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ScienceDirect
Biochimie 91 (2009) 19–29
BIOCHIMIE
www.elsevier.com/locate/biochi

Review

Antimicrobial properties of lactoferrin

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Received 19 March 2008; accepted 23 May 2008
Available online 5 June 2008

Abstract

Milk is a vital nutritional source for the offspring of all mammals, including humans. In addition to its nutritional value, it is a rich source of proteins including lactoferrin. Lactoferrin is a truly multifunctional protein that has been studied extensively over the past decades. It is best known for its ability to bind iron, which eventually led to the discovery of its antibacterial activity. In addition, lactoferrin has demonstrated potent antiviral, antifungal and antiparasitic activity, towards a broad spectrum of species. It is also considered to be an important host defense molecule during infant development. In this review, we focus on the antimicrobial activities of lactoferrin with particular emphasis on antibacterial and antiviral activities, although its antifungal and -parasitic activity are also discussed.

Keywords: Lactoferrin; Antibacterial; Antiviral; Antifungal; Antiparasitic

1. Introduction

Antimicrobial proteins and peptides are produced by a wide variety of organisms as their first line of defense [1], and are found in large quantities in all secretory fluids. The most abundant antimicrobial proteins include lysozyme, collectin [2,3] and lactoferrin (for a comprehensive review see [4] and Baker et al., (2009). The antimicrobial activity of these proteins is related to bacterial lysis or opsonization of the pathogen, for example, mannose-binding proteins' interaction with HIV [5] and neutralization of influenza A virus by surfactant protein A [6]. Lactoferrin is truly a multifunctional protein (for review see [7–10]) and it is known to work as an opsonin to promote bacterial clearance [11], but this activity has not been described for viruses. It seems likely that the main physiological function of lactoferrin is to bind iron, and this was initially identified as a feature of the protein that contributed to its antibacterial activity, by sequestering iron, a necessary nutritional requirement for most bacterial pathogens, and thus inhibiting growth of a broad spectrum of bacterial strains [12–15]. Lactoferrin can also inhibit viral infections (Table 1) [16–28] of both naked [26,29–31], and enveloped viruses [18,20,23–25,32–39], and the activity is primarily exerted during an early phase of the viral infection. Iron saturation does not appear to influence the antiviral activity [24,25,27] of lactoferrin, in contrast to its antibacterial activity. The interplay between lactoferrin and different cellular lactoferrin receptor molecules (for review see [40]), could be of great importance for the antimicrobial activity, but this is outside the scope of this review. In addition to antiviral and antibacterial activity, lactoferrin also inhibits fungal [41,42] and parasitic infections [43]. This review provides an overview of the direct antimicrobial functions of the milk protein lactoferrin, namely its antibacterial, antiviral, antifungal and antiparasitic activity.

2. Antibacterial activity

Sequestering of iron from bacterial pathogens, thus inhibiting bacterial growth, was one of the first antimicrobial properties discovered for lactoferrin (Table 1) [12,13]. This was believed to be the sole antimicrobial action of lactoferrin for a long time, and was supported by several studies demonstrating that only apo-lactoferrin possessed antibacterial activity, and that this activity was reduced upon iron saturation [44–46]. It

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doi:10.1016/j.biochi.2008.05.015

