BioMetals **17:** 285–289, 2004.

Neonatal small bowel epithelia: enhancing anti-bacterial defense with lactoferrin and *Lactobacillus* GG

Michael P. Sherman[1,*], Stephen H. Bennett[1], Freda F.Y. Hwang[1] & Camille Yu[2]
[1]*School of Medicine; University of California; Davis, California U.S.A.*
[2]*School of Medicine; Washington University, St. Louis, Missouri U.S.A.*
[*]*Corresponding author (Tel: (530) 752-3441; Fax: (530) 752-6215; E-mail: mpsherman@ucdavis.edu)*

Abstract

Background and Aims. Extremely preterm human infants have increased susceptibility to small bowel infection. We hypothesized that early colonization of the immature small intestine with *Lactobacillus* GG (LGG), and use of a recombinant lactoferrin (rhLF) to promote growth of LGG, would enhance gut defenses against enteroinvasive *Eschericheria coli*.
Methods. Newborn rat pups were treated with nothing, intra-gastric LGG, or rhLF + LGG on days 3 and 4 of life. Gut colonization by LGG was quantified in lavaged jejunal and ileal fluid and gut wall homogenates on day 5 of life. Separate studies used similarly treated litters of newborn rats that were infected late on day 4 of life with *E. coli* [10^{12} CFU/kg]. Sixteen hours later, the numbers of *E. coli* were measured in small bowel fluid and gut wall homogenates.
Results. Control pups initially had lactic acid bacteria colonize the bowel, but these bacteria were not LGG. Pups treated with LGG or rhLF + LGG had significantly higher numbers of LGG in the ileum versus jejunum. Contrary to our hypothesis, rhLF did not augment LGG colonization. After *E. coli*-related gut infection, planktonic [lavage fluid] and epithelia-adherent growth [gut wall homogenates] of *E. coli* in the small bowel were most effectively reduced by pre-treatment with rhLF and LGG ($P < .05$).
Conclusion. Prophylactic therapy with recombinant human lactoferrin and the probiotic, *Lactobacillus* GG, act to enhance defenses against invasive *E. coli* in the nascent small intestine. We suggest that rhLF and LGG are therapeutic agents that may reduce necrotizing enterocolitis and gut-related sepsis in preterm human infants.

Introduction

Forty-five percent of the late deaths in neonatal intensive care units [NICUs] are caused by infection (Gaynes *et al.* 1996, Stoll *et al.* 1996). These deaths occur primarily in extremely preterm infants. The intestine is an important source of the pathogenic bacteria that cause either necrotizing enterocolitis [NEC] or gut-related sepsis. It is proposed that abnormal bacterial colonization of the intestine causes NEC in preterm infants (Claud 2001).

It is proposed that pregnant women have an enteric bacterial flora that has genetic compatibility with their intestinal epithelia (Hooper 2001). These investigators also suggest this maternal flora is best-suited to colonize the sterile gut of their newborn infant. In doing so, this maternally-acquired gut flora provides commensal bacteria that promote growth and maturation of the neonatal intestine. Breastfeeding allows close contact between mother and her infant and mammary milk has biofactors that help establish bifidobacteria as a predominant intestinal bacterium (Harmsen *et al.* 2000). Bifidobacteria have been used to mitigate NEC in a neonatal rat model (Caplan *et al.* 1999). The healthy term neonate has close maternal contact, leaves the hospital shortly after birth, and is breast fed. The birth of an extremely preterm infant interrupts early nutrition with maternal milk and close maternal contact, and the intestinal flora of this infant is acquired from the NICU.

Here we propose that the major whey protein in milk (Lönnerdal 1995), lactoferrin [LF], will enhance

colonization of the small intestine with a commonly-used probiotic, *Lactobacillus* GG [LGG] (Vanderhoof 2002). Lactobacilli are another member of the 'bifidus flora' in the stool of breast-fed term infants (Harmsen *et al.* 2000). We propose LF and LGG will act together to create a healthy bowel microbiota, promote the maturation of newborn gut epithelia and facilitate emergence of nascent host defenses in the small intestine.

