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Anti-Invasive Activity of Bovine Lactoferrin against *Listeria monocytogenes*

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ABSTRACT

We have investigated the possible role of bovine lactoferrin in protecting the intestinal epithelium from bacterial infections, using as an in vitro model enterocyte-like cell lines HT-39 and Caco-2 infected with a food-borne pathogen, *Listeria monocytogenes.* When infection occurred in the presence of 1 mg/ml of bovine lactoferrin, in the form of apolactoferrin or iron- or manganese-saturated forms, the adhesion of bacteria to eukaryotic cells was unaffected, but the number of internalized bacteria was reduced by 42- to 125-fold. The possibility of a toxic effect of lactoferrin was excluded, because bovine lactoferrin was used at nonbactericidal and noncytotoxic concentrations.

Key words: Lactoferrin, *Listeria monocytogenes,* cell invasion

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Listeria monocytogenes is a gram-positive facultative intracellular pathogenic bacterium which grows between 3 and 50°C *(16, 29, 30).* It is responsible for severe systemic infections in several animal species and in humans *(14).* Most of the outbreaks of listeriosis have clearly been shown to be associated with the ingestion of contaminated food *(12). Listeria monocytogenes* has been found in a wide range of dairy products, in meats, egg products, vegetables, and seafood. Many reports indicate that this microorganism can survive for long periods and multiply during storage of refrigerated foods at 4°C *(30).* In addition, this bacterium exhibits high thermal resistance *(3)* as a stable characteristic. Studies devoted to pathogenicity have demonstrated that the intestine is the usual site of entry of this bacterium *(27).*

In vitro, *L. monocytogenes* enters the human enterocyte-like cell line Caco-2 *(13),* mouse embryo fibroblasts, and other cultured epithelial cells *(25).* Using these different models of infection, the invasion process of this bacterium has been well characterized *(7, 9, 22).* The entry into susceptible cells appears to be mediated by an 88-kDa protein, internalin, coded by the *inlA* gene. The steps of invasion of *L. monocytogenes, i.e.,* escape from vacuoles, intracellular survival and growth, and consequent cell-to-cell spread, require the synthesis of a hemolytic cytolysin, listeriolysin, coded by the *hly* gene *(2).*

Lactoferrin is an iron-binding protein with a molecular mass of about 80 kDa which is synthesized by neutrophils and glandular epithelial cells *(5),* and its role in human defense against intestinal infections has been recognized *(4).* Lactoferrin has a high affinity for and binds iron and other metal ions. When lactoferrin is saturated with metal ions different from iron (e.g., manganese), the overall physico-chemical properties of the protein are not changed, with the sole exception of a small perturbation of the metal binding-site conformation (*(5)* and references therein). Although a protective effect of lactoferrin on the entry process of an invasive *E. coli* HB101 (pRI203) strain has been reported previously *(20),* up to now little information has been available concerning the role of lactoferrin in the first steps of the pathogenesis of invasive bacteria.

In this paper, in order to add further information on the effect of lactoferrin on a food-borne gram-positive intracellular facultative pathogen, human enterocyte cell lines (Caco-2 and HT-29) which better mimic an in vivo environment such as intestinal epithelium, were infected with a *L. monocytogenes* ATCC 7644 strain in the presence and absence of bovine lactoferrin. The ability of this natural compound to significantly decrease invasion by *L. monocytogenes* of cultured intestinal cells of both lines is reported. This effect was demonstrated to be independent of metal saturation of lactoferrin and of its antibacterial activity.

MATERIALS AND METHODS

Bacterial strain

Listeria monocytogenes ATCC 7644 (serotype 1), a clinical isolate from a patient suffering meningitis, was used. This strain was routinely subcultured using listeria selective base (Oxford formulation) (OXOID, Unipath, Basingstoke, Hampshire, England).

