioso y autoinmunitario. Se discuten brevemente los adelantos en el empleo de la lactoferrina como agente inmunomodulador, así como los efectos secundarios observados en ensayos clínicos en relación con la administración de la lactoferrina. En su conjunto, la actividad biológica de la lactoferrina es el resultado de los efectos combinados de otros factores innatos y adquiridos. La disponibilidad comercial reciente de lactoferrina recombinante, permitirá la realización de ensayos clínicos dirigidos a evaluar la inocuidad y eficacia de formulaciones simples o combinadas de la lactoferrina con otras moléculas bioactivas. Enfermedades que como el sida, la hepatitis C crónica, la sepsis bacteriana, la artritis, y las infecciones bacterianas asociadas a pacientes diabéticos . que carecen de tratamientos efectivos en la actualidad, y que pueden ser moduladas potencialmente por la lactoferrina. se consideran los objetivos primarios de estos ensayos. Los adelantos en la investigación de la lactoferrina requieren la evaluación clínica rigurosa de los múltiples efectos observados *in vitro,* como criterio de evaluación final de su valor real como agente antimicrobiano y para el diagnóstico.

Palabras claves: agente antibacteriano, agente antiviral, endotoxemia, hierro, inflamación, inmunomodulación, lactoferrina

*I*ntroduction

The protective effects of breast-feeding have been epidemiologically confirmed by its significant impact on the reduction of infant mortality and morbidity associated with gastrointestinal and respiratory infections. The effects of the milk components transferred are not limited to passive protection, as they also influence the maturation of the mucosal immune system of lactating infants, inducing long-lasting and enhanced protection against mucosal infections and better responses to vaccination in early and later life, as well as reducing the risk of some autoimmune, oncogenic and allergic diseases [1]. Milk antimicrobial components act at mucosal surfaces in combination with innate and adaptive immune factors. They are generally protease-resistant, have multiple defense functions through non-inflammatory mechanisms, and their concentrations in milk are inversely correlated with their production in lactating infants and are poorly represented in non-primate milk.

Recognition of this protective potential and the absence of similarly safe and effective pharmaceutical tools for prophylaxis of microbial infections in new-borns have promoted intensive research of milk immunoactive factors [2].

The high concentration of lactoferrin in colostrum and mature human milk singles it out for research of immunoactive milk components. This monomeric high-affinity iron-binding glycoprotein. estimated pI » 9.7, 77. 80 kD. occurs in all body fluids at different concentrations, from high values in early colostrum (37.5. 87.5 mM) and mucosal secretions, to the lowest ones in human blood (~20 nM). The intake of lactoferrin in a healthy breast-fed full-term infant ranges between 260 mg/kg/day during the first month to 125 mg/kg/day by the fourth month, which suggests an important role in the beneficial effects of breast-feeding [3]. As a result, lactoferrin has become a focus of interest for medical, pharmaceutical and research communities.

Iron plays multiple and important biological functions in all living species and is consequently of pivotal importance in host-parasite relationships (Table 1). Eukaryotes have three main functional groups of proteins involved in iron metabolism: a) oxygen-binding proteins, b) proteins catalysing redox reactions and c) iron-complexing proteins. They carry out essential functions for life ranging from cellular respiration to DNA replication. Lactoferrin and transferrin are the major iron-binding proteins (IBP) dealing with extracellular iron.

Table 1. Role of iron and iron-dependent enzymes in microbial and mammalian biochemical processes [4].

Biochemical function	Mammals	Micro-organisms
Oxygen transport	Haemoglobin and myoglobin	Haemoglobin-like proteins and leghaemoglobin
Oxygen metabolism	Tryptophan-2,3-dioxygenase. Tryptophan hydrolase, etc.	Tryptophan dioxigenase, Catechol dioxygenase, etc.
Peroxide and superoxide metabolism	Catalase, peroxidase	Superoxide dismutase, catalase, peroxidase
Electron transfer	Cytochromes and iron-sulphur proteins	Cytochromes and iron-sulphur enzymes, etc.
Aconitase-IRP	Synthesis of transferrin receptor, ferritin and haemoglobin	Not present
DNA replication	Ribonucleotide reductase	Ribonucleotide reductase

