

Protective Proteins in Mammalian Milks: Lactoferrin Steps Forward

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Educational Gaps

1. In clinical trials, lactoferrin (LF) shows promise for reducing mortality and preventing neonatal infections, particularly late-onset sepsis and necrotizing enterocolitis.
2. Until the U.S. Food and Drug Administration approves of LF-containing preparations to ward off neonatal infection, colostrum feeding should be encouraged for preterm infants as soon as possible after birth because of its high LF concentrations.

Abstract

Human milk is the ideal nutrient for neonates. Breastfeeding exposes neonates to maternal microflora, provides host protection, and has proteins that mediate immune system development. Lactoferrin (LF) is the major whey protein in mammalian milk, and its multifunctional characteristics have shown importance in preventing infections. Ferric iron binding and natural peptide antibiotic properties of LF likely promote a healthy intestinal microbiome that prevents bacterial translocation and mediates optimal epithelial growth and differentiation. An established asset of LF is stimulation of naïve dendritic cells; this initiates the emergence of neonatal Th1 helper cells, thereby reversing the Th2 bias associated with pregnancy. Moreover, LF promotes development of Peyer patches, which leads to enhanced IgA secretion into the intestinal lumen. The anti-inflammatory properties of LF involve its binding of endotoxin and other proinflammatory molecules released by intestinal pathogens. LF also blocks receptors that microbes use for epithelial invasion, and thus LF mitigates a proinflammatory response by the host during infection. These properties of LF are known from basic science research and preclinical investigations, and they have resulted in the enteral use of bovine and recombinant human LF to prevent neonatal late onset sepsis. Bovine LF has been shown to reduce the incidence of late onset sepsis in extremely preterm infants, but Food and Drug Administration approval of LF for use in the NICU has not taken place. Because LF is currently available only for scientific investigations, the feeding of a mother's milk is encouraged shortly after birth because the concentration of LF is highest in colostrum.

Abbreviations

bLF:	bovine lactoferrin
CFU:	colony-forming unit
ELBW:	extremely low birth weight
FDA:	Food and Drug Administration
GRAS:	generally regarded as safe
hLF:	human lactoferrin
IL:	interleukin
LF:	lactoferrin
LFcin:	lactoferricin
LGG:	<i>Lactobacillus rhamnosus</i> GG
LOS:	late onset sepsis
LPS:	lipopolysaccharide (endotoxin)
NEC:	necrotizing enterocolitis
RCT:	randomized clinical trial
rhLF:	recombinant human lactoferrin
TLR:	Toll-like receptor

Objectives After completing this article, readers should be able to:

1. Describe the biochemical structure and multifunctional properties of LF.
2. Appreciate the actions of LF on the immune system and understand why this nutrient protein confers host protection in neonates.
3. Understand the immunoregulatory and anti-inflammatory characteristics of LF.
4. Learn about the preclinical and clinical studies that have resulted in the use of LF in the NICU as a preventive strategy against infections.

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5. Comprehend that feeding colostrum from the day of birth and sustained human milk feeding thereafter is vital to host protection in the neonate and prevention of infection in hospitalized preterm infants.

Introduction

Extremely preterm infants have a risk of infection during hospitalization that ranges from 58% for infants born at 22 weeks of gestation to 20% when birth occurs at 28 weeks of gestation. (1) For those infants who survive late onset sepsis (LOS) or necrotizing enterocolitis (NEC), the outcomes include an increased occurrence of neuro-developmental disabilities. (2) Billions of dollars are spent each year because LOS or NEC extends the hospital stay and requires long-term rehabilitation of surviving infants. The problem of infections in NICUs brought about a workshop convened at the National Institute of Child Health and Human Development to discuss preventive strategies. (3) Breastfeeding is considered an established approach to reduce infection in preterm infants because of its protective nutrients (4); however, the ability of a mother to provide colostrum for her extremely preterm infant in the hours and days after birth is limited. (5) Furthermore, immature infants may not tolerate enteral feedings. An armamentarium of proteins with immunologic properties is present in mammalian milk, (6) but determining the relative importance of these nutrients in promoting neonatal immunity is daunting. In the past two decades, lactoferrin (LF) has emerged as a key mediator of enhanced neonatal immunity and host protection. LF is the major whey protein in milk, and the concentration is highest in colostrum and then declines over time during breastfeeding. (7) LF, and peptides derived from its proteolytic digestion, have pleiotropic functions including antimicrobial, immunostimulatory, anti-inflammatory and antitumor activities, the regulation of cell growth and differentiation (especially in intestine, bone, and hematopoietic tissues), and enzymatic, signal transduction, and antioxidant properties. (8)(9)(10) This review will discuss the basic mechanisms by which LF enhances innate and adaptive immunity and protects infants from infection. Preclinical and clinical investigations revealing how LF prophylaxis prevents infection are described. These studies reveal LF may soon become the first mammalian milk protein used as a nutritional supplement to prevent infections in the NICU.

