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Lactoferrin: an important host defence against microbial and viral attack

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Abstract. The first function attributed to lactoferrin (Lf), an iron binding protein belonging to the non-immune natural defences, was antimicrobial activity that depended on its capacity to sequester iron. Iron-independent microbicidal activities, requiring direct interaction between this cationic protein and microbial surface components, were later demonstrated. Many other anti-microbial and anti-viral functions have since been ascribed to Lf. In mucosal secretions, iron and Lf modulate the motility and aggregation of pathogenic bacteria. Lf inhibits bacterial adhesion on abiotic surfaces through ionic binding to biomaterials, or specific binding to bacterial structures or both. Lf inhibition of bacterial adhesion to host cells requires Lf binding to bacteria and/or host cells. Lf hinders microbial internalization by binding to both glycosaminoglycans and bacterial proteins which can be degraded by Lf-mediated proteolysis. Moreover, Lf internalisation and localisation to the host cell nuclei could modulate bacterial entry into cells through gene regulation. Finally, the capability of Lf to exert antiviral activity, through its binding to host cells and/or viral particles, strengthens the idea that it is an important brick in the mucosal wall, effective against both microbial and viral attacks.

Key words. Lactoferrin; iron; adhesiveness; invasiveness; bacterial aggregation; biofilm; viruses.

Introduction

Lactoferrin (Lf) displays many biological functions related to the host pre-immune defence system [1, 2]. Among these, its antibacterial activity was the first to be discovered [3, 4]. The Lfs from several mammalian species have been shown to inhibit the growth of a number of bacteria, including human and/or animal pathogenic strains of *Escherichia coli, Salmonella typhimurium, Shigella dysenteriae, Listeria monocytogenes, Streptococcus* spp., *Vibrio cholerae, Legionella pneumophila, Klebsiella pneumophila, Enterococcus* spp., *Staphylococcus* spp., *Bacillus stearothermophilus* and *Bacillus subtilis* [5–9]. The in vitro antibacterial results were extended to in vivo observations in the classic paper by Bullen et al. [10] on the protective effect of Lf towards *E. coli* 0111 infection in newborn guinea-pigs. Clear experimental evidence has been accumulated to show that oral administration of Lf reduces bacterial infections in the gastrointestinal tract [8, 11–13] while promoting the growth of bacteria with low iron requirements such as *Lactobacillus* and Bifidobacteria, which are generally believed to be beneficial to the host [14]. In contrast, intraperitoneally, intravenously or intramuscularly administered heterospecific Lf is rapidly cleared from the body of experimental animals, showing little or no protective effect against bacterial infections [15–17].

The mechanisms by which Lf exerts its antibacterial effects were initially assumed to be solely bacteriostatic, arising from its iron-withholding ability. A much wider range of antibacterial activities have been discovered over subsequent years, however, many involving direct action on the bacteria themselves, and to these have been

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added protective effects against viruses, parasites and other harmful species. In this review we discuss current knowledge and perspectives across this whole range of protective activities.

Bacteriostatic activity related to iron withholding

All bacteria require iron for growth, and there is thus a strong correlation between iron availability and virulence. In mucosal secretions, which are a first line of defence against microorganisms, iron limitation in healthy humans hinders bacterial growth. Conversely, increased iron levels, such as occur in some pathologies, favour bacterial virulence. Lf, as a key component of such secretions, thus has an important role to play.

The Lf found in secretions is almost entirely in its iron-free 'apo-Lf' form [18] but has the ability to bind iron, as Fe(III), extremely tightly. In fact, Lf binds two Fe^{3+} ions per molecule, with an affinity and stability much higher than that of transferrin, the iron transport protein in serum [19, 20]. The presence of apo-Lf in the extracellular compartments within the host body or on host mucosal surfaces thus maintains the level of available iron below that required to support microbial growth [21]. Indeed, studies have shown that iron sequestration by apo-Lf can effectively inhibit the growth of many bacterial species [5]. However, this antibacterial activity of Lf due to iron deprivation is merely bacteriostatic, since bacterial growth is only delayed by iron deprivation and can be completely restored after iron supplementation. In addition, most pathogenic bacteria can overcome Lf-generated iron deprivation, acquiring iron by means of two principal systems that counteract host iron-binding proteins: either secreting small iron chelators or acquiring iron directly from host transferrin and Lf.