Table 1
Biological activity of lactoferrin

Activity	Target	Mode of action	References
Gram-positive bacteria	*S. mutans*	Iron-independent interaction with bacterial cell surface	[47–49]
	S. epidermidis	Interaction with lipoteichoic acid on bacterial surface	[62]
	S. epidermidis	Prevents biofilm formation – probably through iron sequestering	[81]
Gram-negative bacteria	*E. coli*, *S. typhimurium*	Cation chelators, damaging the bacterial membrane, altering the outer membrane permeability, resulting in a release of LPS	[54,56]
	H. influenzae	Altering bacterial virulence – degrading IgA1 and Hap	[65]
	S. flexneri	Disrupt bacterial type III secretion system – degrading IpaB and IpaC	[72,73]
	E. coli	Disrupt bacterial type III secretion system – degrading EspA, EspB and EspC	[73–75]
	S. typhimurium	Interaction with the bacterial surface	[76]
	P. aeruginosa	Prevents biofilm formation – probably through iron sequestering	[79,82,83]
	B. cepacia	Prevents biofilm formation – probably through iron sequestering	[80]
	B. cenocepacia	Prevents biofilm formation – probably through iron sequestering	[83]
Enveloped viruses	HSV	Targets adsorption/entry – contradicting results whether there is a direct effect on the viral particle or not	[23,24,100]
	HCMV	Targets adsorption/entry – no effect on the viral particle	[20,32,34]
	VSV	Upregulation of machrophage interferon α/β expression	[147]
	Hepatitis B	Targets cellular molecules interfering with viral attachment/entry	[19]
	Hepatitis C	Targets viral envelope protein E1 and E2 – blocks entry	[21,35,39,140]
	Hepatitis G	Unknown	[21]
	HIV	Targets V3 loop in envelope protein gp120 – blocks CXCR4- or CCR5-attachment	[17,25,38,102]
	Feline herpes virus-1	Targets viral attachment/entry	[16]
	Sindbis virus	Targets adsorption/entry – no effect on the viral particle	[105]
	Semliki Forest virus	Targets adsorption/entry – no effect on the viral particle	[105]
	RS-virus	Unknown	[171]
	Hantavirus	Targets adsorption/entry (not heparan sulphate) – no effect on the viral particle	[36]
Naked viruses	Rotavirus	Viral interaction – prevents hemaglutination and attachment to cellular receptors	[103]
	Poliovirus	Targets viral adsorption/competes for viral receptor interaction	[30]
	Adenovirus	Targets viral adsorption/binds viral protein III and IIIa.	[29,104,141]
	Enterovirus (EV71 and Echovirus 6)	Targets viral adsorption – binds both cellular receptors and the viral surface protein VP1. Inhibits apoptosis	[22,143,144]
Yeast and fungi	*C. albicans*, *C. tropicalis*, *C. krusei*, *C. guilliermondii*, *C. parapsilosis*, *C. glabrat*	Cell wall perturbation	[150–153]
	A. fumigatus	Iron sequestering	[155]
Parasites and other eukaryotic microbes	*P. berghei*	Targets host cell entry	[167,168]
	P. carinii	Iron sequestration	[43]
	E. histolytica	Probably linked to iron sequestration	[160]
	B. caballi	Iron sequestration	[161]
	B. equi		

was later demonstrated that lactoferrin is also able to kill *Streptococcus mutans* through an iron-independent mechanism [47], an effect hypothesized to result from direct interaction of lactoferrin with the bacterial cell surface (Table 1) [48,49].

Crystal structure studies of lactoferrin have demonstrated that the protein has large cationic patches on its surface (Fig. 1) [50], facilitating direct interaction with anionic Lipid A, a component of the lipopolysaccharide (LPS) of Gram-negative bacteria [51–53]. Such interaction can damage the bacterial membrane, altering the outer membrane permeability and resulting in the release of LPS [54]. This effect was easily inhibited by divalent cations like Mg^{2+} and Ca^{2+}, leading Ellison et al. [55] to hypothesize that lactoferrin could work as a cation chelator like EDTA [56], which also is known to induce LPS release from bacterial membranes. Direct binding of Ca^{2+} by lactoferrin has recently been confirmed, strengthening the cation chelator hypothesis [57], thus also explaining the broad antibacterial spectrum of lactoferrin [58,59]. However since many other polycations including lactoferricin, a cationic peptide fragment of lactoferrin, competitively displace divalent cations from LPS in a process preceding so-called self promoted uptake [1], it is possible that lactoferrin also displaces rather than chelates divalent cations from LPS.

By damaging the bacterial membrane, lactoferrin is able to increase the antibacterial effect of commercial drugs like rifampicin [54]. Synergy has also been demonstrated between lactoferrin, lysozyme and other proteins secreted on the mucosal surface [60,61], with potential advantages to host defenses. The proposed mechanism is that lactoferrin interacts with lipoteichoic acid on the surface of *Staphylococcus epidermidis* resulting in a decrease in the negative charge in the membrane, thus allowing lysozyme to reach the cell wall-associated peptidoglycan, that is buried deeper in the membrane [62]. Bacteriophages are also known to be potent antibacterial agents. *In vivo* models of mice infected intravenously with either *E. coli* or *S. aureus* demonstrated that the combined effect of lactoferrin and bacteriophages reduced the numbers of recovered bacteria significantly more than either agent alone [63]. Supporting evidence of synergy between lactoferrin and bacteriophages has been demonstrated in a patient suffering from a prolonged antibiotic-resistant external ear infection [64].