Natural Antibiotic, Antimicrobial Properties

LF is an 80 kDa iron-binding glycoprotein of the transferrin family that is secreted into many body fluids and is an

important component of the mammalian innate immune system. (8)(9)(10)(11) LF is released into mammalian milk as an iron-free, apo-protein that can bind two ferric ions at specific sites (Fig 1). Citrate present in human milk and a pH less than 4 are associated with ferric iron binding to LF. (12) In 1972, Bullen et al (13) reported that LF restricted the in vitro and in vivo growth of *Escherichia coli*. This bacteriostatic function of LF was attributed to the uptake of ferric ions. LF was proposed to compete with siderophores of enteric bacteria. Siderophores are used to acquire iron for microbial growth. (14) Brock has challenged the bacteriostatic actions of LF, (12) and research reveals certain bacteria use LF to actually take up iron. (14) Figure 2 reviews the binding of ferric iron to LF and processes utilized by humans to compete for and obtain intracellular iron via the divalent metal transporter (DMT-1) in the duodenum. (15) This pathway is contrasted with mechanisms used by enteric bacteria to acquire iron. (16) LF with bound ferric iron is designated holo-LF, and it is a closed molecule with greater resistance to proteolysis. The resistance of LF to enzymatic digestion suggests an alternative process may be used by intestinal epithelia to transport iron. The intelectin receptor, a specific transporter of LF, may be that mechanism. (17) In fact, alternative explanations have emerged that describe the antimicrobial effects of LF in animals and man. A potent microbicide called lactoferricin (LFcin) is released during pepsin-related proteolysis of human LF (hLF) in

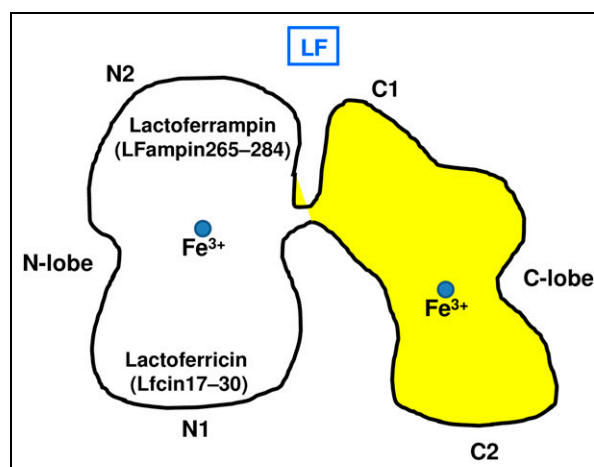


Figure 1. Lactoferrin (LF) structure. The bilobed nature of the protein is displayed. The ferric iron-binding sites are shown in the N- and C-lobes. Each lobe has two subdomains. The amino acid sequence locations of its two peptide antibiotics are depicted in the N-lobe. LF has enzymatic (ribonuclease), nucleotide-binding (RNA and DNA), and heparin-binding characteristics. Glycosylation of the LF molecule is not shown.