As regards the first system, many bacteria synthesize and secrete small iron-chelating molecules (siderophores) that can compete with Lf for insoluble Fe^{3+} ions, thus reversing the growth-inhibiting activity of the protein. Siderophores can be regarded as microbial virulence factors, and highly invasive strains usually possess a very efficient mechanism of iron transport [22, 23]. Another mechanism developed by pathogenic bacteria for iron acquisition in the host involves iron removal from hemin (the oxidation product of the heme released from hemoglobin). Lf can efficiently compete with bacteria for hemin iron [24], although many Gram-negative pathogenic bacteria possessing hemin iron-acquisition systems can acquire iron directly from transferrin and Lf [23, 25, 26]. Direct iron acquisition from transferrin and Lf involves binding of either transferrin or Lf to two different surface bacterial receptors and is found in highly host-adapted bacterial species [27]. The Lf receptor is composed of two proteins: Lf-binding proteins A and B (LbpA and LbpB) [28, 29]. A recently proposed model suggests that the LbpA-Lf interaction induces conformational changes in Lf which results in lowering the affinity of the Lf C-lobe for iron [30].

Bactericidal activity not related to iron withholding

As indicated above, iron deprivation generated by Lf may only delay microbial growth; however, the classic paper of Arnold et al. [31] reported a bactericidal activity of human Lf, subsequently demonstrated to be distinct from its iron-withholding activity [32, 33]. Experiments carried out with either Lf or ovotransferrin (an avian Lf homologue) suggested that direct binding of these proteins to bacteria is involved [34–36], though the high density of charges present at the surface of the Lf molecule [37] can easily induce non-specific binding of Lf to biological structures of either bacteria or hosts. The molecular mechanisms of this bactericidal activity of Lf, which is not related to iron withholding, appears to be quite similar for either Gram-negative or Gram-positive bacteria, resulting in both cases in a perturbation of bacterial membranes.

In Gram-negative bacteria, it was observed that Lf specifically binds to porins present on the outer membrane [38] and induces the rapid release of lipopolysaccharides (LPSs) which is known to enhance bacterial susceptibility to osmotic shock, to lysozyme and to other antibacterial molecules [39]. In mediating LPS release, Lf appears to act in two ways. First, it is a polycationic molecule, with the maximal density of surface positive charge located in the N-terminal region [37]. Most of the iron-independent antibacterial activity of Lf is concentrated into a cluster of positively charged residues near the N-terminus of the Lfs from many mammalian species [40–43]. This positive cluster binds to the lipid A part of LPS molecules present on the outer membrane of clinically relevant bacterial species [44, 45], acting therefore in a way similar to that of a number of polycationic compounds, such as polymyxin B nonapeptide [46]. In addition, following the observation that LPS release induced by Lf is neutralised by high calcium concentration in the medium, Ellison et al. [47] hypothesised that Lf could be broadly active as a calcium chelator such as EDTA, which is known to induce LPS release [48]. Rossi et al. [49] demonstrated that Lf can indeed bind Ca^{2+}, releasing significant amounts of LPS from Gram-negative bacteria without the need of direct contact with bacteria.

The molecular mechanism of the bactericidal effect of Lf towards Gram-positive bacteria is very likely to be similar to that of cationic and amphipathic antibacterial peptides. These peptides act by binding to Gram-positive bacteria through electrostatic interactions between the negatively charged lipid matrix of the target mem-

branes and cationic amino acid residues; after binding, amphipathic residues perturb the non-polar membrane interior due to hydrophobic interactions [50, 51]. In fact, for Lf the region responsible for the bactericidal activity towards Gram-positive bacteria appears to correspond to the same N-terminal region of Lf where, in many mammalian species, several hydrophobic amino acid residues are located in close proximity to the cationic residues [41]. Conclusive support for the importance of the N-terminal region of Lf for iron-independent antibacterial activity comes from the observation that amphipathic, cationic peptides obtained from the N-terminus of either human or bovine Lf are severalfold more active than the parent proteins towards both Gram-negative and Gram-positive bacteria [52–54].

Antibacterial activity related to proteolysis

A surprising new antibacterial activity of Lf has recently been discovered with reports that human Lf displays proteolytic activity towards some bacterial virulence factors, decreasing the pathogenicity of certain microorganisms. Degradation of *H. influenzae* IgA1 protease was observed when *H. influenzae* was cultured in human milk as the sole source of nutrient [55]; Qiu et al. [56] found that human Lf causes the proteolytic degradation of both the IgA1 protease and the Hap adhesin and that a serine-protease-like activity seems to be located in the N-lobe of human Lf. Epitope distribution after exposure to human Lf indicated that a large portion of the IgAb membrane spanning region is removed [57] and that the cleaved site(s) appear to be located in an arginine-rich region [58].

