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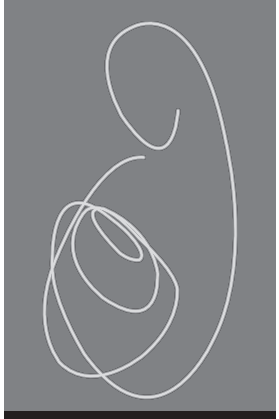

CHAPTER 5

Host-Resistance Factors and Immunologic Significance of Human Milk

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Some of the most dramatic and far-reaching advances in the understanding of the immunologic benefits of human milk have been made using newer techniques to demonstrate the specific contribution of the numerous "bioactive factors" contained in human milk (Table 5-1). The multifunctional capabilities of the individual factors, the interactive coordinated functioning of these factors, and the longitudinal changes in the relative concentrations of them for the duration of lactation make human milk unique. The immunologically active components of breast milk make up an important aspect of the host defenses of the mammary gland in the mother; at the same time, they complement, supplement, and stimulate the ongoing development of the infant's immune system.[107-109]

The explosion of research on all the immunologic properties and actions of breast milk in the last 10 years makes it impossible to summarize all the important aspects of what we now know about the immunologic benefits of breast milk. The recently developed technologies of genomic studies using microarrays and proteomics promise to continue this rapid expansion of knowledge on the biology of the mammary gland, human milk, and the infant's developing immune system. This chapter emphasizes the important concepts of these immunologic benefits and refers the interested reader to the most recent literature for more extensive information on the many specific components.

Overview

The immunologic benefits of human milk can be analyzed from a variety of perspectives:

1. Reviewing the published information on the protection of infants from specific infections that compares breastfed and formula-fed infants.
2. Comparing documented deficiencies in infants' developing immune systems and the actions of bioactive factors provided in breast milk.
3. Examining the proposed function of the active components contained in human milk: antimicrobial, antiinflammatory, and immunomodulating.
4. Considering the nature of the different factors: soluble, cellular, and hormone-like.
5. Examining the contribution of breast milk to immune function of mammary glands and infants as an evolutionary process.
6. Determining the site of the postulated action of the specific factors (e.g., in the breast or in the infant) at the mucosal level (respiratory tract or gastrointestinal [GI] tract) or at the systemic level.
7. Classifying the factors relative to their contribution to the constitutive defenses (innate immunity) versus the inducible defenses (adaptive immunity) of the infant's immune system.
8. Clarifying the mechanism of action of the proposed immunologic benefit (the mucosal-associated lymphoid tissue formation of the

TABLE 5-1 Immunologically and Pharmacologically Active Components and Hormones Observed in Human Colostrum and Milk

Soluble	Cellular	Hormones and Hormonelike Substances
Immunologically specific	Immunologically specific	Epidermal growth factor
Immunoglobulin	T lymphocytes	Prostaglandins
sIgA (11S), 7S IgA, IgG, IgM IgE, IgD, secretory component	B lymphocytes	Relaxin
		Neurotensin
	Accessory cells	Somatostatin
	Neutrophils	Bombesin
T-cell products	Macrophages	Gonadotropins
Histocompatibility antigens	Epithelial cells	Ovarian steroids
		Thyroid-releasing hormone
	Additional cells	Thyroid-stimulating hormone
Nonspecific factors	Stem cells	Thyroxine and triiodothyronine
Complement		Adrenocorticotropin
Chemotactic factors		Corticosteroids
Properdin (factor P)		Prolactin
Interferon		Erythropoietin
α-Fetoprotein		Insulin
Bifidus factor		Cytokines
Antistaphylococcal factor(s)		Interleukins
Antiadherence substances		
Epidermal growth factor		
Folate uptake enhancer		
Antiviral factor(s)		
Migration inhibition factor		
Gangliosides		
Nucleotides		
Antisecretory factor		
Spermine		
Soluble CD14		
Carrier proteins		
Lactoferrin		
Transferrin		
Vitamin B_{12}–binding protein		
Corticoid-binding protein		
Enzymes		
Lysozyme		
Lipoprotein lipase		
Leukocyte enzymes		

Modified from Ogra PL, Fishaut M: Human breast milk. In Remington JS, Klein JO, editors: *Infectious Diseases of the Fetus and Newborn Infant,* ed 4, Philadelphia, 1995, Saunders.

bioactive factors at the level of the mucosa and their subsequent action at the breast or in an infant).

9. Considering the contribution of human milk to the development of an infant's immune system relative to potential long-term immunologic benefits, such as protection against allergy, asthma, autoimmune disease, or inflammatory bowel disease.

Protective Effect of Breast Milk

The protective effect of breast milk against infection was documented as early as 1892 in the medical literature by data proving that milk from various species, including humans, was protective for offspring, containing antibodies against a vast number of antigens.[271]

BOX 5-1. Breastfeeding Definitions

Any breastfeeding	**Full breastfeeding**	Exclusive human breast milk only	Infant ingests no other nutrients, supplements, or liquids
		Almost exclusive	No milk other than human milk; only minimal amounts of other substances such as water, juice, tea, or vitamins
	Partial breastfeeding	High partial	Nearly all feeds are human milk (at least 80%)
		Medium partial	A moderate amount of feeds are breast milk, in combination with other nutrient foods and nonhuman milk (20%-80% of nutritional intake is human breast milk)
		Low partial	Almost no feeds are breast milk (less than 20% of intake is breast milk)
	Token		Breastfeeding primarily for comfort; nonnutritive, for short periods of time, or infrequent
Never breastfed	Infant never ingested any human milk		

From Lawrence RM and Pane CA: Human breast milk: current concepts of immunology and infectious diseases, *Curr Probl Pediatr* 37:1-44, 2007, Table 1, page 10.

Veterinarians have long known the urgency of offspring receiving the early milk of the mother. Death rates among human newborns not suckled at the breast in the Third World are at least five times higher than among those who receive colostrum and mother's milk. The evidence that a lack of breastfeeding and poor environmental sanitation have a pernicious synergistic effect on infant mortality rate has been presented by Habicht[101] after studying 1262 women in Malaysia.

The evidence that breastfeeding protects against infections in the digestive and respiratory tracts has been reported for several decades.[269] However, many of the older studies were criticized for flawed methodology and because they were performed in "developing countries," where the risk for infection due to poor sanitation was expected to be higher.[13,101,116] Various researchers have proposed specific criteria for assessing the methodology of studies reporting on the protective effects of breast milk, clearly identifying measurable outcomes and the definition of breastfeeding, with other methods to limit bias and to control for confounding variables.[13,46,147,149] More recent studies, which have incorporated many of the proposed methodologic criteria, continue to document that breastfeeding protects infants against diarrhea, respiratory infections, and otitis media.* Individual papers report protection against urinary tract infections and neonatal sepsis.[6,109,220,275] Several papers document the decreased risk for dying in infancy associated with exclusive or predominant breastfeeding in Pakistan, Peru, Ghana, India, Nepal, and Bangladesh.[4,6,9,62,179] A systematic review by the Bellagio Child Survival Study Group predicted that exclusive breastfeeding for 90% of all infants through 6 months of age could prevent 13% of the childhood deaths occurring younger than 5 years of age.[133] A recent review on human breast milk documents the evidence for protection against infectious diseases from breastfeeding for resource-rich and resource-poor countries.[153]

*References 7, 15, 43, 51, 55, 122, 167, 199, 208, 223, 226, 243, 278.