Materials and methods

Specific pathogen free Sprague Dawley dams and their suckling pups arrived at our institution two days after birth. Recombinant human lactoferrin [rhLF] was produced as previously described (Ward 1995) and was provided by Agennix, Inc. in sterile phosphate buffered saline at a concentration of 100 mg/mL. Agennix, Inc. tested the lots of rhLF used in these studies for the percentage of bound ferric iron. The percentage of bound iron was consistently around 12%. *Lactobacillus casei* sps. *rhamnosus* [LGG] was obtained as a powder [Culturelle] and suspended in sterile phosphate buffered saline at 10^9 colony-forming units [CFU] per mL. The numbers of LGG were confirmed by serial dilution and pour plate methods on Man Rogosa Sharpe [MRS] agar (Alander 1999).

An initial study examined early colonization of the small bowel with LGG. Pups were given the following treatments on days 3 and 4 of life: nothing, LGG [two daily intra-gastric doses at a concentration of 10^7 CFU/kg], and LGG + rhLF (500 mg/kg/d in two intra-gastric doses]. Three litters were randomly assigned to each experimental group. Litter size was limited to 8 pups. Six pups from each litter were randomly selected for bacteriologic study, while the remaining two pups were used for histologic studies of the small intestine. On the 5th day of life, sterile technique was used to isolate the small bowel from the ligament of Treitz to the ileocecal value. The bowel was divided into nearly equal lengths at its transition point, thereby identifying the proximal jejunum and distal ileum. The jejunum and ileum were lavaged with 250 μL of sterile saline and the effluent aseptically collected. Effluents and homogenized proximal and distal bowel segments [in 1 mL of sterile saline] underwent quantitative culture for LGG on MRS agar using anaerobic chambers with 5% CO_2 at 35 °C. After 72 h of incubation, CFUs of LGG and other lactic acid bacteria [LAB] were determined and normalized to gram weight per cm length of small bowel segment. The remaining pups had their jejunal and ileal segments fixed *in situ* with Formalin^{TM}. These bowel segments were embedded in paraffin, and 4 μm sections were stained with hematoxylin and eosin. These sections were assessed for alterations in microscopic structure.

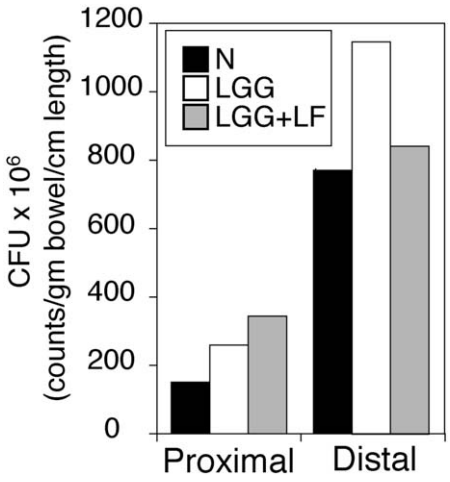

Fig. 1. The Effect of the *Lactobacillus* GG or *Lactobacillus* GG + Recombinant Human Lactoferrin Prophylaxis on the Numbers of Lactic Acid Bacteria in the Proximal and Distal Small Bowel of Neonatal Rats. The proximal [jejunum] and distal [ileum] small bowel site of sampling is shown on the 'x' axis. The colony-forming units [CFU] of lactic acid bacteria [LAB] isolated on Man Rogosa Sharpe agar is shown on the 'y' axis. The internal key shows the assigned treatment: N = nothing, LGG = *Lactobacillus* GG, and LGG + rhLF = *Lactobacillus* GG + recombinant human lactoferrin.

A subsequent study used the same pre-treatments before litters were infected at 1800 on day of life 4 with $\sim 1 \times 10^{12}$ CFU/kg of *Escherichia coli*. Sixteen hours after infection, proximal and distal small bowel segments were aseptically isolated. Lavage samples and gut wall homogenates were prepared as described above. Serial dilutions of lavage and bowel homogenates were made and the numbers of *E. coli* were enumerated using standard pour plate technique and MacConkey's agar. After 48 h of incubation, CFUs of *E. coli* were counted and normalized to gram weight per cm length of small bowel segment. Histologic sections from the three groups of pre-treated and infected newborn rats were also examined for microscopic change.