It has also been demonstrated that the N-terminal lobe of lactoferrin possesses a serine protease-like activity. Studies have shown that lactoferrin is able to proteolytically degrade IgA1 and Hap, two autotransported proteins of *Haemophilus influenzae*, thus attenuating the virulence and preventing colonization [65]. Further studies have revealed that lactoferrin is able to cleave proteins in arginine-rich regions, and that the protease active site is situated in the N-terminal lobe [66]. Numerous bacterial strains have developed an ability to infect human cells. When sensing the presence of potential target cells, these bacterial strains start to secrete virulence proteins using their complex type III secretion systems [67–71]. Lactoferrin has the ability to degrade some of these proteins, such as IpaB and IpaC secreted by *Shigella*. These proteins normally form a complex in the host cell membrane, and are key components responsible for bacterial invasion; thus their degradation leads to inhibition of bacterial uptake into host cells [72,73]. Analogous effects are observed for enteropathogenic *Escherichia coli* where lactoferrin causes loss and degradation of several type III secretion proteins (EspA, EspB and EspC), thus inhibiting bacterial virulence, blocking bacterial adherence, and inducing actin polymerization in HEp2 cells [73–75]. Similarly it has

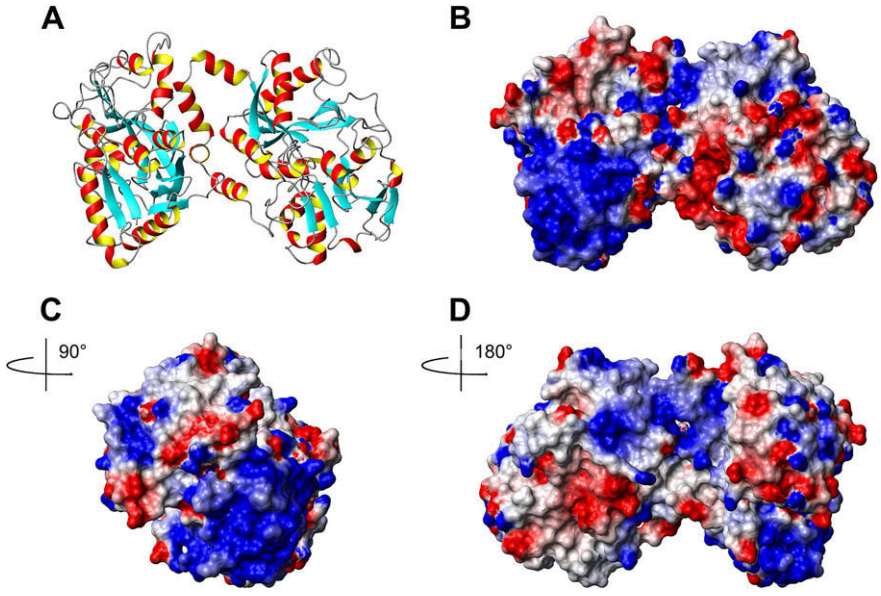

Fig. 1. Lactoferrin structure. (A) Crystal structure of bovine lactoferrin (PDB code 1BLF) [169] presented as a ribbon diagram, illustrating the blue β-strands and red and yellow α-helices. (B) A charge distribution plot of lactoferrin in the same orientation as (A), colored blue, white and red, corresponding to net positive, neutral and negative charge, respectively. This illustrates the highly cationic N-terminal portion of the protein in the bottom left corner. Charge distribution plot of lactoferrin from diagram B rotated (C) 90° and (D) 180° around the *Y*-axis. All the figures have been prepared with use of the graphic program MolMol 2K.2 [170].

been demonstrated that both adhesion and invasion of *Salmonella typhimurium* into HeLa cells can be inhibited in the presence of lactoferrin, possibly due to direct interaction between lactoferrin and the bacterial surface [76]. Lactoferrin may also oppose bacterial invasion of host cells through direct interaction with the bacteria or bacterial target molecules on the host cell surface (for review see [77]).

