Effect of bovine lactoferricin on enteropathogenic Yersinia adhesion and invasion in HEP-2 cells

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Bovine lactoferricin, a pepsin-generated antimicrobial peptide from bovine lactoferrin active against a wide range of bacteria, was tested for its ability to influence the adhesion and invasion of Yersinia enterocolitica and Yersinia pseudotuberculosis in HEP-2 cells. The addition of non-cytotoxic and non-bactericidal concentrations of lactoferricin to cell monolayers before infection, under different bacterial growth experimental conditions, was ineffective or resulted in about a 10-fold increase in bacterial adhesion, whereas, in bacteria grown in conditions allowing maximal inv gene expression, a 10-fold inhibition of cell invasion by lactoferricin was observed. To confirm that the anti-invasive activity of lactoferricin was exerted against invasin-mediated bacterial entry, experiments were also performed utilizing Escherichia coli strain HB101 (pRI203), harbouring the inv gene from Y. pseudotuberculosis, which allows penetration of mammalian cells. Under these experimental conditions, lactoferricin was able to inhibit bacterial entry into epithelial cells, demonstrating that this peptide acts on inv-mediated Yersinia species invasion. As the inv gene product is the most important virulence factor in enteropathogenic Yersinia, being responsible for bacterial adherence and penetration within epithelial cells of the intestinal lumen and for the subsequent colonization of regional lymph nodes, these data provide additional information on the protective role of lactoferricin against bacterial infection.

INTRODUCTION

Enteroinvasive Yersinia species are Gram-negative bacteria capable of infecting animals and humans. Human infections usually arise from ingestion of contaminated foodstuffs (Hoogkamp-Korstanje, 1996). These pathogenic species contain a 70 kb plasmid and chromosome genes encoding a number of virulence factors, of which outer-membrane proteins are the most important (Isberg, 1990). Among these, the inv gene product, the invasin protein, is especially important in the early phases of intestinal infection. In fact, this protein allows the adherence and penetration of cells exposed at the surface of the intestinal lumen and the subsequent colonization of regional lymph nodes (Isberg & Falkow, 1985). The potential outcomes of Yersinia species infections vary widely, ranging from enteric diseases, such as mild diarrhoea, enterocolitis, mesenteric lymphadenitis, pseudoappendicitis and chronic ileitis, to septicaemia (Bottone, 1997; Cornelis, 1998).

Abbreviations: bLfcin, bovine lactoferricin; GAGs, glycosaminoglycans.

Bovine lactoferricin (bLfcin) is a peptide released from bovine lactoferrin upon gastric pepsin cleavage (Tomita et al., 1991). It is active against different pathogens such as bacteria (Bellamy et al., 1992a), fungi (Bellamy et al., 1993), protozoa (Turcany et al., 1995; Isamida et al., 1998) and viruses (Andersen et al., 2001; Di Biase et al., 2003). bLfcin consists of 25 amino acid residues (17–41) from the N-terminal region of lactoferrin, and has a net charge of +8 (Bellamy et al., 1992b). This region has a remarkable conformational flexibility that allows it to form an amphipathic structure in solution, which, in turn, can lead to binding of the peptide to negatively charged membrane microbial surfaces (Vogel et al., 2002). While it is well known that lactoferrin is able to hinder adhesion (Qiu et al., 1998; de Araujo & Giugliano, 2001; Oho et al., 2002; Ajello et al., 2002; Fine & Furgang, 2002) and invasion (Longhi et al., 1993; Valenti et al., 1999; Gomez et al., 2003) of different bacterial species into host cells, little information on the influence of lactoferricin towards these relevant functions is available.

In this study, we tested the ability of bLfcin to influence the adhesion and invasion of Yersinia enterocolitica and Yersinia...
**METHODS**

**Cells.** HEP-2 cells (human epidermoid carcinoma cells, larynx), obtained from the ATCC, were grown as previously described (Di Biase et al., 2003) in 5% CO₂ on 24-well tissue culture plates (BD Falcon) by loading 0.5 ml of a suspension of 2 × 10⁶ HEP-2 cells ml⁻¹.

