CHAPTER 2

The Immunological Components of Human Milk

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Contents

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Abstract Breast-feeding is generally accepted as the optimal method of feeding infants. However, we have yet to fully understand the complex mixture of bioactive compounds contained in human milk. Epidemiological studies have indicated that breast-feeding is associated with health benefits in the infant for many immune-related conditions. Breast milk contains various antimicrobial substances, factors that promote immune development, constituents that promote tolerance/priming of the infant immune system, as well as anti-inflammatory components. This chapter identifies and discusses the immunological compounds in human milk and the available evidence for their effect on the immune system of the infant. Current feeding regimens recommended for infants are based primarily on the current understanding of the nutritional requirements of the neonate, but perhaps will be modified to reflect the consequences on immune function both immediate and later in life.

I. INTRODUCTION

In the nineteenth century, the first immunological molecules isolated from human milk were antibodies, giving rise to the notion that breast milk could have a positive influence on the infant's immunity (Bernt and Walker, 1999). One of the earliest studies reporting an association between breast-feeding and a lower incidence of morbidity and mortality during the first year of life involved 20,000 healthy infants born in the Massachusetts General Hospital in 1932 (Grulee *et al.*, 1935). Since that time, studies have consistently reported reduced disease incidence or severity, particularly infectious and gastrointestinal infections, in breast-fed infants compared to those who were not breast-fed (Arifeen *et al.*, 2001; Beaudry *et al.*, 1995; Duffy *et al.*, 1997; Duncan *et al.*, 1993; Feachem and Koblinsky, 1984; Howie *et al.*, 1990; Kramer *et al.*, 2001; Morrow *et al.*, 1999; Palti *et al.*, 1984; Wilson *et al.*, 1998; Yoon *et al.*, 1996).

The beneficial role of breast-feeding on immune function in infancy is well established and has contributed to the rationale for recommending exclusive breast-feeding (defined as the sole consumption of human milk, no other liquids or foods) from within an hour of birth (Morrow and Rangel, 2004) for the first 6 months of life for all healthy women and infants by associations such as the American Academy of Pediatrics,

Health Canada, and the World Health Organization (1997; Canadian Paediatric Society, Dietitians of Canada, & Health Canada, 2005; World Health Organization, 2001). Health organizations recommend that breast-feeding be continued while the infant consumes complementary foods during the first year of life as desired by mother and child (1997; Canadian Paediatric Society *et al.*, 2005; World Health Organization, 2001).

For a variety of reasons, including insufficient milk production, work/school or travel demands, convenience or perception of breast-feeding, or drugs/illness of mom or baby, some mothers choose to feed breast milk substitutes alone or in combination with breast milk (Lewallen *et al.*, 2006). Although there have been many modifications in the nutrient composition of infant formula aimed at improving immune function, such as fortifying with nucleotides (Hawkes *et al.*, 2006) or long-chain polyunsaturated fatty acids (LCPUFAs) (Field *et al.*, 2000), they have only begun to provide the immune benefits conferred by human milk.

Human milk is a synergistic package of essential nutrients and bioactive components. Epidemiological studies have demonstrated that consumption is associated with health benefits for many immune-related conditions (Table 2.1). Breast milk contains the nutrients necessary to support the development of the infant's immune system as well as other components that defend against infection. This includes various antimicrobial substances, factors that promote immune development, constituents that promote tolerance and the priming of the infant immune system, as well as anti-inflammatory components. The purpose of this chapter is to discuss the evidence for the immune benefits of human milk.

TABLE 2.1 Breast-feeding reduces the risk of developing various immune-related conditions

Condition	References
Necrotizing Enterocolitis	Lucas and Cole, 1990
Coeliac disease	Akobeng *et al.*, 2006
Type 1 diabetes	Malcova *et al.*, 2006
Type 2 diabetes	Owen *et al.*, 2006
Obesity	Arenz *et al.*, 2004
Atherosclerosis	Martin and Abraham, 2005
Breast cancer	Potischman and Troisi, 1999
Hodgkin's lymphoma	Davis, 1998
Rheumatoid arthritis	Karlson *et al.*, 2004
Eczema	Kull *et al.*, 2005
Allergy	van Odijk *et al.*, 2003
Respiratory tract Infection	Chantry *et al.*, 2006
Asthma	Kull *et al.*, 2004

II. EFFECT OF BREAST-FEEDING/HUMAN MILK ON INFECTIOUS DISEASES AND DEFENSES

A. Evidence for benefits on infectious diseases

Young infants, in both developed (Brandtzaeg, 2003) and developing countries (Bernt and Walker, 1999), are at high risk for gastrointestinal infections (especially diarrhea). Diarrheal diseases are a leading cause of morbidity and mortality in developing countries (Bernt and Walker, 1999; Morrow and Rangel, 2004). It accounts for 22% of all deaths in children under 5 years (Morrow and Rangel, 2004; Morrow *et al.*, 2005) and claims five million children per year or ~500 deaths per hour (Brandtzaeg, 2003). The protective effect of breast milk on diarrheal disease has been described in many countries around the world (Ahiadeke *et al.*, 2000; Arifeen *et al.*, 2001; Clemens *et al.*, 1999; Quigley *et al.*, 2006; Ruiz-Palacios *et al.*, 1990; Scariati *et al.*, 1997; Wang *et al.*, 2005; Yoon *et al.*, 1996). Breast-feeding is currently the most effective intervention for preventing morbidity and mortality caused by infectious disease in young children (Bernt and Walker, 1999; Kramer *et al.*, 2003; Morrow *et al.*, 2005; Newburg *et al.*, 2004). The protective effect of breast-feeding is enhanced by longer duration and exclusivity of breast-feeding (Kramer *et al.*, 2003). A WHO meta-analysis of 35 studies from 14 countries found an average four- to fivefold decrease in the incidence of diarrhea during the first 6 months of life among infants who were exclusively breast-fed compared with infants who were not breast-fed at all (Morrow and Rangel, 2004). Breast-feeding has been identified by public health agencies as a cost-effective strategy for reducing the burden of childhood diarrheal disease worldwide (Morrow and Rangel, 2004).

B. Antimicrobial components in human breast milk (Table 2.2)

1. Immunoglobulins

The intestinal mucosa is favored as portals of entry by most infectious agents and secretory IgA (sIgA) is the major immune defense in the intestine. Although the newborn infant produces very low quantities of immunoglobulins (Ig), breast milk is a rich source of sIgA with lesser amounts of sIgG and sIgM (Hanson and Korotkova, 2002; Koenig *et al.*, 2005; Leon-Sicairos *et al.*, 2006; Lonnerdal, 2003). The IgA in human milk is synthesized by resident B-cells in the mammary gland (Kolb, 2002) that have migrated from the mother's intestine (Hanson and Korotkova, 2002). IgA is synthesized as a dimer and joining chain and, similar to IgA in the intestine, is linked to a secretory component and secreted into breast milk via the enteromammary pathway (Lonnerdal, 2003; Morrow and Rangel, 2004). This packaging makes it relatively resistant to proteolytic enzymes

