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Human Breast Milk and the Gastrointestinal Innate Immune System

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INTRODUCTION

The epithelial layers and mucus secretions of the pulmonary, genitourinary, and gastrointestinal (GI) systems all provide a complex mechanical barrier and an inherent defense against pathogens that constantly threaten the human body. Evidence suggests that these systems do not work independently, but form what is referred to as the mucosal immunologic system, an integrated network of tissue, cells, and signaling molecules.[1] Of the 3 systems, the lining of the GI tract provides the largest interface with the external environment (200–300 m^2). Although it was long believed to exist solely for food digestion and nutrient absorption, it is now known that the responsibilities of the intestinal system are diverse and critical to host defense. This amazing organ has evolved an elaborate defense system to protect the human body from continuous threats of numerous disease-causing agents and commensal bacteria present at an impressive number (1×10^{14} CFU).[2] At no time in life is this function more important than shortly after birth. The infant's abrupt introduction to life outside the uterus and exposure to antigens forces the GI tract to adapt quickly and commence its crucial duties. But the neonate's adaptive immune system is naive, and the developmental immunologic immaturity leaves the newborn in a state of vulnerability and at increased risk for serious infection. Components of the intestinal innate immune system do not rely on memory and can act with a preformed, nonspecific response.

Feeding exclusively with human milk is recommended for the first 6 months of life[3] and provides unique components and nutrients, leading to optimal nutrition, growth, and development of the newborn infant.[4] The benefits of human breast milk and its association with healthier babies have been intermittently noted over the past few thousand years.[5] In 1934, Grulee and colleagues[6] showed that formula-fed infants had higher morbidity and mortality when compared with breastfed infants. More recently, breast milk has been associated with a decreased incidence of necrotizing enterocolitis (NEC),[7] gastroenteritis,[8]

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severe respiratory illness,[9] otitis media,[10–14] and urinary tract infections.[15] The unique and dynamic composition of human milk not only supplies optimal nutrients but also contributes an abundance of bioactive factors,[16] which support and enhance the deficient immunologic system of the newborn.

In this article, selected factors in breast milk and how they either act alone to provide innate protection or augment GI innate immune function are reviewed. First, a broad and brief overview of innate immunity within the intestinal system is provided. Then, individual constituents present in human breast milk and the variety of mechanisms by which they exert their effects and afford protection to the newborn infant are discussed.

THE INNATE IMMUNE SYSTEM OF THE GI TRACT

The complex immune system of the intestine can be divided into 2 broad categories: innate and adaptive immunity. Although the innate arm, as its name implies, is present from birth and capable of immediate protection at the local level, the adaptive immune system of the gut is initially naive and needs time to generate an appropriate response and memory. Although much of our focus is on the components of innate immunity in the gut, it is important to remember that this system does not work in isolation. The information it gathers communicates with the adaptive immune system, allowing the 2 to work in concert to provide optimal protection for the host. The innate defense system of the intestine can be broken down into 3 main components: the secreted mucus layer within the gut lumen, a single intestinal epithelial cell (IEC) layer, and the underlying lamina propria.

Mucus Layer

Large, highly glycosylated proteins called mucins are secreted by specialized goblet cells,[17,18] also known as mucin-secreting cells, and are the primary component of mucus. The mucus layer, which is present throughout the GI tract, provides protection, lubrication, and compartmentalization, minimizing contact between the epithelium and commensal bacteria. Mucins secreted by salivary glands coat food and assist with esophageal transit.[19] The mucus layer in the stomach plays a role in protecting the epithelium from the harsh acidic environment.[19] The gel-forming mucin, MUC2, is the most predominant mucin in both the small and large intestine.[20] There is 1 unattached layer of mucin in the small intestine, which acts as a physical and chemical barrier, preventing pathogenic bacteria from contacting the intestinal epithelia.[21] The colon has 2 distinct mucus layers, with the outer layer containing many bacteria and the inner layer being resistant to bacterial penetration.[22] Attached to the apical side of enterocytes in the small intestine is a separate, thin layer of mucus, made up of transmembrane mucins. This layer is commonly referred to as the glyco-calyx; it affords protection to the IECs by means of a physical barrier and plays a role in cellular signaling.[23] An abnormal mucus layer may lead to both acute and chronic intestinal diseases and has been shown to be associated with colitis in a murine model.[24]