**Lactoferrin.** bLfcin, isolated according to Recio & Visser (1999), was dissolved in minimal essential medium (MEM; Gibco), pH 7.2, at a concentration of 16 mg ml⁻¹, sterilized by filtration with 0.45 µm filters (Millipore) and stored at −20°C in 1 ml aliquots. The maximal non-cytotoxic dose of bLfcin was determined as previously described (Di Biase et al., 2003).

**Bacterial strains.** Y. enterocolitica serovar 0:3 and Y. pseudotuberculosis serovar 0:1, both harbouring the pYV virulence plasmid (Oxoid), were isolated from patients with gastroenteritis and sepsis, respectively. *Yersinia* species were cultured in brain heart infusion (BHI) broth (Oxoid) and subcultured on trypticase soy agar (TSA; Oxoid). *Escherichia coli* strain HB101 (pRI203), kindly provided by S. Falkow, Stanford Medical School, CA, USA, was used in this study. *E. coli* HB101 (pRI203) carries a recombinant plasmid, pRI203, containing a 3.2 kb *Y. pseudotuberculosis* chromosomal DNA fragment that renders it invasive for cultured animal cells (Isberg, 1990). *E. coli* HB101 (pRI203) was routinely grown in trypticase soy broth (TSB; Oxoid). Ampicillin was added to the culture at a final concentration of 50 µg ml⁻¹, to select for and to maintain the recombinant invasive plasmid.

**Antibacterial activity.** Bacteria (1 × 10⁷ c.f.u. ml⁻¹) were incubated at 37°C for 2 and 24 h in MEM or PBS supplemented with different concentrations of bLfcin. After these periods, the reaction mixtures and appropriate controls were plated on TSA to determine the number of c.f.u.

**Bacterial adhesion and invasion assays.** Adhesion and invasion assays were performed as described previously (Di Biase et al., 2000). *Yersinia* species were grown overnight at 28°C (to allow maximal expression of invasin) or at 37°C (to induce weak expression of the inv gene and maximal expression of other adherence and/or invasion factors such as YadA). *E. coli* HB101 (pRI203) was grown overnight at 28°C to allow expression of the inv gene located on the pRI203 plasmid (Isberg & Falkow, 1985). For adhesion assays, cells were infected at an m.o.i. of 100 c.f.u. per cell for 2 h at 4°C. After infection, cell monolayers were washed carefully five times with MEM to remove unattached bacteria, lysed by the addition of cold 0.1% Triton X-100 and plated on TSA to determine the number of bound bacteria by performing c.f.u. counts. Adherence was expressed as the number of associated bacteria per 100 HEP-2 cells. For invasion assays, cells were infected at an m.o.i. of 100 c.f.u. per cell for 2 h at 37°C. After this period, cells were washed five times with MEM, and gentamicin diluted in fresh medium was added to each well at the final concentration of 50 µg ml⁻¹ for *Y. enterocolitica* and *E. coli* HB101 (pRI203) and 25 µg ml⁻¹ for *Y. pseudotuberculosis*, to kill extracellular bacteria. After incubation for 1 h at 37°C, the cells were washed with MEM, trypsinized, lysed by addition of cold 0.1% Triton X-100 and plated on TSA to determine the number of viable intracellular bacteria. Invasiveness was expressed as the number of internalized bacteria per 100 HEP-2 cells.

**Electron microscopy.** For scanning electron microscopy, HEP-2 cells, grown on glass coverslips for 24 h at 37°C, were infected with *Y. enterocolitica* or *Y. pseudotuberculosis* (100 c.f.u. per cell) in the presence or in the absence of bLfcin (0.5 mg ml⁻¹). After 2 h incubation at 4°C, cells were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) at room temperature for 20 min. Following post-fixation in 1% osmium tetroxide for 1 h, cells were dehydrated through graded ethanol, critical-point-dried in CO₂ and gold coated by sputtering. Observations were performed with a Cambridge 360 scanning electron microscope.

For transmission electron microscopy, HEP-2 cells infected with bacteria (100 c.f.u. per cell) in the presence or absence of bLfcin (0.5 mg ml⁻¹) for 2 h at 37°C were harvested and processed as previously described (Di Biase et al., 2000).

**Statistical analysis.** Statistical analysis was performed using Student’s t-test for unpaired data. Data were expressed as the mean ± SD and P values <0.05 were considered significant.

