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Effect of lactoferrin on enteric pathogens

Theresa J. Ochoa[a,b,*] and **Thomas G. Cleary**[b]

[a] Universidad Peruana Cayetano Heredia, Instituto de Medicina Tropical, Lima, Peru

[b] University of Texas School of Public Health, Center for Infectious Diseases, Houston, TX, USA

Abstract

Much has been learned in recent years about the mechanisms by which breastfeeding improves child health and survival. However, there has been little progress in using these insights to improve pediatric care. Factors that are important for protecting the breast fed infant might be expected to decrease the adverse effects of weaning on diarrhea, growth, and development. Lactoferrin, an iron-binding protein with multiple physiological functions (anti-microbial, anti-inflammatory, and immunomodulatory), is one of the most important proteins present in mammalian milk. Protection against gastroenteritis is the most likely biologically relevant activity of lactoferrin. Multiple *in vitro* and animal studies have shown a protective effect of lactoferrin on infections with enteric microorganisms, including rotavirus, *Giardia*, *Shigella*, *Salmonella* and the diarrheagenic *Escherichia coli*. Lactoferrin has two major effects on enteric pathogens: it inhibits growth and it impairs function of surface expressed virulence factors thereby decreasing their ability to adhere or to invade mammalian cells. Thus, lactoferrin may protect infants from gastrointestinal infection by preventing the attachment by enteropathogens in the gut. Recently several clinical trials in children have started to address this issue. Whether lactoferrin can prevent a significant portion of diarrheal disease remains to be determined.

Keywords

Lactoferrin; Diarrhea; Enteric bacteria; Enteric viruses; Enteric parasites

1. Introduction

Diarrheal disease is still one of the most important public health problems in developing countries despite advances in understanding and management that have occurred in recent years. Diarrhea is one of the leading causes of death in children under 5 years of age. The WHO estimates 10.6 million deaths yearly in this age group. Diarrhea accounts for 18% of these deaths or approximately 2 million diarrheal deaths each year [1]. Multiple episodes of acute diarrhea and persistent diarrhea seriously affect growth, nutritional status and cognition [2–4].

Breastfeeding is the most effective intervention for protecting infants and toddlers from diarrhea and all causes of mortality [5]. Multiple studies have shown that exclusive breast-feeding, and to a lesser extent partial breast-feeding, protects against acute and persistent diarrhea. The protective effect of human milk is due both to the multiple anti-infective, anti-inflammatory, and immunoregulatory factors transmitted through milk, including secretory

* Corresponding author. Instituto de Medicina Tropical, Universidad Peruana Cayetano Heredia, Av. Honorio Delgado 430, San Martin de Porras, Lima 33, Peru. Tel.: +51 1 482 3910; fax: +51 1 482 3404. *E-mail address:* theresa.j.ochoa@uth.tmc.edu (T.J. Ochoa)..

antibodies, glycans (oligosaccharides and glycoconjugates), lactoferrin, leukocytes, cytokines and other factors produced by the mother's acquired and innate immune systems [6,7] and the decrease in pathogen exposure resulting from ingestion of an uncontaminated source of nutrients.

Lactoferrin is the second most abundant protein in human milk. It is a glycoprotein consisting of a single polypeptide chain of about 80 kDa with two globular lobes each containing an iron-binding site. It is found in most exocrine secretions including milk, tears, saliva, intestinal mucus and genital secretions, and in the specific granules of neutrophils. Multiple activities (anti-microbial, anti-inflammatory, immunomodulatory) have been described although relevance of each of these putative mechanisms in humans remains to be proven [8,9]. Human and bovine lactoferrin are well characterized. They consist of 691 and 689 amino acids, respectively; the sequence identity is 69% [10]. The 3-D structures of bovine and human lactoferrin are very similar. The concentration of lactoferrin in human milk is 5.3 ± 1.9 mg/mL in colostrum, and approximately 1 mg/mL after the first month of lactation [6]. In contrast, the concentration of lactoferrin in bovine milk is very low (1.5 mg/mL in colostral whey and 20−200 mg/mL in milk) [11]. Although differences in structural and biochemical properties exist, the bioactivity of these lactoferrins, as assessed in vitro and in animal models, is quite comparable [11]. The potential activity of these two lactoferrins for enteric viruses, parasites and bacteria, and the relevant human data are summarized below.

2. Effect of lactoferrin on enteric viruses

Multiple studies have shown that apo-lactoferrin (iron-depleted lactoferrin) and holo-lactoferrin (iron-saturated lactoferrin) from bovine milk suppress rotavirus attachment and replication in vitro [12–14]; however, not all investigators have been able to confirm an effect [15]. The effect of lactoferrin on other viral enteritis organisms has not been fully evaluated. There are data showing that lactoferrin interferes with feline calicivirus infection by blocking viral attachment in vitro [16]. However, there are no data evaluating the effect of lactoferrin on the human enteric caliciviruses (sopoviruses or noroviruses). Likewise there are data showing that lactoferrin prevents adenovirus replication [17] and that the antiviral activity is located in the N terminus [18,19]. However, these studies have focused on respiratory rather than enteric adenoviral serotypes.