Antimicrobial peptides (AMPs), a critical element in the chemical response of the innate immune system, are released into the mucus layer of the intestine. These small peptides (20–40 amino acids long) are secreted by the Paneth cell, a pyramidal columnar exocrine cell located at the base of the crypts of Lieberkühn, and can respond to a threat within a matter of

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hours. The continual release of AMPs by Paneth cells maintains the relatively sterile environment of the intestinal crypt, where the intestinal epithelial stem cells reside. When stimulated by inflammatory mediators, AMPs are also secreted into the lumen to help with mucosal defense.[23] They have microbicidal activity against a wide range of pathogens, including many gram-negative and gram-positive bacteria, fungi, protozoa, and viruses.[18] Paneth cell dysfunction has been shown to lead to decreased clearance of pathogenic *Escherichia coli*.[25] Several AMPs are present in the neonate's intestine. Some of the most important and abundant are α-defensin, β-defensin, lysozyme, and LL-37 (a member of the cathelicidin family).

IECs

The intestinal epithelial layer is made up of 4 different types of cells: absorptive enterocytes, hormone-secreting enteroendocrine cells, mucus-secreting goblet cells, and antimicrobial-secreting Paneth cells. These cells mature from a common pluripotent stem cell located in the base of the crypts. This single layer of highly polarized IECs sits below the mucus layer, creating a physical barrier that is anchored by junctional proteins. They are also responsible for sampling intraluminal contents, which instigates transcellular signaling and transcription of genes, resulting in a defense response via the release of cytokines and chemokines and subsequent attraction of leukocytes. This function is mediated by multiple pattern recognition receptors (PRRs), critical for the identification of foreign elements such as peptidoglycan, lipo-proteins, viral DNA, and commensal microflora. The remarkable ability of these receptors to distinguish between harmful and helpful bacteria with subsequent appropriate signaling is critical to intestinal homeostasis.[26] Toll-like receptors (TLRs) are the predominant type of PRR found on the apical side of IECs. Another group of PRRs that cooperate with TLRs are the intracellular NOD-like receptors (NLRs). NOD1 is expressed by IECs, and NOD2 is found in monocytes, dendritic cells, and Paneth cells.[23]

Tight junctions (TJs) regulate paracellular permeability and maintain separation of tissue compartments by sealing the intercellular space[27,28] and are an essential component of the epithelial barrier. A breakdown in the functioning of TJs and, subsequently, the intestinal immune barrier has been implicated in the pathogenesis of idiopathic inflammatory bowel disease,[29,30] infectious enteritis, and NEC.[31] Three types of proteins make up TJs: occludins, claudins, and junctional adhesion molecules. Although not much is known about the occludin proteins, it is known that the family of claudin proteins control the size, strength, and specificity of the ions that can pass through the epithelium.[23] In addition to TJs, adherens junctions are present on the lateral side of the epithelial cells and facilitate intercellular signaling.