Protease-like activity of recombinant human Lf, inhibited by classical serine protease inhibitors, has been also reported by Ochoa and Cleary [59]. They observed that Lf treatment of *Shigella flexneri* 5 strain M90T impaired invasiveness [60] by inducing release and degradation of invasion plasmid antigens B and C (IpaB and IpaC) [61, 62]. They also found that Lf blocks Enteropathogenic *E. coli* adherence, hemolysis and induction of actin polymerisation in HEp2 cells as a result of the Lf-mediated degradation of *E. coli* secreted proteins A, B and D (EspABD) [63, 64].

Massucci et al. [65] characterised in vitro the proteolytic activity of bovine Lf towards synthetic substrates. The substrate specificity is similar to that of trypsin, and serine protease inhibitors inhibit this catalytic activity, although the values of the catalytic parameters (k_{cat}, K_m) are several orders of magnitude lower than those of trypsin. Interestingly, less than 10% of the Lf molecules appear to possess proteolytic activity, as indicated by a serine protease affinity column. Similar results were obtained with recombinant human Lf [G. Antonini, unpublished results]. However, several attempts, carried out both experimentally and by molecular dynamics calculations, to identify the active site and the putative natural substrates, were ineffective. In conclusion, both the very low values of the catalytic constants for the hydrolysis of synthetic substrates and the lack of identification of the active site geometry demand further investigations before attributing the proper biological significance to this unexpected Lf activity.

Influence of Lf on bacterial aggregation and biofilm formation

A recent addition to the repertoire of antibacterial activities of Lf has been the discovery that it can exert other effects through its influence on bacterial aggregation and biofilm formation. Different effects have been shown with respect to respiratory infections and oral infections.

Lf and respiratory infections

Cystic fibrosis (CF), as well as being associated with alterations in the influx and efflux of chloride and sodium ions, results in very high concentrations of iron in sputum (median value of 6.3×10^{-5} M) [66]. This increase in iron content, as well as inducing the generation of reactive oxygen species (ROS) which contribute heavily to lung disorders, enhances the growth and colonisation of *Pseudomonas aeruginosa* and *Burkholderia cepacia*, two motile Gram-negative pathogens that are a major source of morbidity and mortality of CF patients. For both organisms, biofilm formation is a major contributor to virulence.

Peptides and proteins of natural non-immune defences, including Lf, play a crucial role in combating such infections. A striking recent discovery was that apo-Lf, by chelating iron, inhibits *P. aeruginosa* adhesion and biofilm formation through activation of a specialised form of motility named switching [67]. Like *P. aeruginosa*, free-living forms of *B. cepacia* also show a noticeable motility under iron-limiting conditions. On the other hand, iron availability, or the addition of iron-saturated bLf, induces abundant *P. aeruginosa* and *B. cepacia* aggregates, evolving into biofilm [68]. In CF patients, however, these protective effects of Lf are compromised [69]. The hLf concentration increases in infection and inflammation processes and is found in sputum of CF and chronic bronchitis patients at higher concentrations than in normal subjects [70]. It is offset, however, by the high iron concentration (6.3×10^{-5} M), which can saturate hLf (1×10^{-5} M) in CF patient sputum, thus preventing Lf from exerting its pivotal function in inhibiting biofilm formation [67, 68]. The functions of hLf may also be influenced by the enhanced concentration of NaCl in

the airways secretions of CF patients or by bacterial and host cell proteolytic enzymes, highly active in the acidic environment of the lungs of CF patients [71].

In infection and inflammation sites, the pH drops (<4.5) due to the metabolic activity of invading bacteria or of stimulated leucocytes. Even at this pH, however, hLf, unlike Tf, retains high affinity for iron and can bind iron released from transferrin, resulting in the iron-saturated form. These data highlight the importance of defining the Lf activity in the mucosal secretions of healthy humans and illness patients, taking into account its degree of saturation and iron concentration.

Lf and oral infections

Among mucosal secretions, saliva represents another interesting model to investigate the influence of iron and Lf concentrations on bacterial aggregation and biofilm formation. In human saliva, the iron content ranges from 0.1 to 1.0 μM depending on meals but can increase for gingival bleeding or other oral pathologies, including periodontitis. The physiological level of hLf in saliva varies from 5 to 20 μg/ml, reaching 60 μg/ml during infections and inflammations. In the human oral cavity, *Streptococcus mutans*, the principal etiological agent of dental caries, exerts its pathogenesis through its adhesion and aggregation capability. Recently, in a saliva pool well defined for iron and Lf content, apo-bLf was found to enhance *S. mutans* aggregates and biofilm formation, whereas iron-saturated bLf decreases aggregation and biofilm development [72]. These results can explain the observation that saliva of caries-resistant patients favours the clearance of bacteria through a high aggregation efficiency and very low adhesion-promoting activity.