Biofilm formation, which represents a colonial organization of bacterial cells, is a well studied phenomenon, especially for *Pseudomonas aeruginosa* where it has been proposed to occur in patients suffering from cystic fibrosis. Through biofilm formation, bacteria also become highly resistant to host cell defense mechanisms and antibiotic treatment [78]. However, interestingly, lactoferrin inhibits biofilm formation of *P. aeruginosa* at concentrations lower that those needed to kill the bacteria or prevent its regular growth [79]. Another organism that provides a challenge to cystic fibrosis patients is *Burkholderia cepacia*, which is highly intrinsically resistant to antibiotics. However, growth of *B. cepacia* in both planktonic and biofilm cultures can be inhibited by physiological concentrations of lactoferrin. It has also been demonstrated that lactoferrin can enhance the susceptibility of *B. cepacia* to rifampicin [80]. Biofilms of *S. epidermidis* becomes more susceptible to lysozyme and vancomycin if treated with lactoferrin. [81]. It is well known that some bacterial strains require high levels of iron to form biofilms. Thus lactoferrin as an iron chelator has been hypothesized to effectively inhibit biofilm formation through iron sequestration [82]. Addition of iron or iron-saturated lactoferrin to the media has also been demonstrated to stimulate aggregation and biofilm formation in both *P. aeruginosa* and *B. cepacia*, confirming this hypothesis [83].

The importance of iron for bacterial growth, in combination with the iron sequestering ability of host components like lactoferrin [12,13], have stimulated bacterial strains to develop strategies to overcome iron depletion. Under iron-restricted conditions, a number of Gram-negative bacterial pathogens have developed mechanisms for acquiring iron from iron-saturated lactoferrin. The mechanism involves the binding of lactoferrin to specific heterodimeric lactoferrin receptors (e.g. LbpA and LbpB) on the bacterial surface [84,85]. It has been proposed that lactoferrin binding to LbpA results in a conformational change in the C-lobe of lactoferrin resulting in the release of iron into the bacterial periplasmic compartment where it interacts with iron-binding proteins that mediate transport into the cell [86]. *Streptococcus pneumoniae* has been specifically demonstrated to recognize and bind human lactoferrin, using a surface receptor homologous to pneumococcal surface protein A, and it has been suggested that *S. pneumoniae* may use this receptor to overcome iron limitation at mucosal surfaces [87]. Pneumococcal surface protein A interaction with lactoferrin also reduces the antibacterial activity of lactoferrin by reducing its accessibility to the bacterial membrane [88]. Analogous lactoferrin receptors have been identified on the surface of *Helicobacter pylori* [89]. However, judging from *in vivo* experiments it appears that the combined addition of bovine lactoferrin and probiotics to the standard triple therapy (i.e. omeprazole, clarithromycin, amoxicillin) for *H. pylori* improves the eradication rate and reduces side effects of this antibiotic treatment [90,91]. The contradicting results from Tursi et al., which demonstrated no significant improvement of the *H. pylori* eradication by lactoferrin, may be due to a limited patient population [92].

In *Escherichia coli* it has been shown that lactoferrin interacts with the two porins, OmpF and OmpC, in a mechanism that delivers iron to the bacteria [93]. *Mycoplasma pneumoniae* has a highly specific receptor for recognizing and binding lactoferrin, but not the closely related transferrin [94]. In addition, lactoferrin receptors have also been identified on *Neisseria gonorrhoeae* [95], *Neisseria meningitides* [96] and on non-encapsulated *Haemophilus influenzae* [97] and *Haemophilus somnus* [98].

Some bacteria have also developed defense mechanisms against lactoferrin. *Vibrio vulnificus'* swarming is tightly regulated by expression of the *vvpE* gene, encoding a metalloprotease VvpE. It has been demonstrated that this bacterial protease is also able to destroy two important components of mucosal immunity, i.e. IgA and lactoferrin. These results suggest that VvpE is a key player for surface adhesion and colonization of *V. vulnificus*, by inactivating IgA and lactoferrin [99].