3. Effect of lactoferrin on enteric parasites

Human lactoferrin, bovine lactoferrin and its derived N-terminal peptides have been demonstrated to have giardicidal activity and to cause ultrastructural changes in *Giardia* trophozoites, suggesting that lactoferrin may be an important non-immune component of host mucosal defense against *Giardia* [20,21]. Similarly, both holo- and apo-lactoferrin can bind to and kill *Entamoeba histolytica* trophozoites [22,23]. There are no investigations of the effect of lactoferrin on cryptosporidium or cyclospora.

4. Effect of lactoferrin on enteric bacteria

Iron-binding related-growth inhibition or cell death was once thought to be the major antibacterial activity of lactoferrin. For example, human apo-lactoferrin has a bactericidal effect on a variety of microorganism (gram-positive, gram-negative microbes, rods and cocci, facultative anaerobes and aerotolerant anaerobs) [17]; bovine apo-lactoferrin inhibits growth of enteric bacteria (*Shigella*, *Salmonella*, EPEC, STEC and EAEC), by binding ferric iron resulting in bacteriostasis. However, in recent years more complex mechanisms have been described. Lactoferrin not only sequesters iron that is essential for bacterial growth, it binds to the lipid A portion of lipopolysaccharide (LPS) on the cell surface disrupting the bacterial cell membrane [24,25]. Lactoferrin thus decreases virulence of some of the major enteropathogens

by interfering with surface-expressed virulence factors. For example, bovine lactoferrin inhibits *Yersina* spp entry into epithelial cells [26] and induces apoptosis [27]. Other bacterial enteropathogens likewise have complex interactions with lactoferrin.

4.1. Shigella

Recombinant human lactoferrin (11% iron-saturated) (Agennix, Houston, TX) impairs virulence of *Shigella* by its interaction with the type III secretion system. This complex machine secretes virulence proteins in response to sensing the presence of mammalian target cells. Lactoferrin binds to *Shigella flexneri* [28] and decreases invasiveness of *S. flexneri* in HeLa cells [29] associated with release and degradation of invasion plasmid antigens B and C, proteins that are responsible for entry of *Shigella* into mammalian cells [30,31]. Recombinant human lactoferrin also protects rabbits from *Shigella flexneri*-induced inflammatory enteritis [30]. Both gross and microscopic evidence of inflammatory changes in *Shigella* infected rabbits are attenuated by lactoferrin.

4.2. Salmonella

Lactoferrin binds to *Salmonella typhimurium* [32]. Bovine lactoferrin (10e20% iron saturated) has an iron-dependent bacteriostatic effect on *Salmonella typhimurium* [33]. Bovine and human lactoferrin inhibit adherence and invasion of *Salmonella* to tissue culture cells [33,34].

4.3. Enterotoxigenic Escherichia. coli (ETEC)

Lactoferrin binds to diarrheagenic *E. coli* [35]. Binding to *E. coli* colonization factors [36] causes inhibition of hemagglutination [37] and inhibition of adherence of ETEC to epithelial cells in vitro and to intestinal mucosa of germfree mice in vivo [38].

4.4. Enteropathogenic E. coli (EPEC)

In the case of EPEC, both human (Agennix, Houston, TX) and bovine lactoferrin (Tatua Cooperative Dairy Co, Morrinsville, New Zealand), block EPEC adherence to tissue cultured cells [39,40], and significantly decrease EPEC-induced actin polymerization in HEp2 cells [40]. Recombinant human lactoferrin causes loss and degradation of *E. coli* secreted proteins A, B and D (EspABD), especially EspB. These secreted proteins are components of the type III machinery and are known to be key elements in EPEC pathogenesis [41]. EspB plays a central role in pathogenesis both by its membrane lytic and its myosin-binding functions. EspB is responsible for microvillus distortion and close bacterial attachment (the attaching and effacing lesion) [42]. Studies using purified EspB demonstrated that human recombinant lactoferrin has a direct proteolytic effect on EspB that can be prevented by serine pro-tease inhibitors. A synthetic peptide of the N-terminal 33 amino acids of lactoferrin caused loss of cell-associated EspB but in contrast to the whole lactoferrin molecule, the N-terminal peptide did not degrade EspB [43].

The studies in the *Shigella* and EPEC model systems suggest that lactoferrin acts on the type III secretion system in a two step process. It first causes loss of surface expressed virulence antigens from the bacteria into the surrounding media. We have hypothesized that this is due to lactoferrin binding lipid A and inducing changes in bacterial cell surface protein-LPS interactions. The second step involves degradation of virulence proteins via lactoferrin's serine protease activity.

4.5. Enterohemorrhagic or Shigatoxin producing E. coli (EHEC/STEC)

Both human and bovine lactoferrins block attachment of EHEC/STEC to HEp2 cells [44]. Because epithelial attachment is mediated by a type III secretory system, we evaluated the

effect of lactoferrins on EspB. We found that both lactoferrins caused a rapid loss of cell-associated EspB into the media, not affected by presence or absence of ferric iron saturation.

