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The antimicrobial activity of lactoferrin: Current status and perspectives

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'This paper is dedicated to the memory of Eraldo Antonini, eminent biochemist, prematurely deceased twenty years ago, on March 19th, 1983'

Abstract

Lactoferrin (Lf) is a multifunctional iron glycoprotein which is known to exert a broad-spectrum primary defense activity against bacteria, fungi, protozoa and viruses. Its iron sequestering property is at the basis of the bacteriostatic effect, which can be counteracted by bacterial pathogens by two mechanisms: the production of siderophores which bind ferric ion with high affinity and transport it into cells, or the expression of specific receptors capable of removing the iron directly from lactoferrin at the bacterial surface. A particular aspect of the problem of iron supply occurs in bacteria (e.g. Legionella) which behave as intracellular pathogens, multiplying in professional and non professional macrophages of the host. Besides this bacteriostatic action, Lf can show a direct bactericidal activity due to its binding to the lipid A part of bacterial LPS, with an associated increase in membrane permeability. This action is due to lactoferricin (Lfc), a peptide obtained from Lf by enzymatic cleavage, which is active not only against bacteria, but even against fungi, protozoa and viruses. Additional antibacterial activities of Lf have also been described. They concern specific effects on the biofilm development, the bacterial adhesion and colonization, the intracellular invasion, the apoptosis of infected cells and the bactericidal activity of PMN. The antifungal activity of Lf and Lfc has been mainly studied towards Candida, with direct action on Candida cell membranes. Even the sensitivity of the genus tricophyton has been studied, indicating a potential usefulness of this molecule. Among protozoa, *Toxoplasma gondii* is sensitive to Lf, both *in vitro* and *in vivo* tests, while Trichomonads can use lactoferrin for iron requirements. As to the antiviral activity, it is exerted against several enveloped and naked viruses, with an inhibition which takes place in the early phases of viral invection, as a consequence of binding to the viral particle or to the cell receptors for virus. The antiviral activity of Lf has also been demonstrated in *in vivo* model invections and proposed for a selective delivery of antiviral drugs. The new perspectives in the studies on the antimicrobial activity of Lf appear to be linked to its potential prophylactic and therapeutical use in a considerable spectrum of medical conditions, taking advantage of the availability of the recombinant human Lf. But the historical evolution of our knowledge on Lf indicates that its antimicrobial activity must be considered in a general picture of all the biological properties of this multifunctional protein.

Antibacterial activity

Bacteriostatic activity

As to bacteria, the initial studies on the activity of lactoferrin were carried out on its capacity of sequestering the iron necessary for bacterial nutrition and, by consequence, on its growth inhibiting effect (Bullen *et al.* 1972). The bacteriostatic action of lactoferrin has been demonstrated against a wide variety of bacteria and has long been known to be of key importance in host defence mechanisms (Weinberg 1993). Bacteriostasis is usually a temporary antimicrobial measure and it can be counteracted by bacterial pathogens which have been able to achieve acquisition of iron from host by means of two principal systems (Ratledge & Dover 2000).

The first is represented by the synthesis of small chelators, called siderophores, which bind ferric ion with high affinity and transport it into cells through a specific membrane receptor (Braun & Killmann 1999). With only a few exceptions bacterial siderophores can be grouped into three structural

classes: the hydroxamates, the catecholates, and the hydroxycarboxylates. Their production takes place under conditions of iron deprivation and can be either chromosome- or plasmid-mediated.