DOSE-RESPONSE RELATIONSHIP

One of the important considerations relative to measuring the immunologic benefits of breast milk is the exclusivity and duration of breastfeeding. The basic concept is identifying a dose-response relationship between the amount of breast milk received by an infant during the period of observation and the immunologic benefit gained with greater exclusivity and duration equaling greater volume of breast milk or "dose." Dr. Labbok and Krasovec[149] have carefully defined breastfeeding in terms of the patterns of breastfeeding relative to the amount of supplementation with formula or other fluids or foods (full/nearly full, medium or equal, low partial, or token) to standardize the use of equatable terms in different studies. Box 5-1 outlines these definitions of the "amount" of breastfeeding.[153] Raisler et al[227] referred to a dose-response relationship when they studied the effect of "dose" of breast milk on preventing illness in more than 7000 infants. "Full breastfeeding" was

associated with the lowest rates of illness (diarrhea, cough, or wheeze) and even children with "most" or "equal" breastfeeding had evidence of lower odds ratios of ear infections and certain other illnesses. A number of other long-term studies demonstrated greater protection from infection with increased exclusivity of breastfeeding and durations of at least 3 months.* A couple papers demonstrated a "dose" effect relative to decreased occurrence of late onset sepsis in very low-birth-weight infants[73] and premature infants[245] associated with the infants receiving at least 50 mL/kg per day of mother's milk compared with receiving other nutrition. The current recommendations from the American Academy of Pediatrics reinforce the importance of the dose-response relationship between breastfeeding and the benefits of breastfeeding when it recommends exclusive breastfeeding for the first 6 months of life and at least partial breastfeeding after the introduction of solid foods for an additional 12 months or longer.[1] Another important consideration relative to exclusive breastfeeding is the potential effect of other foods and fluids in an infant's diet that could negatively influence immunologic benefits and infection-protective effects at the level of the GI mucosa.

Developmental Deficiencies in Infants' Immune Systems

The human immune system begins forming and developing in the fetus. Newborn infants' immune systems are immature and inadequate at birth. Immune systems rapidly adapt in the postnatal period related to the natural maturation of the skin and mucosal barriers and in response to the exposure of infants to inhaled and ingested antigens and microbial agents in the extrauterine environment. Infants' immune systems develop throughout at least the first 2 years of life. Overall, infants have limited abilities to respond effectively and quickly to infectious challenges, which explains infants' ongoing susceptibility to infections.† Box 5-2 lists most of the better understood deficiencies in infants' immune systems. An extensive discussion of these developmental immune deficiencies affecting infants is presented by Lawrence and Pane.[153] The B-lymphocytes and immunoglobulin production are deficient in the amount and specificity of antibodies produced. There is limited isotype switching and slow maturation of the antibody response to specific antigens (polysaccharides).[114,177] The systemic cell-mediated immune response, including effector and memory T cells is functionally limited in its response in infants.[253,273,280] Neutrophil activity in infants is also developmentally delayed, which directly contributes to infants' susceptibility to invasive bacterial infections during the first months of life.[158,172,250,258,281] The complement system in infants is characterized by low levels of

BOX 5-2. Developmental Defects in Newborns

Phagocytes (function matures over the first 6 months of life):
- Limited reserve production of phagocytes in response to infection
- Poor adhesion molecule function for migration
- Abnormal transendothelial migration
- Inadequate chemotactic response
- Qualitative deficits in hydroxyl radical production
- Decreased numbers of phagocytes reaching the site of infection

Cell-mediated immunity:
- Limited numbers of mature functioning (memory) T cells (gradual acquisition of memory T cells throughout childhood)
- Decreased cytokine production: IFN-α, IL-2, IL-4, IL-10
- Diminished NK cell cytolytic activity (matures by 6 months of age)
- Limited antibody-dependent cytotoxic cell activity
- Poor stimulation of B cells (subsequent antibody production, isotype switching)

B-Lymphocytes and immunoglobulins:
- Limited amounts and repertoire of active antibody production
- Poor isotype switching (primarily IgM and IgG1 produced in neonates)
- IgG1 and IgG3 production is limited (matures at 1 to 2 years of age)
- IgG2 and IgG4 production is delayed (matures at 3 to 7 years of age)
- Serum IgA levels are low (less than adult levels through 6 to 8 years of age)
- Deficient opsonization by immunoglobulins
- Poor response to T-cell independent antigens (polysaccharides) (matures at 2 to 3 years of age)

Complement cascade:
- Decreased function in both the classical and the alternative pathways
- Insufficient amounts of C5a

From Table 2, page 22, Lawrence RM, Pane, CA: Human breast milk: current concepts of immunology and infectious diseases, *Curr Probl Pediatr* 37:1-44, 2007.

*References 3, 14, 51, 55, 59, 60, 61, 122, 167, 208, 223, 243, 278.

†References 45, 84, 85, 87, 120, 279.

complement components, and both the "classical" and alternative pathways have limitations for complement activation.[2,63,251,277] Numerous immune components are produced in limited amounts in infancy, including complement, interferon-γ, secretory immunoglobulin (Ig) A, interleukins (IL-3, IL-6, IL-10), tumor necrosis factor (TNF)-α, lactoferrin, and lysozyme.[45,87]

Relative to these various immune deficits in infants, one can find various bioactive and immunomodulating factors in breast milk that are potentially capable of complementing and enhancing the development of infants' mucosal and systemic immune systems.[87,110] This concept of bioactive and immunomodulating factors in breast milk is an important area of evolving research that has been extensively reviewed in the literature.[87,88,110,144] The most intense focus of this research centers on the effects of human milk on infants' GI tract.[85,201]

Bioactive Factors

The bioactive factors being studied are as diverse as proteins (lactoferrin, lysozyme, etc.), hormones (erythropoietin, prolactin, insulin, etc.), growth factors (epithelial growth factor, insulin-like growth factor, etc.), neuropeptides (neurotensin, somatostatin, etc.), cytokines (TNF-α, IL-6, etc.), anti-inflammatory agents (enzymes, antioxidants, etc.), and nucleotides (see Table 5-1). In the past, it was adequate to point to the lists of factors (especially immunoglobulins) to "explain" the immunologic benefit of breast milk. Today, it is necessary to understand the multifunctional and dynamic action of these individual factors, their specific mechanisms of action on the innate, the adaptive, and the mucosal immune system, and their role in direct infection protection, in the normal development of infants' immune systems, and their contribution against potentially harmful inflammation.

From an evolutionary perspective, maternal antibody is transmitted to the fetus by different pathways in different species.[87,159,261] An association has been recognized between the number of placental membranes and the relative importance of the placenta and the colostrum as sources of antibodies. By this analysis, horses, with six placental membranes, pass little or no antibodies transplacentally and rely totally on colostrum for protection of foals. Humans and monkeys, having three placental membranes, receive more of the antibodies via the placenta and less from the colostrum. The transfer of IgG in humans is accomplished by active transport mechanism of the immunoglobulin across the placenta. Secretory IgA (sIgA) immunoglobulins are found in human milk and provide local protection to the mucous membranes of the GI tract. Other investigations have established that the mammary glands and their secretion of milk are important in protecting the infant not only through the colostrum but also through mature milk from birth through the early months of life.

Although the predominance of IgA in human colostrum and milk had long been described, the importance of this phenomenon was not fully appreciated until the discovery that IgA is a predominant immunoglobulin present in mucosal secretions of other glands in addition to the breast.

Mucosal Immunity

Mucosal immunity has become the subject of extensive research.[21,22,120,201] It is clear that considerable traffic of cells occurs between mucosal epithelia and secretory or lymphoid tissue sites. The data support the concept of a general system of mucosa-associated lymphoid tissue (MALT), which includes the gut, lung, mammary gland, salivary and lacrimal glands, and the genital tract (Figure 5-3). Through the immune response of MALT, a reaction to an immunogen at a mucosal site may be an effective means of producing immunity at distant sites. Antibodies against specific antigens found in milk have also been found in the saliva, evidence for transfer of protection to two different distant sites simultaneously. Evidence suggests that the mammary glands may act as extensions of the gut-associated lymphoid tissue (GALT) and possibly the bronchiole-associated lymphoid tissue. The ability of epithelial surfaces exposed to the external environment to defend against infectious agents has been well documented for the GI, genitourinary, and respiratory tracts.[142] sIgA and secretory IgM (sIgM) produced through the adaptive response of the mucosal-lymphoid immune system act by blocking colonization with pathogens and limiting the passage of harmful antigens across the mucosal barrier. Activated B cells and cytokines pass to the mammary gland where they contribute to the production of sIgA in breast milk. Direct contact between the antigen and the lymphoid cells of the breast is unlikely.[202] Peyer patches, tonsils, and other MALT structures appear to be well developed at birth.[24] Nevertheless, the actual effective production of sIgA to various antigens presented to infants' mucosal surfaces (respiratory and GI tracts) is still inadequate to protect against infection. A breastfeeding infant, as part of the maternal-infant dyad exposed to the same antigens via their mucosal services, can receive protective sIgA and sIgM in the mother's breast milk produced by the mother's MALT (Figure 5-1).