Apo-hLf also induces aggregation of an anaerobic Gram-negative bacterium, *Porphyromonas gingivalis,* which is associated with periodontitis [73]. However, in periodontitis patients, the high iron concentration and the presence of hemin, which can form complexes with hLf, together with Lf degradation by bacterial enzymes [74], could be responsible, in vivo, for the lack activity.

The contrasting effects of iron-limiting conditions on aggregation, biofilm formation or motility of *S. mutans, P. gingivalis, P. aeruginosa* and *B. cepacia* reflect important differences in the nature of the habitat to be colonized and in the expression of virulence among different bacterial genera. Recently, Weinberg reported that human Tf and Lf, as well as at least six low molecular mass iron chelators, could be developed as inhibitors of bacterial biofilm. Moreover, he asserts that biofilm formation is 'a process in some, but not all, bacterial systems which requires a higher level of iron than is needed for growth and it is suppressed by specific iron chelators' [75]. Although cell density-dependent control of gene expression is employed by many bacteria for regulating biofilm formation, the experiments with Lf emphasise that the way by which bacteria increase their density is not always identical. For this reason, it is crucial to know the different bacterial responses to environmental stimuli, including iron and Lf, which, in apo- or iron-saturated form, can influence bacterial density in markedly different ways.

Inhibition of bacterial adhesion on abiotic and cell surfaces by Lf

Abiotic surfaces

Microbial adhesion and subsequent colonisation, resulting in biofilm formation on abiotic surfaces such as medical devices, represents a serious problem that can lead to illness and death. Efforts to reduce microbial adhesion, using new materials or compounds inhibiting microbial adhesion, have had modest success once applied to the patient. Consequently, it could be very helpful to discover other compounds able to hinder microbial adhesion.

The ability of Lf, in both apo- and iron-saturated form, to inhibit the adhesion of *S. mutans* to hydroxyapatite (HA), mimicking tooth surface [76], is an interesting and novel function. The demonstration that Lf inhibits the adhesion of *S. mutans* to a salivary film and HA through residues 473–538 of its C-lobe [77], further helped to understand this activity, which is unrelated to its iron-binding properties. Both apo- and iron-saturated bLf also inhibit adhesion of free and aggregated *S. mutans* cells to a dental polymer when Lfs were pre-coated to dental polymer or bound to both dental polymer and bacterial cells [72]. Both apo-bLf and apo-hLf, but not iron-saturated Lfs, also inhibit the attachment on HA of *Prevotella nigrescens* by binding to both HA and bacteria [78]. In other studies, hLf has been shown to inhibit the adhesion of *Actinobacillus actinomycetemcomitans* and *Prevotella intermedia* to reconstituted basement membrane (Matrigel), through ionic binding, and *P. intermedia* to bacterial adhesins by a specific binding of hLf [79]. The influence of hLf on bacterial adhesion on an abiotic surface (contact lens) is also shown by the much lower number of adherent *P. aeruginosa* cells on hLf-coated lenses than is observed in the absence of hLf [80].

The different nature of abiotic surfaces, the varying microbial adhesion mechanisms and the different in vitro experimental conditions could explain the different results obtained for inhibition of bacterial adhesion by apo- or iron-saturated hLf or bLf, which in some cases requires only ionic binding to biomaterials, and in others specific binding to bacterial structures, or both.

Cell surfaces

The ability of microbes to adhere, colonise and form biofilm on host cells is a crucial step in the development and

persistence of infections. In addition, the high resistance of microbial biofilm to natural defence mechanisms and to antibiotics makes it imperative to discover compounds able to prevent bacterial adhesion. A large number of Gram-positive and Gram-negative bacteria possess specific adhesins that mediate the adhesion process on epithelial host cells. Although hLf and bLf, hLfcin and bLfcin are all able to bind to Gram-negative and Gram-positive bacterial surfaces [36], as well as to host cells, by binding to glycosaminoglycans (GAGs) [81] and in particular to heparan sulphate (HS) [82], there is growing evidence that Lf can prevent adhesion through other mechanisms as well.