TABLE 2.2 Compounds in milk with antimicrobial properties

Immunoglobulins	Free secretory component
Lysozyme	Oligosaccharides
Lactoperoxidase	Fatty acids
Nucleotide-hydrolyzing antibodies	Maternal leukocytes and cytokines
κ-casein	sCD14
α-lactoglobulin	Complement and complement receptors
Haptocorrin	β-defensin-1
Mucins	Toll-like receptors
Lactadherin	Bifidus factor
Anti-secretory lectins	Prebiotics

in the infant's gut (Lonnerdal, 2003; Morrow and Rangel, 2004) enabling the neonate to orally acquire this immune defense from their mothers. sIgA together with lactoferrin comprises ~30% of protein in milk (Hanson *et al.*, 2003a). The concentration of sIgA is reported to be highest in colostrum with the concentration decreasing during the first month to remain stable throughout lactation (Hanson and Korotkova, 2002; Morrow and Rangel, 2004). It has been estimated that an exclusively breast-fed infant ingests daily 0.5–1.0 g of sIgA (Hanson *et al.*, 1994). (Table 2.2)

sIgA provides microbial defense in three ways: preventing bacteria and viruses from attaching to mucosal surfaces (immune exclusion), neutralizing microbial toxins, and increasing virus excretion (Van de Perre, 2003). Through all of the above processes, sIgA would prevent both the establishment of bacterial colonies in the intestine and/or translocation across the mucosal barrier, thereby preventing an inflammatory response that would be both energy costly and damaging to the infant. More recently, it has been reported that specific sIgA can also limit the inflammatory effects initiated by other antibody reactions (Jackson and Nazar, 2006), interfere with growth factors (e.g., iron) and enzymes necessary for pathogenic bacteria and parasites (Dickinson *et al.*, 1998), and facilitate mucosal immunity by promoting antigen uptake by M-cells in the gut-associated lymphoid tissue (GALT) (Mantis *et al.*, 2002).

sIgA antibodies have been identified in breast milk against bacterial pathogens such as *Escherichia coli* (Manjarrez-Hernandez *et al.*, 2000), *Vibrio cholerae*, *Campylobacter*, *Shigella*, *Giardia lamblia*, *Haemophilus influenzae*, and *Clostridium difficile* (Hanson and Korotkova, 2002; Lonnerdal, 2003; Manjarrez-Hernandez *et al.*, 2000); against viruses such as *Rotavirus*, *Cytomegalovirus*, HIV, *Influenza virus*, respiratory syncytial virus, and pneumococcus (Filteau, 2003; Lonnerdal, 2003); and against yeasts such

as *Candida albicans* (Filteau, 2003; Hanson *et al.*, 2003a; Lonnerdal, 2003). sIgA against maternal microflora and dietary proteins have also been identified in human milk (Hanson *et al.*, 2003a). Bacterial translocation of intestinal microflora in breast-fed infants is limited due to the presence of sIgA coating the bacteria (reviewed in Hanson and Yolken, 1999). This provides a potential mechanistic explanation for the reduced neonatal septicaemia associated with breast-feeding (Ashraf *et al.*, 1991; Bhutta and Yusuf, 1997; Winberg and Wessner, 1971).

It has been reported that IgG from the milk of healthy mothers has nucleotide-hydrolyzing activity that is not related to the activity of known enzymes (Semenov *et al.*, 2004). Human milk appears to contain a mixture of antibodies, some hydrolyzing ATP/AMP and others hydrolyzing only AMP (Semenov *et al.*, 2004). It has been suggested that these antibodies neutralize bacterial and viral DNA and RNA by forming complexes with them (Semenov *et al.*, 2004). Although these antibodies may play a protective role in breast milk and enhance the passive immunity of neonates, more research is needed to fully determine their biological role (Semenov *et al.*, 2004).

2. Lactoferrin and related peptides

Lactoferrin is the major whey protein present in breast milk (Teraguchi *et al.*, 1996) with many microbicidal properties (Leon-Sicairos *et al.*, 2006). The concentration of lactoferrin in milk has been reported as 1 g/liter in mature milk and 7 g/liter in colostrum (Houghton *et al.*, 1985). The concentration of lactoferrin in breast milk is controlled by the reproductive hormones prolactin and estrogen (Ward *et al.*, 2005). Lactoferrin has been demonstrated to resist digestion in the infant gut as it has been recovered intact from the stool of breast-fed infants (Bernt and Walker, 1999). Lactoferrin acts mainly in an iron-free state (apo-lactoferrin) and its microbicidal activity is reported to increase in proportion to its concentration in milk (Leon-Sicairos *et al.*, 2006).

Lactoferrin is a well-established, iron-binding protein (Bernt and Walker, 1999; Lonnerdal, 2003) that can bind two ferric ions at a time, and it is believed that its ability to withhold iron from pathogens explains a great part of its antimicrobial ability (Bernt and Walker, 1999; Jackson and Nazar, 2006; Lonnerdal, 2003; Morrow and Rangel, 2004; Ward *et al.*, 2005). The chelation of iron can also induce ''twitching'' by iron-requiring pathogens, which is a specific bacterial surface motility that prevents bacteria from forming biofilms (Singh *et al.*, 2002). These biofilms are encased surface communities that are resistant to host defense mechanisms and antibiotics (Singh *et al.*, 2002).

The demonstration that the bacteriostatic activity of lactoferrin can be independent of the level of iron saturation (Arnold *et al.*, 1980) suggests that lactoferrin has other antimicrobial mechanisms. Lactoferrin has been

reported to possess direct cytotoxic activity against bacteria, viruses, and fungi (Bernt and Walker, 1999; Gomez *et al.*, 2003). Bovine lactoferrin was reported to protect against *Rotavirus* by preventing virus attachment to intestinal cells and inhibiting a postabsorption step, possibly through the withholding of calcium, which is necessary for the formation of new viral particles (Seganti *et al.*, 2004). The glycans on lactoferrin have been demonstrated to also function as receptors for type 1 fimbrial lectin of *E. coli* (Teraguchi *et al.*, 1996).

It has been suggested that lactoferrin may only confer beneficial immune effects when consumed in the form of breast milk (Lonnerdal, 2003). When added to infant formula, lactoferrin may be affected by its prior bioactivity; how it was added (blended or dissolved); and extent of heat treatment of the formula (Lonnerdal, 2003). There is evidence that lactoferrin can be inactivated by invading pathogens or even enhance microbial pathogenicity. For example, the pneumococcal surface protein A of *Streptococcus pneumoniae* was reported to bind to lactoferrin and protect the bacteria from the killing action of lactoferrin (Ward *et al.*, 2005).

A portion of the bactericidal activity of lactoferrin appears to reside in its pepsin-breakdown products lactoferricin B and H (Clare *et al.*, 2003; Kolb, 2002). Lactoferricin has antibacterial properties against a wide range of gram-positive and gram-negative bacteria such as *Listeria*, *E. coli*, *Salmonella*, and *Campylobacter* (Shah, 2000), without inhibiting the growth of bifidobacteria (Leon-Sicairos *et al.*, 2006). Lactoferricin has been demonstrated to inhibit the attachment of bacteria (such as enteropathogenic *E. coli*) to intestinal cells (Zhang *et al.*, 1999) and have cytotoxic activity against viruses (Plaut *et al.*, 2000) and fungi (Andersson *et al.*, 2000). Specifically, lactoferricin B has been shown to disrupt liposomes, causing leakage and fusion of vesicles and enabling the peptide to cross the membrane barrier and make contact with cytoplasmic targets (Ulvatne *et al.*, 2001). This is supported by data from Haukland *et al.* (2001), who observed lactoferricin B in the cytoplasm of both *Staphylococcus aureus* and *E. coli*.