The protective properties of human milk can be divided into cellular factors and humoral factors

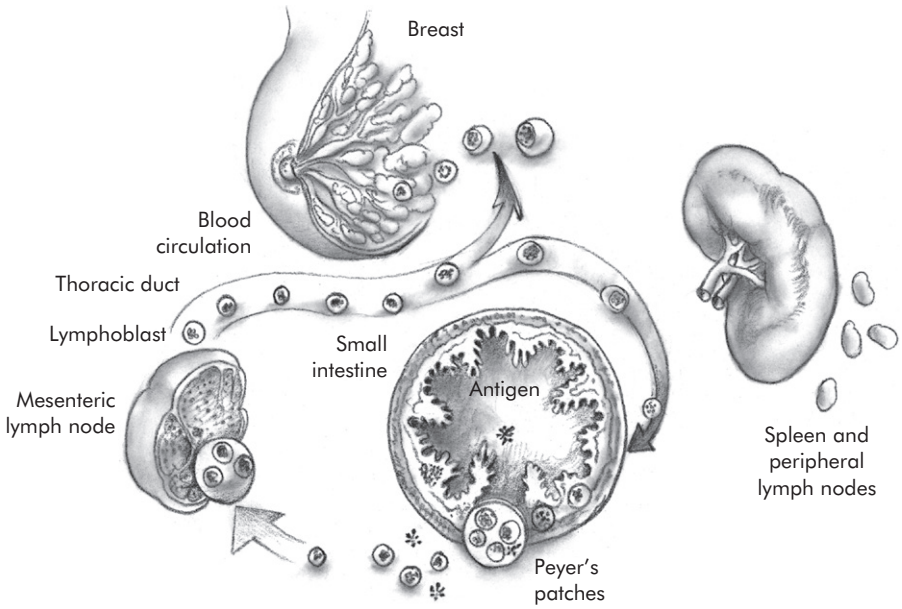

Figure 5-1. Schema of mechanism by which progeny of specifically sensitized lymphocytes originating from gut-associated lymphoid tissue may migrate to and infiltrate mammary gland and its secretions, supplying breast with immune cells. (Modified from Head JR, Beer AE: The immunologic role of viable leukocytic cells in mammary exosecretions. In Larson BL, editor: *Lactation, vol 4, Mammary Gland/Human Lactation/Milk Synthesis*, New York, 1978, Academic Press.)

for facility of discussion, although they are closely related in vivo. A wide variety of soluble and cellular components and hormone-like agents have been identified in human milk and colostrum (see Table 5-1). Although the following discussion separates these elements, it is important to emphasize that the constituents of human milk are multifunctional and their functioning in vivo is interactive and probably coordinated and complementary.

Cellular Components of Human Colostrum and Milk

Cells are an important postpartum component of maternal immunologic endowment. More than 100 years ago, cell bodies were described in the colostrum of animals. As with much lactation research, further study of colostral corpuscles was undertaken by the dairy industry for commercial reasons in the early 1900s. This research afforded an opportunity to make major progress in the understanding of cells in milk. Initially, it was thought that these cells represented a reaction to infection in the mammary gland and were even described as "pus cells."

It has become clear that the cells of milk are normal constituents of colostrums in all species. Cells include macrophages, lymphocytes, neutrophils, and epithelial cells, and they total approximately 4000/mm^3. Cell fragments and epithelial cells were examined by electron microscope in fresh samples from 30 women by Brooker.[27] He found that the membrane-bound cytoplasmic fragments in the sedimentation pellet outnumbered intact cells. The fragments were mostly from secretory cells that contained numerous cisternae of rough endoplasmic reticulum, lipid droplets, and Golgi vesicles containing casein micelles. Secretory epithelial cells were found in all samples and, after the second month postpartum, began to outnumber macrophages. Ductal epithelial cells were about 1% of the population of cells for the first week or so and then disappeared. All samples contained squamous epithelial cells originating from galactophores and the skin of the nipple.

LEUKOCYTES

Living leukocytes are normally present in human milk.[142] The overall concentration of these leukocytes is of the same order of magnitude as that seen in peripheral blood, although the predominant cell in milk is the macrophage rather than the neutrophil. Macrophages compose about 90% of the leukocytes, and 2000 to 3000/mm^3 are present. Lymphocytes make up about 5% to 10% of the cells (200 to 300/mm^3), which is a much lower concentration than in human blood.[91] The number of cells found in human milk increases with mastitis. Both large and small lymphocytes are present. By indirect immunofluorescence with anti–T-cell antibody to identify thymus-derived lymphocytes, it has been shown that 50% of human colostral lymphocytes are T cells, and in human milk up to 80% of the lymphocytes are T cells.[276]

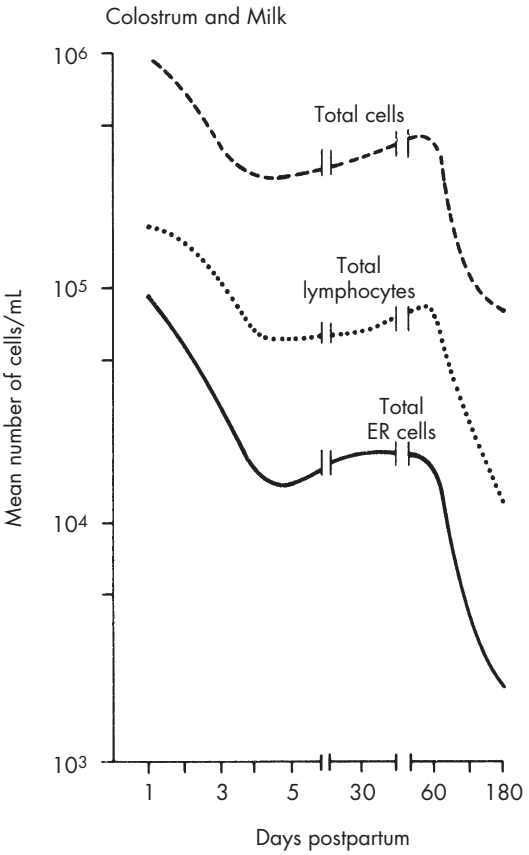

Figure 5-2. Geometric mean concentration of total cells, lymphocytes, and erythrocyte rosette-forming cells (ER) in colostrum and milk of 200 lactating women. (Modified from Ogra SS, Ogra PL: Immunologic aspects of human colostrum and milk. I. Distribution characteristics and concentrations of immunoglobulins at different times after the onset of lactation, *J Pediatr* 92:546, 1978.)

Immunofluorescence procedures to detect surface immunoglobulins characteristic of B lymphocytes identified 34% as B lymphocytes.

The number of leukocytes and the degree of mitogenic stimulation of lymphocytes sharply decline during the first 2 or 3 months of lactation to essentially undetectable levels, according to Ogra and Ogra (Figure 5-2).[92] Enumeration of the total cell numbers in milk has been difficult, but when various techniques are compared (Coulter electronic particle counter, visual cell counting with special stains, filter trapping with fluorescent detection, and automated fluorescent cell counting), stains for deoxyribonucleic acid (DNA) were superior to the other techniques.