Prokaryotes possess several mechanisms to overcome iron deprivation imposed by the host in extracellular fluids. Some bacteria reduce IBP-bound ferric iron to its less tightly bound ferrous form [5] and/or express proteases to degrade IBP, thus providing iron for bacterial growth. Generally, host-restricted bacteria such as *Neisseria meningitidis* express outer membrane receptors capable of specifically sequestering iron by direct binding of IBP, whereas most biphasic bacteria (i.e. both environmental and pathogenic) such as *Escherichia coli* secrete siderophores, low-molecular-weight organic compounds with extremely high affinity for ferric iron which compete with the host IBP and shuttle iron back to the bacterial cells. Some biphasic and ubiquitous pathogens such as *Staphylococcus aureus* produce siderophores and IBP-receptors as well. Different combinations of these mechanisms may occur in different pathogens under specific conditions. The implications of iron metabolism in host-parasite relationships were comprehensively reviewed recently by Aguila and Brock [4].

We will critically review the antibacterial and antiviral activities of lactoferrin in relation to its structure and functions and the use of lactoferrin for diagnosis of gastrointestinal inflammation, and will briefly mention its immunomodulatory and anti-inflammatory effects as well. Finally, we will critically discuss the current and potential pharmaceutical applications of lactoferrin in each of the topics mentioned above, and the most promising areas for future research are specifically highlighted.

*L*actoferrin: Structure-functional Properties

Lactoferrin has three major structural properties directly related to its biological activity [6]. Firstly, it is organised in two homologous lobes, with one iron-binding site each, whose capacity to bind ferric iron with high affinity even at the low pH values common in certain body locations is crucial for its microbiostatic effects. Lactoferrin binds ferric iron (Fe3+) reversibly but with a high affinity (KB~1020), whereas its affinity for ferrous iron (Fe2+) is much lower (KB~103) and requires the binding of one bicarbonate ion with each iron ion bound, a synergism thought to be unique in biology. Lactoferrin does not participate in iron transport, but functions to keep concentrations of biologically available iron at levels insufficient to support microbial growth in tissue fluids. It may occur in four forms: iron-free (apo-form), iron-loaded N-lobe, iron-loaded C-lobe, and iron-saturated (holo-form), with the apo- and partially saturated N-lobe forms being predominant *in vivo*. Apo- and holo-lactoferrin have different spatial conformation. Iron saturation enhances the conformational stability and resistance to denaturation compared to the apo-form.

Secondly, the presence of two glycosylation sites is related to resistance to mucosal proteases and variations in the glycosylation patterns between lactoferrins from bovine and human origins have been implicated in the different antimicrobial capacities of these two otherwise almost identical proteins (86% amino acid homology).

Thirdly, the highly basic N-terminus mediates the binding to several eukaryotic and prokaryotic structures and has been implicated in bactericidal and antiviral effects, as well as in the immunomodulatory and antiendotoxic effects ascribed to this protein.

*A*ntibacterial Properties

Iron is critical for growth and full expression of virulence factors by bacterial pathogens *in vivo*. Thus, protection by host defense mechanisms correlates with the capacity of host IBP to minimise free-iron concentrations. The high ferrochelating capacity of lactoferrin is the functional basis for most of its broad spectrum antimicrobial effects [7].

However, many siderophilic bacteria evade the microbiostatic effect of lactoferrin *in vitro* depending on the experimental conditions and growth culture media used. This phenotypic trait thought to be correlated with virulence was recently observed in several clinical *Staphylococcus aureus* isolates from Havana hospitals (Aguila A *et al.,* manuscript in preparation) (Table 2).

Table 2. Antimicrobial effects of human lactoferrin [1, 3, 4, 8].

Microbiostatic effect	Microbicidal effect	No effect
Klebsiella pneumoniae	*Haemophilus influenzae*	*Helicobacter pylori*
Enteropathogenic *E. coli* in milk	Non-enteropathogenic *E. coli*	Enteropathogenic *E. coli* grown in LB
Neisseria catharralis	*Legionella pneumophila*	*Neisseria gonorrhoeae* FA19
Shigella flexneri	*Pseudomonas aeuroginosa*	*Bifidobacteria* spp.
Shigella sonnei	*Streptococcus mutants*	*Lactobacillus* spp.
Salmonella typhimurium	*Streptococcus pneumoniae*	*Streptococcus agalactiae*
Salmonella wien 1	*Vibrio cholerae*	*Streptococcus uberis*
Staphylococcus aureus	*Candida albicans*	*Staphylococcus aureus(*)*
Vibrio cholerae		
Mycobacterium tuberculosis		