In addition to the synthesis of siderophores, some highly host-adapted bacterial species have developed a specific pathway that removes iron directly from lactoferrin at the cell surface. This process takes place through the action of specific outer membrane receptors that bind and remove iron directly from lactoferrin or transferrin. The synthesis of these receptors is modulated by bacterial iron availability. In the genera *Neisseria* and *Moraxella* the binding between lactoferrin and the bacterial cell is mediated through interaction with a bacterial membrane protein distinct from the transferrin-binding proteins, as demonstrated by isolation of mutants deficient in transferrin binding or lactoferrin binding (Blanton *et al.* 1990) and by analysis of the different compositions of the two ligands (Schrivers & Lee 1989). Recent studies carried out in *Moraxella bovis* (Yu & Schrivers 2002) have demonstrated that lactoferrin receptors are heterodimers composed of two sub-units of identical molecular weight: LbpA and LbpB. LbpA are integral membrane proteins, whereas LbpB are thought to be cell surface-exposed lipoproteins and they function according to the suggested topology model for receptor-ligand complex in the outer membrane.

A particular aspect of the supply of iron is that occurring in bacteria which behave as intracellular pathogens, multiplying in the macrophages of the host. An interesting example is offered by *Legionella pneumophila* in which only three years ago Liles *et al.* (2000) identified a nonclassical siderophore, called legiobactin. In *L. pneumophila* Byrd & Horwitz (1989, 1991) studied the effect of lactoferrin on intracellular multiplication in non-activated and interferon gamma-activated monocytes. They demonstrated that the internalization of apo-lactoferrin into monocytes leads to intracellular iron chelation, thus preventing the growth of the bacterium. On the contrary, the internalization by monocytes of iron-saturated lactoferrin provides an iron source for bacterial growth and can inhibit the anti-microbial activity of interferon gamma. The inhibiting activity of lactoferrin in the apo-form or saturated with different metals has also been studied in our Department (Goldoni *et al.* 2000) following the infection of non-professional phagocytes by *L. pneumophila*. The intracellular multiplication of the bacterium was inhibited not only by apo-lactoferrin, but also by lactoferrin saturated with manganese and zinc, whereas lactoferrin saturated with iron enhanced the intracellular growth.

Bactericidal activity

This schematic representation of the antibacterial activity of lactoferrin as a battle of chelating agents, soon appeared too simple to explain many experimental data. Already in 1977 Arnold *et al.* had suggested that lactoferrin could exert an antibacterial action distinct from a simple iron deprivation. And I remember that in the 80's in Rome our group, on the basis of experiments carried out separating lactoferrin from bacteria by a dialysis membrane, or immobilizing the same protein on Sepharose 4B, or testing the activity of Zn-lactoferrin, postulated a direct interaction between lactoferrin and bacterial surface (Dalmastri *et al.* 1988) (Orsi *et al.* 1988). Our research had been encouraged by the late Professor Eraldo Antonini and carried out with him from 1977 with a series of investigations on conalbumin, which had been taken as a model for the activity of transferrins. In experimental infection of newborn guinea pigs with *E. coli* O111 we demonstrated the protective effect of conalbumin orally administered (Antonini *et al.* 1977).

A more detailed information of the direct action of this protein on bacteria was given by the observation made by Ellison *et al.* (1988, 1990) who reported that apo-lactoferrin binds to the outer membrane of Gram-negative bacteria. This binding produces the rapid release of lipopolysaccharides with an associated increase in membrane permeability. A direct binding to the lipid A part of lipopolysaccharide was reported by Appelmelk *et al.* (1994). In particular the binding takes place to the phosphate group within the lipid A part, inducing a rigidification of the acyl chains of lipopolysaccharides, as demonstrated by Brandenburg *et al.* (2001).

An analogous mechanism of antibacterial action is exerted also by lactoferricin, a peptide obtained by Tomita *et al.* (1991) upon gastric pepsin cleavage of lactoferrin and active against a wide range of Gram+ and Gram− bacteria (Bellamy *et al.* 1992). Lactoferricin is derived from the amino-terminal region of lactoferrin of different animal species and bovine lactoferrin appears to be the most efficacious (Vorland 1999). The mechanism of action of lactoferricin has been only partially clarified. The peptide binds to lipopolysaccharide in Gram− bacteria and to techoic acid in Gram+ bacteria (Vorland 1999). From there it is assumed that it is brought to the cytoplasmic mem-

brane, where it exerts its effect by disintegrating the cytoplasmic membrane. This action can be explained by the conformational flexibility off the lactoferricin portion in the lactoferrin molecule that allows it to form an amphipatic structure in solution. This amphipathic character of lactoferricin (Hwang *et al.* 1998) makes its structure similar to that of a vast number of cationic peptides which exert their antimicrobial activities through membrane disruption (Testa 2002). In addition to its action against bacteria, lactoferricin is effective also towards fungi, protozoa and viruses, which will be discussed later.

