Lactoferrin Protects Rabbits from *Shigella flexneri*-Induced Inflammatory Enteritis

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Shigella species cause bacillary dysentery in humans by invasion, intracellular multiplication, spread to adjacent cells, and induction of brisk inflammatory responses in the intestinal epithelium. In vitro data suggest that lactoferrin, a glycoprotein present in human mucosal secretions, has a role in protection from bacterial enteric infections. We sought to determine the activity of lactoferrin in vivo, using the concentration present in human colostrum, to investigate its effect on the development of clinical and pathological evidence of inflammation in a rabbit model of enteritis. Lactoferrin protected rabbits infected with *Shigella flexneri* from developing inflammatory intestinal disease. Typical histological changes in ill animals included villous blunting with sloughing of epithelial cells, submucosal edema, infiltration of leukocytes, venous congestion, and hemorrhage. Lactoferrin at a concentration normally found in human colostrum blocks development of *S. flexneri*-induced inflammatory enteritis.

Dysentery due to *Shigella* spp. is among the most communicable and severe forms of bacterial gastroenteritis in humans (43). The virulence of *Shigella* is due to its ability to invade, multiply, spread intracellularly, and induce inflammation within the intestinal epithelium (33). Most of the genes responsible for invasion of eukaryotic cells are located on a 230-kb virulence plasmid (1). Entry into mammalian cells is regulated by a plasmid-encoded type III secretory system located on a 31-kb locus that codes for the invasion plasmid antigens IpaA, IpaB, IpaC, and IpaD. This secretory mechanism is activated upon contact with epithelial cells (49). Upon entry into host cells, bacteria gain access to the cytoplasm by lysing the phagocytic vacuole and rapidly multiplying and spreading (26, 32, 41)

Epidemiologic studies have shown that breast-feeding decreases the severity of *Shigella* sp. infection in infants who become colonized early in life (2, 12, 13, 20, 22, 25). Immune and nonimmune components of milk may be relevant to this protection. Among the nonantibody factors is lactoferrin, an iron-binding glycoprotein of 78 kDa that is resistant to proteolytic enzymes (10). However, epidemiologic data cannot separate the effect of lactoferrin from those of other potentially protective human milk factors. Lactoferrin, antilipopolysaccharide (anti-LPS) and anti-invasion plasmid antigen secretory immunoglobulin A (IgA), lysozyme, and oligosaccharides may each play a role in protection of infants from shigellosis. Although multiple effects of lactoferrin have been demonstrated in vitro, there are no animal model studies demonstrating a role in the gut in vivo. The objective of these studies was to determine whether lactoferrin at concentrations found in human colostrum (0.125 mM) could prevent the development of clinical and pathological changes in an in vivo rabbit model of inflammatory enteritis.

MATERIALS AND METHODS

Lactoferrin was obtained from Agennix Inc. Lactoferrin is expressed in *Aspergillus awamori* as a glucoamylase fusion polypeptide secreted into the medium and processed to mature human lactoferrin by an endogenous KEX-2 peptidase. The recombinant protein retains full biological activity (47).

Rabbit infection model. Four-week-old New Zealand White rabbits were challenged with 10^8 CFU of log-phase *Shigella flexneri* serotype 5 strain M90T. Preliminary experiments were done with 10^7 to 5×10^9 CFU before selecting a dose of 10^8 CFU. *S. flexneri* 5 strain M90T, stored in charcoal-yeast extract glycerol at $-70°C$, was grown overnight at 37°C on Congo Red agar to verify the presence of virulence genes. A log-phase culture in brain heart infusion broth (BHI) was centrifuged, washed with 10 mM phosphate-buffered saline (PBS), pH 7.4, and incubated with end-over-end rotation at 37°C for 1 h in PBS in the absence or presence of lactoferrin (0.125 mM). The bacteria were then centrifuged, washed, and resuspended in PBS. The number of bacteria was estimated from a curve relating optical density to CFU of a log-phase culture prior to inoculation into the rabbits. The effect of lactoferrin treatment on bacterial viability was assessed by incubating inocula of lactoferrin-treated or PBS-treated washed organisms, adding them to BHI, and performing serial determinations each hour for the next 3 h of incubation at 37°C.