*Clinical isolates

This resistance has been related to the occurrence of different mechanisms for iron uptake from lactoferrin. Some bacteria such as *Helicobacter pylori* express highly-specific human lactoferrin receptors which, as in other species, have been implicated in bacterial pathogenicity and immunogenicity, suggesting the therapeutic and research potential of lactoferrin [9]. Other bacteria produce siderophores capable of chelating and shuttling iron from lactoferrin to bacteria, while other bacteria such as *E. coli* [5] express iron reductases. A similar activity was recently observed by our group in supernatants of *S. aureus* cultured in a chemically-defined medium, as described in [6]. The reductase activity secreted by both bacteria was completely abolished by proteinase K (1.5 mg/mL) treatment, which suggests its protein nature. Interestingly, transferrin-bound but not lactoferrin-bound iron was reduced when added to supernatants of each bacteria, which provided additional explanation to the higher bacteriostatic effect of lactoferrin compared to transferrin (Aguila A *et al.,* manuscript in preparation). Other bacteria secrete lactoferrin proteases or, as in some clinical isolates of *E. coli,* inhibit lactoferrin binding to cell wall structures. The failure of lactoferrin to limit the growth of bifidobacteria [10] due to the low iron requirements of these species results in the preferential growth of harmless gut flora microbes capable of overgrowing pathogenic enterobacteria, thus having a beneficial effect in host defense as breast-feeding shows only too well [1].

In addition to bacteriostatic activity mediated by iron-chelation, alternative, mainly bactericidal effects have been reported. Complexes of lactoferrin and secretory IgA (sIgA) are bactericidal against Gram-negative and Gram-positive pathogens due to a combination of specific binding by sIgA of bacterial siderophore and

lactoferrin receptors and immunogenic cell wall components along with targeted iron deprivation by sIgA-bound lactoferrin [11]. The effective protection of new-borns against many mucosal pathogens after specific oral vaccination of pregnant women in developing countries suggests that this may be a common mechanism of mucosal protection [1].

Direct binding of lactoferrin to cell wall structures such as *E. coli* outer membrane porins [12], *Haemophilus influenzae* colonisation factors [13] and the lipid A moiety of lipopolysaccharide (LPS) interferes with vital functions and/or increases cell wall permeability, which eventually helps killing Gram-negative bacteria. The presence of other innate immune factors such as lysozyme and lactoperoxidase capable of damaging cell wall structures enhances and broadens the antibacterial effects of lactoferrin.

Phagocytosed bacteria are subjected to lactoferrin microbicidal effects, such as iron-catalysed generation of oxygen-reactive metabolites, withholding of iron released from lysed bacteria in phagolysosomes, and synergism with bactericidal cationic peptides of activated neutrophils [14, 15].

Lactoferrin regulates the production and function of neutrophils and monocytes, modulating their antimicrobial activity either via the regulation of iron availability or by increasing the phagocytosis of lactoferrin-coated micro-organisms by binding to its receptors on phagocytic cells [16].

The sequence containing the first 47 N-terminal amino acids of lactoferrin is termed lactoferricin and comprises the highly cationic structure responsible for the binding of lactoferrin to several of the aforementioned bacterial and eukaryotic structures, thus mediating lactoferrin bactericidal effects [17]. The specific activity of lactoferricin is higher than that of the intact molecule, does not depend on iron saturation, and follows a biphasic kinetics with significant killing in the first hour, and a slower rate thereafter. The mechanism is thought to be mediated by direct interaction with anionic cell wall structures facilitated by the lower molecular mass of lactoferricin (~ 26 times lower than lactoferrin), which permits an easier access to bacterial structures without steric restrictions. This activity is partially inhibited at high concentrations of iron and other cations, reinforcing the non-specific electrostatic nature of its interactions with bacterial structures [11].

The bactericidal activity of lactoferrin hydrolysates against clinical isolates of *S. aureus* was completely lost when the bacteria were incubated in media capable of sustaining bacterial growth. Whether this is due to the capacity of sublethally damaged cells to recover their structural integrity if grown in a nutrient-rich medium, is a subject of future research. Our results indicate that the staphylostatic activity of lactoferrin is mainly dependent on its ferrochelating properties and completely abolished by addition of iron to culture media, thus suggesting a minor role for the bactericidal effects mentioned above [18].