3. Antiviral activity

The antiviral activity of lactoferrin has been investigated in great detail. Pioneer work demonstrated that only enveloped viruses were affected, and that this activity was due to either inhibition of virus–host interaction e.g. hepatitis B virus (HBV) [19], herpes simplex virus (HSV) [100] (for review see [101]) and human cytomegalovirus (HCMV) [20] or direct interaction between lactoferrin and the viral particle e.g.; feline herpes virus (FHV-1) [16], hepatitis C virus (HCV) [21,35], hepatitis G virus (HGV) [21] and human immunodeficiency virus (HIV) [17,25,38] (for review see [102]) (Table 1). However, recently it has also been demonstrated that naked viruses like rota-, polio-, adeno- and enterovirus [22,29,30, 103,104] are susceptible to inhibition by lactoferrin (Table 1). In all cases studied, it appears that lactoferrin exhibits its antiviral activity at an early phase of the infection process [16,17,19,22,29,30,35,100,102–105]. *In vitro* studies also demonstrated that lactoferrin exhibits synergy, in combination with zidovudine, against HIV-1 [106]. A synergistic antiviral activity was also observed for HSV-1 and HSV-2 when acyclovir was used in combination with lactoferrin [107,108]. In clinical trials on a limited set of HCV patients, it was demonstrated that lactoferrin significantly reduces the HCV RNA titer, and contributes to the effectiveness of a combined therapy with interferon and ribavirin [109]. Oral administration of lactoferrin has also led to promising improvement in the immune responses of antiretroviral therapy-naïve children suffering from HIV [110].

3.1. Antiviral mode of action

A broad panel of experimental assays has been developed for lactoferrin mode of action studies. Pre-incubation of human or

bovine lactoferrin with the host cell appears to be essential for its antiviral activity against a spectrum of viruses, e.g. HBV, HS-adapted Sindbis virus, Semliki Forest virus, HCMV, HSV-1 and HSV-2 [19,20,100,105]. Time of addition studies demonstrated that 5–10 min pre-incubation of lactoferrin with the host cell was sufficient to prevent HCMV infection, even when lactoferrin was removed after the addition of the virus [20]. Expression of both early and late HCMV antigens, as well as production of infectious viral progeny, were effectively inhibited by both human and bovine lactoferrin and did not relate to the presence of bound Fe^{3+} [3]. Complementary studies demonstrated that the anti-HCMV activity of both lactoferrins was abolished if lactoferrin was added after viral penetration, thus leading to the conclusion that lactoferrin acted at the level of virus adsorption or penetration [34].

In addition to this, no significant change in the antiviral activity of either human or bovine lactoferrin was observed upon pre-incubation of lactoferrin with HSV-1 or HSV-2 prior to infection, which was interpreted to indicate that the antiviral activity of lactoferrin is exerted through interaction with cellular rather than viral targets [100]. Conversely, Marchetti et al. [23,24] suggested that lactoferrin prevents HSV entry in part by binding to the virus particles; however these mechanisms need not be exclusive, and may reflect the different experimental conditions. Electron micrographs have confirmed that lactoferrin must be located at the cell surface to exert antiviral activity against HSV [100]. It has also been demonstrated that lactoferrin remains at the cell surface after exposure [100,111,112], which may explain the post-infection effect of lactoferrin that is observed, by plaque reduction assays, for HSV on Vero cells [100].

Lactoferrin-mediated inhibition of viral infection through interference with virus–host cell interactions seems likely to involve widespread host cell surface molecules. Proteoglycans are found in all types of tissue, in intracellular granule secretions [113], extracellular matrix [114], and on the cell surface [115]. They consist of a core protein and one or more covalently attached glycosaminoglycan chains, which are highly sulfated, rendering these molecules amongst the most anionic compounds present at mammalian cell surfaces [116]. This strong net negative charge permits glycosaminoglycans to bind to small cations [117], proteins [118], enzymes [119] growth factors [120–122], cytokines [123], chemokines [124] and lipoproteins [125,126], in addition to a number of pathogens such as viruses [127,128].

One of the most important glycosaminoglycan molecules for virus interaction is heparan sulfate [127,128]. Lactoferrin also binds heparan sulfate with a rather high affinity [129], as a result of its two N-terminal glycosaminoglycan-binding domains [130–132], and this is likely responsible for efficient blocking of viral HSV-1 entry [23,100,133]. The anti-HSV activity of lactoferrin has been investigated with several cell lines, both deficient for and expressing different glycosaminoglycan molecules at the cell surface. It was demonstrated that heparan sulfate at the cell surface is important for lactoferrin-mediated antiviral activity against HSV [100,133]. In these studies, there was no detectable difference in the ability of lactoferrin to block viral entry when pre-incubated with the cells prior to infection or when added after viral attachment (1 h at 4 °C) [100].