Because the CFUs of *E. coli* in the small bowel homogenates and lavages were not normally distributed, differences among groups were determined using the Kruskal Wallis statistic (Glantz 1997).

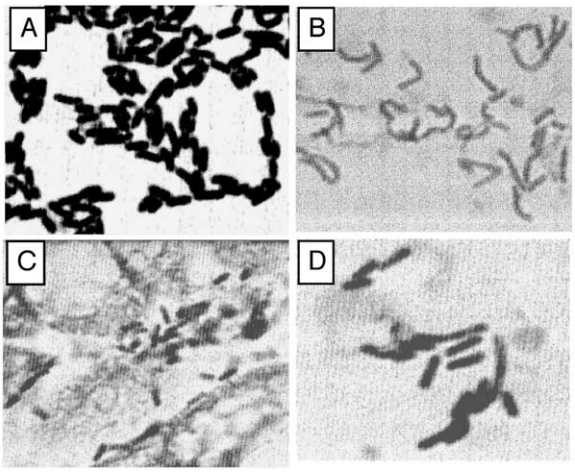

Fig. 2. Identification of Lactobacillus GG [LGG] in Different Samples Taken During the Colonization Studies. Panel A is a Gram stain of LGG in the suspension used to inoculate the stomach and colonize the gut. Panel B is a Gram stain of bacteria [i.e., lactobacilli] found in an ileal lavage of a pup colonized with LGG. Panel C is a tissue section [1000×], and this photomicrograph shows lactobacilli in the mucus layer overlying ileal villi of a pup colonized with LGG. Panel D is a Gram stain of bacteria [i.e., lactobacilli] isolated on MRS agar that had been inoculated with ileal homogenate after LGG therapy.

Results and discussion

When enteral therapy with LGG or LGG + LF is given to neonatal rats, more LAB are isolated in their small bowel homogenates on the 5th day of life (Figure 1). There was more LAB in the ileal homogenates compared to the jejunum. In the jejunum and ileum of pups treated with LGG or LGG + rhLF, the LAB were identified as lactobacilli. Non-treated pups had high numbers of LAB, but they were not identified as LGG. Rather Gram stain analyses and sugar fermentation studies revealed the LAB were mostly *Lactococcus*. The numbers of LAB quantified in the jejunal and ileal homogenates of neonatal rat pups did not differ statistically among the three groups. Figure 2 shows Gram stain results taken from different samples during these studies. The culture results in Figure 1 and the photomicrographs in Figure 2 confirm that LGG given orally to neonatal rats can be recovered from the small bowel as a bacterium with the same culture characteristics and morphology. Review of the microscopic findings revealed the observations in Figure 2 were only consistently seen in the rhLF + LGG-treated pups. The rhLF + LGG-treated pups also had domed villi in their distal ileum. Domed villi are precursors to Peyer's patches, and this finding suggests that a combination of rhLF + LGG, but not either alone, might accelerate the maturation of lymphatic tissue in the distal small bowel. Quantitative stereologic assessment of the ileum from the 3 treated groups must confirm this subjective observation.

The effect of prophylaxis with LGG or rhLF + LGG on the numbers of *E. coli* isolated from the small bowel of neonatal rats following enteral infection is shown in Figure 3. Pups pre-treated with rhLF + LGG had significantly lower numbers of *E. coli* were isolated from the luminal fluid and gut wall homogenates of the jejunum compared to control pups [*E. coli*-infected pups without prophylaxis, $P < .05$]. Pre-treatment with rhLF and LGG was also effective in reducing the numbers of *E. coli* identified in ileal homogenates compared to control pups ($P < .05$). The observation that there were lower numbers of *E. coli* in proximal small bowel lavages and homogenates compared to distal samples suggests jejunal motility may either move more *E. coli* distally or the jejunum has unidentified mechanisms to kill this pathogen before it reaches the ileum. Only a single inoculum of *E. coli* was introduced because our end-point was the numbers of *E. coli* present in the small bowel. No pups died during these investigations compared to an earlier study wherein rhLF protected neonatal rats from death when enteral *E. coli* infection was induced on successive days (Edde 2001).