Additional antibacterial activities

Besides the bacteriostatic and bactericidal activities of lactoferrin, which have been considered up to now in general, there are also some additional or complementary activities which must be taken into account in relation to microbial virulence and pathogenicity. They will be cited on the basis of the different steps which characterize the relationship between the bacterium and the parasitized host.

First of all a reference should be made to a paper (Singh *et al.* 2002) underlining the role that lactoferrin can have in the innate immunity by blocking the biofilm development by the opportunistic pathogen *Pseudomonas aeruginosa*. At concentrations lower than those killing or preventing the growth, and with iron chelating activity, lactoferrin stimulates twitching, a specialized form of surface motility, causing the bacteria to wander across the surface instead of forming clusters or biofilms. This phenomenon must be considered with great interest since the formation of biofilm is a very important step in the colonization of the host.

Concerning the adhesion to the cells of the host, lactoferrin has also been shown to inhibit the attachment of *Helicobacter pylori* to the gastric epithelial cells, probably, as suggested by Dial and Lichtenberger (2002), by interaction of oligomannoside-type glicans present in the lactoferrin molecule and bacterial adhesins which recognize this type of substance.

Moreover, in a series of papers published by Qiu *et al.* (1998), Plaut *et al.* (2000) and Hendrixson *et al.* (2003) it has been demonstrated that lactoferrin attenuates the potential pathogenicity of *Haemophilus influenzae* by cleavage and removing two putative colonization factors, namely the IgA1 protease protein and the Hap adhesin. Lactoferrin acts as a serine protease capable of cleaving arginine-rich sequences and probably this highly specific activity could also be extended towards a variety of microbial virulence proteins in different bacterial species.

Furthermore, it has been observed that lactoferrin, independently of the iron-binding status of the protein, is capable of inhibiting the intracellular invasion by pathogens such as *Escherichia coli, Listeria monocytogenes* and *Shigella flexneri*. In invasive *E. coli* harboring a plasmid with the inv gene from *Yersinia pseudotuberculosis* (Longhi *et al.* 1993) a binding of lactoferrin to HeLa cell membrane as well as to bacterial outer membrane was observed. In *L. monocytogenes* Conte *et al.* (1999) showed that two surface proteins, of approximately 80 and 60 kDa bind to lactoferrin. This year, in *S. flexneri* Gomez *et al.* (2003) have demonstrated that lactoferrin induces degradation of invasion plasmid antigens IpaB and, to a lesser extent, IpaC, the key proteins responsible for bacteria directed phagocytosis in mammalian cells.

The lipid A-binding amino-terminal portion of lactoferrin induces release of invasion antigens but not degradation of IpaBC, which is, however, made susceptible to breakdown by surface expressed protease(s).

Lactoferrin is also able to amplify the apoptotic signals in infected cells, shifting the equilibrium between apoptosis and necrosis towards the programmed cell death, as shown by Valenti *et al.* (1999) in Caco-2 intestinal cells invaded by *L. monocytogenes* and by Ajello *et al.* (2002) in HeLa S3 cells invaded by group A streptococci.

Finally, after bacteria have undergone phagocytosis, lactoferrin may contribute to the bactericidal activity of PMN. This effect takes place by donating the iron required for catalyzing hydroxyl radical production. By reason of the stable binding of iron to lactoferrin even at low pH, the prooxidant activity of the protein is exerted in the intracellular environment of the activated neutrophil where the acidic phagolysosome fuses with the lactoferrin-containing specific granules (Ward *et al.* 2002).