Lamina Propria

A comprehensive review of the innate and adaptive immune functions occurring within the lamina propria is beyond the scope of this article. Further, the gut-associated lymphoid tissue include Peyer patches, isolated lymphoid follicles, and M cells, which are not discussed in this review. Intraepithelial T-cell lymphocytes have also recently been recognized as an important innate immune cell, which is critical for host-microbial homeostasis and protects the gut from injury.[32] The lamina propria contains many innate

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immune cells; the functions of these cells are being elucidated in ongoing animal and human studies. Among these cells are macrophages and dendritic cells; their roles include antigen uptake and transport, induction of T-cell differentiation, stimulation of immunoglobulin production (IgA), and tissue repair.[33] Macrophages and dendritic cells are also important for maintaining tolerance to the commensal microbiota.[33] In addition to different populations of T-cell and B-cell lymphocytes present in the lamina propria, innate lymphoid cell populations have recently been described, including natural killer cells, which are purported to play important roles in producing proinflammatory and regulatory cytokines.[34]

There certainly are other components of the innate immune system, which are not discussed here but are important. One simple example is the acidic and bacteriocidal environment of the stomach, which not only aids in digestion but also decreases the number of viable pathogens reaching the distal intestine. The disruption of this milieu can lead to disease. Multiple studies have revealed an association between the use of histamine 2 blockers, which inhibit gastric acid secretion, and both NEC and late-onset sepsis.[35–38]

INNATE IMMUNITY AND HUMAN BREAST MILK

Human infants are born with certain developmental immune deficiencies.[39] Phagocyte function and responses are immature and inadequate. Antibody production is limited and delayed, and serum IgA levels are far lower than adult levels. Both the classic and alternative pathways of the complement cascade have decreased performance. In addition to nutritive components, the ingestion of human breast milk delivers numerous antipathogenic and antiinflammatory bioactive factors[40] that provide passive protection to the neonate and stimulate maturation of host intestinal defenses. This factor is particularly relevant for premature infants, whose immune defenses are more immature than term neonates. The milk of mothers who give birth prematurely contains higher amounts of phagocytes and secretory immunoglobulin A (sIgA).[41–43] Breast milk is capable of directly modulating the development of the immune system,[44] as breastfed infants have been shown to have a reduced incidence of allergic disease[45] and autoimmune diseases such as Crohn disease[46] and insulin-dependent diabetes mellitus.[47] These collective properties make breast milk the gold standard for providing protective nutrients to the newborn.[48]

The Intestinal Microbial Environment

Colonization of the infant gut with more than 400 species of commensal bacteria lays the foundation for a healthy microbiome, which contributes to immune homeostasis, setting up a symbiotic relationship between colonizing bacteria and the underlying epithelial cells and lamina propria.[49,50] Barrier function, mucin and IgA secretion, inflammation, and homeostatic processes such as proliferation and apoptosis are influenced by these helpful bacteria.[51–56] Their effects on the intestinal immune system are believed to be largely mediated through TLRs present on IECs, which are able to distinguish between commensal bacteria and harmful pathogens.[57] Normal colonization begins at the time of birth with a vaginal delivery, when the infant is exposed to maternal vaginal and colonic bacteria. This process is followed by an exclusive diet of human milk, which contains factors that promote the growth of commensal bacteria. Distinct differences have been shown in the intestinal flora of breastfed and bottle-fed infants.[58]

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Oligosaccharides are nondigestable sugars found in breast milk and are believed to be responsible for promoting the growth of protective bacteria in the colon. They make up approximately 1% of the milk and 10% of the caloric content,[59] although the amount present varies diurnally and with duration of lactation and the infant's gestational age.[60] The presence of a nonnutritional substance at such high concentrations led to the hypothesis that glycans, including oligosaccharides, play a role in protection against disease. Because they are indigestible, oligosaccharides pass through the small intestine and enter the colon. Here, they produce short-chain fatty acids through fermentation, creating a favorable environment for the growth of probiotic bacterial species such as bifidobacteria and lactobacilli. This factor leads to a stable ecosystem in the intestine and augmentation of intestinal host defenses. The stimulation of sIgA-producing plasma cells in the intestine by these commensal bacteria is 1 such example of this symbiotic relationship.[61] Furthermore, glycans can inhibit binding of pathogens to the intestinal cell wall by acting as ligands, attaching to various bacteria, toxins, and viruses.[62]