For rabbit infection the bacteria were delivered in 5 ml of buffer. With each group of animals studied (usually six per day) virulence of the *S. flexneri* was confirmed by Congo Red uptake of the organisms used for inoculation of animals. Lactoferrin was used in a physiologic concentration (0.125 mM). The inoculum was given by orogastric tube to anesthetized rabbits after pretreatment with 7.5 mg of ranitidine to block acid secretion and two 5-ml doses of $NaHCO_3$ (5%) followed by 0.2 mg of loperamide. This method is a modification of a previously described procedure (20). The rabbits were then given ad lib access to PBS with or without lactoferrin (0.125 mM) until sacrifice; the continuation of lactoferrin was meant to simulate the situation that occurs during feeding of human infants. Twelve animals were monitored for 15 days to determine the course of infection; weight and body temperature were determined daily. An additional 68 animals (35 given lactoferrin and 33 given just buffer) were sacrificed after 24 h so that the intestines could be examined grossly and microscopically for evidence of inflammatory enteritis. Macroscopically, inflammation was assessed by presence of edema, erythema, and hemorrhage. For microscopic examination, the most distal ileal Peyer's patch and a 1-cm^2 area on either side of it were fixed in formalin, stained with hematoxylin and eosin, and evaluated by a blinded observer. Each slide was scored for submucosal edema, submucosal hemorrhage, venous congestion, inflammatory submucosal leukocyte infiltrate, and shortening of villi (with one point given for the presence of each finding and zero points given for the absence of each finding; maximum score, 5). The studies

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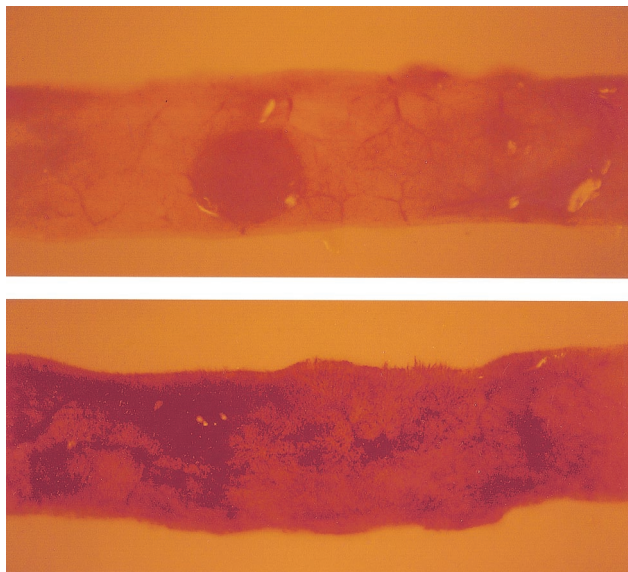
FIG. 1. Macroscopic evidence of severe inflammatory enteritis in a portion of ileum of a rabbit infected with *Shigella* and not receiving lactoferrin treatment (bottom). Normal distal ileum in an infected animal treated with lactoferrin (top).

described in this report were all reviewed and approved by the Animal Welfare Committee of the University of Texas Health Science Center at Houston.

Statistical analysis. Parametric data were analyzed with the Student *t* test. The chi-square test was used for analyzing differences in proportions. Two-tailed tests were used.

RESULTS

Effect of lactoferrin on bacterial viability. Bacteria that had been previously incubated for 1 h at 37°C in lactoferrin, washed, and cultured had a number CFU essentially identical to that observed for those cultures that had been preincubated in PBS. At 1, 2, and 3 h after initiation of culture in BHI, the lactoferrin-treated organism CFU were 2.9×10^8, 4.4×10^8, and 6.2×10^8 compared to the organism that had been incubated in buffer only prior to culture, whose CFU were 2.8×10^8, 4.4×10^8, and 6.8×10^8, respectively. The data are geometric means for seven separate experiments. There is no significant difference between the growth curves.