High doses of LPS result in overproduction of oxygen-free radicals by phagocytic cells and host tissue damage typical of bacterial endotoxemia. LPS triggers these effects after specific binding to its cell receptors through two major mechanisms; firstly, binding to CD14 receptor on macrophages, which is catalysed by the plasma LPS binding protein (LBP) and secondly, LBP/CD14 independent mechanisms such as the binding to neutrophil L-selectin. Lactoferrin and lactoferricin-containing peptides bind directly to LPS lipid A moiety and simultaneously interfere with both pathways (LBP/CD14-dependent and independent) of LPS-mediated activation of phagocytic cells [19, 20], thus providing a potent antiendotoxic effect.

The physiological significance of all these *in vitro* observations related to lactoferricin has not been definitely established yet. However, the generation of lactoferricin-containing peptides by pepsin digestion of lactoferrin occurs *in vivo* [21], and it is likely that other host proteases in phagocytes could generate similar peptides, suggesting that different bactericidal/bacteriostatic combinations of intact lactoferrin and lactoferricin-containing peptides may account for the antibacterial and antiendotoxic effects observed *in vivo*.

The protective effects of lactoferrin against bacterial infections have been corroborated in animal models of infections, such as *S. aureus*-related infectious arthritis [22], systemic staphylococcal infection [23] and endotoxin lethal shock [24]. The increased susceptibility of diabetic individuals to bacterial infections has been related to the abnormally high levels of glucose-modified proteins bearing advanced glycosylation end products (AGEs) in tissues and serum. AGEs bind innate humoral factors such as lactoferrin and lysozyme to completely block their antimicrobial activity. This suggests a potential use of lactoferrin in new approaches for

preventing diabetes-associated infections [25].

The absence of observations in humans and differences in iron metabolism between species [4] highlight the need for controlled clinical trials in humans to assess the actual pharmaceutical and clinical value of the observations reported. The forthcoming availability of commercial recombinant lactoferrin [7], whose safety and innocuity has already been established in preclinical trials in mice and Rhesus monkeys. lactoferrin-knockout mice [26] have been developed. will speed up the pharmaceutical development of lactoferrin and promote a deeper understanding of its mechanisms of action in the near future. Another field of research which deserves especial attention is the use of lactoferrin as a tool for research into the mechanisms of iron-uptake by pathogenic micro-organisms [8], where promising applications in the design of alternative therapeutic and prophylactic approaches and in the development of antigen-defined vaccine formulations may be reasonably predicted [4].

*A*ntiviral Properties

The antiviral activity of milk proteins has been known for more than 20 years [27]. The initial assumption that sIgA is the only factor responsible for these effects was abandoned, as further research on the antiviral effects of milk components singled out lactoferrin as the major antiviral milk component with potent effects against human immunodeficiency virus type 1 (HIV-1) [28], human cytomegalovirus (HCMV) [29], herpes simplex virus types 1 and 2 (HSV 1/2) [30], SA-11 rotavirus [31], poliovirus type 1 (PV-1) [32] and Hepatitis C virus (HCV) [33] (Table 3).

Table 3. Parameters of the antiviral activity of lactoferrin against several human viruses.

Virus	$CC_{50\%}$ (mg/mL)	$IC_{50\%}$ (mg/mL)	SI
HCMV	> 0.250	36.70	> 6.00
HSV-1	> 10.00	12.00	83.33
HSV-2	> 10.00	5.20	1923.00
HIV-1	> 0.250	40.0	> 6.30
HIV-2	> 0.250	> 250	@ 1.00
Rotavirus SA-11	> 30.00	50.00	> 600
HCV-1b	> 2.00	200.00	> 10
HGV	> 2.00	2 000.00	>1.00

$CC_{50\%}$, cytotoxic concentration 50%; $IC_{50\%}$, inhibitory concentration 50%; SI, selectivity index ($CC_{50\%}$ / $IC_{50\%}$).

Three major mechanisms for the antiviral effects of lactoferrin may be proposed. Firstly, the interaction of the highly cationic N-terminus of lactoferrin with negatively-charged cell membrane structures such as glycosaminoglycans [34] competes with enveloped viruses, including HSV, HCMV and HIV, for their binding to glycosaminoglycan receptors on target cells. Consequently, preincubation of cells with lactoferrin results in an increased inhibition of viral replication directly correlating with the time of exposure to lactoferrin. Although addition of lactoferrin after virus entry cannot inhibit initial viral replication, it should not be concluded that lactoferrin is useless against ongoing viral infections, as addition after viral replication does reduce virus infectivity by interfering with the propagation of newly released viral particles to neighbouring cells. The specificity of this mechanism is suggested by the inability of lactoferrin to inhibit cell entry of SA-11 rotavirus,

which does not use cell membrane glycosaminoglycans as cell receptors.