Host cell lines

HT-29 and Caco-2 cells, both deriving from human colonic carcinoma cell lines, were used as model intestinal cells *(26).*

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Caco-2 cells were kindly provided by Dr. Bernardini (Department of Cellular Biology and Development, University of Rome La Sapienza, Italy). HT-29 cells, a human colon adenocarcinoma cell line, were obtained from the American Type Culture Collection (ATCC, Rockville, MA). The cells were grown in a 5% CO_2 incubator as monolayers at 37°C in Eagle's minimum essential medium (MEM) (containing 1.2 g of $NaHCO_3$ per liter, 2 mM glutamine, 100 U of penicillin per ml 0.1 mg of streptomycin per ml, and 10% heat-inactivated fetal calf serum). For HT-29 cells the medium was supplemented with 1% nonessential amino acids.

Lactoferrin

Unsaturated (apo-) and iron- and manganese-saturated bovine lactoferrin (apoLf, Fe^{3+}-Lf and Mn^{2+}-Lf) were kindly supplied by Besnier-Bridel (Laval, France). Lactoferrin purity was verified by sodium dodecylsulfate-polyacrilamide gel electrophoresis (SDS-PAGE); the gels were stained with silver nitrate. Only one band with an apparent molecular weight of 85,000 was found. Lactoferrin concentration was assessed by UV spectroscopy of a 1% solution at 280 nm on the basis of an extinction coefficient of 15.1 *(15)*. Iron saturation of lactoferrin was determined by recording optical absorption at 468 nm before and after addition of several aliquots (up to a total concentration of 10 molar excess) of iron(III) citrate to the protein dissolved in 0.1 M sodium bicarbonate. The iron saturation of Fe^{3+}-Lf corresponded to about 95%. Manganese saturation of lactoferrin was determined by recording optical spectra between 600 and 400 nm before and after addition of iron(III) citrate to the protein dissolved in 0.1 M sodium bicarbonate in order to measure, after saturation with iron, the metal-binding sites void of manganese ions. The manganese saturation of Mn^{2+}-Lf corresponded to about 85%. It was observed by optical spectroscopy that the rate of the exchange of manganese for iron in lactoferrin is very slow at the stoichiometric concentration of iron. During the first hour of reaction less than 5% of manganese-saturated lactoferrin was transformed to iron-saturated lactoferrin in the experimental conditions used for the invasion assay. An extinction coefficient of 0.54 (468 nm, 1% solution) was used for iron-saturated lactoferrin *(15, 17, 24)*. It should be noted that apolactoferrin dissolved at 1 mg/ml in MEM medium was found to be approximately 10% iron saturated.

Chemicals

All reagents (analytical grade) were purchased from Sigma Chemical Co., St. Louis, MO, USA.

Toxicity of lactoferrin towards cultured cells

Cells propagated in tissue-culture clusters were incubated with different concentrations of apo- or iron- or manganese-saturated lactoferrin at 37°C for 60 min in MEM. Then the cell monolayers were washed in Earle's balanced salt solution; fresh medium was added and the cells were examined after a 24-h incubation period at 37°C. Three parameters were examined: cell morphology, viability (as determined by neutral red staining), and yield.

Antibacterial activity of lactoferrin

Listeria monocytogenes cells, cultured overnight at 37°C in listeria selective base medium, were inoculated (approx 1×10^7 cells per ml) in MEM in the presence of apolactoferrin or iron- or manganese-saturated lactoferrin. The determination of viable bacteria was performed after 1 h of contact by counting CFU on listeria selective agar base.

Adhesion assay

HT-29 and Caco-2 confluent monolayers were infected with 1 ml of bacterial suspension (at a multiplicity of infection of 100 bacteria per cell) in logarithmic-phase growth. Generally 1×10^7 bacteria were added to 1×10^5 cultured cells. The adhesion assay was carried out by keeping cells and bacteria in contact for 60 min at 4°C in the presence or absence of 1 mg/ml of apolactoferrin or iron- or manganese-saturated lactoferrin. Loosely bound bacteria were then removed from the cell monolayers by five washings with phosphate-buffered saline. The monolayers were then fixed in methanol and stained with Giemsa. The number of bacteria bound to cell surfaces was evaluated by random counting of 400 cells.

Invasion assay

HT-29 and Caco-2 confluent monolayers were infected with 1 ml of bacterial suspension in logarithmic phase at a multiplicity of infection of 100 bacteria per cell (10^7 bacterial cells per 10^5 cultured cells). The infection was carried out for 60 min at 37°C, in the presence and absence of 1 mg/ml of apolactoferrin or iron- or manganese-saturated lactoferrin.