MACROPHAGES

Macrophages are large-complex phagocytes that contain lysosomes, mitochondria, pinosomes, ribosomes, and a Golgi apparatus. The monocytic phagocytes are lipid laden and were previously called the colostral bodies of Donne. They have the same functional and morphologic features as phagocytes from other human tissue sources. These features include ameboid movement, phagocytosis of microorganisms (fungi and bacteria), killing of bacteria, and production of complement components C3 and C4, lysosome, and lactoferrin. Other milk macrophage activities include the following[219]:

Phagocytosis of latex, adherence to glass
Secretion of lysozyme, complement components
C3b-mediated erythrocyte adherence
IgG-mediated erythrocyte adherence and phagocytosis
Bacterial killing
Inhibition of lymphocyte mitogenic response
Release of intracellular IgA in tissue culture
Giant cell formation
Interaction with lymphocytes

Data suggest these macrophages also amplify T-cell reactivity by direct cellular cooperation or by antigen processing. The colostral macrophage has been suggested as a potential vehicle for the storage and transport of immunoglobin. A significant increase in IgA and IgG synthesis by colostral lymphocytes, when incubated with supernatants of cultured macrophages, has been reported.[221]

The macrophage may also participate in the biosynthesis and excretion of lactoperoxidase and cellular growth factors that enhance growth of intestinal epithelium and maturation of intestinal brush-border enzymes.

The mobility of macrophages is inhibited by the lymphokine migration inhibitor factor, which is produced by antigen-stimulated sensitized lymphocytes. The activities of macrophages have been demonstrated in both fresh colostrum and colostral cell culture, and certain functions are altered compared with their counterpart in human peripheral blood.

POLYMORPHONUCLEAR LEUKOCYTES

The highest concentration of cells occurs in the first few days of lactation and reaches more than a million per milliliter of milk.

Colostrum (1 to 4 days postpartum) contains 10^5 to 5×10^6 leukocytes/mL, and 40% to 60% are polymorphonuclear cells (PMNs). Mature milk (i.e., after 4 days) has fewer cells (see Figure 5-3), approximately 10^5/mL with 20% to 30% PMNs. After 6 weeks, few PMNs are present. The functions of the PMNs normally include microbial killing, phagocytosis, chemotactic responsiveness, stimulated hexose monophosphate shunt activity, stimulated nitroblue tetrazolium dye reduction, and stimulated oxygen consumption.[33] When milk

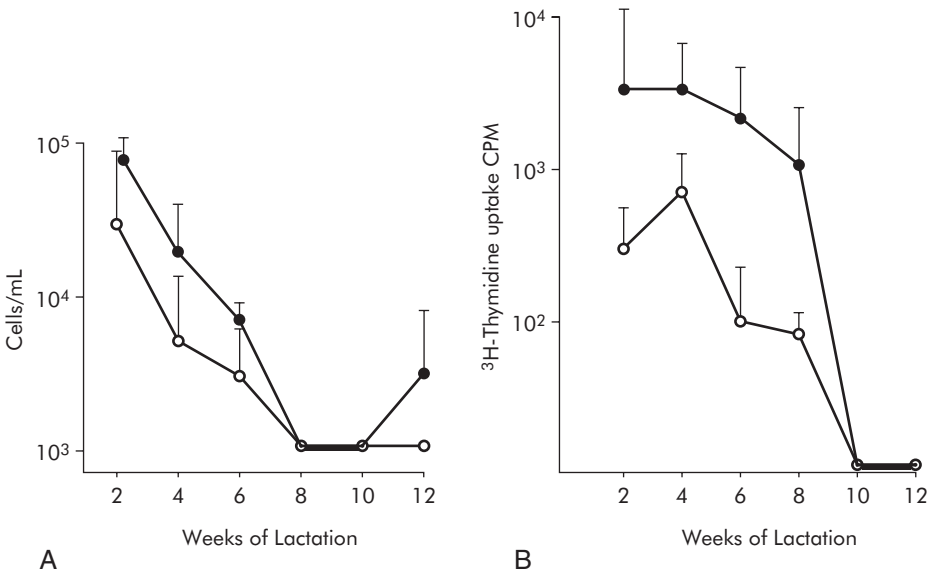

Figure 5-3. **A,** Longitudinal study of numbers of leukocytes. **B,** Longitudinal study of uptake of ^{3}H-thymidine in lymphocytes. Same subjects were examined during second through twelfth week of lactation. Data are presented as mean ± SD of macrophages-neutrophils (•) and lymphocytes (○) in **A** and of stimulated (•) and unstimulated (○) lymphocytes in **B.** (From Goldman AS, Garza C, Nichols BL et al: Immunologic factors in human milk during the first year of lactation, *J Pediatr* 100:563, 1982.)

PMNs are compared with those in the serum, their activity is often less than that of serum PMN cells. Whether milk PMNs actually perform a role in protection of the infant has been studied by many investigators using many techniques. Briefly, animal studies have shown that (1) the mammary gland is susceptible to infection in early lactation, (2) a dramatic increase in PMNs occurs with mammary inflammation, and (3) in the presence of peripheral neutropenia during chronic mastitis, severe infection of the gland occurs. This implies, according to Buescher and Pickering,[33] that the primary function of milk PMNs is to defend the mammary tissue per se and not to impart immunocompetence to the newborn. This may explain the presence of large numbers of PMNs that are relatively hypofunctional early and then disappear over time. Evidence shows that neutrophils found in human milk demonstrate signs of activation, including increased expression of CD11b (an adherence glycoprotein), decreased expression of L-selectin, spontaneous production of granulocyte-macrophage colony-stimulating factor (GM-CSF), and the ability to transform into $CD1^+$ dendritic cells (DCs).[125] Human milk macrophages have the morphology and motility of activated cells. The movement of these cells in a three-dimensional system is greater than that of monocytes, their counterparts in peripheral blood. Such activated neutrophils may play a role in phagocytosis at the level of the mucosa of the GI tract, supplementing infants' poor ability to recruit phagocytes to that site.[137]

LYMPHOCYTES

Both T and B lymphocytes are present in human milk and colostrum and are part of the immunologic system in human milk. T cells are 80% of the lymphocytes in breast milk. Human milk lymphocytes respond to mitogens by proliferation, with increased macrophage-lymphocyte interaction and the release of soluble mediators, including migration inhibitor factor. Cells destined to become lymphopoietic cells are derived from two separate influences, the thymus (T) and the bursa (B) or bursal equivalent tissues. The population of the B cells makes up the smaller part of the total. They synthesize IgA antibody. The term B cell is derived from its origination in a different anatomic site from the thymus; in birds, it has been identified as the bursa of Fabricius. The B cells can be identified by the presence of surface immunoglobulin markers. The B cells in human milk include cells with IgA, IgM, and IgG surface immunoglobulins. B cells transform into plasma cells and remain sessile in the tissues of the mammary gland.

T-CELL SYSTEM

More rapid mitotic activity occurs in the thymus gland than in any other lymphatic organ, yet 70% of the cells die within the cell substance. The thymus is the location for much of the T-cell differentiation and selection and plays a major role in the development of infants' immune systems. Thymosin has been identified as a hormone produced by thymic

epithelial cells to expand the peripheral lymphocyte population. After emergence from the thymus gland, T cells acquire new surface antigen markers. The T cells circulate through the lymphatic and vascular systems as long-lived lymphocytes, which are called the "recirculating pool." They then populate restricted regions of lymph nodes, forming thymic-dependent areas.[276] It is interesting to note that exclusively breastfed infants have a significantly larger thymus than formula-fed infants at 4 and 10 months.[111] The significance of the lymphocytes in human milk in affording immunologic benefits to breastfed infants continues to be investigated. It is suggested that lymphocytes can sensitize, induce immunologic tolerance, or incite graft-versus-host reactions. According to Head and Beer,[115] lymphocytes may be incorporated into sucklings' tissues, achieving short-term adoptive immunization of the neonate.

Studies of the activities of lymphocytes have been carried out by a number of investigators who collected samples of milk from lactating women at various times postpartum, examined the number of cell types present, and then studied the activities of these cells in vitro.[138,142] Ogra and Ogra[203] collected samples from 200 women and measured the cell content from 1 through 180 days (see Figure 5-3). They then compared the response of T lymphocytes in colostrum and milk with that of the T cells in the peripheral blood. T-cell subpopulations have also been shown by surface epitopes to be similar to those in the peripheral blood.