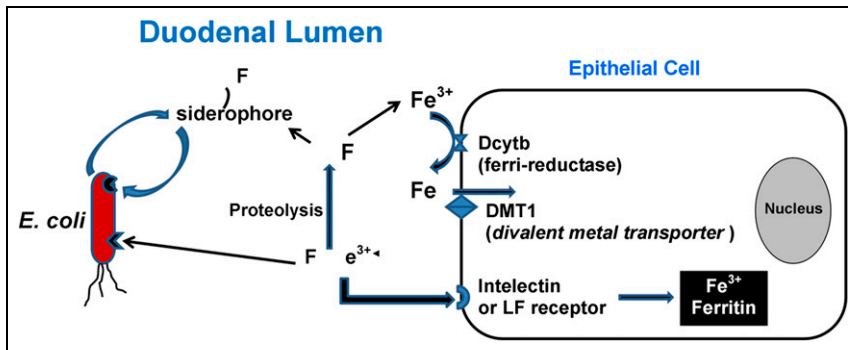


Figure 2. Ferric iron homeostasis in the duodenum. Digestion of LF provides free ferric iron that can be reduced and can be transported inside intestinal epithelia. Alternatively, ferric iron bound to LF can be transported to the intracellular environment via its specific receptor, intelectin. Bacteria can also uptake LF with bound ferric iron. LF competes for free ferric iron with siderophores secreted by enteric bacteria.

the stomach. (18) The protective effects of LFcins include direct microbicidal activities against bacteria, viruses, fungi and parasites. (9)(18) Many microbes killed by LFcins are important pathogens in neonates. Thus, it is prudent for neonatologists to carefully consider the use of H2 blockers or proton pump inhibitors that may interfere with pepsin cleavage of LFcins from LF at a low gastric pH. (19) Figure 1 shows that LFcins are an amino acid sequence near the N-terminus in the N1 domain. Like LF, LFcins bind to the lipid A portion of lipopolysaccharide (LPS), and its binding destabilizes the bacterial membrane and increases permeability. (14)(18)(20) This is similar to the mechanisms associated with other antimicrobial peptides found in nature. (20) Figure 1 shows a second domain in the N lobe of LF that has an amino acid sequence designated “lactoferrampin”; however, this antimicrobial peptide is inactive. By increasing a net positive charge near the C-terminal end of lactoferrampin, a significant increase in bactericidal and candidacidal activity is observed. (21) Although hLF was believed to have antibacterial activity against *E. coli*, subsequent studies reveal this is not correct. (22) Bovine LF (bLF) is at best bacteriostatic. (23) Conversely, bovine LFcins rapidly disrupts the outer membrane of *E. coli*. (23) If hLF or bLF are incubated with lysozyme, another

natural peptide antibiotic present in human milk, (6) rapid killing of *E. coli* takes place. (22)(23) If recombinant hLF (rhLF), lysozyme and *E. coli* are incubated under conditions that simulate fluid in the small intestine, bacterial destruction occurs within 2 hours. (24) In neonates, there is also a synergy between rhLF and pharmaceutical antibiotics that eradicate coagulase-negative staphylococci and *Candida albicans*. (25) Under in vitro and in vivo conditions, antibiotics and LFcins more effectively kill *Pseudomonas aeruginosa*. (26)

In addition to the natural antibiotic effects, Fig 3 outlines nonantibiotic mechanisms that hinder microbes from causing infection. (9)(27)(28) LF blocks bacterial adhesion to host cells by binding to glycosaminoglycans on bacteria and/or host cell membranes. (29)(30) In a unique process, the enzymatic activity of LF degrades proteins associated with the type 3 secretion system of enteric pathogens. The type 3 secretion system uses a needlelike structure as a sensory probe to detect and adhere to the