The two viruses HSV-1 and HSV-2 differ in their interaction with heparan sulfate [134], which in turn may explain their different susceptibility for inhibition with lactoferrin [107]. Recently it was demonstrated that bovine lactoferrin inhibition of HSV-2 entry, in contrast to inhibition of HSV-1, is not due to interference with viral glycoprotein C interaction with heparan sulfate [135]. This is in agreement with other observations demonstrating that heparan sulfate-dependent interaction with target cells differs considerably between HSV-1 and HSV-2 [134,136,137]. Similar results have also been shown for heparan sulfate-adapted Sindbis and Semliki Forest viruses, whereby their ability to infect BHK-21 cells could be effectively blocked by lactoferrin, while non-adapted viruses were not affected [105]. There is evidence that lactoferrin needs to be at the cell surface to block viral entry, and given the rapid partial internalization of lactoferrin [100,112], there is reason to suggest that host cells are able to enact long lasting antiviral immunity. Similar immunity to HSV infection, lasting for several hours, has also been reported for derivatives of dispirotripiperazine [138] that also interact with heparan sulfate.

Heparan sulfate and other glycosaminoglycans are also known to play key roles in HSV cell-to-cell spread, a mechanism crucial for viral escape from the host immune response. Consequently it has been hypothesized that lactoferrin may also interfere with viral cell-to-cell spread. To investigate this, green monkey kidney cells were infected with a low MOI of HSV-1, and following 8 h of infection, pooled human sera and neutralizing antibody were added to the infected cells, in the presence or absence of bovine lactoferrin. The results demonstrated that HSV-1 was able to spread to adjacent cells in the absence of lactoferrin, but this was inhibited by lactoferrin [139]. Human lactoferrin can inhibit HSV cell-to-cell spread, albeit less effectively than bovine lactoferrin. Inhibition of cell-to-cell spread of HSV-2 is less affected by lactoferrin [112].

Not all lactoferrin-inhibitible viruses require heparan sulfate as an attachment receptor on the host cell. Hantavirus infection of Vero E6 cells results in formation of foci, and when treated with lactoferrin, the number of foci are significantly reduced [36]. The antiviral effect was increased when the cells were pre-incubated with lactoferrin, and reduced if the cells were subsequently washed with PBS [36]. High affinity interaction between lactoferrin and heparan sulfate will not be affected by PBS washing, thus indicating that the antihantavirus effect of lactoferrin is due to a weak interaction between lactoferrin and an unknown cell surface molecule.

For some viruses, the viral particle itself appears to be a crucial target for lactoferrin. HCV infection of PH5CH8 cells was effectively inhibited by pre-incubation of bovine lactoferrin with the viral particle prior to infection. Conversely, pre-treatment of the host cells with bovine lactoferrin had no effect on the viral infection rate, indicating that bovine lactoferrin exerted its anti-HCV activity through direct interaction with the viral particle [35]. Similar results have recently been

reported for camel lactoferrin, demonstrating complete inhibition of virus entry when lactoferrin and HCV were pre-incubated together, while lactoferrin pre-incubation with human leukocytes prior to HCV infection had no effect on viral entry [140]. Both human and bovine lactoferrin have been demonstrated to interact directly with two envelope proteins in HCV, E1 and E2 [39]. Similarly, direct interaction between lactoferrin and the virus particle has been demonstrated for HIV, where lactoferrin strongly interacts with the V3 loop of envelope protein gp120. Thus it has been hypothesized that shielding of this domain results in the inhibition of HIV fusion and entry into MT4 cells [38]. Both HIV-1 replication and syncytia (giant cell) formation can also be inhibited in a dose-dependent manner by lactoferrin, and the effect was not dependent on the ferric ion loading of lactoferrin [25]. Supporting studies have demonstrated that bovine lactoferrin can block HIV-1 infection using either CXCR4- or CCR5 receptor, thus clearly targeting the HIV-1 entry process [17].