The greatest number of cells appeared on the first day, with the counts ranging from 10,000 to 100,000/mm^3 for total cells. By the fifth day, the count had dropped to 20% of the first day's count. In addition, the number of erythrocyte rosette-forming cells was determined by using sheep erythrocyte-rosetting technique. The erythrocyte rosette formation lymphocytes constituted a mean 100/mm^3 on the first day and ⅒ of that by the fifth day.

At 180 days, total cells were 100,000/mm^3, lymphocytes were 10,000/mm^3, and erythrocyte rosette formation lymphocytes were 2000/mm^3. The investigators compared the values with those in the peripheral blood of each mother; the levels remained essentially constant.[202] In a similar study, Bhaskaram and Reddy[18] sampled milk over time from 74 women and found comparable cell concentrations. They examined the bactericidal activity of the milk leukocytes and found it to be comparable with that of the circulating leukocytes in the blood, irrespective of the stage of lactation or state of nutrition of the mother.

Ogra and Ogra[203] also studied the lymphocyte proliferation responses of colostrum and milk to antigens. Their data show response to stimulation from the viral antigens of rubella, CMV, and mumps. Analysis of cell-mediated immunity to microbial antigens shows milk lymphocytes are limited in their potential for recognizing or responding to certain infectious agents compared with cells from the peripheral circulation. This is thought to be an intercellular action and not caused by lack of external factors. In contrast, the T cells and B cells have been shown to have unique reactivities not seen in peripheral blood.

Colostral lymphocytes are derived from mature rather than immature T-cell subsets. The distribution of T-cell subsets in colostrum includes both $CD4^+$ and $CD8^+$ cells.[231] The distribution of CD4 cells in colostrum and human milk is lower than in the serum, and fewer CD4 cells exist than CD8 cells.[276] The percentage of CD4 cells is higher than in the serum of either postpartum donors or normal control subjects. No correlation exists with length of gestation and number of cells (normal blood usually contains twice as many $CD4^+$ as $CD8^+$ lymphocytes).[134]

Parmely et al[212] partially purified and propagated milk lymphocytes in vitro to study their immunologic function. Milk lymphocytes responded in a unique manner to stimuli known to activate T lymphocytes from the serum. The authors found milk lymphocytes to be hyporesponsive to nonspecific mitogens and histocompatibility antigens on allogenic cells in their laboratory. They found them unresponsive to *Candida albicans*. Significant proliferation of lymphocytes occurred in response to K_1 capsular antigen of *Escherichia coli*.[118] Lymphocytes from blood failed to respond to the same antigen. This supports the concept of local mammary tissue immunity at the T-lymphocyte level.

More recent experiments in rodents have provided evidence that T lymphocytes that are reactive to transplantation alloantigens can adoptively immunize a suckling newborn. Foster nursing experiments performed in rodents have shown that newborn rats exposed to allogenic milk manifested alterations in their reactivity to skin allografts of the foster mother's strain. In animals, mothers may give their suckling newborn immunoreactive lymphocytes. The influence of maternal milk cells on the development of neonatal immunocompetence has been demonstrated in several different immunologic contexts. Congenitally, athymic nude mice nursed by their phenotypically normal mothers or normal foster mothers had increased survival. The mothers contributed their T-cell–helper activity to the suckling newborn.

Colostral lymphocytes proliferate in response to various mitogens, alloantigens, and conventional antigens. Colostral cells survive in the neonatal stomach and in the gut of experimental animals, some remaining viable in the upper GI tract for a week. No evidence, however, indicates that

transepithelial migration takes place when neonatal mice are foster-nursed by newly delivered animals whose colostral cells were tagged with ^{3}H-thymidine.[33]

Cells in human milk have been studied using the same markers employed with cells in the peripheral blood; 80% of the lymphocytes are T cells that are equally distributed between $CD4^+$ and $CD8^+$ subpopulations, and their T-cell receptors are principally of the α/β type. $CD4^+$ cells are common leukocyte cells of the helper and suppressor-inducer subsets, and $CD8^+$ cells are leukocytes of the cytotoxic and noncytotoxic subsets. T cells in human milk are presumed activated because they display increased phenotypic markers of activation including HLA-DR and CD25 (IL-2 receptor). The majority of T cells in human milk are $CD45RO^+$, consistent with effector and memory T cells.[236,276] These cells are effective producers of interferon-γ, which is consistent with their phenotypic features. Here again, human milk may supplement the infant with a functioning immune cell to compensate for an identified deficiency in the infant, a paucity of memory T cells.

B-CELL SYSTEM

Juto[134] studied the effect of human milk on B-cell function. Cell-free, defatted, filtered colostrum as well as mature breast milk showed an enhancing effect on B-cell proliferation and generation of antibody secretion. This was not seen with formula. Juto suggested that this could represent an important immunologic mechanism. Goldblum et al[82] were able to show a B-cell response in human colostrum to *E. coli* given to the mother orally, which was not accompanied by a systemic response in the mother. This suggests that the breast and breast milk reflect sites of local humoral or cell-mediated immunity, which were initially induced at a distant site such as the gut and transferred via reactive lymphoid cells migrating to the breast. Head and Beer[115] provided a scheme to describe this mechanism (see Figure 5-1). The diagram depicts the progeny of specifically sensitized lymphocytes that originated in GALT, specifically Peyer patches, as they migrate to the mammary gland. As they infiltrate the mammary gland and its secretion, they supply the breast with immune cells capable of selected immune responses. Ogra and Ogra[202,203] suggest that the cells may selectively accumulate in the breast during pregnancy. The responses of milk cells and their antibodies are not representative of an individual's total immunity.[212] Most of these immunocompetent cells, initially stimulated in GALT, recirculate to the external mucosal surface and populate the lamina propria as antibody-producing plasma cells. A substantial number of these antigen-sensitized cells selectively home-in to the stroma of the mammary glands and initiate local IgA antibody synthesis against the antigens initially encountered in the respiratory or intestinal mucosa.[18] More recent work on human-milk–derived B cells demonstrates that breast milk contains activated memory B cells, different than those in the blood. These cells express mucosal adhesion molecules ($\alpha_4\beta_7^{-/+}$, $\alpha4\beta_1^+$, $CD44^+$, $CD62L^-$) suggesting origin in the mammary gland, but similar to GALT-associated B cells.[265] The mucosae-associated epithelial cytokine CCL28 may contribute to migration of and retention of these cells in the mammary gland.[274] This information supports the concept of the mammary gland as an effector site of the mucosal immune system.

The accumulated epidemiologic research support the concept that colostrum and milk provide human infants with immunologic benefits. Both T and B lymphocytes found in breast milk are reactive against organisms invading the intestinal tract. However, the proof of specific viral or bacterial protection secondary to the action of immunologically active B cells has not been demonstrated.

Survival of Maternal Milk Cells

Although it is clear that cells are provided in the colostrum and milk, the effectiveness and impact of these cells on the neonate depend on their ability to survive in the GI tract. It has been demonstrated in several species, including humans, that the pH of the stomach can be as low as 0.5, but the output of hydrochloric acid is minimal for the first few months, as is the peptic activity. Immediately after a feeding begins, the pH rises to 6.0 and returns to normal in 3 hours. The cells from milk tolerate this. Studies in rats have also shown that intact nucleated lymphoid cells are found in the stomach and intestines.[16] These cells, when removed from rat stomachs, are capable of phagocytosis. Lymphoid cells in milk have been shown to traverse the mucosal wall.

When human milk is stored, however, the cellular components do not tolerate heating to 63° C (145.4° F), cooling to –23° C (–9.4° F), or lyophilization. Although a few cells may be identified in processed milk, they are not viable.[83]

Stem Cells

Cregan et al[49] have reported the presence of mammary stem cells in human breast milk based on the demonstration of the cytokeratin 5 mammary stem cell marker. Additionally cells from breast

milk were analyzed after culturing which showed both a multipotent stem cell marker, nestin, and the cytokeratin 5 marker. Although human milk may serve as a source of mammary stem cells in the future, no evidence of an immunologic role for these cells in developing infants currently exists.