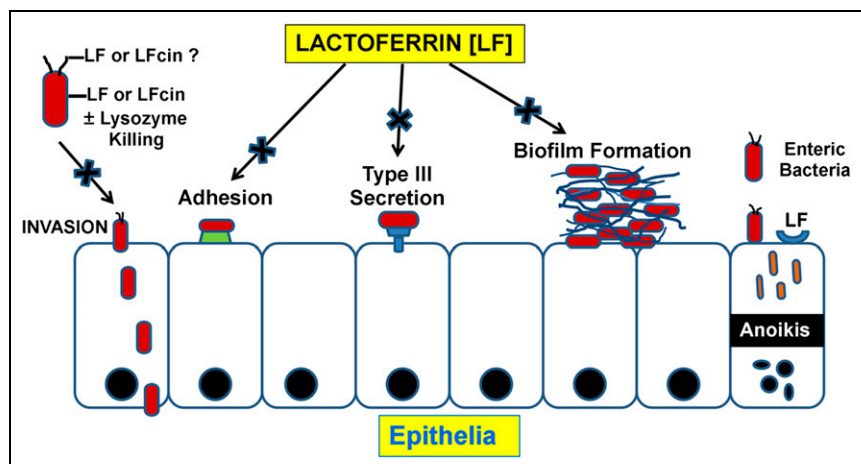


Figure 3. LF-mediated restriction of bacterial invasion of intestinal epithelia. LF can block translocation of intestinal bacteria by killing of the organism in synergy with lysozyme or by release of its peptide antibiotics. There is speculation that LF binds to flagella and hinders invasion by altering bacterial motility. Lactoferrin does attach itself to enteric bacteria or receptors on epithelia and prevents cell entry by inhibiting microbial adhesion. Other LF-associated mechanisms that prevent infection include interference with the bacterial type 3 secretory system or biofilm formation. Anoikis is an LF-associated mechanism that traps bacteria inside epithelia when they undergo apoptosis; apoptosis is followed by release of the dead cell that contains bacteria into the gut lumen.

surface of epithelia and thereafter injects proteins into the cell that enable bacterial entry. (31) LF can prevent bacterial colonization on mucosal surfaces by inhibiting bio-film formation; this mechanism is associated with iron restriction and abnormal bacterial motility. (32) Prophylactic LF fed to neonatal rats before they are enterally infected with *E. coli* initiates a mechanism called anoikis wherein cells containing viable bacteria develop apoptosis. (33) Bacteria trapped inside of apoptotic epithelial cells are shed into the gut lumen and depart the body via peristalsis. LF also binds DNA with high-affinity, but the biologic effects of the LF-DNA interactions are still emerging. (34) Research is needed to define whether LF-DNA binding initiates intracellular events that enhance resistance to microbial invasion. DNA-binding of LF does increase production of interleukin (IL)-1 β . (35) Finally, antiviral effects of LF involve binding to host cells and/or viral particles, thereby obstructing viral entry. (9)(36)

Immunostimulatory Properties

The balance between Th1 and Th2 cell subsets, and their production of specific cytokines, differs during pregnancy compared with normal adults. (37) A Th2 bias in the

maternal intrauterine environment and the fetoplacental unit assures embryonic implantation, proper placental development, and fetal maintenance until term gestation. The Th2 bias established in the fetus, however, is likely responsible for an increased risk of infection during the neonatal period. (38) LF improves Th1 cell function via several mechanisms. The most important action is LF-mediated maturation, differentiation, and proliferation of dendritic cells capable of supporting Th1 differentiation from naïve Th0 cells. (39)(40) Figure 4 shows that LF and foreign antigen exposure increases the maturation of dendritic cells. This process boosts IL-12 production by dendritic cells, an event that reverses the neonatal Th2 bias and makes the Th1 cell population fully functional. (38) LF also recruits and activates antigen-presenting cells and augments antigen-specific immune responses, increases CD4⁺ T cells, and in turn, LF-mediated maturation of Th1 cells improves outcomes during infection. (40)(41)(42) It is important to appreciate that monocyte-derived dendritic cells that are generated in the presence of bLF are not activated by CD83, costimulatory and major histocompatibility complex molecules, or by cytokine and chemokine signaling. (43) Hence, depending on the in vitro or in vivo experimental conditions,

LF is a double-edged sword with either activating or inhibitory functions being predominant.