Secondly, direct binding of lactoferrin to viral structures has been shown in the case of HCV particles grown in PH5CH8 cells (human neoplastic cloned hepatocytes immortalised with SV40 large T antigen) [35], and HIV [36]. In contrast to bacterial systems, the binding to HCV E1 and E2 envelope proteins and HIV gp120 protein was not mediated by the cationic N-terminus. The anti-HCV activity of lactoferrin does not depend on the incubation period, as viral infection is prevented after less than 60 s of preincubation, suggesting that the binding of lactoferrin to viral structures overcomes viral absorption. A similar effect was observed against several HCV genotypes, which indicates the presence of lactoferrin-binding structures regardless of the viral genotype. That these lactoferrin-binding moieties may be a common pattern in the Flaviviridiae family is also suggested by the antiviral activity of lactoferrin against Hepatitis G virus (HGV), thus singling out lactoferrin not only as the second natural substance with antiviral effects against HCV, but also as a drug potentially capable of overcoming the genetic heterogeneity of HCV, which has hampered the development of broadly effective anti-HCV therapeutic drugs and prophylactic vaccines.

The third mechanism involves the antiviral effects of cations released into the cytoplasm of virus-infected cells, and is thought to be mediated by the inhibition of host or viral enzymes involved in intracellular viral replication and assembly, or by the intracellular generation of toxic compounds. The specificity of this mechanism is suggested by the differences in antiviral effects of lactoferrin loaded with different cations [28, 30, 32].

Unexpectedly, lactoferrin-derived peptides seldom showed any significant antiviral effects in any of the systems tested so far, and when observed they were always inferior to the activity of the whole protein [37]. Experimental results indicate that bovine lactoferrin is more active than human lactoferrin in spite of their high structural homology and that the antiviral effect does not seem to be related to the iron withholding activity of lactoferrin, as iron-saturated forms are more effective than the apo-variants. The presence of specific mechanisms for antibacterial and antiviral effects of lactoferrin and variations between different species indicates the versatility of the protective functions of this protein in mammals.

The differences in antiviral activity between human and bovine lactoferrins seem to be due to variations in the amino acid sequences putatively responsible for this activity, as well as in the number of glycosylation sites and disulphide bridges in both proteins [38]. The greater inhibitory activity of iron-saturated forms may be due to an increased affinity for viral cell receptors as result of the conformational changes induced after iron-binding. Apo-lactoferrin showed a lower affinity for Vero cells and consequently inhibition of HSV-1 was reduced in 20% [30]. HCMV replication was inhibited for more than 48 h by holo-lactoferrin whereas apo-lactoferrin. s effects lasted only 60 min. The inhibition period correlated directly with the amount of protein being antigenically identified as lactoferrin thus suggesting that iron or metal binding may increase resistance to proteolysis and denaturation .

Taken together these experiments support the importance of metal saturation of lactoferrin linked to conformational changes, stability and the biological activity of bound metals once released to the cells, and seem to rule out any link between the antiviral effects of lactoferrin and its ferrochelating capacity. The ferrochelating-dependent antibacterial effect of lactoferrin not only does not reduce, but on the contrary increases its antiviral activity, thus providing a structure-functional basis for a much broader antimicrobial activity of this protein.

The clinical significance of this effect is suggested by several lines of evidence. Firstly, in contrast to bacterial infections where plasma concentrations of lactoferrin are increased up to values close to or above the IC50% reported *in vitro,* in HIV-infected individuals and AIDS patients plasma lactoferrin levels decline with the progression of the disease concomitant with an increase in the incidence of opportunistic infections, including HCMV as well [39]. Breastfed HIV-1. infected new-borns had a 10-month longer median incubation time than bottle-fed infants, and much slower progression to AIDS. The administration of dietary whey proteins to HIV-seropositive individuals increased the number of blood mononuclear cells and consequently improved their general health status. Secondly, the surprisingly low rate of vertical transmission of HCV compared to that of HBV may be associated with the increases in plasma lactoferrin during pregnancy and the high intake of lactoferrin during breast-feeding accounting for nearly 3% of new-born total nutrition. Thirdly, lactoferrin antiviral effects have been demonstrated *in vitro* with SI higher than 10 suggesting its pharmaceutical potential (Table 3); as well as in experimental animals infected with HSV-1 [40], HCMV [41] and friend virus complex (FVC) where it acted in synergism with co-administered interferons [42]. A recent clinical trial with chronic HCV

patients indicated the therapeutic potential of lactoferrin as anti-HCV drug [43].