3. Lysozymes and other enzymes

Lysozyme is a major part of the whey protein fraction of human milk (Lonnerdal, 2003) and is found in highest concentration in colostrum (Leon-Sicairos *et al.*, 2006). Human milk contains ~3000 times the concentration of lysozyme as bovine milk and is reported to be more stable (Clare *et al.*, 2003). This protein is hypothesized to play a major role in immune defense of the infant (Hanson *et al.*, 2003a), likely acting in concert with lactoferrin, sIgA, and other antimicrobial compounds (Leon-Sicairos *et al.*, 2006). Lysozyme functions by hydrolyzing β-1,4 linkages of *N*-acetylmuramic acid and *N*-acetylglucosamine on the outer cell wall of gram-positive bacteria (Chipman and Sharon, 1969). It also

appears to function, at least *in vitro*, in a synergistic manner with lactoferrin to kill gram-negative bacteria (Ellison III and Giehl, 1991). Lactoferrin removes lipopolysaccharide (LPS) from the outer cell membrane so that lysozyme can enter and degrade the inner proteoglycan matrix of the membrane (Ellison III and Giehl, 1991). Lysozyme is also reported to have antiviral activity (Lee-Huang *et al.*, 1999).

Human milk contains active lactoperoxidase. The concentration of lactoperoxidase in colostrum and milk is about 11 $\pm$ 45 mg/ml and 13 $\pm$ 30 mg/ml, respectively (Korhonen, 1977). Lactoperoxidase, in association with thiocyanate and hydrogen peroxide, has been shown to inhibit growth of *Streptococcus cremoris* 972 (Steele and Morrison, 1969). This data was supported by Bjorck *et al.* (1975), who demonstrated that the antibacterial properties of lactoperoxidase are closely connected to the oxidation of thiocyanate, both *in vitro* and in milk. Since thiocyanate is found in human saliva, lactoperoxidase may therefore be an effective defense against bacteria found in the mouth and upper gastrointestinal tract (Lonnerdal, 2003).

4. Oligosaccharides

Oligosaccharides are a heterogeneous group of carbohydrates comprising the third most abundant constituent in milk (Dai *et al.*, 2000; Morrow and Rangel, 2004; Newburg, 2000). Like most components of breast milk, they are found in high concentration in colostrum (~22 g/liter) and are less abundant (~12 g/liter) in mature milk (Erney *et al.*, 2000). The types of oligosaccharide present in milk also change during lactation (Newburg, 2000) and the concentration tends to decrease within a feeding (Thurl *et al.*, 1993). Many of these oligosaccharides occur in free form, while others are linked to glycoproteins [e.g., lactoferrin, κ-casein, and sIgA (Kelleher and Lonnerdal, 2001)] or lactose (Newburg, 2000; Shah, 2000). Oligosaccharides are produced by an antigen-independent mechanism in epithelial cells in the mammary gland (Dai *et al.*, 2000). The amount produced by these cells is determined in part by genetics (Newburg, 2000).

Oligosaccharides have been shown *in vitro* to resist hydrolysis by gastrointestinal enzymes (Engfer *et al.*, 2000), indicating that they would be present and remain intact in the small intestine. Due to homology of cell surface receptors for common environmental pathogens and microbes present on the mucosal epithelium, including *E. coli*, *Campylobacter jejuni*, and *S. pneumoniae* (Shah, 2000), it has been shown that that milk-derived oligosaccharides can bind pathogens, preventing attachment and thereby protecting the infant from infection (Holmgren *et al.*, 1983). Recently, Coppa *et al.* (2006) tested various fractions of oligosaccharides *in vitro* and showed that acidity and molecular weight of oligosaccharides appear to determine their efficacy against specific pathogens. Specifically, the action against *Salmonella fyris* was due to acid and neutral low-molecular

weight oligosaccharides, while *V. cholerae* was inhibited primarily by neutral high-molecular weight oligosaccharides (Coppa *et al.*, 2006). Coppa *et al.* (2006) conclude that the protective effect of oligosaccharides stems from a joint action of various compounds that act on multiple different bonds. The prebiotic properties of oligosaccharides will be discussed in detail in a later section.

5. Fats and fatty acids

The fat content of breast milk is 3–4 g/liter in colostrum, transitional, and mature milk, with 93–97% of the lipids in the form of triglycerides (reviewed by German and Dillard, 2006). In breast milk, the milk fat globule is comprised of a triglyceride core bound by a single layer of lipids and further surrounded by a bilayer membrane made up of lipids and proteins, including cholesterol, phosphatidylcholine, and sphingomyelin (German and Dillard, 2006). Milk fats, in addition to their nutritive and developmental benefits, have been demonstrated to provide antimicrobial activity in infant gut (German and Dillard, 2006).

The lipid products of gastric and intestinal lipolysis (primarily medium-chain saturated, long-chain unsaturated fatty acids, and monoglycerides) have been demonstrated to have antiviral, antiprotozoan, and antibacterial properties (German and Dillard, 2006; Isaacs, 2001; Thormar *et al.*, 1987; Welsh *et al.*, 1978). Lauric acid has been described as one of the most antimicrobial fatty acids in milk (reviewed by Hamosh *et al.*, 1999). Caprylic acid (eight carbons in chain length) has been demonstrated *in vitro* to reduce *S. aureus* and *E. coli* in milk (Nair *et al.*, 2005) and the infectivity of *Chlamydia trachomatis* was reduced *in vitro* by incubation with lauric acid (12:0) and caprylic acid (10:0) (Bergsson *et al.*, 1998). In contrast, the triglyceride and diglyceride fractions appear to not have antimicrobial activity (Welsh *et al.*, 1978). Data from Isaacs *et al.* (1995) suggests that monoglycerides with a fatty acid chain of <12 carbons exhibit more antiviral activity than the same free fatty acid. Their data also suggests that medium-chain monoglycerides are more effective against viruses than monoglycerides containing a long-chain (>12 carbons in chain length) fatty acid (Isaacs *et al.*, 1995). Others have identified monocaprylin as a potent antibacterial agent *in vitro* (Bergsson *et al.*, 1998; Nair *et al.*, 2005).

The mechanism for the antimicrobial effects of fatty acids and monoglycerides has not been established but it has been suggested that free fatty acids damage bacteria by disrupting their cell membranes (Bergsson *et al.*, 1998; Hamosh *et al.*, 1999; Thormar *et al.*, 1987) or by changing intracellular pH (Sun *et al.*, 1998). Passive protection might also be provided by components in the milk fat globules (i.e., mucins), which could prevent attachment of pathogens in the infant's stomach (reviewed by Filteau, 2000) and small intestine (Schroten *et al.*, 1992).

6. Components of the maternal innate and acquired immune system

Active, as opposed to only passive, protection against microbial may also be provided by human milk. Theoretically, the leukocytes and cytokines in milk (discussed in detail in a later section) could directly contribute to the effective host defense in the infant's intestine. In support of this, studies have demonstrated phagocytosis of bacteria by leukocytes from breast milk (Williamson and Murti, 1996). Mature milk is reported to contain a soluble form of CD14 (sCD14). sCD14 is a glycoprotein that is part of the LPS recognition complex that binds microbial and other bacterial wall components and is reported to be produced by mammary epithelial cells in concentrations 20-fold higher than serum (Labeta *et al.*, 2000). Recent studies suggest that sCD14 could also act as a ''sentinel'' molecule in the microbial defense of the neonatal intestine (Vidal *et al.*, 2001).

Several complement components (C3 and C4), receptors (CF2, CD21), and activation fragments are found in human milk (reviewed in Ogundele, 2001). It has been suggested that these participate in immune bacteriolysis, neutralization of viruses, immune adherence, immunoconglutination, and enhance phagocytosis in the infant's intestine (Brandtzaeg, 2003; Ogundele, 2001). Human β-defensin-1 (detected in breast milk in concentrations of ~1–10 μg/ml) has been demonstrated to have antimicrobial activity against *E. coli*, but could also be involved in upregulating the adaptive immune system in the gut (Jia *et al.*, 2001). Toll-like receptors play a crucial ''sensing'' role in the early innate immune response to invading pathogens. Although the exact role of the soluble form of the toll-like receptor 2 (TLR2), recently reported in breast milk, is not known, it might be postulated to play a role in protection from microbial infection (LeBouder *et al.*, 2003).