**RESULTS**

**Effect of bLfcin on *Y. enterocolitica* and *Y. pseudotuberculosis* adhesion to HEP-2 cells**

A preliminary set of experiments was carried out in order to determine the maximal non-cytotoxic and non-bactericidal concentration of bLfcin. For this purpose, twofold serial dilutions of protein from 8 mg ml⁻¹ in MEM were incubated with HEP-2 cells for 24 h at 37°C. Under these conditions, up to 2 mg bLfcin ml⁻¹ did not affect any of the cytotoxicity parameters (data not shown). The antibacterial activity of bLfcin on both *Y. enterocolitica* and *Y. pseudotuberculosis* was then investigated. Table 1 shows that bLfcin up to 1 mg ml⁻¹ did not affect bacterial cell viability. The effect of 0.5 mg bLfcin ml⁻¹ on *Y. enterocolitica* and *Y. pseudotuberculosis* adhesion to HEP-2 cells was then tested. For these experiments, bacteria were grown overnight either at 28 or 37°C (as specified in Methods). As shown in Table 2, enteropathogenic *Yersinia* attachment to cell membranes was significantly enhanced by bLfcin when bacteria were grown at 37°C, whereas bLfcin treatment did not influence adhesion.

<table>
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<th>Table 1. Antibacterial activity of bLfcin towards <em>Y. enterocolitica</em> and <em>Y. pseudotuberculosis</em></th>
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<td><strong>bLfcin (mg ml⁻¹)</strong></td>
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of bacteria grown at 28 °C. To confirm that bLfcin treatment did not affect invasin-mediated bacterial adhesion, we carried out experiments using a saturated culture of E. coli HB101 (pRI203) grown at 28 °C; even in this case, bLfcin did not influence adhesion of bacteria (data not shown).

As it has been reported that YadA expression can induce bacterial agglutination (El Tahir & Skurnik, 2001), it could be hypothesized that the higher adhesion observed with Yersinia species grown at 37 °C in the presence of bLfcin could be due to an increase of bacterial aggregation. To verify this hypothesis, the way in which adhesion of bacteria, grown in these experimental conditions, was influenced by bLfcin was visualized by SEM. For these experiments, the adhesion assay was performed for 2 h at 4 °C in the presence or absence of bLfcin. The results obtained showed that, independent of bacterial strain, bLfcin induced enhanced adhesion and bacterial cells, often in division, appeared uniformly distributed on the surface of HEp-2 cells, while clumped bacteria were never observed (Fig. 1).

**Table 2.** Effect of bLfcin (0.5 mg ml⁻¹) on the ability of Y. enterocolitica and Y. pseudotuberculosis to adhere to epithelial cells

HEp-2 cells were infected with bacteria for 2 h at 4 °C. Bacteria were grown overnight at 37 or 28 °C with bLfcin added (+) to cell monolayers or not (−) during the infection period. Standard deviation <0.8.

<table>
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<th>Bacteria</th>
<th>Adhesion efficiency (%)</th>
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<tr>
<td></td>
<td>37 °C/−</td>
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<tr>
<td>Y. enterocolitica</td>
<td>2.9</td>
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<tr>
<td>Y. pseudotuberculosis</td>
<td>5.1</td>
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**Effect of bLfcin against Y. enterocolitica and Y. pseudotuberculosis invasion into HEp-2 cells**

The effect of bLfcin on Y. enterocolitica and Y. pseudotuberculosis invasion was then tested. As invasin represents the primary invasion factor of enteropathogenic Yersinia, for these experiments, bacteria were grown overnight at 28 °C to allow maximal inv expression. As shown in Table 3, enteropathogenic Yersinia internalization into cells was significantly inhibited by bLfcin. Similar results were obtained with saturated cultures of E. coli HB101 (pRI203) grown at 28 °C. To analyse the effect of bLfcin on invasin-independent bacterial internalization, the same experiments were performed with Y. enterocolitica or Y. pseudotuberculosis grown overnight at 37 °C. Under these experimental conditions, bLfcin also inhibited bacterial internalization (data not shown). The internalization of bacteria into target cells was also studied at the ultrastructural level. For transmission electron microscopy experiments, invasion, in the presence or absence of bLfcin, was performed for 2 h at 37 °C. The results obtained confirmed that Y. enterocolitica internalization was strongly inhibited by bLfcin (Fig. 2). Similar results were obtained with the other bacterial strains (data not shown).