Loosely bound extracellular bacteria were then removed from the cell monolayers by five washings. Fresh medium containing 40 μg of gentamicin was added and the cells were incubated for 2 h at 37°C in 5% CO_2. This treatment kills extracellular bacteria but does not affect the viability of intracellular bacteria, because gentamicin is unable to enter eucaryotic cells *(13)*. The intracellular bacteria were cultured on selective medium and CFU were counted after the lysis of the infected monolayers, obtained with the addition to 1 ml of cell suspension of 0.5 ml of ice-cold 0.1% Triton X-100 solution.

Particle agglutination assay

Latex particle suspensions (bead diameter 0.8 μm) (Difco) were coated with apolactoferrin according to the procedure of Naidu et al. *(23)* with minor modifications. *L. monocytogenes* cells grown for 24 h were harvested, washed in 0.02 M potassium phosphate buffer (pH 6.8), and suspended in the same buffer at a concentration of 10^9 cells per ml. Equal volumes of bacterial suspension and latex reagent were placed on a glass slide and gently mixed. The agglutination reaction was read within 5 min.

Other experiments were performed to establish the binding of latex beads coated with apolactoferrin to HT-29 and Caco-2 cells, as previously described *(19)*. The binding of latex beads (5×10^6/ml) to HT-29 and Caco-2 cell monolayers was observed by microscopy after 120 min of incubation of the mixtures at 4°C.

Statistics

Experimental values concerning the antibacterial, antiadhesive, and antiinvasive activity of lactoferrins are means of 3 to 5 independent experiments performed in triplicate. The Student *t* test was used to assess the significance of differences between numerical values.

RESULTS

An initial test of the toxicity of bovine lactoferrin toward cultured cells was performed; the results are reported in Table 1. After the incubation with lactoferrin, the health of the intestinal cells was scored by the examination of the three parameters, cell morphology, viability, and yield. From the data reported in Table 1, it appears clearly that apolactoferrin and iron-saturated bovine lactoferrin at concentrations of up to 4 mg/ml did not produce any damage to HT-29 and

TABLE 1. *Toxicity (on the basis of cell morphology, viability, and yield) of apolactoferrin towards intestinal cultured cells*

Apolactoferrin (mg/ml)	Toxicity apolactoferrin to cultured cells:[a] HT-29	Caco-2
apoLF		
16	+	+
8	+/−	+/−
4	−	−
2	−	−
1	−	−
.5	−	−
.25	−	−
Fe^{3+}-Lf		
16	+	+
8	+/−	+/−
4	−	−
2	−	−
1	−	−
.5	−	−
.25	−	−
Mn^{2+}-Lf		
16	+	+
8	+	+
4	+/−	+/−
2	−	−
1	−	−
.5	−	−
.25	−	−

[a] Three parameters were examined: cell morphology, viability (as determined by neutral red staining), and yield. +, at least one parameter affected in 100% of the cells; +/−, one parameter affected in <50% of the cells; −, none of the parameters was affected. The data were derived from three independent experiments performed in triplicate.

Caco-2 cells, whereas the maximal noncytotoxic concentration for manganese-saturated lactoferrin was 2 mg/ml.

In order to assay the viability of the microbial cells during the invasion experiments, *L. monocytogenes* suspensions (ca. 1×10^7 CFU/ml) in MEM from an overnight culture at 37°C were added to different concentrations of lactoferrins. The data reported in Table 2 show that apolactoferrin and iron- and manganese-saturated lactoferrin exert a weak antibacterial activity only at 8 and 4 mg/ml.