7. Other non-antibody protein defense agents in milk

Many microorganisms express surface molecules (adhesions or lectins), which specifically recognize carbohydrate residues on the epithelial cells. Many of these compounds are believed to act like oligosaccharides by successfully competing with pathogens for the binding sites on the epithelial surfaces of the intestine (Gopal and Gill, 2000). The candidates include lactadherin (Kvistgaard *et al.*, 2004), free secretatory component (de Araujo and Giugliano, 2001), mucins (Peterson *et al.*, 1998; Schroten, 2001), and antisecretory lectins (Hanson, 1998).

Lactadherin is a glycoprotein produced by mammary epithelial cells during lactation and associated with the milk fat globule membrane (Newburg *et al.*, 1998). Its concentration in milk peaks at 0.139 mg/ml immediately postpartum and declines thereafter (reviewed by Kvistgaard *et al.*, 2004). It has been reported to bind to human *Rotavirus*,

thus preventing viral attachment to host cell receptors (Kvistgaard *et al.*, 2004) and its concentration has been inversely associated with *Rotavirus* symptoms in infected breast-fed infants (Newburg *et al.*, 1998). Interestingly, bovine lactadherin was not found to possess antiviral properties (Kvistgaard *et al.*, 2004).

Free secretatory component is abundant in breast milk and may on its own block epithelial adhesion of, and thereby limit infection by enterotoxigenic *E. coli* (de Araujo and Giugliano, 2001), *Salmonella typhimurium* (Bessler *et al.*, 2006), *C. difficile* toxin A (Dallas and Rolfe, 1998), and pneumococcus (Hammerschmidt *et al.*, 1997).

Additionally, several hydrolysis products of κ-casein (specifically glycomacropeptide) isolated from milk have been demonstrated to possess broad antimicrobial activity against several gram-positive and gram-negative bacteria and yeast (reviewed by Kelleher and Lonnerdal, 2001; Liepke *et al.*, 2001). Similarly, some of the polypeptide fractions of α-lactalbumin (LTD1 and LTD2, and LDC) have been demonstrated to have antimicrobial activity against *E. coli*, *Klebsiella pneumoniae*, *S. aureus*, *Staphylococcus epidermis*, *Streptococci*, and *C. albicans* (Pellegrini *et al.*, 1999). Treatment of human milk with pepsin did not yield antibacterial peptides from α-lactalbumin (Pellegrini *et al.*, 1999).

The protein haptocorrin has been shown *in vitro* to resist digestion by proteolytic enzymes (Adkins and Lonnerdal, 2003). Haptocorrin has been reported to inhibit the growth of enteropathogenic *E. coli*, possibly by sequestering vitamin B12 that is required for microbial growth (Adkins and Lonnerdal, 2003).

8. Compounds that promote "healthy" intestinal microflora

The microflora in the gut of infants affects their capacity to resist infection. It is now well documented that the colon of breast-fed infants contains fewer potentially pathogenic bacteria such as *E. coli*, *Bacteroides*, *Campylobacter*, and *Streptococcus*, and more beneficial bacteria such as *Lactobacillus* and *Bifidobacterium*, as compared to formula-fed infants (Kleessen *et al.*, 1995). In addition to the antimicrobial components in human milk that inhibit the growth of pathogenic bacteria, there are bifidus factors (Kunz *et al.*, 1999), prebiotics (Kunz *et al.*, 1999), and compounds with prebiotic activity [i.e., proteins (Lonnerdal, 2003)] that nourish and stimulate the growth of beneficial bacteria. Bifidobacteria produce several acids, including acetic, lactic, and pyruvic (Shah, 2000). Their antimicrobial effects were traditionally thought to stem from their lowering of pH to a range unacceptable for bacterial growth; however, more recent studies have reported that bifidobacteria do not directly produce butyric acid but indirectly stimulate the production of butyric acid through promoting the growth of butyric acid-producing bacteria such as *Roseburia* spp. (Duncan *et al.*, 2004).

Bifidogenic peptides from lactoferrin including hLACFR-Ia, hLACFR-Ib, hLACFR-Ic, hLACFR-IIa, and hLACFR-IIb; hPIGR-Ia and hPIGR-Ib; and fragments of the polyimmunoglobulin receptor hPIGR have been isolated from human milk (Liepke *et al.*, 2002). These peptides were demonstrated to promote the growth of several strains of bifidobacteria that usually colonize infants' intestines and may be more effective than most currently known bifidogenic carbohydrates (Liepke *et al.*, 2002). More specifically lactoferricin has been found to be structurally related to certain bifidogenic peptides and is hypothesized to participate in the colonizing newborn infants' bacterial flora (Liepke *et al.*, 2002). Several oligosaccharides (specifically galacto- and fructo-oligosaccharides) with prebiotic properties have been shown to promote colonization with bifidobacteria when added to infant formula (Boehm *et al.*, 2004). By both providing a metabolic substrate for these bacteria and preventing the attachment of harmful bacteria, oligosaccharides promote growth of beneficial bacteria in the infant intestine (Dai *et al.*, 2000; Morrow and Rangel, 2004; Morrow *et al.*, 2005). Probifidogenic properties have also been found for *N*-acetyl-glucosamine in milk (reviewed in Kleessen *et al.*, 1995). The secretatory component of IgA may also have some prebiotic properties (Liepke *et al.*, 2002). It has also been hypothesized that the LCPUFAs in human milk facilitate the adhesion of probiotics to mucosal surface (Das, 2002). Studies have also identified a potential role for sCD14 during bacterial colonization of the gut (Labeta *et al.*, 2000; Vidal *et al.*, 2001).

III. EFFECT OF BREAST MILK ON IMMUNE DEVELOPMENT

A. Immune development in the infant

The neonatal period is particularly critical as the newborn is exposed to a large number of microorganisms, proteins, and chemicals that they have not previously encountered. Ultimately, resistance to infection relies on a balance between innate and adaptive (antigen driven) immunity. At birth, the cellular components of the immune system are in place, both mucosal and systemic antibody responses can be detected, and most infants can respond appropriately to immunization (Kelly and Coutts, 2000). However, the immune system of the infant is not the same as the adult, but whether one can define the immune system of the infant as ''immature'' or ''immunosuppressed'' in a strict definition is debatable. As little antigen exposure occurs *in utero*, the infant is born with an acquired immune system that is antigen naive [lower level expression of costimulatory molecules such as CD40L and require a greater stimuli for activation (Kelly and Coutts, 2000)]. The limitation of T-cells being able to mount the appropriate immune responses may also relate to deficiencies in antigen presentation by B- and other antigen presenting cells (Kelly and Coutts, 2000).

As a result of the lower responses by T- and B-cells, during the neonatal period, cells of the innate immune system, predominantly macrophages, neutrophils, and natural killer cells, become responsible for the clearance of foreign antigen. However, neonates have been reported to mount a less-efficient mononuclear phagocytic response to numerous pathogens (Marodi, 2006). When the T-cells respond, the neonate displays preferential T-helper 2 (Th2) type cytokine response which is antibody dominant as opposed to a cell-mediated cytokine-mediated T-helper 1 (Th1) response (Cummins and Thompson, 1997). This naivety and altered response by the adaptive arm of the immune system likely contributes to the lower immune competence in the infant (Hanson *et al.*, 2003b). From an immunological standpoint, this naivety translates to the infant's immune cells requiring ''education'' in the period immediately after birth. Postnatal maturation of the immune system is characterized by the development of a balanced Th1/Th2 response (Bjorksten, 1999).