**Table 3.** Effect of bLfcin (0.5 mg ml⁻¹) on the ability of Y. enterocolitica, Y. pseudotuberculosis and E. coli HB101 (pRI203) to invade epithelial cells

HEp-2 cells were infected with bacteria for 2 h at 37 °C in the absence (−) or presence (+) of bLfcin. Standard deviation <0.8.

<table>
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<tr>
<th>Bacteria</th>
<th>Invasion efficiency (%)</th>
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<tr>
<td></td>
<td>(−)</td>
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<tr>
<td>Y. enterocolitica</td>
<td>79</td>
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<tr>
<td>Y. pseudotuberculosis</td>
<td>75</td>
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<td>E. coli HB101 (pRI203)</td>
<td>73</td>
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**DISCUSSION**

bLfcin is an antimicrobial peptide composed of 25 amino acid residues (17–41) derived by pepsin digestion of bovine lactoferrin prevents Yersinia species invasion

**Fig. 1.** Scanning electron microscopy analysis of Y. enterocolitica (a, c) and Y. pseudotuberculosis (b, d) attachment to HEp-2 cells. Bacterial adhesion to target cells, in the absence (a, b) or presence (c, d) of bLfcin, was performed for 2 h at 4 °C. The samples were examined with a Cambridge Stereoscan 360 scanning electron microscope.
lactoferrin, a multifunctional protein in milk responsible for innate defence (Tomita et al., 1991). This peptide is known to inhibit a wide range of micro-organisms such as Gram-negative and Gram-positive bacteria, fungi, protozoa and viruses (Bellamy et al., 1992a, 1993; Turchany et al., 1995; Isamida et al., 1998; Andersen et al., 2001; Di Biase et al., 2003). bLfcin, which exhibits numerous biological activities in common with those of lactoferrin, is known to possess bactericidal properties more potent than the intact protein (Tomita et al., 1991; Bellamy et al., 1992b).

In this study we have examined the effect of bLfcin on enteropathogenic Yersinia—epithelial cell interactions. The enteropathogenic Yersinia species Y. enterocolitica and Y. pseudotuberculosis cause several enteric diseases such as enteritis, diarrhea and lymphadenitis (Bellamy et al., 1993). Pathogenicity of enteropathogenic Yersinia species is mediated by a large number of virulence factors, some of which, such as YadA, are encoded on plasmids, and others by the chromosome. Of the chromosomal virulence factors, the most important are invasin, Ail and the Yst enterotoxin, which, such as YadA, are encoded on plasmids, and others by the chromosome. Of the chromosomal virulence factors, the most important are invasin, Ail and the Yst enterotoxin, which may be a mediator of the diarrhea observed in infants infected with enteropathogenic Yersinia (Delor & Cornelis, 1992). Y. enterocolitica and Y. pseudotuberculosis possess two different non-pilus-associated adhesins, invasin and YadA, anchored to the outer membrane. In both micro-organisms, they mediate initial adhesion, uptake and transfer of the bacteria through M intestinal cells, and establish the extracellular colonization of lymphatic tissues and organs. YadA promotes significant attachment to epithelial cells, professional phagocytes and extracellular matrix proteins, but its contribution to Yersinia species entry into susceptible cells is not significant, being only apparent in the absence of invasin. Invasin has been demonstrated to be the most efficient factor that promotes binding and internalization of Y. pseudotuberculosis into mammalian cells (Isberg et al., 1987) and the primary invasion factor of Y. enterocolitica in tissue-culture invasion models (Miller & Falkow, 1988). As enteropathogenic Yersinia species initiate systemic diseases after attachment to and translocation across the intestinal epithelium, we analysed the effect of bLfcin on YadA- and invasin-mediated bacterial adhesion and invasion of epithelial cells.