TABLE 2. *Antibacterial activity of unsaturated, iron-saturated, and manganese-saturated lactoferrin in Eagle's minimal essential medium*

Lactoferrin (mg/ml)	*Listeria monocytogenes* (CFU/ml)[a]
0	1.0×10^7
apoLF	
8	1.0×10^6 *
4	3.2×10^6 *
2	8.4×10^6
1	9.2×10^6
.5	9.4×10^6
.25	1.2×10^7
Fe^{3+}-Lf	
8	1.3×10^6 *
4	2.4×10^6 *
2	8.5×10^6
1	9.0×10^6
.5	9.3×10^6
.25	1.0×10^7
Mn^{2+}-Lf	
8	1.1×10^6 *
4	5.6×10^6 *
2	8.0×10^6
1	9.5×10^6
.5	1.2×10^7
.25	1.0×10^7

[a] The values are means of three independent experiments performed in triplicate. SD is omitted for clarity. An asterisk indicates statistical significance ($P < 0.01$).

Effect of lactoferrin on L. monocytogenes *adhesion to cultured cells*

HT-29 and Caco-2 confluent monolayers were infected with 1 ml of bacterial suspensions in the logarithmic phase of growth. The adhesion assay was carried out in the presence and absence of 1 mg/ml of apolactoferrin or iron- or manganese-saturated lactoferrin for 60 min at 4°C, a temperature that does not allow bacterial invasion. The number of adherent bacteria was evaluated by optical microscopy; the data obtained are reported in Table 3. It can be observed that in these conditions the adhesion of bacteria to Caco-2 or HT-29 cell surfaces was not affected.

Effect of lactoferrin on L. monocytogenes *invasion into cultured cells*

HT-29 and Caco-2 confluent monolayers were infected with bacterial suspensions in logarithmic-phase growth for 60 min at 37°C in the presence and absence of 1 mg/ml of lactoferrin. Loosely bound extracellular bacteria were killed by addition of gentamicin and the intracellular bacterial CFU were counted on selective medium after the lysis of infected monolayers. Table 4 reports the internalization of *L. monocytogenes* in the host cells. The invasion efficiency of the bacterium was significantly inhibited by apolactoferrin and the iron- and manganese-saturated forms.

TABLE 3. *Effect of unsaturated lactoferrin (apoLf), iron-saturated lactoferrin (Fe^{3+}-Lf) or manganese-saturated lactoferrin (Mn^{2+}-Lf) on the adhesion of* L. monocytogenes *to cultured cells*

Lactoferrin (1 mg/ml)	Adherent *L. monocytogenes* cells per cultured cells:[a] HT-29	Caco-2
None	0.2	0.6
apoLF	0.3	0.4
Fe^{3+}-Lf	0.3	0.5
Mn^{2+}-Lf	0.2	0.4

[a] The values are means of three independent experiments performed in triplicate; SD is omitted for clarity. There are no significant differences ($P < 0.01$).

TABLE 4. *Effect of unsaturated lactoferrin (apoLf), iron-saturated lactoferrin (Fe^{3+}-Lf) or manganese-saturated lactoferrin (Mn^{2+}-Lf) on the invasion of* L. monocytogenes *into cultured cells*

Lactoferrin (1 mg/ml)	% inoculated *L. monocytogenes* CFU invading cultured cells:[a]	
	HT-29	Caco-2
None	2.5	3.2
apoLf	0.02*[b]	0.03*
Fe^{3+}-Lf	0.03*	0.04*
Mn^{2+}-Lf	0.06*	0.05*

[a] Percentages are means of five independent experiments performed in triplicate. SD is omitted for clarity.
[b] Values with an asterisk are significantly different from the control ($P < 0.01$).

Control experiments were performed by preincubating each of the forms of lactoferrin with bacteria or cells separately before the invasion assays. In these experimental conditions, the lactoferrins were ineffective in protecting against *L. monocytogenes* invasion (data not shown).

Binding of lactoferrin to bacteria or intestinal cultured cells

To assess the putative binding of apolactoferrin to *L. monocytogenes* cells, a particle agglutination assay was performed. Bacterial suspensions showed strongly positive particle-agglutination reactions, readily visible within 1 min, with apolactoferrin. Appropriate controls performed with uncoated or ovalbumin-coated latex beads or with bacterial cells heated at 80°C for 20 min showed no particle-agglutination reactions.

Further experiments were performed by keeping apolactoferrin-coated latex beads in contact with Caco-2 or HT-29 semiconfluent monolayers for 120 min at 4°C. Observation by optical microscopy revealed the binding of lactoferrin-coated beads to the cells. The binding was absent with noncoated beads. This result is consistent with that already reported for the binding of bovine lactoferrin to HeLa cells *(19)*.