The infant's intestinal immune system develops rapidly in the early postnatal period as it comes into contact with dietary and microbial antigens in the gut (Brandtzaeg, 2003; Rautava *et al.*, 2002). In addition to eliminating infectious agents and minimizing the damage they cause, the infant's immune system also has to process harmless antigens and has evolved intricate mechanisms to discriminate between antigens with the potential to cause damage and those without (tolerance). Induction of tolerance is believed to occur primarily in the gut and contributed to by unique features of GALT, including specialized B- and T-cells, the production of IgA, and the Th2 response (Mowat, 2003; Strobel, 2001). Failure to regulate tolerance and active immune responses (education of the immune system) can lead to diseases such as food-related allergy, autoimmunity, and inflammatory bowel disorders (Kelly and Coutts, 2000). Human milk contains its own immune system and a wide range of soluble and cellular factors (Table 2.3), which most recently have been demonstrated to produce long-term benefits to the infant by facilitating immune development and maturation (Field, 2005).

TABLE 2.3 Compounds found in human milk that could influence immune development

Maternal immune cells	Long-chain polyunsaturated fatty acids
Macrophages	Other immune components
Neutrophils	Chemokines
T-cells	Other soluble factors
B-cells and their immunoglobulins	Compounds that promote microbiological colonization of the infant's colon
Cytokines	Hormones and bioactive peptides
Nucleotides	

B. Immune cells present in human milk

Depending on the phase and stage of lactation, a variety of leukocytes are present in colostrum ($\sim 4 \times 10^9$/liter) and mature milk ($\sim 10^8$–10^9/liter) (Goldman, 1993; Michie, 1998). Over the course of a single day, it is estimated that a breast-fed baby consumes on average 10^8 immune cells (Michie, 1998). Macrophages (55–60%) and neutrophils (30–40%) dominate over lymphocytes (5–10%) (Goldman, 1993). All of the aforementioned cell types are present in an activated phenotype when found in breast milk (Michie, 1998). It has been suggested that their presence in milk is due to transepithelial migration of leukocytes into mammary tissue that are stimulated by specific chemoattractants in the mammary gland (Michie *et al.*, 1998). Viable leukocytes from milk have been isolated in feces from infants fed human milk (Michie, 1998); thus, it is possible that key surface molecules on these cells remain antigenically intact in the gut. From the following discussion, it can be concluded that the immune cells present in mother's milk play a pivotal role in bridging the gap between birth and the child's development of a fully functional immune system.

1. Macrophages

The macrophages isolated from breast milk express activation markers (i.e., CD11c, Leu-M3, and Leu-M5) (Rivas *et al.*, 1994) are phagocytic, and secrete immunoregulatory factors (reviewed by Field, 2005). Oral administration of macrophages to newborn mice survived for several hours in the gut and some were found to cross the mucosal barrier to reach peripheral lymphoid organs (Hughes *et al.*, 1988). Freshly isolated macrophages from breast milk were recently reported to differentiate into dendritic cells after incubation with IL-4 (Ichikawa *et al.*, 2003). These IL-4-stimulated macrophages were found to be efficient stimulators of T-cells, suggesting their potential role in mediating T-cell-dependent immune responses in the infant (Ichikawa *et al.*, 2003).

2. Neutrophils

Breast milk neutrophils are also present in an activated form, as evidenced by increased levels of CD11b/CD18 and lower expression of L-selectin (Goldman *et al.*, 1998). However, these neutrophils may have a limited functional capacity once secreted into milk as they demonstrate lower adherence, polarity, and motility when in the activated state (Thorpe *et al.*, 1986). Little is known about the impact of milk neutrophils on immune development in the infant, but most researchers suggest that the main role is maternal protection as they have limited functional capacity once they are secreted into milk.

3. T-Cells

The majority of lymphocytes in milk are T-cells (>80%; Wirt *et al.*, 1992). Human milk contains both T-cells and the compounds responsible for activating them (Wirt *et al.*, 1992). Breast milk T-cells differ both in relative abundance and in quality from those found in the peripheral blood compartment (Sabbaj *et al.*, 2005). The higher proportion of $TCR\gamma\delta^{+}CD8^{+}$ (expressing L-selectin, $\alpha4\beta7$ integrin, mucosal addressin cell adhesion molecule-1) as compared to blood suggests that these cytotoxic T-cells ($CD8^{+}$) have selectively homed from the maternal mucosal immune system to the mammary gland (Sabbaj *et al.*, 2005; Wirt *et al.*, 1992). Breast milk $CD4^{+}$ cells are also present in an activated state (expressing activation markers CD40L, sCD30, CD62L, CCR7, IL-2 receptor, human mucosa lymphocyte antigen-1, or late activation protein-1) and express $CD45RO^{+}$, a surface protein associated with immunological memory (Bertotto *et al.*, 1997; Eglinton *et al.*, 1994; Sabbaj *et al.*, 2005).

It has been hypothesized that activated T-cells from maternal origin might compensate for the immature function of neonatal T-cells and promote their maturation. Additionally, activated antigen mature lymphocytes might help compensate for the low antigen presenting capacity of macrophages. In animal models, milk-derived lymphocytes can traverse the neonatal intestine, suggesting that their influence extends beyond the intestine (Jain *et al.*, 1989). Some recent studies have shown that immunophenotypic differences in systemic lymphocyte populations occur following exposure to maternal milk. These differences include a decrease in $CD4^{+}$:$CD8^{+}$ cells, an increase in natural killer cells and an increase in IFNγ production (Hawkes *et al.*, 1999). The functional consequences of a report that breast-fed infants have a thymus twice the size of that of non-breast-fed infants (Hasselbalch *et al.*, 1999) has yet to be explained but supports the role of human milk on T-cell development.

4. B-cells and immunoglobulins

The B-cells account for less than 20% of all lymphocytes in breast milk (reviewed by Field, 2005). IgA, IgG, and IgM are all present in human breast milk (Koenig *et al.*, 2005). Little is known about the potential role of milk B-cells on immune development in the infant but one might hypothesize that these cells could influence the infant's immune system.

C. Cytokines

Cytokines are multifunctional glycoproteins involved in cell communication and immune system activation (Ustundag *et al.*, 2005). Human milk contains an array of cytokines, some in concentrations that could potentially influence immune function. This list includes IL-1β (Grosvenor *et al.*, 1993b; Hawkes *et al.*, 2002c; Ustundag *et al.*, 2005), IL-2 (Bryan *et al.*, 2006;

Ustundag *et al.*, 2005), IL-4 (Bottcher *et al.*, 2000b, 2003), IL-5 (Bottcher *et al.*, 2000b), IL-6 (Bottcher *et al.*, 2000b; Hawkes *et al.*, 2002; Ustundag *et al.*, 2005), IL-8 (Bottcher *et al.*, 2000a, 2003; Grosvenor *et al.*, 1993; Michie *et al.*, 1998; Ustundag *et al.*, 2005), IL-10 (Bottcher *et al.*, 2000b; Garofalo *et al.*, 1995), IL-12 (Bryan *et al.*, 1999), IL-13 (Bottcher *et al.*, 2000b; Bryan *et al.*, 1999), IL-16 (Bottcher *et al.*, 2003), IL-18 (Takahata *et al.*, 2001), TNF-α (Hawkes *et al.*, 2002; Ustundag *et al.*, 2005), TGF-β (Bottcher *et al.*, 2000b; Hawkes *et al.*, 2001), and IFNγ (Bottcher *et al.*, 2000b). The exact source of the cytokines present in the aqueous fraction of breast milk remains to be determined, though it is suspected that the primary source is cells present in the mammary gland (Bryan *et al.*, 2006). However, leukocytes recovered from expressed human milk have been shown to be capable of producing a number of cytokines (Hawkes *et al.*, 2002). The extent to which cytokines survive passage through the infant stomach is largely unknown, but recent work has suggested that some cytokines may be sequestered and protected until they reach the intestine (Calhoun *et al.*, 1999). Although particular cytokines can be in high concentrations in some women's breast milk, in general, the concentrations of cytokines vary widely, change through lactation, and are influenced by the mother's health, making it difficult to assess their roles (individually or together) in the development of the infant's immune system (Erbagci *et al.*, 2005; Ustundag *et al.*, 2005).