Antifungal activity

Regarding the antifungal activity of lactoferrin, the first observation which can be made is that the great majority of research has been carried out on *Candida*, well known as one of the most dangerous opportunistic pathogens. As for bacteria, the anti-*Candida* activity of lactoferrin was initially considered as re-

lated to its ability to bind and sequester environmental iron. But in addition to the iron-chelating activity, a direct interaction between lactoferrin and *Candida* cells was demonstrated in our Department by Valenti *et al.* (1986).

The mechanism of this candidacidal activity is related to alteration in cell surface permeability, since lactoferrin and its derived peptide lactoferricin bind directly to *Candida* cells (Bellamy *et al.* 1993) and show an action on *Candida* cell membrane (Wakabayashi *et al.* 1996). The effect of lactoferrin and lactoferricin has also been demonstrated on the hyphal growth of some azole-resistant strains of *Candida albicans* (Wakabayashi *et al.* 1998).

In addition to all this fundamental research carried out on *Candida*, it is necessary to cite also some investigations by Wakabayashi *et al.* (2000, 2002) and by Yamauchi *et al.* (2000) on dermatophytic fungi belonging to the genus *Trichophyton*. The sensitivity of these parasites to lactoferrin was tested *in vitro* and *in vivo*, in animal models and in patients, indicating another important potential usefulness of lactoferrin.

Antiprotozoal activity

In contrast to the great quantity of research carried out on bacteria, the data available on the antiprotozoal activity of lactoferrin are scarce. *Toxoplasma gondii* appears to be sensitive and it has been suggested that lactoferrin binds as a cationic compound to the strong negative charges which are present on the surface of tachyzoites (Cintra *et al.* 1986). Experiments carried out in murine macrophages and embryonal cells showed that lactoferrin had no effect on the penetration of the parasites, while development of intracellular parasites was inhibited linearly in concentration of lactoferrin (Tanaka *et al.* 1997). Research in experimental murine toxoplasmosis demonstrated that bovine lactoferricin intraperitoneally or orally administered induces resistance to *T. gondii* in mice (Isamida *et al.* 1998). This reduction of the infectivity of sporozoites has also been confirmed in studies carried out in parallel with sporozoites of *Eimeria stiedai* (Omata *et al.* 2001).

Contrary to these results, lactoferrin does not inhibit trichomonads, but in fact can represent an iron source for these parasites. This has been demonstrated for *Trichomonas vaginalis*, a human pathogen (Lehker & Alderete 1992), and for *Tritrichomonas foetus*, an agent responsible for venereal disease of cattle (Tachezy *et al.* 1998). Both these parasites utilize lactoferrin for iron requirements by means of specific receptors which make possible the binding and the successive internalization.

Antiviral activity

As to the antiviral activity of lactoferrin, it was demonstrated much later, but since then many experimental data have been collected, as it appears in the valuable reports by van der Strate *et al.* (2001) and Marchetti & Superti (2001).

Only in a few cases it is reported that lactoferrin failed to prevent virus infection. On the contrary, a long list of virus has been found to be sensitive to the inhibiting action of lactoferrin. This list includes several enveloped viruses such herpes simplex virus 1 and 2 (Hasegawa *et al.* 1994), human cytomegalovirus (Hasegawa *et al.* 1994), human immunodeficiency virus (Harmsen *et al.* 1995), hepatitis B virus (Hara *et al.* 2002), hepatitis C virus (Ikeda *et al.* 1998), respiratory syncytial virus (Grover *et al.* 1997), hantavirus (Murphy *et al.* 2000) and four naked viruses: rotavirus (Superti *et al.* 1997), poliovirus (Marchetti *et al.* 1999), adenovirus (Arnold *et al.* 2002) and enterovirus 71 (Lin *et al.* 2002).