In considering the role of LF in both immune activation and suppression, LF enhances natural killer cell activity by stimulating IL-18 and type 1 interferon production. (44) Calves fed LF plus vitamin A show enhanced epithelial cell maturation and an expanded size of Peyer patches. (45) Mice given enteral bLF have increased synthesis of IgA in Peyer patches, (46) a key intestinal defense during infancy. In other words, LF modulates maturation and differentiation of T cells and B lymphocytes. Also, LF improves the efficacy of bacterial and viral vaccination, (47)(48) and LF may become an adjuvant during the vaccination of infants. (49) Compared with formula-fed infants, it is interesting to speculate that the enhanced immune responses observed in breastfed infants after bacillus Calmette-Guérin (50) and

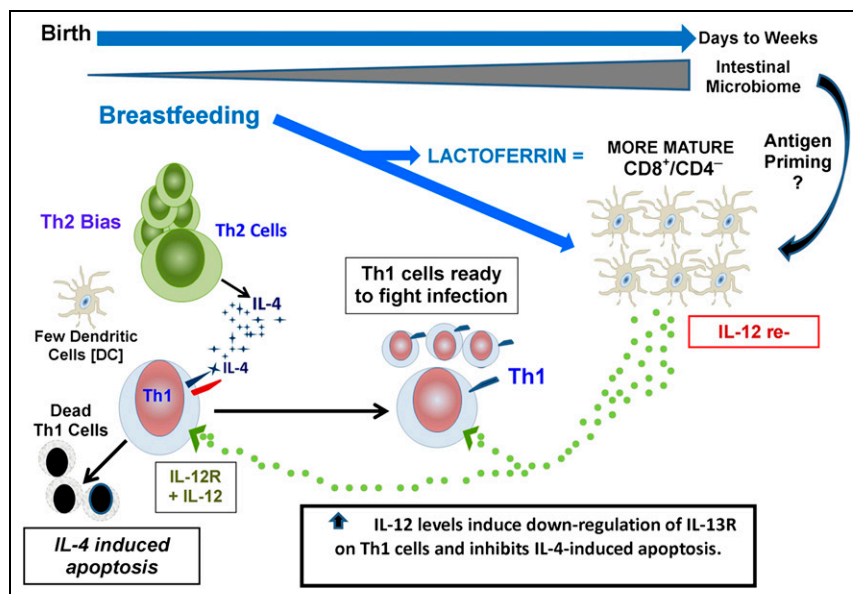


Figure 4. LF-mediated maturation of nascent dendritic cells and reversal of neonatal Th2 bias. A Th2 cell bias in the fetus is known to limit development of the Th1 lymphocyte population during the neonatal period. LF in the gastrointestinal tract probably assists in reversing this immunologic state. LF and antigen exposure (ie, likely intestinal bacteria) expands a functional dendritic cell (DC) population, and in turn, dendritic cells secrete IL-12. This cytokine then plays a key role in the emergence of competent Th1 cells that must be functional during neonatal infection.