The first demonstration of the mucosal protective activity of hLf against injury by adherent bacteria was shown for *E. coli* HB101 carrying the pRI203 plasmid *Yersinia pseudotuberculosis inv* gene. The invasin, the *inv* gene product, promotes both bacterial binding and internalisation into mammalian cells [83]. Pre-incubation of apo- and iron-saturated Lf with bacteria or with cultured cells, carried out at 4 °C to avoid internalisation, did not exert any anti-adhesive activity. Both hLf and bLf were shown, however, to inhibit *E. coli* HB101 (pRI203), *Y. pseudotuberculosis* and *Y. enterocolitica* adhesion through the ability of Lf to bind to both epithelial cells and invasins in a manner unrelated to its iron-binding capability [83, 84]. Lf has also been shown to inhibit the adherence of enterotoxigenic *E. coli* (ETEC) to human epithelial cells and to intestinal mucosa of germfree mice [85], as well as the adhesion of three adhesive diarrheagenic *E. coli* strains (DAEC), enteroaggregative *E. coli* (EAEC) [86] and enteropathogenic *E. coli* (EPEC) [87].

The importance of the sugar residues on Lf is suggested by the observation that whereas native hLf inhibits *Shigella* spp adhesion [88], recombinant hLf (rhLf), with different glycosylation, has no effect on *Shigella flexneri* adhesion to epithelial cells [61]. Another paper suggests that hLf, rhLf and bLf inhibit the attachment of *Helicobacter felix* to the gastric epithelial cells, probably by interaction between oligomannoside-type glycans of Lf and bacterial adhesins which recognise these residues [89].

Although inhibition of bacterial adhesion seems generally to be mediated by Lf binding to both bacterial and host cell surfaces, the surprising demonstration of hLf proteolytic activity [56] has provided an additional mechanism to explain Lf anti-adhesive activity. Thus, inhibition by hLf of the adhesion of EPEC strains [87], which use a type III secretory system to deliver effector proteins into the host cell, has recently been ascribed to hLf-mediated degradation of the secreted proteins, EspA, B, D [63], as well as hLf inhibition of *H. influenzae* and *A. actinomycetemcomitans* adhesion to the degradation of two colonisation factors and of autotransporter proteins, respectively [57, 58, 90].

Although the experimental conditions of the studies reported are different, pre-incubation of Lf with host cells seems never to inhibit Gram-positive and Gram-negative bacterial adhesion, suggesting that Lf binding to GAGs or HS is not crucial. Instead, the inhibition of Gram-positive and Gram-negative bacterial adhesion by Lf seems to require Lf binding to bacteria or a putative Lf-mediated degradation of the adhesins or proteins of the secretory systems, or Lf binding to both bacteria and host cells.

Inhibition of microbial invasion of epithelial cells by Lf

Some mucosal pathogenic bacteria are capable not only of adhering, but also of entering into non-professional phagocytes, such as epithelial cells. Inside host cells, bacteria are in a protective niche in which they can replicate and persist, thus avoiding host defences. Virulence determinants, such as surface proteins able to bind host cells, play a key role in the entry process inside the host cells. Lf has now been shown to inhibit the entry of facultative intracellular bacteria, both Gram-negative and Gram-positive. Moreover, a similar role also appears to apply to some parasites (see later).

The first demonstration of the inhibition of bacterial invasion by bLf involved a recombinant enteroinvasive *E. coli* strain HB101(pRI203) [83]. Bacterial entry into host cells is mediated by the binding of bacterial invasin to a host integrin receptor. The effectiveness of apo- and iron-saturated bLf, and bLfcin, towards *E. coli* strain HB101 (pRI203) invasion is correlated with their ability to bind to both cultured cells and bacterial invasin [83, 84]. Likewise, for *Y. enterocolitica* and *Y. pseudotuberculosis,* grown in conditions allowing maximal invasin synthesis, a 10-fold inhibition of invasion of cultured cells by bLfcin was observed [84]. It appears that the binding of Lf and Lfcin to integrins through the same domains that are targeted by invasin, and to GAGs, can induce a dramatic subversion in bacterial-host cell interaction, thus inhibiting bacterial internalisation [84]. Similar mechanisms apply to inhibition of invasion of the Gram-positive bacteria *L. monocytogens, Streptococcus pyogenes* (GAS) and *Staphylococcus aureus*, i.e. apo- or iron-saturated bLf binding to both bacterial adhesins and host cells [91–93]. The ability of bLf to decrease GAS invasion was also confirmed by an in vivo trial carried out on 12 children suffering from pharingitis and already scheduled for tonsillectomy [92].