Humoral Factors

IMMUNOGLOBULINS

All classes of immunoglobulins are found in human milk. The study of immunoglobulins has been enhanced through the techniques of electrophoresis, chromatographics, and radioimmunoassay. More than 30 components have been identified; of these, 18 are associated with proteins in the maternal serum, and the others are found exclusively in milk. The concentrations are highest in the colostrum of all species, and the concentrations change as lactation proceeds.[173] IgA, principally sIgA, is highest in colostrum. Although postpartum levels fall throughout the next 4 weeks, substantial levels are maintained throughout the first year, during gradual weaning between 6 and 9 months, and even during partial breastfeeding (when infant receives solid foods) in the second year of life (Figures 5-4 and 5-5 and Table 5-2). Specific sIgA antibodies to *E. coli* persist through lactation and may even increase (see Figure 5-4).

The main immunoglobulin in human serum is IgG; IgA content is only one fifth the level of IgG. In milk, however, the reverse is true. IgA is the most important immunoglobulin in milk, not only in concentration but also in biologic activity. sIgA is likely synthesized in the mammary alveolar cells[256] or by lymphocytes that have migrated from Peyer patches in the GI tract or from lymphoid tissue in the respiratory tract via the lymphatics to the breast. Cytokines cause isotype switching of local IgM^+ B cells to become IgA^+ B lymphocytes.[85,248,272] These isotype switched cells travel to the breast where they are transformed into plasma cells producing secretory, dimeric IgA. It is through this "enteromammary" pathway that the mother provides increased amounts of sIgA to the infant against the microorganisms present in the mother's and infant's environment.[261]

Brandtzaeg et al[22] have proposed a model for the transport of IgA (polymeric) and IgM (pentameric), produced by plasma cells, across the secretory epithelium with the formation of sIgA and IgM through binding with the secretory component attached to the epithelial membrane. This occurs in the membrane of mammary epithelial cells during lactation.[23,93]

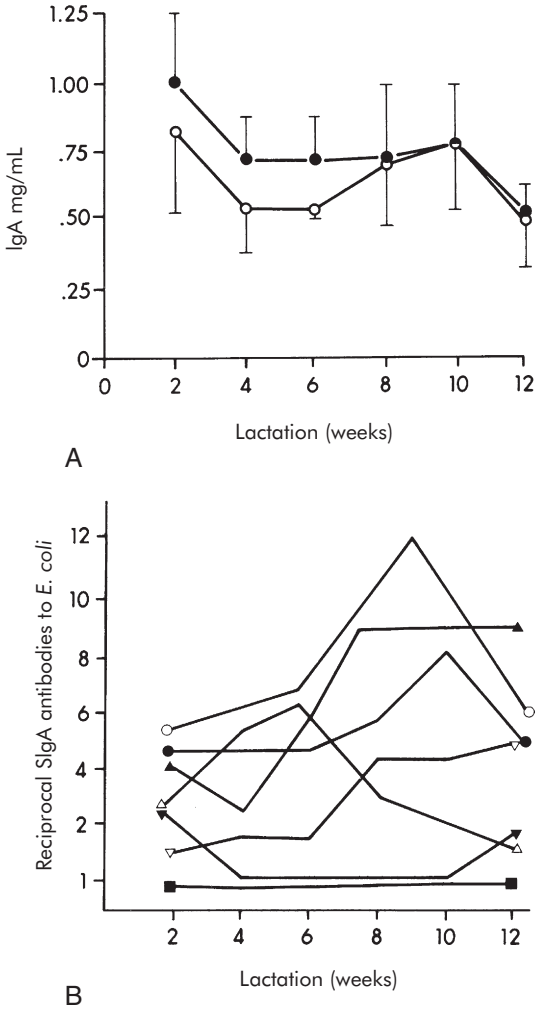

Figure 5-4. Same subjects in Figure 5-2 were examined during second through twelfth week of lactation. Total (•) and secretory IgA (○) data are presented as mean ± SD. The sIgA antibody titers to *E. coli* somatic antigens from each subject are represented by different symbols. **A,** Longitudinal study of total IgA and sIgA. **B,** Longitudinal study of reciprocal of sIgA antibody titers to *E. coli* somatic antigens in human milk. (From Goldman AS, Garza C, Nichols BL et al: Immunologic factors in human milk during the first year of lactation, *J Pediatr* 100:563, 1982.)

Quantitative determinations of immunoglobulins in human milk were made from milk collected at birth to as long as 27 months postpartum by Peitersen et al[213] and by Goldman et al.[92] The IgA content was high immediately after birth, dropping in 2 to 3 weeks, and then remaining constant. Similar observations were made on IgG levels and IgM levels. Ogra and Ogra[202,203] have compared serum and milk levels at various times postpartum. Samples obtained separately from the left and right breasts showed similar values. The levels remained constant during a given feeding and throughout a 24-hour period. In all quantitative determinations,

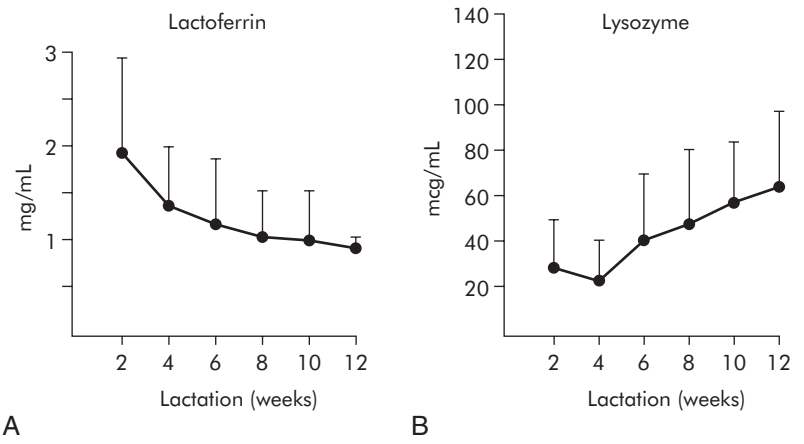

Figure 5-5. Same subjects in Figure 5-2 were examined during second through twelfth week of lactation. Data in longitudinal studies are presented as mean ± SD. **A,** Concentration of lactoferrin progressively decreased through first 8 weeks (r = 0.69) (2 vs. 8 weeks; $p < 0.02$), but not thereafter. **B,** In contrast, lysozyme levels steadily increased from fourth through twelfth week (r = 0.76) (4 vs. 12 weeks; $p < 0.01$). (From Goldman AS, Garza C, Nichols BL et al: Immunologic factors in human milk during the first year of lactation, *J Pediatr* 100:563, 1982.)

TABLE 5-2 Concentrations of Immunologic Components in Human Milk Collected During Second Year of Lactation

Component	Duration of Lactation (mo)		
	12	13-15	16-24
IgA (mg/mL)			
Total	0.8 ± 0.3	1.1 ± 0.4	1.1 ± 0.3
Secretory (sIgA)	0.8 ± 0.3	1.1 ± 0.3	1.1 ± 0.2
Lactoferrin (mg/mL)	1.0 ± 0.2	1.1 ± 0.1	1.2 ± 0.1
Lysozyme (μg/mL)	196 ± 41	244 ± 34	187 ± 33
sIgA antibodies (reciprocal titers to *E. coli* somatic antigens)	5 ± 6	9 ± 10	6 ± 3

From Goldman AS, Goldblum RM, Graza C: Immunologic components in human milk during the second year of lactiation, *Acta Paediatr Scand* 72:461, 1983.

Data are presented as the mean ± SD.

IgA is the predominant immunoglobulin in breast milk, constituting 90% of all the immunoglobulins in colostrum and milk.

Ogra and Ogra[202-204] studied the serum of postpartum lactating mothers and nonpregnant matched control subjects and noted that the individual and mean concentrations of all Ig classes were lower in the postpartum subjects. The levels were statistically significant for IgG; they were 50 to 70 mg higher in the nonpregnant women.

Immunoglobulin levels, particularly IgA and IgM, are very high in colostrum and drop precipitously in the first 4 to 6 days, but IgG does not show this decline. The volume of mammary secretion, however, increases dramatically in this same period; thus the absolute amounts of immunoglobulins remain more nearly constant than it would first appear. Local production and concentration of IgA and probably IgM may take place in the mammary gland at delivery.