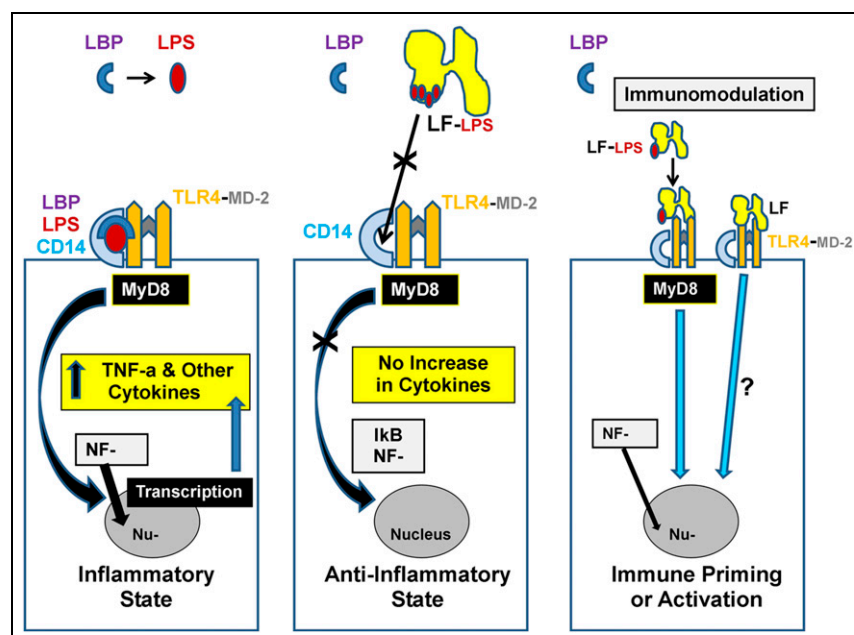


Figure 5. Modulation of inflammation by LF in the intestinal environment. (52) The first epithelial cell shows how LPS from enteric pathogens activates inflammation via the TLR4 receptor. In the second epithelia cell, LF interdicts this process by binding LPS in the environment, provided the LPS concentration is not excessive. In the third epithelial cell, low concentrations of LPS bound to LF may provide a priming signal through TLR4 (and maybe the intelectin receptor); this signal informs intestinal epithelia or immune cells of impending danger. Whether LF without bound LPS can prime or activate intestinal epithelia or cellular immune defenses in the gut via TLR4 needs additional study. Abbreviations: LBP= LPS binding protein; NFκB= nuclear factor kappa B; TNF= tumor necrosis factor.

measles-mumps-rubella (51) vaccinations might be attributed to the presence of LF in mother's milk.

Anti-inflammatory Properties

The mechanisms by which LF quiets, primes, or enhances inflammation are summarized in Fig 5. (52) In the immature infant who has an emerging intestinal microflora, orally administered LF may protect the bowel from inflammation. Pathogen-associated molecular patterns (53) are potent activation signals related to the inflammatory response. LPS, flagellin, and bacterial CpG DNA are among microbial signals present in the intestine, but LF binds these agonists and blocks their attachment to Toll-like receptors (TLRs). (10) In Fig 5, LF is shown engaging TLR4 with either low amounts of LPS or no LPS. (24) (52)(54) Depending on the LF to LPS ratio in an environment, no inflammation, cellular priming for host defense, or a significant inflammatory response may occur. This is an illustration of the immunoregulatory actions of LF.

LPS is a major bacterial toxicant that attaches to the N-terminal subdomain of LF. When LPS is given by

intraperitoneal injection, hLF suppresses tumor necrosis factor-α, IL-1β, and IL-6 release from mononuclear cells. (55) LF inhibits the binding of LPS to L-selectin and the production of hydrogen peroxide by neutrophils. (56) LF also blocks sCD14-LPS-induced expression of E-selectin, ICAM-1 (intercellular adhesion molecule-1), and IL-8 by human umbilical endothelial cells. (57) When piglets are fed LF before they were challenged with intravenous endotoxin, a significant reduction in mortality is seen compared with animals fed bovine albumin. (58) The anti-inflammatory effects of LF may be why feeding this whey protein to extremely preterm infants improves clinical outcomes.

Preclinical Studies in Neonatal Animals

Commercial amounts of pharmaceutical grade rhLF became available in 1995, (59) but a clinical application did not exist. One potential use for rhLF was to supplement human milk feeding immediately after birth. During

this period, very preterm infants take little colostrum compared with term infants. To evaluate the usefulness of rhLF, it was given enterally as a prophylactic immunotherapy to neonatal rats. (24) Rats were used because their milk has minuscule amounts of LF. (7) On days 1 and 2 of life, rats received 350 mg/kg per day of rhLF by gastric gavage, an amount of LF consumed daily during human breastfeeding. (11) On the eves of days 3 and 4, pups were infected with intragastric *E coli* at 10¹² colony-forming units (CFU)/kg. On day 5 of life, blood and liver cultures were obtained, and a Likert scale measured health status. The cumulative illness score was significantly higher in controls versus rhLF-treated rat pups. Animals given the placebo had a sixfold higher death rate compared with rhLF-treated neonatal rats. The CFUs of *E coli* in the blood of control rat pups were more than three logs higher compared with rhLF-treated neonates. Because rhLF was not given right before infection, and because the infective dose was very large, one can propose the effect of rhLF was mediated by an immunostimulatory action rather than an antimicrobial process. These findings

indicate that rhLF may have an application when caring for very preterm infants in the NICU.