Interestingly, treatment of *S. flexneri* with rhLf, even if unable to inhibit adhesion, impairs the invasiveness of cultured cells by inducing release and degradation of antigens, IpaB and IpaC, responsible for invasion [61]. Bovine Lf also seems to activate the secretion of IpaB and IpaC from an enteroinvasive *E. coli* (EIEC) strain,

under conditions in which iron availability is low due to iron chelation by bLf separated from bacteria by a dialysis membrane. Small bLf-derived components, diffusing across the dialysis membrane, also contribute to the release of Ipa proteins. When bacteria are exposed to bLf, the activation of secretion is due neither to bLf-induced damage of the EIEC outer membrane, nor to increased transcription of the *mxi* operon. Low iron availability, due to the iron-chelating ability of Lf, may be an environmental signal perceived by enteroinvasive microorganisms that modulates secretion of virulence proteins [94].

Finally, a different mechanism applies for the anti-invasive activity of hLf against *A. actinomycetemcomitans*, which is due to the cleavage of Aae, an autotransporter which mediates its binding to epithelial cells [90].

In all reported studies, with different intracellular microorganisms in different in vitro models, Lf, in apo- or iron-saturated form, exerts an inhibiting activity against the microbial internalisation. In contrast to inhibition of bacterial adhesion, Lf binding to GAGs of host cells seems crucial in inhibiting bacterial internalization. An intriguing hypothesis arises from the observation that hLf can be internalised and localised to the nucleus, as observed for human erythroleukemic and intestinal cells, and that it binds to a specific DNA sequence and activates the transcription of a specific gene [95 and references therein]. This suggests that Lf could act, through gene regulation, on some functions of epithelial cells, including the rearrangement of cytoskeleton crucial for bacterial internalisation. In contrast, the internalisation and localisation to the cytoplasm of hTf [95], in addition to its anionic charge, could explain why this transferrin does not exert the functions exerted by Lf.

Inhibition of viral infections by Lf

The antiviral activity of hLf was first demonstrated in mice infected with the polycythemia-inducing strain of the Friend virus complex (FVC-P) [96]. Since 1994, potent antiviral activity of hLf and bLf has been demonstrated against both enveloped and naked viruses. The following discussion is not exhaustive but illustrates a common theme of Lf binding to surface proteins on virus or host cells or both.

Cytomegalovirus (CMV)

In vitro antiviral activity of hLF and bLf against human CMV has been reported, independent of Lf iron status or of the presence of sialic acid [97]. When negatively charged groups were added to Lf by succinylation, the antiviral potency was mostly decreased, whereas the addition of positive charges to Lf through amination of the protein resulted in increased anti-HCMV activity [98]. In in vivo experiments, administration of bLF before murine CMV infection completely protected BALB/c mice (but not athymic nude mice) from death [99]. In these Lf-treated mice, a significant increase in the activity of NK cells was observed. In another experiment, Lf treatment led to a 10-fold reduction in the final virus titres at 4 weeks after infection of immunocompromised rats [100]. These data indicate that Lf may exert its effects by inhibition of viral entry rather than stimulation of the immune system.

Herpes simplex virus (HSV)

The initial attachment of HSV to cells occurs through binding of the viral glycoprotein(s) gC or gB to HS of host cells. In the absence of HS, virus can bind to chondroitin sulfate proteoglycans (CS), although with lower efficiency [97]. Both hLf and bLf, independent of iron status or sialic acid content, have been shown to inhibit infection and replication of HSV-1 in human embryo lung cells [97]. Other studies have confirmed the activity of hLf against HSV-1 infection of cultured cells and shown that bLf, Mn-bLf and Zn-bLf are potent inhibitors of HSV-1 and HSV-2 infections through their binding to host cells and HSV particles [101, 102]. Likewise, bLf fragments corresponding either to the C-lobe (residues 345–689) or to a large portion of the N-lobe (residues 1–280) prevented entry of HSV-1 into the host cell in a similar manner to full-length bLf, although a smaller part of the N-lobe (residues 86–258) did not [103, 104].

There is evidence that inhibition of HSV-1 infectivity by Lf is dependent on its interaction with cell surface GAG chains of HS and CS on host cells [105]. Thus, bLf inhibition of HSV-1 was less effective using GAG-deficient cells or cells treated with GAG-degrading enzymes. Furthermore, Lf inhibition was less for viral mutants devoid of glycoprotein C (gC), indicating that bLf interferes with the binding of viral gC to cell surface HS and/or CS on GAG-expressing cells. Lf bound directly to both HS and CS isolated from these cells, as well as to commercial preparations of GAG chains. Topical administration of 1% bLf, prior to the virus inoculation, suppressed HSV-1 infection in the mouse cornea, but it did not inhibit propagation of the virus [106].