IgE and IgD have also been measured in colostrum and milk. Using radioimmunoassay techniques, colostrum was found to contain concentrations of 0.5 to 0.6 IU/mL IgE in 41% of samples and less in the remainder.[10] IgD was found in all samples in concentrations of 2 to 2000 mg/dL. Plasma levels were poorly correlated. The findings suggest possible local mammary production rather than positive transfer. The question of whether IgE or IgD antibodies in breast milk have similar specificities for antigens as the IgA antibodies in milk remains unanswered.[171] Keller et al[139] examined the question of local mammary IgD production and its possible participation in a mucosal immune system by comparing colostrum and plasma levels of total IgD with specific IgD antibodies. From their work comparing colostrum/plasma ratios for IgG, IgD, and albumin and measuring IgD against specific antigens, the authors reported evidence for IgD participation in the response of the mucosal immune system, with increases in total IgD and IgD against specific antigens found in colostrum.

To address the question of total quantities of immunologic components secreted into human milk per day and available to an infant, Butte et al[36] measured the amounts of sIgA, sIgA antibodies to *E. coli*, lactoferrin, and lysozyme ingested per day and per kilogram per day in the first 4 months of life (Figures 5-6 through 5-10). Lactoferrin, sIgA, and sIgA antibodies gradually declined in amount ingested per day and per kilogram per day.

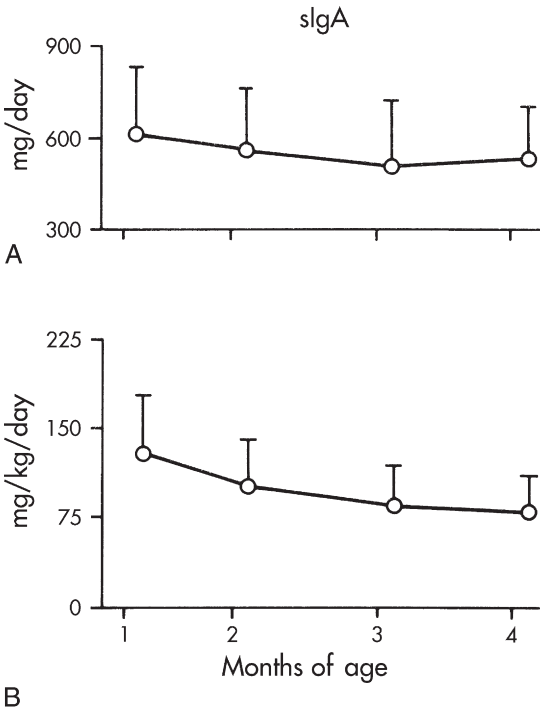

Figure 5-6. Amounts of sIgA and sIgA antibodies to *E. coli* somatic antigens in human milk ingested per day **(A).** Per kilogram per day **(B).** Data are presented as mean ± SD. (From Butte NF, Goldman RM, Fehl LM et al: Daily ingestion of immunologic components in human milk during the first four months of life, *Acta Paediatr Scand* 73:296, 1984.)

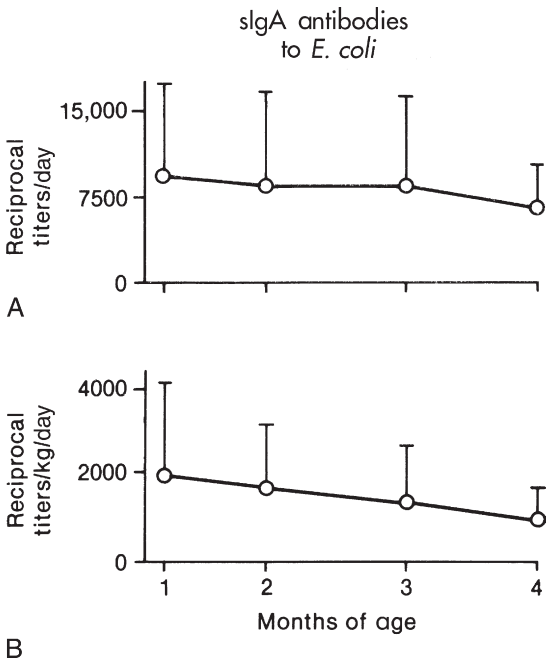

Figure 5-7. Amounts of sIgA antibodies to *E. coli* somatic antigens in human milk ingested as reciprocal titers per day **(A). (B).** Per kilogram per day. Data are presented as mean ± SD. (From Butte NF, Goldman RM, Fehl LM et al: Daily ingestion of immunologic components in human milk during the first four months of life, *Acta Paediatr Scand* 73:296, 1984.)

Lysozyme, in contrast, rose during the same period in total amount available and amount per kilogram per day. The authors[36] suggest that production and secretion of these immunologic factors by the mammary gland may be linked to the catabolism of the components at an infant's mucosal tissues. When the concentrations of sIgA, IgG, IgM, α_1-antitrypsin, lactoferrin, lysozyme, and globulins C3 and C4 were compared in relationship to parity and age of the mother, no consistent trend was observed. When maturity of the pregnancy was considered, however, mean concentrations of all these proteins were higher, except for IgA, when the delivery was premature. Because several proteins in human milk have physiologic function in infants, Davidson and Lönnerdal[53] examined the survival of human milk proteins through the GI tract. Crossed immunoelectrophoresis showed that three human milk proteins transversed the entire intestine and were present in the feces: lactoferrin, sIgA, and α_1-antitrypsin.

Miranda et al[176] reported on the effect of maternal nutritional status on immunologic substances in human colostrum and milk. Maternal malnutrition was characterized as lower weight-to-height ratio, creatine/height index, total serum proteins, and IgG and IgA. In malnourished mothers, the colostrum contained one third the normal concentration

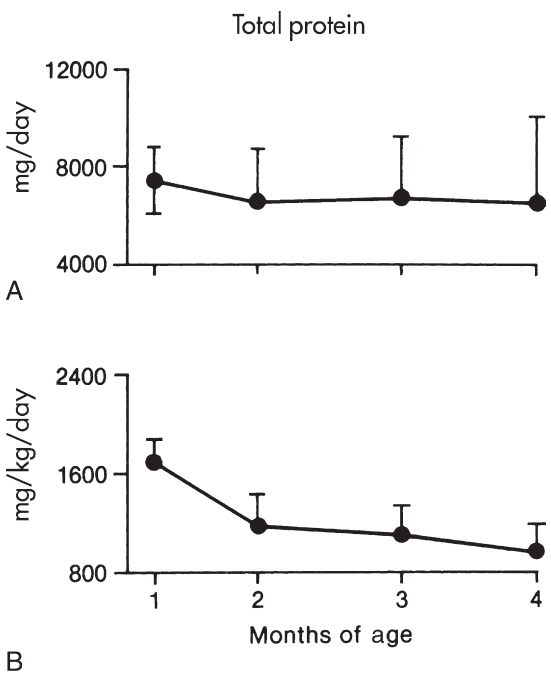

Figure 5-8. Amount of total protein in human milk ingested per day **(A). (B).** Per kilogram per day. Data are presented as mean ± SD. (From Butte NF, Goldblum RM, Fehl LM et al: Daily ingestion of immunologic components in human milk during the first four months of life, *Acta Paediatr Scand* 73:296, 1984.)