The mechanisms by which rhLF, LGG or rhLF + LGG enhance neonatal intestinal host defenses and alter the colonization or infection of the small bowel with *E. coli* are poorly understood. It has been suggested LF acts as a bacteriostatic agent against *E. coli* in the developing intestine, and this effect is proposed to be mediated by iron sequestration (Bullen 1972). Since the ferric iron content of rat milk is low, intestinal bacteriostasis of *E. coli* caused by chelation of ferric ions seems improbable. Moreover, the amount of prophylactic rhLF introduced into the gut before infection was far too low to hinder the intestinal growth of *E. coli* via an iron withholding defense because the inoculum of *E. coli* was so large. In this model, other anti-bacterial mechanisms related to rhLF and LGG prophylaxis likely explain our findings. Currently, the immuno-stimulatory and/or immunomodulatory effects of rhLF and LGG probably account for their actions at epithelial surfaces (Ward 2002, Macfarlane 2002).

Pre-treatment with rhLF and LGG also showed a beneficial effect when the histologic sections were reviewed. The distal ileum of infected pups without

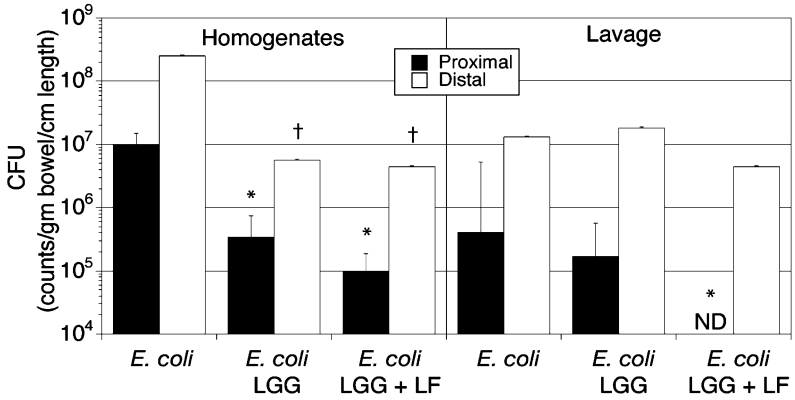

Fig. 3. The Effect of Lactobacillus GG or *Lactobacillus* GG + Recombinant Human Lactoferrin Pre-treatment on Bowel Infection Caused by *Escherichia coli.* Jejunal [proximal] and ileal [distal] lavage fluid and homogenates were cultured. These sites of study are identified in the internal legends. The treatment group and its associated infection with enteral *E. coli* are shown on the 'x' axis. LGG means pre-treatment with Lactobacillus GG. rhLF means pre-treatment with recombinant human lactoferrin. Log bacterial counts are shown on the 'y' axis. The results are based on the colony-forming units (CFU) of *E. coli* identified on MacConkey's medium. CFUs are standardized per g of bowel tissue per cm of bowel length. ND means *E. coli* were not detected at that serial dilution. The * indicates a $P < .05$ compared to control [*E. coli*-infected pups]. The † designates a $P < .05$ compared to control [*E. coli* infected-pups].

pre-treatment was often edematous with ballooning enterocytes. Again, stereologic methods are needed to quantify the volume and surface area of the villi, thereby confirming these subjective observations.