The antiviral mechanism of lactoferrin appears to be equally complex for the naked viruses, in that lactoferrin has been demonstrated to inhibit replication of rota-, polio- and adenovirus in a dose-dependent manner [29,30,103]. Apolactoferrin (iron-free) can bind to the rotavirus particle and prevent both hemaglutination and virus binding to cellular receptors [103]. This antiviral activity was gradually inhibited by saturation with Fe^{3+}, Fe^{2+} or divalent cations such as Mg^{2+} or Zn^{2+}, with the latter being more inhibitory [26]. Antiviral activity towards poliovirus generally requires the presence of lactoferrin during the viral adsorption step, although zinc-saturated lactoferrin strongly inhibits viral infection when added after viral internalization [30]. Inhibition of adenovirus replication also requires addition of lactoferrin before or during the viral adsorption step [29]. Lactoferrin activity against adenovirus infection in HEp2 cells involves competition for viral glycosaminoglycan receptors on the host cells, which is mediated through the N-terminal half of the protein, which is sufficient for inhibition [141]. Further studies have demonstrated that this neutralization of adenovirus is due to direct interaction between lactoferrin and the structural viral proteins, III and IIIa [104]. However, a strict species and cell specificity has been demonstrated for lactoferrin to inhibit adenovirus infection. For example, human lactoferrin has been shown to facilitate adenovirus entry into A549 cells rather than inhibiting viral entry, in a process unrelated to the presence of cellular glycosaminoglycans like heparan sulfate, or the coxsackie and adenovirus receptor, CAR [142]. A similar but much weaker effect was demonstrated for bovine lactoferrin. The cytopathic effect of enterovirus 71 (EV71) in human embryonal rhabdomyosarcoma cells is also inhibited by both bovine and human lactoferrin. However, ongoing infections are resistant to inhibition, suggesting that the antiviral activity of lactoferrin is exerted at the level of viral adsorption [22]. It was demonstrated that lactoferrin interacts with both the host cell membrane and VP1 protein on the surface of the EV71 particle [143]. Echovirus 6, another member of the enterovirus family, can infect green monkey kidney cells, which subsequently die as a result of apoptosis. This programmed cell death is also inhibited by lactoferrin [144]. These results indicate that lactoferrin interacts directly with the echovirus capsid, possibly leading to stabilization of the virion's conformation and rendering it resistant to uncoating. Thus echovirus 6 inhibition is dependent on lactoferrin interaction with viral structural proteins rather than cellular glycosaminoglycans [145].

Lactoferrin can also modulate the host cell response to viral pathogens. It has been demonstrated that lactoferrin can work as a double-edged sword, preventing HIV uptake by dendritic cells, while on the other hand complexing with natural anti-HIV antibodies, thus enhancing HIV attachment on dendritic cells [146]. An even more sophisticated response is reported in mouse peritoneal macrophages treated with bovine lactoferrin after infection with vesicular stomatitis virus (VSV). In this model, the virus yield was significantly reduced and the antiviral effect was due to the induction of interferon-α/β expression resulting in inhibition of viral replication, rather than inhibition of entry or direct viral inhibition [147].

4. Antifungal activity

Candida can colonize mucosal surfaces in healthy individuals and is considered to be analogous to a commensal organism that can also become an opportunistic pathogen when the host fails to control it. The growth of *Candida* is normally strictly controlled by several non-specific host factors, e.g. immunoglobulin A, lysozyme and histatins, secreted on mucosal surfaces [148,149]. Lactoferrin is also secreted on mucosal surfaces and demonstrates species-dependent antifungal activity against *Candida* [150] with the following decreasing order of susceptibility: *C. tropicalis* > *C. krusei* > *C. albicans* > *C. guilliermondii* > *C. parapsilosis* > *C. glabrata*, with the last species being the most resistant. The antifungal mode of action of lactoferrin was proposed to be due to cell wall perturbation [150], as confirmed by cryo-scanning electron microscopy which revealed drastic changes to the cell wall, resulting in surface blebs, swelling and cell collapse [151]. Similar cell wall damage has been reported by Nikawa et al., after *Candida* exposure to both human and bovine lactoferrin [152,153], and it was concluded that the candidacidal activity of human lactoferrin is due to direct interaction of the protein with the fungal cell surface, rather than iron sequestration [154]. Conversely, it has been demonstrated that iron sequestration by lactoferrin is important for host defense against *Aspergillus fumigatus* [155].