sIgA

In the human adult, large amounts of sIgA are produced daily by plasma cells in the gut and transported into the intestinal lumen. This abundant antibody coats both harmful and commensal microorganisms, preventing colonization and penetration of the mucosal barrier, and it may even be able to inactivate certain viruses.[63] In the full-term newborn gut, plasma cells responsible for producing sIgA are absent for about 10 days after birth, and it takes up to 30 days postpartum for the neonatal intestine to produce levels of sIgA that are sufficient for protection.[64] To compensate for this deficiency, maternal milk contains large amounts of sIgA, which accounts for 90% of total immunoglobulins in milk. More than 50 years ago, it was discovered that there was up to 12 g/L of sIgA in human colostrum and 1 g/L in mature milk.[65] When secreted by the infant's gut, sIgA can be considered a part of the innate immune system, but when sIgA is ingested in mother's milk, it works through a unique system of immunity whereby the infant acquires protection from enteric pathogens to which the mother is exposed. First, within the mother's intestine, a novel enteric pathogen is presented to the dendritic cell. Next, activated T lymphocytes stimulate B lymphocytes, inducing the production of IgA by plasma cells at the basolateral side of the mammary epithelial cell. IgA is then transported across the epithelial cell attached to the polyimmunoglobulin receptor. On the apical side, the complex is cleaved, and dimeric sIgA is secreted into the milk, conferring immunity to the nursing infant.[66]

Selected Bioactive Proteins in Breast Milk with Antipathogenic Activity

Lactoferrin—This multifunctional, iron-binding glycoprotein possesses many anti-infective properties that act as part of the innate immune system and is present in mature human breast milk at concentrations of 1 to 3 g/L and in colostrum at 7 g/L.[67] It also occurs naturally in most exocrine fluids such as tears, saliva, bile, and pancreatic secretions. A recent study performed in very low birth weight infants showed that administration of bovine lactoferrin (LF), which is nearly homologous with human LF, either alone or in combination with *LGG*, can reduce the incidence of late-onset sepsis caused by bacteria and invasive fungal infections.[68,69] Antiviral properties have also been shown against a wide

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range of viruses, including human immunodeficiency virus, cytomegalovirus, herpes simplex virus, hepatitis B and C, adenovirus, and rotavirus.[70]

Many modes of action have been discovered by which LF acts to provide protection to the neonate, including its high affinity for iron, which may limit the amount of iron available to bacteria and other microorganisms. When LF is exposed to pepsin in the stomach, a potent antimicrobial agent is produced called lactoferricin, which is capable of killing a wide range of pathogens and, in particular, disrupts the cell membrane of gram-negative bacteria.[71] Another factor contained in breast milk, lysozyme, acts together with LF in the stomach to kill gram-negative bacteria.[72] Intact LF is passed into the small intestine and can bind to multiple receptors, including TLRs and CD14, blocking the adherence of pathogens to the intestinal epithelium.[73] Other beneficial actions of LF in the intestine include initiation of apoptosis in infected IECs,[74] promotion of growth of commensal bacteria,[75] stimulation of proliferation and differentiation of IECs,[76] and a reduction in inflammatory cytokine production through inhibition of nuclear factor κB activation in monocytes.[48,77] LF continues to be at the forefront in the fight against systemic infections and NEC in premature infants. There are multiple ongoing clinical trials studies looking at the effects of either bovine LF or human recombinant LF.