Research reveals that formula containing bLF establishes a “bifidus flora” in infants. (60) Additionally, an enteral supplement of bifidobacteria reduces NEC in a neonatal rat model of the disease. (61) For these reasons, we examined whether enteral prophylaxis with *Lactobacillus rhamnosus* GG (LGG) and rhLF lessens intestinal infection caused by *E. coli*. (62) Newborn rat pups received no treatment, LGG, or rhLF + LGG by gavage on days 3 and 4 of life. On the eve of day 4, pups were challenged with enteral *E. coli* (10^{12} CFU/kg). The numbers of *E. coli* were measured in small bowel fluid and in intestinal wall homogenates 16 hours later. Nonadherent (bowel lavage fluid) and epithelia-adherent (gut wall homogenates) revealed growth of *E. coli* in the small bowel was significantly reduced by rhLF and LGG. Based on these results, we propose rhLF and LGG reduces bacterial translocation and might prevent gut-related bacteremia or NEC in human preterm infants.

Clinical Studies in Premature Infants

By using a design similar to the rhLF and LGG study described above, Manzoni et al (63) performed a randomized clinical trial (RCT) in 11 Italian NICUs. The research protocol fed a placebo, bLF, or bLF and LGG to very low birth weight infants. Overall, control infants had a 17.3% rate of LOS, whereas newborns fed bLF had a 5.9% rate, and infants given bLF and LGG had a 4.6% incidence of LOS. Extremely low birth weight (ELBW) infants were found to have the lowest incidence of LOS. On the other hand, LOS in infants weighing 1,001 to 1,500 g at birth did not reach statistical significance. Mortality associated with LOS was found in 0 of 153 infants treated with bLF, 1 of 151 who received bLF + LGG, and 8 of 168 infants in the placebo group. Invasive fungal disease was reduced significantly in ELBW infants given bLF compared with bLF and LGG. NEC $\geq$ stage 2 was significantly lower in ELBW infants who were fed bLF + LGG compared with bLF alone. When all results are considered, there is no clear benefit when an infant receives bLF + LGG compared with bLF alone. A larger study population may delineate differences between the two prophylactic treatments. Manzoni et al did not observe safety concerns in feeding bLF, but the investigators acknowledge that the study was underpowered to detect adverse events. There was no long-term follow-up that determines neurodevelopment among infants receiving the different treatments. Clearly, the initial report by Manzoni et al provides exciting findings, but the results must be confirmed with additional clinical trials.

In that regard, a National Institutes of Health-funded and Food and Drug Administration (FDA) Investigational New Drug-approved study is nearing completion in the United States. This RCT feeds rhLF to infants with birth weights of 750 to 1,500 g (Talactoferrin Oral Solution for Nosocomial Infection in Preterm Infants; ClinicalTrials.gov identifier: NCT00854633). rhLF is started on the day of birth and continues through 28 days of age. The enteral dose of rhLF is 150 mg/kg per dose given twice daily. The rhLF consumed by an infant is similar to that taken during breastfeeding. (11) By comparison, the Italian RCT gave bLF at a dose of 100 mg per day. The Phase 1/Phase 2 study of rhLF is evaluating safety and collects pilot information on whether the rate of LOS is trending downward.

bLF has a GRAS (generally regarded as safe) designation from the FDA. (64) GRAS relates to food supplements and assumes there are no claims of a therapeutic benefit. A GRAS designation is based on a less rigorous review by the FDA compared with a licensed drug. When requesting an investigational new drug designation before the performance of an RCT, information on in vitro and in vivo safety and effectiveness of an experimental agent must be presented to and approved by the FDA. Final drug licensure is based on data provided to the FDA after completing RCTs. Neonatal caregivers are eagerly anticipating the licensure of bLF or rhLF once safety and effectiveness research reveals consistent prevention of late-onset neonatal infections.