The results of our studies demonstrate that, at non-cytotoxic and non-bactericidal concentrations, bLfcin treatment of epithelial cells resulted in about a 10-fold increase in bacterial adhesion, in bacteria grown under conditions in which inv is poorly expressed, and was ineffective on bacterial adhesion, under bacterial growth conditions ideal for inv expression. As the inv gene product represents the most important translocation factor of enteropathogenic Yersinia species, we analysed the anti-invasive activity of bLfcin by focusing our attention on this virulence determinant. For this purpose, in invasion experiments, we utilized bacterial growth conditions that allowed maximal inv expression rates. It is well known that inv expression is regulated in response to pH, growth phase and temperature. In vitro, inv is maximally expressed at 26–28 °C, pH 8.0 or 37 °C, pH 5.5 (Pepe et al., 1994) in early stationary phase, whereas, at 37 °C, pH 8.0, inv is weakly expressed (Revell & Miller, 2000). Moreover, as the inv locus of enteropathogenic Yersinia is sufficient to convert a non-invasive E. coli K-12 strain into one that is able to penetrate cultured mammalian cells (Young et al., 1990), we have included in our studies E. coli carrying recombinant plasmid pRI203 containing the inv gene. In all of these experiments, we observed that bLfcin strongly protected epithelial cells from bacterial invasion, suggesting that bLfcin acts on inv-mediated Yersinia species invasion

As for many other enteric pathogens that bind host-cell integrin receptors (this interaction facilitating extracellular adhesion of the micro-organism or internalization by the
host cell), invasin promotes enteropathogenic *Yersinia* species attachment and entry into host cells by binding to at least five different members of the β1 integrin receptor superfamily (Dersch & Isberg, 2000). The interaction of invasin with its receptors has been well characterized, whereas little is known about the mechanism by which invasin induces bacterial uptake. Efficient internalization needs tight binding of bacteria to host cells and uptake is controlled by both integrin receptor density and affinity of binding. For example, low-affinity ligands can promote bacterial adhesion to host cells without internalization (Tran Van Nhieu & Isberg, 1993).

It has been demonstrated that lactoferricin is able to bind glycosaminoglycans (GAGs), long polyanionic carbohydrate chains, and, in particular, heparin (Shimazaki et al., 1998). GAGs covalently linked to a protein core are termed proteoglycans, and are involved in several important functions such as cell attachment, proliferation, migration, morphogenesis and receptor-mediated endocytosis (Ho et al., 1997; Poole, 1986). GAGs link extracellular matrix proteins and, in particular, fibronectin. Fibronectin is a large adhesive extracellular matrix protein with two similar sub-units joined near the C terminus. This heterodimeric glycoprotein contains multiple binding domains and can bind with different ligands such as heparin, collagen and fibrin. Moreover, fibronectin mediates the adherence of a wide variety of micro-organisms in a ligand–receptor-mediated manner (Hook et al., 1989), and interacts with α5β1 integrin through the same domains as invasin does (Isberg & Barnes, 2001).

It is likely that the binding of lactoferricin to heparin allows a dramatic subversion in the interactions between GAGs, fibronectin and integrins. In particular, as GAGs bind primarily to heparin-binding extracellular matrix molecules such as fibronectin, covering or masking potential binding ligands for enteropathogenic *Yersinia* species, the higher attachment to cells by bacteria expressing YadA could be explained by improved access to fibronectin. On the other hand, the binding of lactoferricin to GAGs can also influence the affinity of the binding between invasin and integrins, allowing bacterial adhesion but not efficient internalization. It is also possible that lactoferricin can induce inhibition of integrin-receptor clustering, because such clustering is predicted to be sufficient to transmit an internalization signal.

As entry into epithelial cells allows enteropathogenic *Yersinia* species to attack the host and to gain access to other tissues within the host, allowing the initiation of systemic disease, our results provide additional information on the protective role of lactoferricin against bacterial infection of the gastrointestinal tract. It must be considered that Jones et al. (1994) showed that lactoferricin, despite its rapid *in vitro* activity against a wide range of pathogens, has a marked sensitivity to physical variables and changes in medium constituents, suggesting that this peptide could be less active *in vivo*. Although our experimental conditions differ from those in their study, having studied the effect of lactoferricin on bacteria–cell interactions and not its direct activity on the micro-organism, we cannot rule out the possibility that ionic strength or pH changes could influence the anti-invasive activity of lactoferricin. Further experiments will be carried out to confirm this new potential antimicrobial application of this peptide.

**ACKNOWLEDGEMENTS**

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