Human immunodeficiency virus (HIV)

Native bLf or hLf (iron saturation 10–20%) inhibits the HIV-1-induced cytopathic effect. When negatively charged groups were added to Lf by succinylation, there was a strong antiviral effect on HIV-1 and HIV-2, whereas addition of positive charges to Lf through amination resulted in a loss of anti-HIV activity [98, 107]. Both native Lf and the charged-modified protein bind

strongly to the V3 loop of the gp120 envelope protein, resulting in inhibition of virus-cell fusion and entry of the virus into cultured cells [108]. Both HIV-1 replication and syncytium formation were also inhibited efficiently, in a dose-dependent manner, by apo-, Fe^{3+}-, Mn^{2+}- or Zn^{2+}-saturated bLf when added prior to HIV infection or during the viral adsorption step, thus that Lf blocks HIV binding to or entry into cultured cells [108]. Modest inhibition of HIV infection was obtained with bLfcin, indicating that other domains within the native bLF protein may be required to inhibit HIV-1 entry [109].

Human hepatitis C (HCV) and human hepatitis B (HBV) viruses

bLF and hLf effectively prevented HCV infection in cultured human hepatocytes. Direct interaction between hLF or bLf and HCV occurs through the E1 and E2 envelope proteins [110]. Thus, preincubation of HCV with bLF resulted in inhibition of HCV infection, whereas preincubation of bLF with the cells gave no inhibitory effect [111]. Lf also prevents HBV infection in a susceptible human hepatocyte cell line. Unlike HCV, preincubation of the cell with bLF or hLF was required to prevent HBV infection of cells [112]. Lf may well be among the candidates for an anti-HBV reagent that could prove effective in treatment of patients with chronic hepatitis.

Rotavirus

Apo- and iron-saturated bLf inhibit the replication of rotavirus, in a dose-dependent manner, with apo-Lf being the most active. Apo-Lf hinders virus attachment to cell receptors by binding to the viral particles, thus preventing both rotavirus hemagglutination and viral binding to susceptible cells. Moreover, rotavirus antigen synthesis and yield in intestinal cultured cells was markedly inhibited when Lf was added during the viral adsorption step or when it was present in the first hours of infection, suggesting that it interferes with the early phases of rotavirus infection [113]. Manganese- or zinc-saturated bLf had slightly decreased activity, compared with apo- or iron-saturated bLf, and the removal of sialic acid enhanced the anti-rotavirus activity. Experiments with tryptic fragments of bLf identified a large fragment (86–258) and a small peptide (324–329: YLTTLK) that could inhibit rotavirus, although to a lower extent than undigested bLf [114].

Adenovirus

hLf and bLf, in native and apo form, and Fe^{3+}-, Mn^{2+}- and Zn^{2+}-saturated bLf, added before or during the viral adsorption step, or during the entire replicative cycle, were effective against adenovirus type 2 infection in cultured cells, with bLf showing the lowest EC50 (median effective concentration) values and the highest selectivity [115]. As HS is involved in the binding of adenovirus type 2 [116], the N-lobe, which binds GAGs on HS, can bind to HS in competition with viral receptors, unlike the C-lobe, which cannot. The anti-adenovirus activity of bLfcin alone is sufficient to prevent infection [117]. Extraction of adenovirus proteins also showed that bLf interacts strongly with two structural proteins of 86 and 66 kDa, corresponding to the viral polypeptides III and IIIa that mediate viral attachment to integrin cell receptors and internalisation [118]. Thus, bLf exerts its anti-adenovirus activity by the binding to both cell receptors and viral polypeptides III and IIIa.

Antifungal and anti-parasitic activities

Both hLf and bLf, as well as the bLf-derived peptide lactoferricin, have well documented in vitro activity towards human pathogenic fungi, above all *Candida albicans* and several other *Candida* species. However, minimum growth-inhibitory concentrations reported for Lf are much higher than those of most antifungal drugs currently available and vary markedly from study to study, probably due to different assay conditions and to significant intra-species variation in susceptibility to the protein [32, 119–121]. Bovine Lf has been shown to be fungicidal for six Candida species, in decreasing order of efficacy, *C. tropicalis* > *C. krusei* > *C. albicans* > *C. guilliermondii* > *C. parapsilosis* > *C. glabrata*, the latter being the most resistant to Lf [120]. This effect was related to Lf adsorption to the *C. albicans* cell surface rather than iron deprivation [122], a suggestion subsequently supported in several reports demonstrating cell wall damage [120, 123, 124]. Recently, it has been reported that the candidacidal activity exerted by hLf is dependent on the extracellular cation concentration and on the cellular metabolic state, although an extensive membrane permeabilisation was not observed [125]. In addition, recent studies on experimental candidiasis suggest that bovine Lf works by some host-mediated mechanisms of action rather than by a direct antimicotic activity [126].