Both human and bovine lactoferrin show this inhibiting activity, which is exerted not only by intact lactoferrin but also, in many cases, by enzymatic digests of the molecule, as observed for herpes simplex virus (Siciliano *et al.* 1999), cytomegalovirus (Andersen *et al.* 2001), adenovirus (Di Biase *et al.* 2003) and rotavirus (Superti *et al.* 2001).

The effect on viral infection does not appear related to the iron withholding from the environment and in many cases was shown to take place even with metal-saturated isoforms of lactoferrin, but the reason for this is unknown (van der Strate *et al.* 2001).

As to the mechanism of action of lactoferrin on viruses, it is generally accepted that the inhibiting activity takes place in the early phases of viral infection, rather than inhibiting virus replication after the target cell has been infected. For many susceptible viruses, a direct binding of lactoferrin to viral particles occurs. Another mechanism for the antiviral activity is binding to host cell molecules which the virus uses as a receptor or a co-receptor.

The antiviral activity of lactoferrin has also been demonstrated in a few *in vivo* models. The first observation was made by Lu (Lu *et al.* 1987) who noticed

that lactoferrin prolonged the survival rates in mice infected with Friend virus complex. Fujihara & Hayashi (1995) observed the inhibition of herpes simplex virus-1 infection to mouse cornea by bovine lactoferrin topically administered prior to virus inoculation. Shimizu *et al.* (1996) reported the protective effect of bovine lactoferrin saturated with iron against the infection by murine cytomegalovirus.

Finally, in human subjects, Tanaka *et al.* (1999) showed that bovine lactoferrin inhibits hepatitis C virus viremia in chronic hepatitis C patients and this result was confirmed in a subsequent paper by Iwasa *et al.* (2002) in patients with high viral loads and hepatitis C virus genotype 1b. In addition to its direct effect on virus particles or their target cells, lactoferrin can also exert an indirect action through its influence on immune cells (van Hooijdonk *et al.* 2000), demonstrated *in vivo* against the Friend virus complex and murine cytomegalovirus. The capacity of lactoferrin for binding specifically to many virus particles or viral receptors has also suggested a possible use of this protein for a selective delivery of antiviral drugs (van der Strate *et al.* 2001).

Future perspectives

All the studies cited demonstrate the enormous amount of research carried out on this subject and the current status of our knowledge on the antimicrobial activity of lactoferrin. As to the future, an important development will be offered by the potential prophylactic and therapeutic use of lactoferrin or its derived peptides in a wide spectrum of medical conditions, taking a great advantage from the availability of the recombinant form of human lactoferrin. An interesting perspective which can be foreseen is the use of lactoferrin or its derived peptides in combination with other antimicrobial compounds. As to bacteria, there is in general the problem of the limitation of a possible use of lactoferrin in association with antibacterial drugs only for pathogens incapable of utilizing lactoferrin as an iron source (Weinberg 2001).

Much more promising appears, on the contrary, the case of the combination of lactoferrin with antimycotic drugs, since fungi are not able to extract iron from lactoferrin. As to *Candida*, the problem of the infections caused by these microorganisms in immunocompromised patients is always present, since these subjects develop one or more fungal infections during their illness. Fungal infections are widely treated with triazole antifungal agents, such as fluconazole. Unfortunately long term therapies have led to the emergence of fluconazole-resistant strains which are resistant not only to the other azoles but also to amphotericin B (Kelly *et al.* 1996). Therefore it has been proposed to support the activity of the common antifungal agents with other compounds which represent non specific host defence factors.

Trials made with lactoferrin showed an encouraging synergistic effect on the growth of several *Candida* species by the combined action of fluconazole and lactoferrin (Kuipers *et al.* 1999), and this effect was not only demonstrated by lactoferrin but even by lactoferricin (Wakabayashi *et al.* 1998). Another interesting case is that of *Pneumocystis carini*, an important pathogen responsible for morbosity and mortality in patients with AIDS, in which the combined use of lactoferrin and antimicrobial agents showed a significant decrease in cystic and trophic forms, by the combination of lactoferrin and clarithromycin (Cirioni *et al.* 2000).

