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Lactoferrin disruption of bacterial type III secretion systems

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Abstract

Many Gram-negative bacteria share a closely related mechanism for secretion of virulence proteins. This complex machine, the type III secretion system, secretes virulence proteins in response to sensing the presence of target mammalian cells. We have found that recombinant human lactoferrin impairs the function of this system in two model organisms: *Shigella* and Enteropathogenic *E. coli* (EPEC). In the case of *Shigella*, there is loss and degradation of two proteins secreted by the type III mechanism, invasion plasmid antigens B and C (IpaB and IpaC); these proteins normally form a complex that causes *Shigella* to be taken up by host mammalian cells. In the case of EPEC, lactoferrin causes loss and degradation of *E. coli* secreted proteins A, B and D (EspABD) particularly EspB. These proteins are components of type III machinery and are known to be key elements of EPEC pathogenesis. Studies using purified EspB demonstrated that lactoferrin has a direct proteolytic effect on EspB that can be prevented by serine protease inhibitors. A synthetic peptide of the N-terminal 33 amino acids of lactoferrin caused loss of cell associated EspB but, unlike the whole lactoferrin molecule, did not caused degradation of EspB. Thus, in both model systems, brief exposure to lactoferrin causes loss and degradation of type III secretion system virulence proteins.

Introduction

Pathogens including *Shigella, Salmonella*, Enteropathogenic *E. coli* (EPEC), Shigatoxin producing *E. coli*, Enteroinvasive *E. coli, Yersinia enterocolitica, Yersinia pseudotuberculosis, Yersinia pestis, Bordetella pertussis, Bordetella bronchiseptica, Chlamydia pneumoniae, Chlamydia trachomatis* and *Pseudomonas aeruginosa* share a closely related secretion mechanism for injecting virulence proteins into host cells (Jarvis *et al.* 1995, Kaniga *et al.* 1995, Blocker *et al.* 1999, Buttner *et al.* 2002, Feldman *et al.* 2003). This mechanism is called the type III secretion system. It works by sensing the presence of mammalian cells and secreting virulence proteins in response. For many of these organisms a needle complex has been demonstrated; this needle is used for injection of bacterial proteins into host cell. It is stabilized on the bacterial surface by interaction with both the inner and outer membranes. We hypothesized that disruption of outer membrane integrity and function by lactoferrin could alter the relationship between the needle and the outer membrane, thereby impairing secretory function. This paper summarizes some of our recent observations on the role of recombinant human lactoferrin in interfering with the type III secretion system and new data supporting a hypothesis for the mechanism.

We previously found that lactoferrin treatment of *Shigella flexneri* 5 strain M90T impaired invasiveness (Gomez *et al.* 2001) by inducing release and degradation of invasion plasmid antigens B and C (IpaB and IpaC), proteins that are responsible for uptake of *Shigella* by mammalian cells (Gomez *et al.* 2002, Gomez *et al.* 2003). Antipain, chymostatin, and soybean trypsin inhibitor blocked breakdown of *Shigella* virulence antigen IpaC and partially blocked proteolytic digestion of IpaB. To determine whether these observations in the *Shigella* model might have relevance for ad-

ditional organisms that secrete virulence proteins via a type III secretion apparatus, we evaluated the effect of lactoferrin on a well characterized EPEC strain (E2348/69).

We found that lactoferrin blocks EPEC adherence and induction of actin polymerization in HEp2 cells, and blocks EPEC-induced hemolysis (Ochoa *et al.* 2003). The mechanism of this inhibition was lactoferrin-mediated degradation of the *E. coli* secreted proteins A, B and D (EspABD), particularly EspB. EspA normally forms a tube between the bacteria and the host cell. EspB and EspD are delivered at the end of this tube where they make a pore through which bacterial proteins are delivered directly into gut epithelial cells. The proteolytic effect of lactoferrin on EspB was prevented by serine protease inhibitors (Antipain, chymostatin and soybean trypsin inhibitor). To further characterize the effect of lactoferrin on EPEC's virulence proteins, we focused on the lipid A binding N-terminal 33 amino acids segment of lactoferrin, that is known to be the high affinity domain for binding to LPS.