Although other factors in the immune defense against viral infections are necessarily involved, the role of lactoferrin as a non-specific immune defense factor should not be overlooked. There are several fundamental topics of research which will repay further research efforts, such as the effect of HCV genotype on the antiviral ability of lactoferrin, the antimicrobial activity of lactoferrin in simultaneous bacterial and viral infections, the specific role of the mechanisms described in its antiviral effects, the intracellular effects, if any, of lactoferrin and/or its digested forms and the bound cations in DNA and RNA virus replication.

The future of chronic HCV therapy should include the administration of multiple drugs capable of reducing the levels of hepatic iron associated with tissue-damaging oxidative stress as well as inhibiting viral replication [44]. The capacity of lactoferrin to synergise/potentiate the effects of concomitantly administered antiviral drugs such as interferons [42] and zidovudine [45] has already been demonstrated. The current commercial availability of recombinant human lactoferrin and interferons creates special conditions for evaluation of a combined therapy with these two antiviral drugs in chronic HCV patients, which may be regarded as the only new therapeutic approach against HCV, which is based on natural substances in the short term.

A Biochemical Marker of Gastrointestinal Inflammation

Neglect of the pioneering work of Willmore and Shearman [46] showing the diagnostic potential of fecal "cytodiagnosis" in bacillary and amoebic dysentery led to the widespread use of unreliable, time-consuming and limited techniques for diagnosis of diarrhoeas of infectious origin for more than half century. Re-examination of leucocytes in the stools of volunteers during clinical trials of enteric vaccines established a significant correlation (> 89%) between the detection of fecal leucocytes and the occurrence of invasive bacterial infections. The complete absence of leucocytes in cases of viral, enterotoxin-associated non-invasive, and non-specific diarrhoeas firmly established the suitability of this low-cost approach for eliminating the need for fastidious, often impractical and always expensive isolation of enteric bacteria, as well as increasing epidemiological efficacy of antibiotic therapy by allowing early and correct prescription, thus eliminating potential outbreaks due to the spread of infectious bacteria into the environment [47].

Widespread diagnostic use of fecal leucocytes faced several limitations such as the need for immediate, effective *in situ* analysis of fresh cup, rather than swab samples, the destruction of leucocytes either by toxins or other stool components, as well as the availability of a skilled microscopist and the unavoidable subjectivity of microscopic detection of leucocytes.

The identification of lactoferrin in stool samples stored or transported even under aggressive leucocyte-destroying conditions, and the strong direct correlation between the concentration of fecal lactoferrin and the number of fecal leucocytes in fresh stool samples, provided the basis for the development of a semi-quantitative latex agglutination assay for diagnosis of gastrointestinal inflammation of infectious origin [48].

Subsequent development of the lactoferrin latex agglutination assay extended the applicability of lactoferrin as a reliable biochemical marker for a wide range of inflammatory diarrhoea caused by *Salmonella* spp. *Shigella* spp., *Campylobacter* spp., *Clostridium difficile*, and enterohaemorragic *E. coli*. A critical bibliographic evaluation of all tests currently used for screening of bacterial diarrhoea showed that lactoferrin measurement is the most accurate diagnostic index test [49].

The clear association between the increase of polymorphonuclear cells in gastrointestinal inflammation, and thus the production of lactoferrin, extended its use to the diagnosis of gastrointestinal inflammation of non-infectious, rather autoimmune inflammatory bowel diseases such as ulcerative and ischaemic colitis, Crohn. s disease, etc. [50].

The unexpected inflammatory origin of diarrhoea observed in volunteers orally vaccinated with genetically attenuated *Vibrio cholerae* strains was first suspected after detection of increased levels of fecal lactoferrin, thus opening the application of lactoferrin-detection assays in vaccine development [6].