Conclusions

Recently, the multifunctional characteristics of LF have been appreciated. In vitro experiments and in vivo animal studies suggest that LF may enhance neonatal immunity and provide host protection. This assumption is supported by the high concentrations of LF in colostrum from human mothers and the reduced incidence of bacterial infections seen in breastfed versus formula-fed preterm infants. Because many preterm infants cannot consume sufficient quantities of a mother’s colostrum immediately after birth, LF has been isolated from bovine milk or hLF has been produced using recombinant technology. An RCT using bLF has been completed, and an RCT using rhLF is nearly complete. Each study reviews the safety and efficacy of these biologic agents to prevent LOS in very preterm infants. While waiting for FDA approval of one or more LF-containing preparations to prevent infections in preterm infants, we recommend feeding of colostrum as soon as possible after birth because of its high LF content. Although a very preterm infant may be taking nothing by mouth, colostrum should be well tolerated. Thereafter, a mother’s milk should provide long-term nutrition for

infants because LF and other components in milk accelerate maturation of the nascent intestinal and systemic immune systems.

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American Board of Pediatrics Neonatal-Perinatal Medicine Content Specifications

- Know the immunologic constituents in human milk and their physiologic effects.
- Know the role of fibronectin and lactoferrin in host defense mechanisms.



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NeoReviews Quiz

New minimum performance level requirements

Per the 2010 revision of the American Medical Association (AMA) Physician's Recognition Award (PRA) and credit system, a minimum performance level must be established on enduring material and journal-based CME activities that are certified for AMA PRA Category 1 Credit™. In order to successfully complete 2012 NeoReviews articles for AMA PRA Category 1 Credit™, learners must demonstrate a minimum performance level of 60% or higher on this assessment, which measures achievement of the educational purpose and/or objectives of this activity.

Starting with 2012 NeoReviews, AMA PRA Category 1 Credit™ can be claimed only if 60% or more of the questions are answered correctly. If you score less than 60% on the assessment, you will be given additional opportunities to answer questions until an overall 60% or greater score is achieved.

1. Lactoferrin is a whey protein that confers protection against infections. Clinical trials are currently examining the benefit of supplementing a premature infant's diet with lactoferrin. Of the following, the dietary component with the highest amount of lactoferrin is:
 - A. Colostrum
 - B. Cow's milk formula
 - C. Hind breast milk
 - D. Mature breast milk
 - E. Soy formula
2. Lactoferrin is a glycoprotein that is secreted into many body fluids, including mammalian milk. Of the following, lactoferrin is least likely to bind to:
 - A. DNA
 - B. Ferric iron
 - C. Heme
 - D. Heparin
 - E. Ribonuclease
3. Lactoferrin is an important mediator that enhances neonatal immunity and protects infants from infection. Of the following, the most likely mechanism by which lactoferrin protects infants from infection is the:
 - A. Antibiotic effect after proteolysis to lactoferricin
 - B. Delayed development of Peyer's patches
 - C. Direct oxidant effect on bacteria
 - D. Release of iron to decrease intestinal epithelial growth
 - E. Suppression of nascent dendritic cells
4. Manzoni and colleagues examined the potential benefit of lactoferrin supplementation. In this randomized clinical trial, VLBW infants were fed bovine lactoferrin, bovine lactoferrin combined with *Lactobacillus rhamnosus* GG, or a placebo. Of the following, the most likely outcome that was unaffected by lactoferrin supplementation in this trial was:
 - A. Chronic lung disease
 - B. Fungal infection
 - C. Late-onset sepsis
 - D. Mortality
 - E. Necrotizing enterocolitis
5. Bovine lactoferrin is a compound that has a GRAS (Generally Recognized As Safe) designation from the US Food and Drug Administration. Of the following, the most accurate interpretation of the GRAS designation is that it:
 - A. Allows a compound to be used for any purpose without requiring a randomized clinical trial
 - B. Refers to an ingredient in food that is recognized as safe
 - C. Denotes that the compound went through a safety review that was as thorough as a licensed drug
 - D. Indicates that the compound is recognized as safe based on in vitro data only
 - E. Suggests that studies have determined safety and therapeutic benefit of the compound