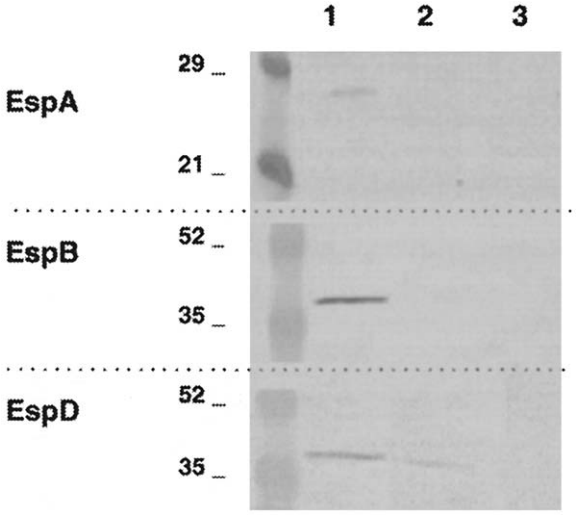

Fig. 1. Composite Western Blot showing the effect of lactoferrin on cell associated EspABD. EPEC were grown in DMEM/HEPES (Lane 1, control) or in lactoferrin (Lane 3) and bacterial pellets were evaluated for the presence of EspABD at 3 hours. EPEC grown in the absence of lactoferrin were exposed to lactoferrin at 3 hours and immediately after bacterial pellets were evaluated (Lane 2). Lactoferrin immediately decreased EspA and EspB; it also decreased EspD, but less dramatically.

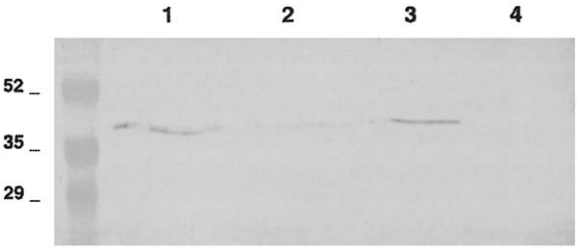

Fig. 2. Effect of lactoferrin and peptide (N-terminal 33 amino acids of lactoferrin) on cell associated EspB. EPEC were grown in DMEM/HEPES for 4 hours at 37 °C. At that time DMEM/HEPES (Lane 1), peptide (0.125 mM) (Lane 2), peptide (0.025 mM) (Lane 3) or lactoferrin (0.125 mM) (Lane 4) were added to the media and immediately after the bacterial pellets were evaluated by Western blot for the presence of EspB. Cell associated EspB was undetectable in the presence of lactoferrin, and decreased at the higher concentration of the peptide.

Methods

Reagents

Recombinant human lactoferrin (11% iron saturated), prepared as previously described (Ward *et al.* 1995), was provided by Agennix Inc. Lactoferrin was used in the concentration range found in human colostrum and milk (approximately 10 mg/mL [0.125 mM] and 1 mg/mL [0.0125 mM], respectively) and diluted in DMEM/HEPES.

A peptide consisting of the N-terminal 33 amino acids of lactoferrin (GRRRRSVQWCAVSQPEATKC FQWQRNMRKVRGP) was synthesized (SynPep, Dublin, CA). It was used at the same molar concentration as lactoferrin (0.125–0.0125 mM) and diluted in DMEM/HEPES.

Bacterial strain

EPEC 0127:H6 strain E2348/69 was used. Bacteria from overnight growth in LB were inoculated (1.7×10^5 CFU/mL) in DMEM with 25 mM HEPES, pH 7.4, and incubated at 37 °C in a 5% CO_2 incubator.