Clinical status. Twelve animals monitored for 2 weeks after *Shigella* inoculation all survived. Diarrhea did not develop, perhaps reflecting the loperamide used to slow intestinal motility and promote development of infection. There were no significant differences in their weights during the 2 weeks. The only significant difference was that the animals not given lactoferrin had a significantly higher body temperature [39.9 ± 0.2°C (mean ± standard error of the mean) versus 38.9 ± 0.2°C, $P < 0.01$] 24 h after inoculation. Subsequent temperature measurements were not significantly different. Adult New Zealand White rabbit body temperature range is normally 38.9 to 39.6°C (29). The animals not given lactoferrin developed ruffled fur and were less active during the initial febrile period.

Macroscopic changes of enteritis. Groups of animals were sacrificed 24 h after inoculation in order to look for evidence of enteritis. Gross evidence of inflammatory changes (Fig. 1) developed significantly more often in rabbits infected without
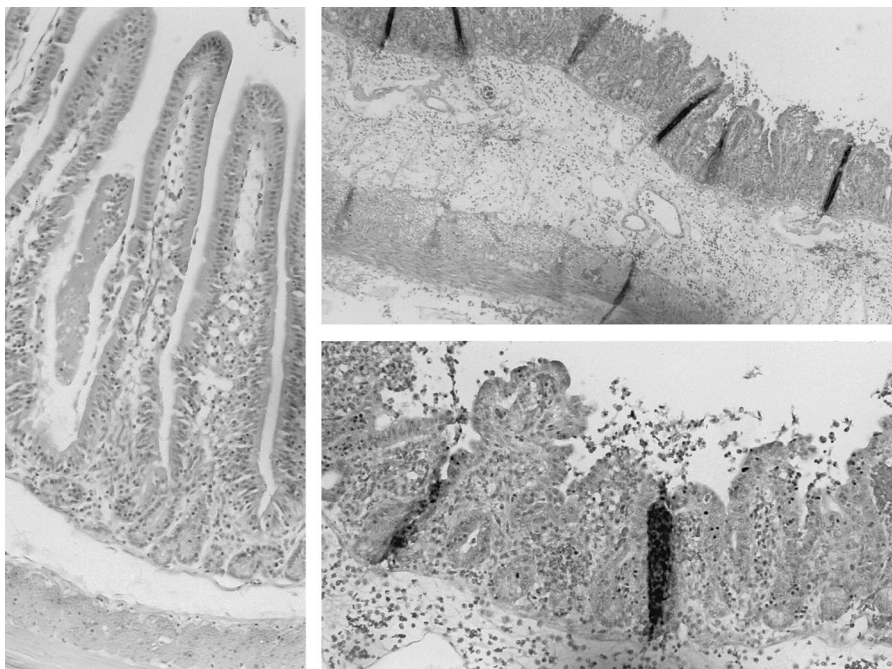

FIG. 2. The bottom left panel shows the normal histological appearance of a section of ileum of a rabbit infected with *Shigella* and receiving lactoferrin treatment. (score, 0) The other panels show typical histological changes in a section of ileum of ill rabbits infected with *Shigella* and not receiving lactoferrin treatment (right top, low-power magnification; bottom, high-power magnification) (score 5) showing blunting of villi, submucosal edema, hemorrhages, and severe inflammation.

lactoferrin treatment (22 of 33 [67%]) than in those infected with lactoferrin treatment (4 of 35 [11%]) ($P < 0.001$).

Microscopic evidence of inflammatory changes. Fig. 2 shows the histology of an infected rabbit given lactoferrin and an infected animal not given lactoferrin. Typical histological changes in animals not given lactoferrin included villous blunting with sloughing of epithelial cells, submucosal edema, infiltration of leukocytes, venous congestion, and hemorrhage (Fig. 2). The degree of inflammation interpreted by a blinded observer was significantly less in the infected animals treated with lactoferrin than in those not given lactoferrin [inflammation score, 2.1 ± 0.3 versus 3.8 ± 0.3 (mean $\pm$ standard error of the mean); $P < 0.001$].