Lysozyme—This antibacterial enzyme is present in breast milk at relatively high concentrations. It can act alone to degrade bacteria by cleaving β,1-4 glycoside linkages in their cells walls.[78] As mentioned earlier, the activity of lysozyme can be increased through its relationship with LF. This expansion of its capabilities is accomplished when LF disrupts the outer membrane of gram-negative bacteria, such as *Salmonella typhimurium* and *E coli*. Lysozyme can then enter the bacteria and destroy it.[72]

Caseins—This family of highly glycosylated proteins makes up about 40% of the protein present in human milk and has immunologic activity in the newborn. β-casein is the predominant casein found in human milk. A synthetic peptide of β-casein has been shown to stimulate the expression of MUC2 genes and increase the numbers of goblet cells and Paneth cells in the small intestine of a rat pup model.[79] As discussed earlier, MUC2 is the most prevalent mucin in the mucus layer of the small intestine and provides protection through multiple mechanisms. κ-Casein is a minor casein subunit in breast milk.[80] It can act as a receptor analogue, preventing the attachment of bacteria to mucosal epithelium[81] and inhibit binding of *Helicobacter pylori* to human gastric mucosa in vitro.[82]

Cytokines and Chemokines Found in Human Milk

The gut of the newborn lacks the ability to respond appropriately to foreign pathogens and, more specifically, the capacity to produce a contained inflammatory response. There is a tendency toward excessive inflammatory signaling, as shown in immature IECs when exposed to inflammatory stimuli such as interleukin 1β (IL-1β), tumor necrosis factor α, and lipopolysaccharide, with an increased release of IL-8,[48,83] a chemokine known to stimulate neutrophil recruitment.[84] Cytokines are responsible for mediating, regulating, and modulating immune responses. Human breast milk contains a significant amount of this diverse group of signaling molecules, which help control the inflammatory response. For

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example, the antiinflammatory cytokine, IL-10, is present in breast milk[85] and believed to be critical for intestinal homeostasis and protection of the host. IL-10–deficient mice develop chronic enterocolitis,[86] and human infants with defects in the genes encoding IL-10 receptor subunit proteins have severe early-onset colitis.[87] With regard to NEC, IL-10 knockout mice have increased intestinal inflammation and increased apoptosis of IECs when exposed to hypoxia and formula feeding,[88] and the feeding of maternal milk in a rat model led to a reduction in the severity of NEC and increased intestinal IL-10.[89] Claud and colleagues[83] found that IL-10 and transforming growth factor β (TGF-β) both decreased IL-8 secretion by fetal human enterocytes in vitro.

The TGF-β family of immunoregulatory cytokines have been shown to be involved in wound healing, the inhibition of inflammation by decreasing the production of proinflammatory cytokines, and the regulation of lymphocytes, natural killer cells, dendritic cells, macrophages, and granulocytes.[90] Neonates have decreased expression of TGF-β,[91] but maternal milk supplies sufficient levels of the much-needed cytokine.[92] Exogenous supplementation can have a significant impact on the developing mucosal immune system, through its effects on oral tolerance and regulatory T cells. Infants breastfed by mothers with increased levels of TGF-β in breast milk have a decreased risk of wheezing and atopic dermatitis in childhood.[93,94] In direct relation to the innate immune system, TGF-β can also initiate local production of IgA in the gut, providing additional protection.[95]

Development and Repair of the GI Epithelium

With exposure to multiple factors in amniotic fluid and human breast milk, growth and differentiation of the intestinal epithelium peak shortly after birth. Epidermal growth factor (EGF) is a peptide that augments IEC proliferation and differentiation[96] and is secreted by multiple cells throughout the GI system into the intestinal lumen. EGF is supplied by amniotic fluid throughout pregnancy, whereas the infant in the postnatal period relies on the significant concentrations of EGF found in human milk and colostrum. Milk from mothers who have delivered an extremely premature infant contains 50% to 80% more EGF when compared with milk from mothers with full-term infants,[97] leading to speculation that EGF may be one of the reasons why human milk is protective against NEC.[98] Enteral administration of EGF resulted in a 50% reduction of NEC in a rat model.[99] More specifically, EGF has been associated with increased goblet cell density and MUC2 production in the ileum, and normalization in the expression of the intestinal epithelial TJ proteins, occluding and claudin, resulting in improved intestinal barrier function.[98,100] Another protein found in human milk that is capable of contributing to the development of the epithelium is LF. In addition to its antibacterial activity discussed earlier, experiments in human intestinal cell lines have shown that LF, which peaks in colostrum, induces cell proliferation at high concentrations and cell differentiation at low concentrations.[76]