The intake of cytokines, through human milk, has the potential to influence the maturation and development of immune cells in the infant. Neonates have a limited ability to produce many of the cytokines found in human milk (Field *et al.*, 2001). For example, maternal cytokines (TGF-β, IL-6, and IL-10) in milk could contribute to the development and differentiation of IgA-producing cells (Bottcher *et al.*, 2000b) and maturation of the naive intestinal immune system (Donnet-Hughes *et al.*, 2000). For example, TGF-β is present in milk and has been proposed to stimulate appropriate maturation of naive infant intestinal immune system (Hanson *et al.*, 2003b). Maternal cytokines (TGF-β, IL-6, and IL-10) in milk are believed to contribute to the development and differentiation of the infant's IgA-producing cells (Bottcher *et al.*, 2003; Kalliomaki *et al.*, 1999; Mowat, 2003) and IL-6 is hypothesized to enhance the development of the infant's mucosal immunity (Brandtzaeg, 2003). Additionally, the cytokines present in milk may assist both the transport of maternal leukocytes into milk (Ustundag *et al.*, 2005) and across the infants gut epithelium (Michie, 1998). Unfortunately, most of the research on milk cytokine activities has been conducted *in vitro* and there are many factors in breast milk that could either facilitate or inhibit cytokine activities (i.e., adhesion molecules and soluble receptors and antagonist receptors for cytokines (reviewed by Filteau, 2001) that are not accounted for in these studies.

D. Hormones and bioactive peptides

Many hormones, growth factors, and partially digested milk peptides have been detected in human milk, including cortisol, sex hormones (estrogen, progesterone, and derivatives), thyroid hormones (Buts, 1998), parathyroid hormone-related peptiden (Escande *et al.*, 2001), adrenocorticotropic hormones, gastrointestinal regulatory peptides (gastrin, GIP, GRP, neurotensin, peptide YY, somatostatin, substance P, VIP, bombesin; Berseth *et al.*, 1990), erythropoietin, gonadotropin, human-chorionic gonadotropin, insulin, leptin (Ilcol *et al.*, 2006), adiponectin (Martin *et al.*, 2006), hypothalamus-related hormones (GnRH, GHRH, GH, prolactin, TRH, TSH; Groer, 2005) (Buts, 1998), prolactin and procalcitonin, growth factors (EGF, IGF-1, IGF-II, NGF; Buts, 1998; Hirai *et al.*, 2002) and their binding proteins, α-lactalbumin, β-lactoglobulin, and lactoferrin (LF) (reviewed by Aggett *et al.*, 2003; Groer, 2005; Lonnerdal, 2003). Although little is known about the activity of these compounds on the naive immune system of an infant when delivered orally, one would suspect that they impact immune development in some synergistic manner.

E. Nucleotides

Nucleotides are present in human milk and encompass ~2–5% the non-proteinaceous nitrogen present in breast milk (Buts, 1998). Dietary nucleotides are reported to benefit the systemic immune system by promoting lymphocyte proliferation, NK activity, macrophage activation, and by producing a variety of other immunomodulatory factors (reviewed by Aggett *et al.*, 2003; Buts, 1998). Feeding nucleotide-supplemented formula to full- and preterm infants improved responses to immunizations, promoted T-cell maturation, and reduced the risk of diarrheal disease (reviewed by Aggett *et al.*, 2003). Although the mechanisms remain somewhat unclear, animal studies suggest that dietary nucleotides promote a Th1 response and modulate maturation and differentiation of T- and B-cells (Aggett *et al.*, 2003). The immune benefits of nucleotides in milk are somewhat debatable as a recent study failed to demonstrate that nucleotide supplementation (5 mg/100 kcal) to formula fed infants had any clinical advantageous to immune development in healthy term infants (Hawkes *et al.*, 2006).

F. Long-chain polyunsaturated fatty acids (LCP)

It is well established that dietary (n-6) and (n-3) LCP modulate Th1 and Th2 immune cell responses generation in the adult (Calder and Grimble, 2002). Docosahexaenoic acid (DHA) and arachidonic acid (AA) constitute a relatively small fraction of the total fatty acids in human breast milk, but have recently been suggested to participate in immune development

(Field *et al.*, 2001). Adding the LCP AA and DHA to preterm formula resulted in lymphocyte populations and cytokines more similar to human milk-fed infants than to infants who received unsupplemented formula (Field *et al.*, 2001).

G. Other immune components

Human milk is also reported to contain chemokines and other soluble immune factors, including granulocyte-colony stimulating factor (Calhoun *et al.*, 1999), monocyte-chemotactic protein 1 (Eglinton *et al.*, 1994), sFas ligand (Takahata *et al.*, 2001), and RANTES (Bottcher *et al.*, 2000a, 2003; Michie *et al.*, 1998). If any of these compounds were to come in contact with the infant's naive immune system, they could impact immune development. For example, granulocyte-colony stimulating factor is important for the growth and differentiation of neutrophils (Bell, 2006). Monocyte-chemotactic protein 1 can act as chemoattractant for Th1 cells (Shiratsuchi *et al.*, 2007), RANTES play a role in T-cell activation and differentiation (Takahata *et al.*, 2001) and serve as a chemoattractant for basophils and eosinophils and induces chemotaxis (Bottcher *et al.*, 2000b).

Osteoprotegerin (OPG) is found in mammary gland epithelial cells and in human milk at concentrations that are up to 1000 times higher than that found in human serum (Vidal *et al.*, 2004b). The gavage experiments of Vidal and colleagues in neonatal rats suggest that milk OPG survives gastrointestinal passage, crosses the intestinal epithelium, and can enter the pup's circulation (Vidal *et al.*, 2004a). OPG can bind to TNF-related apoptosis-inducing ligand (TRAIL) and induce caspase-dependent apoptosis of primarily Th1 cells (Vidal *et al.*, 2004a), which is hypothesized to be important in regulating Th1/Th2 balance in the infant's developing immune system (Vidal *et al.*, 2004a).

The concentration of sCD14 is 100–1000 times higher in breast milk than in serum (Snijders *et al.*, 2006). sCD14 can act as an acute phase protein and may also regulate T-cell activation (Snijders *et al.*, 2006). *In vitro* sCD14 is able to stimulate B-cell growth and differentiation (Filipp *et al.*, 2001). The presence of sCD14 in breast milk may serve to activate B-cells of the neonatal innate immune system before the infant acquires a full T-cell repertoire to respond to immunocompromising situations (Filipp *et al.*, 2001).

H. Compounds that promote microbiological colonization of the infant's colon

A major factor in the development of mucosal immunity in the infant is exposure to the microbial flora colonizing the gut (Brandtzaeg, 1996). Unlike pathogens which induce strong activation of immune defense

mechanisms, bacterial antigens from the indigenous microflora, enhanced by breast-feeding (see earlier section), have the potential to accelerate development of the infant's own mucosal barrier function and alter maturation of the infants' systemic immune system (Boehm *et al.*, 2004; Filteau, 2000; Forchielli and Walker, 2005). This is illustrated in studies of germ-free animals, which after intestinal colonization rapidly expand their immune systems (reviewed by Hanson *et al.*, 2003b). Bacterial colonization of the colon has also been reported to be essential in establishing a balance between Th1 and Th2 immune responses (Furrie, 2005). The mechanisms by which microbes influence the phenotype and function of lymphoid cells associated with the GALT are not known, but have been hypothesized to be complex and involve microbial antigen uptake and processing (Kelly and Coutts, 2000).