The antifungal activity of lactoferrin can be regulated by the metabolic state of the fungus. Experiments have demonstrated that the fungicidal activity of lactoferrin was significantly reduced under anaerobic growth conditions, in the presence of mitochondrial inhibitors and at low extracellular concentrations of Na^+, K^+, Ca^{2+} and Mg^{2+} [156]. The antifungal activity of lactoferrin has also been reported to be considerably lower than the activity of commercially available antifungal drugs. However, the combined use of lactoferrin and several commercial drugs, e.g. clotrimazole, fluconazole, amphotericin B and 5-fluorocytosine, demonstrates additive or synergistic activity [150,157]. Recombinant human

lactoferrin given prophylactically conveyed significantly improved survival in an *in vivo* rat model of co-infection with *C. albicans* and *S. epidermis* [158]. It has also been indicated that lactoferrin can mediated its antifungal activity through the stimulation of host cell immune mechanisms both *in vitro* and *in vivo* [159].

5. Activity against other microbes

A fairly new aspect of the properties of lactoferrin is its activity against a range of other eukaryotic microbes including parasites. To date it has been suggested that lactoferrin possesses antiparasitic activity towards *Pneumocystis carinii* through iron sequestration [43]. There is also evidence supporting a similar mechanism towards the amoeba *Entamoeba histolytica*. Both human and bovine lactoferrin have demonstrated the ability to kill this amoeba in a concentration-dependent manner. The antimicrobial activity was inhibited by both Fe^{2+} and Fe^{3+} and other divalent cations like Mg^{2+} and Ca^{2+} [16]. Similar results were demonstrated for bovine lactoferrin against the *in vitro* growth of *Babesia caballi* and *B. equi* [161].

Interestingly, to counteract iron sequestering by lactoferrin, some parasites have evolved to benefit from this process. Studies with *Tritrichomonas foetus* have demonstrated that when this organism is grown under iron limitation, lactoferrin has the ability to enhance the growth of the parasite, by functioning as an iron source. Lactoferrin is also taken up and released from the parasite in an energy-dependent mechanism [162]. Similar mechanisms of iron acquisition from lactoferrin have been demonstrated for *Tritrichomonas vaginalis* [163]. Direct interactions between lactoferrin and the parasite have also been demonstrated for *Toxoplasma gondii* [164], and although this interaction appears to have no direct cytotoxic effect on the parasite and no obvious effect on the level of parasite entry into the host cell, it appears that human lactoferrin triggers an unknown antiparasitic mechanism in infected CaCo-2 epithelial cells [165].

Plasmodium spp. invasion of cultured cells requires that the pathogen protein circumsporozoite recognizes and binds to host cell heparan sulfate. Lactoferrin is known to interact strongly with heparan sulfate [129], and therefore it has been suggested that the antiplasmodulium activity of lactoferrin results from blocking of this receptor [166]. The circumsporozoite protein from *Plasmodium berghei* has also been demonstrated to bind to low-density-lipoprotein receptor-related protein. However, *P. berghei* invasion of heparan sulfate deficient cells can be effectively inhibited with lactoferrin, indicating that lactoferrin might also bind and block parasite interaction through the low-density-lipoprotein receptor-related protein [167]. Lactoferrin also demonstrates additive or synergistic activity with clinically used antiparasitic compounds [168].

6. Conclusion

Lactoferrin has antibacterial activity towards a spectrum of different bacterial pathogens, through iron sequestration, membrane destabilization, targeting of bacterial virulence mechanisms and host cell invasion strategies. The broad spectrum antiviral activity of lactoferrin is primarily related to inhibition of viral host cell interaction through blocking of host cell heparan sulfate or interaction with viral surface proteins. The antifungal effect of lactoferrin is predominantly linked to iron sequestration and destabilization of the fungal membrane, whereas the antiparasitic activity of lactoferrin may have similarities to its antiviral mode of action, but appears to be mechanistically distinct. Overall the antimicrobial mode of action of lactoferrin is strongly dependent on experimental conditions, demonstrating its tremendous ability to exercise a diverse range of antimicrobial effects.

Acknowledgements

The authors greatly acknowledge Dr Pamela Hamill for valuable suggestions and linguistic assistance. The authors acknowledge funding from the Canadian Institutes of Health Research and the Advanced Foods and Material Network of Centres of Excellence for their peptide research. RH holds a Canada Research Chair.

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