The non-quantitative character of the lactoferrin latex agglutination method limits its use in the diagnosis of gastrointestinal inflammation in suckling new-borns, and likelihood ratios should be used for diagnosis of invasive inflammatory diarrhoea in field trials in epidemic conditions [51], thus highlighting the need for the

development of alternative quantitative methods. Due to the clinical and economic importance of rapid diagnosis of inflammatory diarrhoea in this age group and in travellers to endemic countries, widespread use of equally simple, but quantitative methods for lactoferrin detection in hospital and domestic settings may be predicted.

*I*mmunoregulatory Properties of Lactoferrin

As well as having antibacterial and antiviral effects, lactoferrin may also directly affect the immune system. A wide variety of activities on various components of the immune system have been reported. These include interaction with B [52] and T [53] lymphocytes, macrophages [54] and monocytes [55]. Although these interactions are said to be mediated by specific receptors, none has been well characterised or cloned, and it is likely that relatively non-specific interactions of cell surface molecules with the basic N-terminal region of lactoferrin are involved [50, 56]. Lactoferrin can enhance lymphocyte proliferation [57] and NK cell activity [58], but the mechanism is unknown and is probably not related to iron metabolism since lactoferrin cannot deliver iron to cells, as shown by our group [59], whereas others have reported that lactoferrin inhibits lymphocyte proliferation [60]. Furthermore, lactoferrin hydrolysates (containing the lactoferricin peptide) were found to enhance B lymphocyte proliferation, whereas the intact protein was inhibitory [61], suggesting again a key role for the basic N-terminal region. *In vitro* activation of macrophages by lactoferrin has also been reported [62].

The above described phenomena are all *in vitro* and the mechanism by which lactoferrin carries out these effects is generally unknown, so it is difficult to assess their importance *in vivo*. In addition, there is far less evidence for *in vivo* immunomodulatory effects. Local administration of lactoferrin was found to reduce the severity of joint inflammation in mouse models of arthritic disease [22], and transgenic mice expressing human lactoferrin showed a bias towards a Th1 response following infection with *S. aureus* (JH Brock, unpublished data). This might relate to lactoferrin. s iron-withholding properties, as iron deficiency is known to favour Th1 responses.

Another aspect of the interactions of lactoferrin with the immune system is the readiness with which it gives rise to autoantibodies. Anti-lactoferrin autoantibodies have been reported in a wide variety of diseases [63], and these can cause neutrophil activation [64] and interfere with lactoferrin iron-binding [65]. Why lactoferrin should be highly auto-antigenic is not known, but it has been proposed that it may result from priming by bovine lactoferrin in cow. s milk [64]. It has been reported that human milk proteins fail to elicit oral tolerance after feeding [66].

At present, therefore, there is no clear role for a potential clinical use of lactoferrin as an immunomodulating agent, except, perhaps, in arthritis. The propensity of lactoferrin to give rise to autoantibodies should be addressed before contemplating clinical applications.

Conclusions

Breast-feeding has a significant prophylactic effect against infectious diseases at the epidemiological level, and iron deprivation is the main basis of this protective activity. Lactoferrin as the major ferrochelating agent present in human milk is crucial for its antimicrobial effects. As with any component of the immune system, the final outcome of the biological activity of lactoferrin is the result of combined effects with other innate and adaptive immune factors. In this regard, the availability of recombinant lactoferrin is cardinal for clinical trials addressing the innocuity and efficacy of single or combined formulations of lactoferrin with other biologically active molecules, such as interferons, antimicrobial drugs, etc. Diseases such as AIDS, chronic HCV, bacterial sepsis, arthritis, and bacterial infections in diabetic subjects, which lack an effective treatment and have the potential to be modulated by lactoferrin, may be considered as targets for such trials.

The use of lactoferrin as an immunomodulating agent still awaits deeper understanding of the mechanisms of action, and although no clear application can be envisaged yet, some studies indicate a potentially negative side effect of lactoferrin *in vivo* (i.e. autoantibody production), which needs to be carefully considered prior any clinical application. Lactoferrin also has promising applications in basic research into the mechanisms of iron-uptake by microbial pathogens, with potential implications in the design of new clinical and pharmaceutical approaches, as well as in the diagnosis of gastrointestinal inflammation of infectious and autoimmune origin. Research on lactoferrin has reached a stage where a rigurous clinical evaluation of the multiple effects

observed *in vitro* would provide a definitive answer to its actual protective and diagnostic value.

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Note
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