Furthermore, a possible field of application of lactoferrin in combination with specific chemotherapeutic agents is also the treatment of latent or persistent viral infections which are typical of immunosuppressed hosts. This is the case of infections caused by HIV in which the therapeutic protocol is getting more and more elaborate and the synergistic effect of zidovudine and lactoferrin has already been demonstrated (Viani *et al.* 1999). Also in the case of cytomegalovirus infections a combination of lactoferrin with the anti-cytomegalovirus drug cidofovir has been proposed (van der Strate *et al.* 2001).

This effect *in vitro* can even be increased *in vivo* by the stimulatory effect produced by lactoferrin not only on NK cells, but also in monocytes and granulocytes (Levay & Viljoen 1995, Lonnerdal & Yver 1995).

This phenomenon recalls the interactions which have been observed between lactoferrin and other important immunological factors. As pointed out by Weinberg (2001), several investigators have noted the joint presence of lactoferrin and lysozyme in milk, specific granules of polimorphonucleates, tears and tubotympanum mucus. And in *in vitro* tests a synergy between lactoferrin and lysozyme was shown towards various bacteria (Ellison & Giehl 1991, Leitch & Willcox 1998).

Moreover, in the past it was observed that lactoferrin may act synergistically with complement (Rainard 1993) and with antibodies (Stephens *et al.* 1980, Dolby & Stephens 1983).

All these data confirm that lactoferrin is a complex and multifunctional protein, a component of natural immunity, and that the study of its antimicrobial activities must always be taken into account in a more general picture of the host's defence.

Considering historically the evolution of our studies on lactoferrin, it is possible to find some singular common characteristics between lactoferrin and another very important type of biological molecule, which is interferon. This class of molecules was discovered in 1957 as antiviral agents and it is still now a very important antiviral drug, representing, as in the case of hepatitis C virus infection, the fundamental therapeutic agent. But during the years it was discovered that interferons are also able to inhibit the proliferation of cancer cells. Due to these combined properties of inducing antiviral resistance and regulating cell growth, interferons are used as therapeutic agents for the treatment of both viral infections and cancer. Interferons are also able to modulate the immune response and this has led to their clinical use in diseases with underlying immunological etiologies, including forms of multiple sclerosis (Williams 2000).

A similar development of studies has been observed with lactoferrin.

After the discovery of this molecule it was first thought that its function was typical of an iron transporter. Later on the attention of researchers was attracted by its chelating activity and iron scavenging properties, which proposed its function as a typical bacteriostatic molecule, capable of playing an important role as a first line of defence against microbial infections.

It was successively ascertained that the antimicrobial activity was not only due to an iron chelation but involved a more direct action against potential pathogens. This action has a wide spectrum and is shown against bacteria, fungi, protozoa and virus.

Further studies devoted to define more precisely the mechanism of this antimicrobial action showed that as in those Russian dolls called 'matrioska', in addition to the bacteriostatic activity of the whole molecule, related to its iron-chelating effect, an even stronger activity can be exerted by isolated amino-terminal basic peptides which can be liberated by pepsin cleavage and designated as lactoferricin.

These peptides have a different mechanism of action and can be liberated by enzymatic cleavage which can take place not only by the action of gastric pepsin but even of pepsin-like proteases in neutrophilic phagolysosomes (Weinberg 2001).

The antimicrobial activity of lactoferrin is in fact the result of a long evolutionary process of a molecule which acts in a complex picture. This includes effects on the production of cytokines, on the immune cells and a general modulatory action on inflammatory response. In conclusion, lactoferrin must be considered not simply as a primary factor of host defence in mammals, but even more in general as a polyvalent regulator, which accomplishes its task by interacting with several components involved in infectious or inflammatory processes.

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