IV. TOLERANCE

Infancy is a time where there is a fine balance between an antigen response that results in tolerance (suppression) and one that results in sensitization (priming). The direction of the response is influenced by the nature, dose, and frequency of exposure of the antigen, all of which can be influenced by the maternal diet, as well as the age, genetic polymorphisms, and immunological status of the infant (Mayer and Shao, 2004). Breast-feeding is thought to promote oral tolerance in the infant (van Odijk *et al.*, 2003), and exclusive breast-feeding may even prevent or delay the onset of atopic illness such as allergies (Bernt and Walker, 1999). This phenomenon is particularly noticeable in infants with atopic heredity (i.e., infants whose parents suffer from atopic illness such as asthma or allergies) (van Odijk *et al.*, 2003). The relationship between breast-feeding and allergic disease is reviewed in van Odijk *et al.* (2003).

As much as 5–8% of the population in industrialized nations suffers from gastrointestinal allergies (Bernt and Walker, 1999). Food allergies may originate as a result of a failure to effectively develop tolerance (Korotkova *et al.*, 2004). Dietary proteins that have been detected in breast milk include ovalbumin (egg), β-lactoglobulin (cow's milk), gliadin (wheat), and Ara h1/Ara h2 (peanuts) (reviewed in Palmer and Makrides, 2006). Although it is well established that dietary antigens/potential allergens are present in human milk, the consequences on the infant's immune system are not clear. Animal studies have shown that tolerance to food proteins can be transferred to an offspring via maternal milk (Korotkova *et al.*, 2004). The allergic response (high IgE production) in young rats has been suppressed by administering milk from immunized dams (Roberts and Turner, 1983). It is hypothesized that breast milk promotes tolerance to dietary and microflora via the immunosuppressive

cytokines (i.e., IL-10 and TGF-β) and antigens present in breast milk (Brandtzaeg, 2003).

A. Compounds found in breast milk that may be involved in the induction of tolerance

1. TGF-β and IL-10

Tolerization is an active process that often involves the downregulation of the immune response through the secretion of the cytokines like TGF-β and IL-10 (Harbige and Fisher, 2001). TGF-β is present in human milk (Saito *et al.*, 1993) and can be absorbed by the infant gut rapidly (Letterio *et al.*, 1994). Radiolabeled TGF-β that was administered by gavage into the stomachs of 5-day-old mice is later found in various tissues including the lung, heart, liver, and kidney, suggesting that TGF-β may influence sites beyond the gut (Letterio *et al.*, 1994). Low levels of TGF-β in breast milk have been related to an increased risk of atopic illness in infants, which supports the role of this cytokine in the development of tolerance (Laiho *et al.*, 2003). Other dietary nutrients may influence the concentration of TGF-β in human milk as levels of TGF-β were reported to be positively correlated to PUFA content and negatively correlated to SFA content (Laiho *et al.*, 2003).

IL-10 is found in both the aqueous and lipid layer of human milk (Garofalo *et al.*, 1995). There is some evidence that the IL-10 present in human milk is bioactive (Garofalo *et al.*, 1995). Human milk inhibited the proliferation of human blood lymphocytes *in vitro* and this inhibition was lessened by adding antihuman IL-10 antibodies along with the milk (Garofalo *et al.*, 1995). IL-10 plays a role in the synthesis of IgA (Dunstan *et al.*, 2004), though the process of IgA synthesis is thought to be initiated by TGF-β (Ogawa *et al.*, 2004). Consumption of human milk is thought to lead to greater IL-10 production in the infant (Dvorak *et al.*, 2004). Whether greater IL-10 production by mucosal immune cells promotes oral tolerance remains to be determined.

2. N-6 and n-3 fatty acids

In human breast milk, there are roughly 10 different LC-PUFA consistently detected, representing both the n-3 and the n-6 series and including AA and DHA (Koletzko *et al.*, 2001). However, LA is the primary milk PUFA (Koletzko *et al.*, 2001). Maternal intake of PUFA prior to (Koletzko *et al.*, 2001) and during lactation is reflected in breast milk PUFA content (Hawkes *et al.*, 2002). For example, DHA-rich tuna oil supplementation was found to increase the DHA content of breast milk (Hawkes *et al.*, 2002).

Membrane phospholipid fatty acid composition in infants is strongly influenced by maternal diet (Korotkova *et al.*, 2004) and can alter the

function of the immune cells (Field *et al.*, 2000). Hence, changes in the infant's diet via changes in maternal diet could influence neonatal development of tolerance and sensitization (Korotkova *et al.*, 2004). Low n-6/n-3 ratios (due to a higher content of n-3) in breast milk have been related to a lower risk of atopic illness in the breast-fed infant (Hanson *et al.*, 2003b). In particular, animal studies suggest that the essential fatty acid content of the maternal diet significantly affects the development of immunological tolerance to food antigens in the suckled offspring (Korotkova *et al.*, 2004).

Immunoregulatory benefits have been attributed to n-3 PUFA (reviewed in Palmer and Makrides, 2006). The magnitude of these benefits may be inversely proportional to the age of the individual (reviewed in Palmer and Makrides, 2006). In other words, dietary intervention during critical early stages of immune development (i.e., in uterus or via breast milk) before the establishment of allergic responses is thought to be most preferable (reviewed in Palmer and Makrides, 2006). Cohort studies have demonstrated lower levels of n-3 PUFA in the milk of mothers whose infants showed symptoms of atopic illness before the age of 18 months (reviewed in Palmer and Makrides, 2006). Preliminary data from studies on n-3 PUFA supplementation during the perinatal period appear promising (reviewed in Palmer and Makrides, 2006). However, the completion of current and planned n-3 LC-PUFA interventions will allow for solid conclusions to be drawn (reviewed in Palmer and Makrides, 2006).

B. Priming of the immune system

A balance between tolerance and sensitization (priming) is necessary for the gut immune system to discriminate between harmless antigens and those associated with pathogenic and nonpathogenic microbes (Harbige and Fisher, 2001). Antigen exposure via mother's milk has been shown to prime the immune response of the suckling pup against that antigen in rats (Strobel, 2001). Clinical trials have shown that breast-fed babies have enhanced specific antibody titer to some, but not all vaccines (Hanson *et al.*, 2003b; Kelly and Coutts, 2000). The ability to transfer vaccinations from mother to infant via her milk is of great interest because it could eliminate potential problems associated with directly vaccinating the infant (Gust *et al.*, 2004). One possible explanation for the ability to immunize infants with their mother's milk has been attributed to the presence of anti-idiotypic antibodies in the breast milk (Hanson *et al.*, 2003a). Anti-idiotypic antibodies are antibodies with specificity against other autologous (i.e., from the same individual) antibodies (reviewed in Field, 2005). Therefore, anti-idiotypic antibodies, if present in breast milk, could have the capability of priming the infant's antibody response against the antigen the idiotype is directed to (Van de Perre, 2003).

V. ANTI-INFLAMMATORY FACTORS IN HUMAN MILK

Inflammation is a necessary part of the immune response that helps protect the infant from infection (reviewed by Calder, 2006). The inflammatory response traps pathogens and signals the arrival of immune cells to destroy the antigen. However, this process results in a great deal of ''collateral'' damage of healthy tissue if it is not controlled. Gastrointestinal infections like necrotizing enterocolitis or those caused by *Rotavirus* can result in severe damage to intestinal tissue due to an over active inflammatory response. A decreased risk of developing necrotizing enterocolitis in preterm breast-fed infants compared to preterm formula fed infants was reported in a prospective multicenter study of 926 preterm infants (Lucas and Cole, 1990). A study by the Department of Pediatrics at the University of Turin found that breast-fed infants were less likely to contract or suffer complications due to *Rotavirus* infection compared to their formula-fed counterparts (Gianino *et al.*, 2002). These studies indicate that breast milk protects the infant from infection, which could be due in part to anti-inflammatory components in the human milk ensuring an appropriate and effective immune response of the infant.