Molecular mechanims of Lf antiparasitic activity are even more complex. Antiparasitic activities of Lf appear often to involve interference with iron acquisition by some parasites, e. g. *Pneumocystis carinii* [127,128], while Lf appears to act as a specific iron donor in other parasites such as *Tritrichomonas foetus;* in the latter case, Lf could be expected to enhance infection [129]. It was recently reported that two *Trypanosoma brucei* proteins bound human serum transferrin as well as human Lf and bovine Lf [130], and preincubation of *Toxoplasma gondii* and *Eimeria stiedai* sporozoites with a Lf-derived peptide, lactoferricin, reduces their infectivity in animal models [131].

Lf antiparasitic activity is also, sometimes, mediated by interaction with host cells. Thus, iron-saturated Lf enhances intramacrophage killing of *Trypanosoma cruzi* amastigotes [132] and decreases intra-erythrocytic growth of *Plasmodium falciparum* [133]. Lf is able to inhibit the invasion of cultured cells by *Plasmodium* spp. sporozoites through specific binding to HS [134]. In the case of *Plasmodium berghei*, Lf reduces invasion by inhibiting the binding of the plasmodial CS protein, with or without HS, suggesting the possibility that Lf can also bind to the same site on LDL receptor-related protein (LRP) as the CS protein [135].

Conclusions

The protective effect of Lf towards microbial and viral infections has been widely demonstrated in a large number of in vitro studies, although few clinical trials have been carried out. Nevertheless, Lf can be considered not only a primary defence factor against mucosal infections, but also a polyvalent regulator, which interacts with several microbial, viral and host components involved in infectious processes.

In addition to the microbistatic or microbicidal functions that were initially attributed to Lf, both related and unrelated to its capacity to sequester iron, many other functions relevant to microbial and viral infections have now been ascribed to this protein.

In mucosal secretions, Lf, by sequestering iron, modulates the motility, aggregation and biofilm formation of pathogenic bacteria. The contrasting effects of iron-limiting conditions on aggregation and biofilm formation of different bacterial genera reflect important differences in the nature of the habitat to be colonised and in the expression of bacterial virulence genes. Thus, for *S. mutans* the cell density required for biofilm formation can be reached through microbial aggregation without multiplication, in the presence of apo- or native-Lf, whereas for *P. aeruginosa* and *B. cepacia* multiplication and biofilm formation are favoured by iron supplied from iron-saturated Lf.

Lf inhibits bacterial adhesion on abiotic surfaces through ionic binding to biomaterials, or specific binding to bacterial structures or both. Just as bacterial adhesion on abiotic surfaces can lead to serious illness and death, so the ability of microorganisms to adhere to host cells is a crucial step in the development of disease. Bacteria bound on cell surfaces multiply, aggregate, form biofilm, become resistant to natural immunity, phagocytic and antibiotic killing, and can release toxins damaging the epithelium, thus encouraging microbial invasion. Lf interference with bacterial structures, leading to the inhibition of bacterial adhesion to host cells, is thus important to counteract microbial virulence and to hinder microbial persistence in the host. Although Lf can bind to host cell GAGs and HS, pre-incubation of Lf with host cells is unable to inhibit the adhesion of Gram-positive and Gram-negative bacteria, suggesting that Lf binding to GAGs or HS is not crucial. Instead, inhibition of bacterial adhesion seems to require Lf binding to bacteria or to both bacteria and host cells, or a putative Lf-mediated degradation of the adhesins or proteins of the secretory systems.

Lf, in apo- or iron-saturated form, exerts an inhibiting activity against microbial internalisation in target cells. In contrast to its effects on bacterial adhesion, Lf binding to host cell GAGs appears to be crucial in inhibiting bacterial internalisation. The observation that Lf can be internalised and localised to the nucleus offers the intriguing suggestion that Lf could act on some functions of epithelial cells crucial for bacterial internalisation, through gene regulation.

Finally, the capability of Lf to exert antiviral activity, through its binding to host cells and/or viral particles, strengthens the idea that this glycoprotein is an important brick in the mucosal wall, effective against both microbial and viral attacks.

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