DISCUSSION

Listeria monocytogenes is the first reported gram-positive bacterium able to invade intestinal cells and is responsible for severe infections in humans associated with the ingestion of various contaminated foods *(14)*. The incidence of listeriosis appears to be on the increase worldwide. It has been shown that the susceptibility of the human population is increasing, as well as the number and types of foods in which this bacterium is able to survive and grow. In particular, *L. monocytogenes* has been found in a wide range of dairy products, and among these cheese has been extensively examined for its association with food-borne listeriosis *(12)*.

The in vitro and in vivo antimicrobial effects of human lactoferrin towards gram-negative and gram-positive bacteria have been reported by several authors *(1, 4, 5, 6, 10, 11, 28)*. Among the antibacterial mechanisms proposed, it has been suggested that the action of this protein takes place by chelating iron required for bacterial growth *(5)* or by a direct bactericidal effect *(1)*. Since lactoferrin is present in most mucosal secretions, including those of the gastrointestinal tract, we examined the effect of this biological molecule on the virulence properties of an invasive gram-positive bacterium, *L. monocytogenes,* for which the usual site of entry is the intestine.

In our study an interesting finding was obtained when lactoferrin at physiological pH and noncytotoxic concentration was added to enterocyte cell lines Caco-2 and HT-29 together with bacteria during the infection period (60 min at 37°C in the invasion assays). In these conditions a protective effect of lactoferrin against bacterial invasion was clearly demonstrated. This effect does not appear to be related to the iron-witholding ability of lactoferrin since similar results were obtained with apolactoferrin or iron- or manganese-saturated lactoferrin. In fact, manganese-saturated lactoferrin, although it cannot bind or release iron, displays physico chemical properties similar to those of the protein in the unsaturated or iron-saturated forms. A direct antibacterial effect is also excluded since lactoferrins under the above described experimental conditions and at the concentrations used did not exert any antibacterial activity.

This protein is already known to be able to bind in a quantitative fashion to various host cells *(10)* and bacteria *(28)*. In this report we demonstrated the binding of this protein to *L. monocytogenes* as well as to Caco-2 and HT-29 cell surfaces. A decrease in bacterial invasion occurs when lactoferrin, bacteria, and cells are simultaneously present during the infection step. Lactoferrin, being a cationic molecule (pI 8.5), might inhibit the early interactions between bacteria and host cells, since it shares some common properties with the family of polycationic agents *(11)*. However, when lactoferrin is bound to both bacteria and cells, the lack of inhibition of bacterial adherence to the intestinal cultured cells leads to the hypothesis that electrostatic interactions, which govern adhesion in the invasion process, are not hindered. It can be suggested therefore that the surface molecules (such as lipoteichoic acids), which generally mediate the adhesion of gram-positive bacteria to cellular plasma membranes in a nonspecific way *(8)*, do not represent the interaction site of lactoferrin. If the iron-deprivation mechanism, the direct antibacterial effect, and the electrical features of lactoferrin are not involved in its protective action against *L. monocytogenes,* it can be hypothesized that a more specific mechanism is involved. Lactoferrin could probably interact at the level of the trasmembranal bacterial proteins belonging to the internalin family *(13)* that allow the entry process of *L. monocytogenes.*

Our data were obtained in experimental systems which mimic intestinal epithelium and provide additional information on the protective role of lactoferrin against bacterial infections in the gastrointestinal tract. Further studies will be necessary to elucidate the complex mechanism of action of lactoferrin and its influence in in vivo infections mediated by intracellular facultative gram-positive pathogens. In mature

human breast milk the lactoferrin concentration is between 1 and 3 mg/ml *(18)*, while in bovine milk and dairy products the lactoferrin content is much lower, ranging from 0.2 to 0.02 mg/ml *(15, 21)*. Therefore, the addition of bovine lactoferrin, which is commercially available, could improve the healthfulness of those foods which could be contaminated by *L. monocytogenes*, serving as a barrier to prevent the invasion of intestinal cells.

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