Finally, the rationale for developing this model to study the maturation of gut host defenses should be discussed briefly. Rats were used because their milk lacks lactoferrin (Masson 1971). Despite this finding, human lactoferrin stimulates the growth of rat enterocytes (Nichols 1989). Neonatal rats are the accepted animal model to study NEC, the devastating intestinal disease of extremely preterm human infants (Caplan *et al.* 1999). It is reported that abnormal bacterial colonization leads to excessive toxin production by enteric bacteria and the toxin in turn is associated with platelet activating factor release, a mediator of distal small bowel ischemia. These studies are designed to test the potential use of these enteral therapies to prevent abnormal bacterial colonization of the nascent small intestine in the preterm infant. We suggest that this prophylactic approach will mitigate the sequence of events that cause NEC and gut-related bacteremia in preterm human infants.

Acknowledgements

This research was supported by a grant from the Children's Miracle Network. The investigators thank Agennix, Inc. for providing rhLF. The authors have no financial interest in Agennix, Inc.

References

Alander M, Satokari R, Korpela R, *et al.* 1999 Persistence of colonization of human colonic mucosa by a probiotic strain, *Lactobacillus rhamnosus* GG, after oral consumption. *Appl Environ Microbiol* **65**, 351–354.

Bullen JJ, Rogers HJ, Leigh L. 1972 Iron-binding proteins in milk and resistance to *Escherichia coli* infection in infants. *Br Med J* **1(792)**, 69–72.

Caplan MS, Miller-Catchpole R, Kaup S *et al.* 1999 Bifidobacterial supplementation reduces the incidence of necrotizing enterocolitis in a neonatal rat model. *Gastroenterology* **117**, 577–583.

Claud EC, Walker WA. 2001 Hypothesis: inappropriate colonization of the premature intestine can cause neonatal necrotizing enterocolitis. *FASEB* **15**, 1398–1403.

Edde L, Hipolito RB, Hwang FFY, Headon DR, Shalwitz RA, Sherman MP. 2001 Lactoferrin protects neonatal rats from gut-related systemic infection. *Amer J Physiol: Gastroenterol Liver Physiol* **281**, G1140–G1150.

Gaynes RP, Edwards JR, Jarvis WR *et al.* 1996 Nosocomial infections among neonates in high-risk nurseries in the United States. National Nosocomial Infections Surveillance System. *Pediatrics* **98**, 357–361.

Glantz SA. 1997. Primer of Biostatistics. New York, McGraw-Hill.

Harmsen HJM, Wildeboer-Veloo ACM, Raangs GC *et al.* 2000 Analysis of intestinal flora development in breast-fed and formula-fed infants using molecular identification and detection methods. *J Pediatr Gastroenterol Nutr* **30**, 61–67.

Hooper LV, Gordon JI. 2001 Commensal host-bacterial relationships in the gut. *Science* **292**, 1115–1118.

Lönnerdal B, Iyer S. 1995 Lactoferrin: molecular structure and biological function. *Annu Rev Nutr* **15**, 93–110.

Macfarlane GT, Cummings JH. 2002 Probiotics, infection and immunity. *Curr Opin Infect Dis* **15**, 501–506.

Masson PL, Heremans JF. 1971 Lactoferrin in milk from different species. *Comp Biochem Physiol* **39B**, 119–129.

Nichols BL, McKee, Putman M, Henry JF, Nichols VN. 1989 Human lactoferrin supplementation of infant formulas increases

thymidine incorporation into the DNA of rat crypt cells. *J Pediatr Gastroenterol Nutr* **8**, 102–109.

Stoll BJ, Gordon T, Korones SB, *et al.* 1996 Late-onset sepsis in very low birth weight neonates: a report from the National Institutes of Child Health and Human Development Neonatal Research Network. *J Pediatr* **129**, 63–71.

Vanderhoof JA, Young RJ. 2002 Probiotics in pediatrics. *Pediatrics* **109**, 956–958.

Ward PP, Piddington CS, Cunningham G, Zhou X, Wyatt RD, Conneely OM. 1995 Development of a system for production of commercial quantities of human lactoferrin: a broad-spectrum natural antibiotic. *Nature/Biotechnology* **13**, 498–503.

Ward PP, Uribe-Luna S, Conneely OM. 2002 Lactoferrin and host defense. *Biochem Cell Biol* **80**, 96–102.