Assays for lactoferrin-induced release of virulence proteins

EPEC were grown in DMEM/HEPES in the presence or absence of lactoferrin for up to 7 hours. EPEC grown in the absence of lactoferrin were exposed to lactoferrin (0.125 and 0.0125 mM) or to the peptide (0.125 and 0.025 mM) at 3 or 4 hours and immediately after the bacterial pellets were evaluated by Western

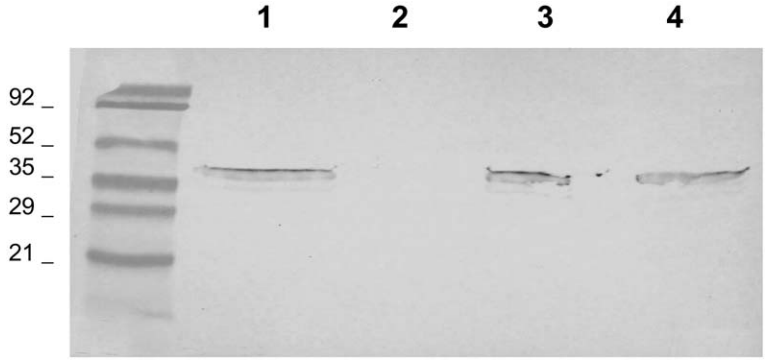

Fig. 3. Proteolytic effect of lactoferrin on EspB. Purifed EspB (2 μg/mL) was incubated with DMEM/HEPES (Lanes 1 and 3, controls), with lactoferrin (0.0125 mM) (Lane 2) or with the peptide (N-terminal 33 amino acids of lactoferrin, 0.0125 mM) (Lane 4) for 4 hours at 37 °C. The Western blot demonstrated that lactoferrin degraded EspB, while the N-terminal peptide left it intact.

blot for the presence of cell associated EspABD. Human polyclonal (EspA) or mouse monoclonal (EspB and EspD) antibodies were used with appropriate conjugates.

Studies with purified EspB

EspB was purified as previously described (Ochoa *et al.* 2003). Pure EspB (2 μg/mL) was incubated with DMEM/HEPES in the presence or absence of lactoferrin (0.0125 mM) or the peptide (0.0125 mM) for 4 hours at 37 °C . EspB remaining after incubation was assessed by Western blot.

Results

Lactoferrin decreased cell associated EspABD

EPEC in DMEM/HEPES had detectable cell-associated EspABD at 2–3 hours of growth, peaking at 5–6 hrs (data not shown). Lactoferrin decreased cell-associated EspB at every time point and less striking, also decreased EspA and EspD (data shown only for the 3-hour time point, Figure 1, Lane 3). To determine whether low levels of cell associated virulence proteins reflected decreased production or loss, EPEC were grown in DMEM/HEPES for 3 hours, at which time lactoferrin was added and the amount of EspABD in the bacterial pellets determined. Lactoferrin immediately decreased EspA and EspB; it also decreased EspD although less dramatically (Figure 1, Lane 2). Thus lactoferrin caused a rapid release of virulent proteins from bacterial cells. Since the most dramatic effect of lactoferrin was on EspB, we focused on this protein for the following experiments.

The N-terminal 33 amino acids (peptide) of lactoferrin decreased cell associated EspB

EPEC were grown in DMEM/HEPES for 4 hours, at which time lactoferrin or the peptide were added and the amount of cell associated EspB was determined. EspB was undetectable in the presence of lactoferrin. The peptide at high concentration also decreased the amount of cell associated EspB (Figure 2).

The N-terminal 33 amino acids (peptide) of lactoferrin did not caused degradation of EspB.

Pure EspB was incubated with DMEM/HEPES in the presence or absence of lactoferrin or the peptide. The amount of EspB remaining after incubation was determined by Western Blot. Lactoferrin completely degraded EspB, while the peptide left it intact (Figure 3).