4.6. Enteroaggregative E. coli (EAEC)

Lactoferrin purified from human milk (Sigma-Aldrich Co. St. Louis, MO), recombinant human lactoferrin (Agennix, Houston, TX) and bovine lactoferrin (Tatua Cooperative Dairy Co, Morrinsville, New Zealand), inhibit the aggregative adherence “stacked-brick pattern” of EAEC in tissue cultured cells [45,46]. Bovine lactoferrin also inhibited EAEC biofilm formation and increased autoagglutination, further suggesting that surface adhesins were affected. We have histidine tagged dispersin, a virulence protein involved in the aggregative adherence phenotype, and the surface fimbria (AAF-II). We found that lactoferrin-induced loss and degradation of AAF-II but not of dispersin [46].

5. Clinical studies of lactoferrin's effect on enteric pathogens

Multiple studies of bovine lactoferrin supplementation have been conducted in infants to determine its effect on fecal flora and iron status [47–50]. However, these studies typically have not addressed the effect on pathogenic flora. Of interest, no side effects have been noted related to use of bovine lactoferrin in infants.

A clinical trial of 298 Japanese children less than 5 years of age showed that daily consumption of 100 mg of bovine lactoferrin-containing products (Morinaga Milk Industry Co., Tokyo, Japan) vs. placebo for 3 months, had no effect on the incidence of rotavirus gastroenteritis; however, there was a significant lower frequency and duration of vomiting and diarrhea in the lactoferrin group ($p < 0.05$) [51]. Similarly, a placebo-controlled trial of bovine lactoferrin (850 mg/L) (DMV International, Delhi, NY) supplementation for 12 months in 52 bottle-fed infants in the United States showed no difference in the diarrhea incidence or duration. Of interest, there were significantly fewer lower respiratory tract illness and higher hematocrits in the lactoferrin group [52].

A study of 140 Peruvian children with acute watery diarrhea and dehydration showed that adding recombinant human lactoferrin and lysozyme (Ventria Bioscience, Sacramento, CA) to oral rehydration solution reduced the duration of diarrhea (3.7 days vs. 5.2 days, $p = 0.05$) and relapse after 48 h (9% vs. 19%) [53].

We recently conducted a small community-based randomized double blind placebo controlled trial comparing twice daily oral supplementation with bovine lactoferrin (10−20% iron saturated, 1 g/day) (Tatua Cooperative Dairy Co, Morrinsville, New Zealand) versus placebo for 9 months in 52 Peruvian children 12−36 months of age [54]. Despite the small number of children enrolled in this study, several potentially useful observations were made related to bovine lactoferrin supplementation. There was a decreased *Giardia* burden ($p < 0.01$) and better height-for-age z-score in the lactoferrin group ($p < 0.05$), as well as a trend toward shorter duration of diarrheal episodes related to lactoferrin intake.

6. Discussion

Lactoferrin clearly has two major effects on bacterial enter-opathogens. It binds iron and limits growth under low iron conditions and it disrupts surface expressed virulence proteins, typically causing their loss and degradation. The iron binding activity has been reviewed extensively over recent years. The effects of lactoferrin on the bacterial surface have been a focus of recent studies. The effect of lactoferrin on surface anchored proteins may relate to the fact that after binding to phosphates of lipid A, the acyl chains of LPS become rigid; this rigidity of the acyl chains changes the lipid A aggregate structure from inverted cubic to multilamellar [25]. Such

a change may affect the hydrophobic interactions of surface anchored proteins with acyl groups or the charge related interactions with phosphates of LPS. The loss of surface anchored type III secretory system virulence proteins after expose to lactoferrin may be a result of disruption due to such interactions. Teleologically, such disruptions may be difficult for bacteria to develop resistance to and thus circumvent. Proper function of a complex machine such as the type III secretory system depends on dozens of interacting proteins; single mutations may not easily restore virulence. Multiple simultaneous mutations might be required for emergence of lactoferrin resistance in such bacteria. This complexity may explain lactoferrin's continuing biologic activity despite eons of interactions with bacteria. Alternatively, bacterial resistance might be achieved by changing the structure of LPS; however, this may be equally problematic from the bacteria's point of view given the multitude of surface anchored proteins that must interact with LPS. Thus, lactoferrin resistance has not developed with even simple systems such as the adherence fimbria of enteroaggregative *E coli*; such fimbria are shed after exposure to lactoferrin despite the fact that such bacteria must have encountered lactoferrin many times over the years. Much of the LPS interaction and surface disruption is the result of the N-terminal cationic amino acid residues since shedding of virulence proteins can be demonstrated with N-terminal peptides. In contrast, the role of the serine protease activity in these events remains of uncertain significance. Our studies show that lactoferrin's effect on type III secretory system virulence proteins occurs in seconds to minutes while the protease activity can be demonstrated only much later. The N-terminal cationic amino acids may also be responsible for some of the antiparasitic effects of lactoferrin although not specifically demonstrated for major parasitic enteropathogens such as *Giardia* or *Entamoeba histolytica* [55].

Although protection against gastroenteritis is the most likely biologically relevant activity of lactoferrin, it is still not clear whether lactoferrin can prevent a significant portion of diarrheal disease. Clinical trials in children have started to address this issue.

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