There have been very few experimental studies on the anti-inflammatory properties of human milk. Neutrophils are the main immune cells involved in the inflammatory process and *in vitro* studies have shown that human milk can limit the oxidative injury produced by them (reduced cytochrome *c* and consumed H_2O_2) (Grazioso and Buescher, 1996). Human milk also lowered the enzymatic activity of neutrophils (both myeloperoxidase and β-glucuronidase) (Grazioso and Buescher, 1996). Using an animal model of inflammation to test the anti-inflammatory activity of human milk *in vivo*, researchers found that injecting colostrum along with carrageenan into subcutaneous air sacs in rats resulted in a suppressed inflammatory response compared to injecting carrageenan alone (Murphey and Buescher, 1993). Another group of researchers induced colitis in rats with an acetic acid enema to test the anti-inflammatory properties of a human milk diet. The rats that were fed a human milk diet had lower colonic myeloperoxidase activity (indicating less neutrophil infiltration) compared to rats fed chow or an infant formula-based diet (Grazioso *et al.*, 1997). The explanation for the prophylactic nature of human milk is not currently known. However, some components of human milk have potential anti-inflammatory effects; these include cytokines (as well as their receptors and antagonists), antioxidants, antiproteases, and fatty acids (Garofalo and Goldman, 1999). The literature pertaining to the anti-inflammatory properties of these compounds in human milk will be discussed in the following sections.

A. Cytokines

1. IL-10

IL-10 inhibits the production of pro-inflammatory cytokines, providing the necessary balance to ensure that the inflammatory response is limited to destroying the pathogen and not healthy tissue. *In vivo* evidence of the necessity of IL-10 as an anti-inflammatory cytokine is provided by genetically altered mice that are not able to produce IL-10. These mice mount an immune response to the normal microflora in their gut, but without the IL-10 to suppress the inflammation, they develop enterocolitis (similar to ulcerative colitis and celiac disease in humans) (Sydora *et al.*, 2003). This suggests that IL-10 in human milk might help regulate aberrant immune responses in the infant.

2. TGF-β

The anti-inflammatory properties of TGF-β are reviewed by Donnet-Hughes *et al.* (2000), but briefly TGF-β inhibits the production of inflammatory cytokines and promotes the healing of intestinal cells damaged by injury or infection. A feeding trial examining the effectiveness of a polymeric diet (supplemented with TGF-β) in the management of pediatric crohn's disease provides the most convincing *in vivo* evidence of the anti-inflammatory properties of TGF-β (Fell *et al.*, 2000). The enteral diet containing high levels of TGF-β resulted in decreased mucosal IL-1 mRNA (pro-inflammatory cytokine) and clinical remission of in 79% of the children (Fell *et al.*, 2000).

3. IL-1 receptor antagonist

The IL-1 receptor antagonist (IL-1ra) is present in human milk. IL-1ra limits inflammation by competing with IL-1 (pro-inflammatory cytokine) for receptor binding (Dinarello, 1995). The reduced inflammatory response in rats with colitis fed human milk compared to chow or formula was similar to the inflammatory response in rats fed infant formula supplemented with IL-1ra (Grazioso *et al.*, 1997). These results suggest that the IL-1ra content of human milk contributes to its anti-inflammatory properties.

4. Other compounds with potential to regulate inflammation

Soluble TNF-α receptors bind to TNF-α (a pro-inflammatory cytokine), limiting its activity (Buescher and Williams-Koeppen, 1998). Platelet-activating factor acetylhydrolase (PAF-AH) degrades PAF, thereby limiting the gastrointestinal mucosal injury caused by PAF (Furukawa *et al.*, 1993).

B. Antioxidants

Free radicals, or reactive oxygen species, are produced during the normal metabolic activity of cells (Sharda, 2006). These free radicals can damage cells by lipid peroxidation and alteration of protein and/or nucleic acid structures leading to oxidative stress (Sharda, 2006). Antioxidants in both milk and formula prevent significant lipid oxidation, breast milk suppresses oxidative DNA damage better than formula does; however, expressed milk is more sensitive to degradation than formula (Turoli *et al.*, 2004). We have yet to identify all of the compounds in human milk that have antioxidant properties; however, there are several antioxidants in human milk that can scavenge free radicals and thereby limit the damage caused by oxidative stress. These compounds include α-tocopherol (Romeu-Nadal *et al.*, 2006), β-carotene (Sakurai *et al.*, 2005), cysteine (Darragh and Moughan, 1998), ascorbic acid (Sakurai *et al.*, 2005), catalase (Friel *et al.*, 2002), and glutathione peroxidase (Friel *et al.*, 2002). The ability of human milk to resist oxidative stress is greater than formula (Friel *et al.*, 2002). *In vitro* studies have shown that human milk degrades the naturally occurring hydrogen peroxide as well as that produced by neutrophils. This is possibly due to the catalase content of human milk (Grazioso and Buescher, 1996).

Lactoferrin has been shown to inhibit the production of proinflammatory cytokines (IL-6 and TNF-α) as well as inflammatory mediators (nitric oxide, granulocyte-macrophage colony stimulating factor; reviewed by Hanson *et al.*, 2003a; Lonnerdal, 2003). The anti-inflammatory activity of lactoferrin is generally attributed to its ability to search out free iron, which is a potent oxidizer.

C. Anti-Proteases

Inflammatory cells produce proteases, which allow the cells to enter the affected area. Some pathogens also produce proteases in order to enter the body. Human milk contains active protease inhibitors (e.g., α-1-antitrypsin, α-1-antichymotrypsin, and elastase inhibitor) that can limit the ability of pathogens to gain entry into the body and limit the inflammation caused by the inflammatory response (Lindberg *et al.*, 1982).

D. LCPUFAs

The effects of LCPUFAs in inflammation have been reviewed by Calder (2006). Briefly, it is hypothesized that the effects of LCPUFA n-3 fatty acids on immune function are mediated by their ability to compete with the metabolism of the n-6 fatty AA. AA can be metabolized into the pro-inflammatory prostaglandin-E_2 (PGE_2) or leukotriene-B_4 (LTB_4).

Prostaglandin E_2 is one of the most important prostaglandins formed as it initiates the typical sensations associated with inflammation: pain, fever, and swelling (Wahle *et al.*, 2004). The metabolism of AA to yield PGE_2 and LTB_4 can be inhibited by DHA, thereby decreasing the capacity of immune cells to synthesize eicosanoids from AA. DHA will then give rise to PGE_3 and LTB_5, which are considered less biologically potent than the eicosanoids derived from AA. However, their activities have not yet been fully investigated.

VI. CONCLUSIONS

Human milk is a complex mixture of interacting compounds, of which the composition differs not only between women but also within the lactation period. Although it is well documented that breast milk provides antimicrobial defense to the infant, research is still in its infancy in our understanding of the importance of the many minor components in this complex nutritional supplement on neonatal immune development, tolerance, and prevention of the inflammatory response. These gaps in our knowledge will be a fruitful area of research for nutritionists for many years. Current feeding regimens recommended for infants are based primarily on the current understanding of the nutritional requirements of the neonate, but perhaps will be modified to reflect the consequences on immune function both immediate and later in life.

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