Discussion

The studies in these model systems suggest that lactoferrin acts on the type III secretion system in a two step process. The first step involves stimulation of virulence antigen secretion. We have determined that both lactoferrin and its N-terminal peptide caused rapid depletion of cell-associated virulence proteins in *Shigella* and EPEC. This could reflect lipid A-induced changes in the bacterial cell surface. It is known that lactoferrin binding to the phosphate groups of lipid A

is associated with the acyl chains becoming more rigid so that they are more tightly packed (Brandenburg *et al.* 2001). Such rigid packing could affect the stability or function of Type III secretion system needle complex proteins that must be anchored in and traverse the outer membrane. The second step demonstrated in these studies involves lactoferrin-mediated proteolysis. In both *Shigella* and EPEC, lactoferrin degrades critically important virulence antigens. The proteolytic activity is susceptible to inhibition by serine protease inhibitors. In the *Shigella* model, proteolysis is demonstrated only when bacteria are present (presumably reflecting surface localization and activity of lactoferrin), while in the EPEC model proteolysis can be shown even with purified virulence proteins in the absence of binding to the bacterial surface. The difference in these models may be due to the resistance of the IpaBC complex to degradation once the proteins have become associated. IpaB and IpaC may be susceptible to lactoferrin-mediated degradation only prior to complex formation.

The type III secretion system is a virulence machine shared by both *Shigella flexneri* and EPEC. Many other organisms shared closely related type III secretion systems. It remains to be determined whether lactoferrin affects the function of type III secretion systems in these many organisms. Lactoferrin may have therapeutic potential as an agent useful against an array of pathogens possessing this conserved machinery. Lactoferrin may also prove to be a useful tool to further understand the interactions of this important machinery with outer membrane lipopolysaccharide.

Acknowledgements

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References

Blocker A, Gounon P, Larquet E *et al.* 1999. The tripartite type III secretion of *S. flexneri* inserts IpaB and IpaC into host membranes. *J Cell Biol* **147**, 683–93.

Brandenburg K, Jurgens G, Muller M *et al.* 2001. Biophysical characterization of lipopolysaccharide and lipid A inactivation by lactoferrin. *Biol Chem* **382**, 1215–25.

Buttner D, Bonas U. 2002. Port of entry–the type III secretion translocon. *Trends Microbiol* **10**, 186–92.

Feldman MF, Cornelis GR. 2003. The multitalented type III chaperones: all you can do with 15 kDa. *FEMS Microbiol Lett* **219**, 151–8.

Gomez HF, Herrera-Insua I, Siddiqui MM *et al.* 2001. Protective role of human lactoferrin against invasion of *Shigella flexneri* M90T. *Adv Exp Med Biol* **501**, 457–67.

Gomez HF, Ochoa TJ, Herrera-Insua I *et al.* 2002. Lactoferrin protects rabbits from *Shigella flexneri*-induced inflammatory enteritis. *Infect Immun* **70**, 7050–3.

Gomez HF, Ochoa TJ, Carlin LG *et al.* 2003. Human lactoferrin impairs virulence of *Shigella flexneri. J Infect Dis* **187**, 87–95.

Jarvis KG, Giron JA, Jerse AE *et al.* 1995. Enteropathogenic *Escherichia coli* contains a putative type III secretion system necessary for the export of proteins involved in attaching and effacing lesion formation. *Proc Natl Acad Sci (USA)* **92**, 7996–8000.

Kaniga K, Trollinger D, and Galan JE. 1995. Identification of two targets of the type III protein secretion system encoded by the inv and spa loci of *Salmonella typhimurium* that have homology to the *Shigella* IpaD and IpaA proteins. *J Bacteriol* **177**, 7078–7085.

Ochoa TJ, Noguera-Obenza M, Ebel F *et al.* 2003. Lactoferrin impairs type III secretory system function in Enteropathogenic *Escherichia coli. Infect Immun*, **71**, 5149–5155.

Ward PP, Piddington CS, Cunningham GA *et al.* 1995. A system for production of commercial quantities of human lactoferrin: a broad spectrum natural antibiotic. *Biotechnology* **13**, 498–503.