DISCUSSION

There are many activities that have been attributed to lactoferrin based on in vitro studies. Lactoferrin has been thought to protect against gram-negative bacteria in a variety of ways. Lactoferrin has both bacteriostatic (37, 39, 44) and bactericidal activity (4, 5, 16). It chelates iron required for bacterial growth. In iron-deficient media, this results in bacteriostasis. However, the antibacterial activity of lactoferrin is not due solely to its iron-binding capacity. A pepsin-derived fragment of lactoferrin, lactoferricin, has iron-independent bactericidal activity that is associated with release of LPS (17, 18, 51). An 11-residue peptide (FQWQRNMRKVR) is responsible for the bactericidal activity (6). Lactoferrin binds to the phosphate group of the lipid A moiety of LPS (3, 9). There are both high-affinity and low-affinity LPS binding sites in lactoferrin (14). Lactoferrin also binds to outer membrane proteins (porins) of *Escherichia coli* and *Salmonella* and *Shigella* species, thereby destabilizing the bacterial exterior surface (19, 34, 38, 46). Tissue culture models suggest that lactoferrin inhibits adhesion of enterotoxigenic *E. coli*, enteropathogenic *E. coli*, diffusely adherent *E. coli*, and enteroaggregative *E. coli* (27, 35, 36). Lactoferrin also blocks hemagglutination caused by enterotoxigenic *E. coli* (23). However, there is little proof that these in vitro observations regarding diarrheagenic *E. coli* have any relevance for these or other enteropathogens in a living animal.

There are few in vivo demonstrations of lactoferrin's relevance. Oral lactoferrin reduces *E. coli* urinary tract infection in mice (24), and intravenous lactoferrin improves survival during *E. coli* sepsis in mice (52). It is generally assumed that human milk protects from bacterial intestinal infection in part due to lactoferrin, but there are no animal model studies evaluating its effect in isolation. It is unclear whether lactoferrin protects rabbits from inflammatory enteritis by one of the above previously described mechanisms or some other process. A remarkable feature of the studies of *Shigella*-induced enteritis is the decrease in inflammation noted in the lactoferrin-treated rabbits. Lactoferrin has been noted to have anti-inflammatory effects in several model systems. (7, 28, 52) The decrease in intestinal inflammatory findings we observed might have been due to direct effects of lactoferrin either on the bacteria or on the gut. Data strongly suggest that the anti-inflammatory effect of lactoferrin is due to its binding lipid A (3) and soluble CD14. By preventing LPS binding to LPS binding protein and membrane CD14 (15), lactoferrin decreases secretion of multiple cytokines (interleukin-6 [IL-6], IL-1, and tumor necrosis factor

alpha) by macrophages (13, 30, 31). However, this mechanism is very unlikely to be responsible for the effects we observed in this rabbit model. The intestinal macrophages of rabbits (50), like those of humans (42), lack CD14 so that a role for lactoferrin-mediated blockade of LPS-induced inflammation in shigellosis seems untenable. Indeed, the lack of CD14 receptors on gut macrophages is probably an important factor in the lack of inflammation in normal intestine despite massive exposure to LPS. The data currently suggest that although LPS may contribute to the inflammatory response in shigellosis (8), it is not the primary inflammatory stimulus. *Shigella* spp. induce inflammation via effects of IpaB on IL-1β-converting enzyme, not via LPS release and LPS binding protein/membrane CD14-mediated cytokine induction. *Shigella* spp. are taken up by M cells (40, 48) and infect Peyer's patch macrophages where IpaB binds to interleukin-1β-converting enzyme (11, 45). Apoptosis of macrophages, T cells, and B cells then occurs (54–56). Release of IL-1α and IL-1β evokes an inflammatory reaction that causes polymorphonuclear leukocytes to migrate through the epithelium into the lumen, disrupting the epithelium and allowing massive entry of bacteria into the submucosa with further tissue destruction (53). Organisms taken up at Peyer's patches should have been able to induce a local inflammatory reaction if IpaB were present. The absence of inflammation therefore suggested that IpaB had been affected by lactoferrin treatment. Lactoferrin decreases invasiveness of *S. flexneri* (21). Lactoferrin causes loss and degradation of invasion plasmid antigens (21a).

Although it is not clear which of the biologic activities of lactoferrin is responsible for protection, these studies clearly show that at the intact gut level, lactoferrin protects against development of inflammatory enteritis. These data suggest that if the human gut behaves like that of the rabbit, lactoferrin may play an important role in protection of breast fed infants from bacillary dysentery and may merit study as a potential agent for therapy of *Shigella*-induced inflammatory enteritis.

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Editor: J. D. Clements