Other Active Components in Breast Milk

Leukocytes—During early lactation, human milk contains large amounts of macrophages (up to 80% of total cells present), and an infant may consume up to 10^{10} maternal leukocytes per day.[16] Breast milk phagocytes, which are believed to be derived from maternal peripheral blood monocytes, possess unique functional features. One study showed

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that after phagocytosis of breast milk components, the phagocytes were capable of spontaneously producing granulocyte-macrophage colony-stimulating factor and differentiating into dendritic cells.[101] There is speculation that these cells possess many more functions that we do not yet know about.

Triglycerides—The fat or triglyceride found in human milk is a key constituent for infant nutrition and growth. It also has an additional function. When the triglyceride enters the stomach, it is digested by lingual and gastric lipases. This process releases free fatty acids and monoglycerides. These products act as a part of the innate immune system in the stomach and provide immediate protection to the newborn infant through their lytic effect on various viruses and some antibacterial and even antiprotozoal activity, specifically against *Giardia* (Table 1).[39,66,102]

SUMMARY

The neonatal intestine faces many changes, including adaptation from a sterile intra-uterine environment to one in which a diverse microbial population outnumbers human cells 10 to 1. To maintain homeostasis, it must protect the host from potential noxious and infectious stimuli and tolerate the diverse commensal microbes that colonize the entire gut. Furthermore, the gut must also perform important digestive and absorptive functions. Human breast milk contains many components that aid neonatal gut function and development. Understanding both neonatal gut immunity and how breast milk components influence its development and function are areas of active investigation. Future studies in this field are needed to develop targeted strategies to prevent and treat neonatal gut injury and infection, particularly in extremely low birth weight and premature infants.

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KEY POINTS

- Newborns infants are in a susceptible immunologic state after birth, with an immature adaptive immune system, making them reliant on their innate immune system for protection.
- The gastrointestinal innate immune system is comprised of many components. The acidic environment in the stomach and the mucus layer of the small intestine provide an initial barrier. The intestinal epithelial cells create a physical barrier and are involved in signaling to the underlying tissue. The lamina propria is rich in immune cells and contributes greatly to intestinal defense.
- In addition to providing optimal nutrition to infants, human breast milk has an abundance of bioactive factors that act as a part of the innate immune system of the gastrointestinal tract. Some factors have intrinsic properties that act as part of the defense system, whereas others enhance the ability of the gastrointestinal tract to defend the host.

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Table 1

Selected components present in human breast milk that act as part of the GI innate immune system

Component in Breast Milk	Action	Reference
Oligosaccharides (or prebiotics)	Promote growth of commensal bacteria Directly bind pathogenic bacteria and viruses	62
Secretory IgA	Coats harmful and commensal bacteria, preventing penetration of the epithelial barrier	63
LF	Binds iron within gut and limits its availability to microorganisms Produces lactoferricin when exposed to pepsin Binds receptors, interfering with pathogen binding to the epithelial barrier Stimulation of proliferation and differentiation of IECs Reduces inflammatory cytokine production Induces IEC proliferation and differentiation	48,71,73,76,77
Lysozyme	Degrades bacterial cell walls	78
Casein proteins	Increase numbers of goblet cells, Paneth cells, and expression of MUC2 genes Can act as receptor analogues	79,81
IL-10	Attenuates inflammation in the gut	83
TGF-β	Stimulates local production of sIgA in the gut Regulation of multiple types of immune cells	95
EGF	Increases goblet cell density and MUC2 production in ileum Normalizes expression of TJ proteins	98,100
Free fatty acids and monoglycerides	Antiviral, antibacterial, and antiprotozoal activity in stomach	39,66,102