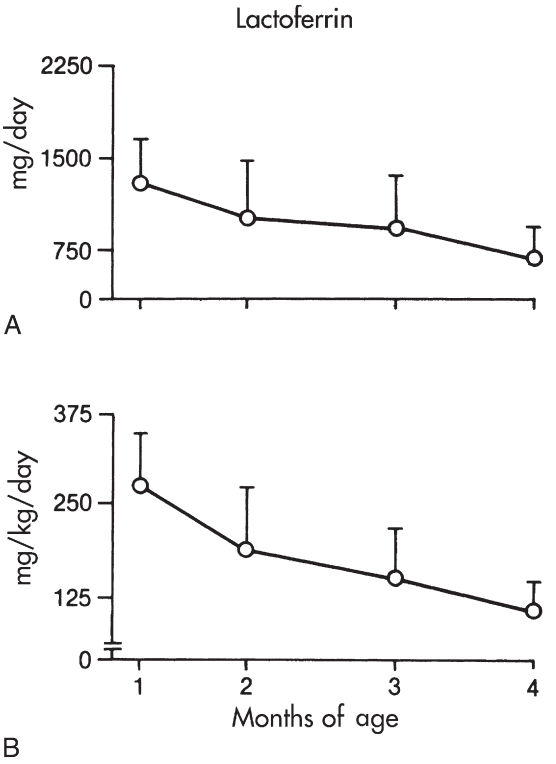

Figure 5-9. Amount of lactoferrin in human milk ingested per day **(A). (B).** Per kilogram per day. Data are presented as mean ± SD. (From Butte NF, Goldblum RM, Fehl LM et al: Daily ingestion of immunologic components in human milk during the first four months of life, *Acta Paediatr Scand* 73:296, 1984.)

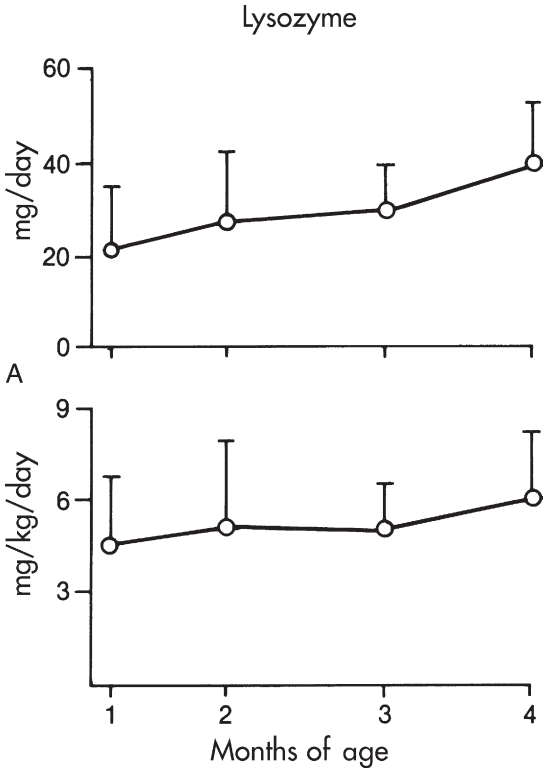

Figure 5-10. Amount of lysozyme in human milk ingested per day **(A). (B).** Per kilogram per day. Data are presented as mean ± SD. (From Butte NF, Goldblum RM, Fehl LM et al: Daily ingestion of immunologic components in human milk during the first 4 months of life, *Acta Paediatr Scand* 73:296, 1984.)

of IgG, less than half the normal level of albumin, and lower IgA and complement C4. Lysozyme, complement C3, and IgM levels were normal. Levels improved with development of mature milk and improvement in maternal nutrition. According to one report in 2003, moderate exercise during lactation does not affect the levels of IgA, lactoferrin, or lysozyme in breast milk.[165] Immunologic components contained in human milk during the second year of lactation become a significant point as more infants are nursed longer. For a longitudinal study of lactation into the second year by Goldman et al,[89] women were included who had fully breastfed their infants for 6 months to a year, and were continuing to partially breastfeed. Samples were collected by fully emptying the breast by electric pump. Table 5-2 summarizes the concentrations of the measured factors. No leukocytes were detected. Concentrations of total IgA and sIgA, lactoferrin, and lysozyme were similar to those 7 to 12 months postpartum and during gradual weaning. sIgA antibodies to *E. coli* were produced in the second year, demonstrating significant immunologic benefit to the infant with continued breastfeeding.[89] IgA, IgM, and IgG were measured in nursing women from the beginning of lactation and simultaneously in the feces of their children by Jatsyk et al[130] at the Academy of Medicine in Moscow. They reported IgA to be very high in the milk and rapidly increasing in the feces. IgG and IgM levels, however, were low in both milk and feces. In normal full-term bottle-fed infants, IgA appeared in the feces at 3 to 4 weeks of age but at much lower levels than in breastfed infants. Koutras[146] reported that in the first 8 weeks of life increased amounts of sIgA are found in the stools of breastfed infants compared with formula-fed infants. The authors ascribed this phenomenon to the presence of sIgA in human milk and a stimulation of the local GI production of immunoglobulin.

Savilahti et al[242] measured serum levels of IgG, IgA, and IgM in 198 infants at 2, 4, 6, 9, and 12 months of age. By 9 months, the exclusively breastfed infants had IgG and IgM levels significantly lower than those who had been weaned early (before 3.5 months) to formula. Six infants were still exclusively breastfed at 12 months, and their IgA levels had also lowered to levels found at 2 months with bottle feeders. Infection rates

were similar. Two months after the children were weaned to formula, the IgG and IgM levels were comparable. Iron and zinc levels were the same in all children.

SPECIFICITY OF IMMUNOGLOBULINS

sIgA antibodies have been identified in human milk that recognize a large variety of microorganisms. The sIgA antibodies that recognize bacteria, viruses, parasites and fungi are listed in Table 5-3. Some sIgA antibodies recognize various bacteria, including *E. coli, Shigella, Salmonella, Campylobacter pylori, V. cholerae, Haemophilus influenzae, Streptococcus pneumoniae,* streptococcus group B, type III, *Staphylococcus aureus, Clostridium difficile, Clostridium botulinum, Klebsiella pneumonia,* and *Listeria monocytogenes*. Some sIgA antibodies recognize *Entamoeba histolytica, Giardia, Strongyloides stercoralis,* and *Candida albicans*.[85,196] The list of viruses for which sIgA antibodies exist in human milk is equally long including enteroviruses (poliovirus, coxsackie, and echovirus), cytomegalovirus (CMV), herpes simplex virus, human immunodeficiency virus (HIV), Semliki Forest virus, respiratory syncytial virus (RSV), rubella, reovirus type 3, rotavirus, measles, Norovirus, and porcine coronavirus. IgG and IgM antibodies also exist in human milk against CMV, RSV, and rubella as well as IgE antibodies against parvovirus B19. Noguera-Obenza and Cleary[196] reviewed the role of breast milk sIgA in providing protection for infants against various agents specifically causing bacterial enteritis.

STABILITY OF IMMUNOGLOBULINS

Preservation of human milk at −20° C for up to 3 months does not decrease significantly the levels of IgA, IgG, IgM, C3, C4, lactoferrin, or lysozyme.[65,70,160,205] The preservation of sIgA, IL-6 and TNF-α with freezing at 4° C or −20° C was recently confirmed by Hines et al.[117]

A variety of different heat treatments have been applied to milk to protect against bacterial contamination or to protect against infection with specific infectious agents (especially HIV and CMV). Heat treatments include low-temperature, short-time 56° C for 15 minutes; Holder pasteurization 62.5° C for 30 minutes; high-temperature, short-time 70° to 73° C for 15 seconds; boiling 100° C for greater than 1 minute; sterilization, variable time periods, Pretoria pasteurization 56° to 62.5° C for approximately 15 minutes[131]; flash heating 56° C for approximately 6 minutes with a peak temperature at 72° C[127,128]; and microwave heating, with milk temperatures of 20° to 77° C for 30 seconds.[225] Boiling or sterilization essentially destroys 100% of immunologic activity. sIgA and lysozyme activities drop by 20% with Holder pasteurization and by 50% at 65° C. Neither low-temperature, short-time nor high-temperature, short-time reduces the sIgA or lysozyme content markedly. IgG and IgM are greatly reduced by Holder pasteurization.

sIgA differs antigenically from serum IgA. IgA can be synthesized in the nonlactating as well as in the lactating breast. It is a compact molecule and resistant to proteolytic enzymes of the intestinal tract and the low pH of the stomach. sIgA present in human milk is primarily manufactured by plasma cells in the mammary gland, modified in its translocation across the mammary epithelia and only minimally produced by the cellular lymphocytes in milk. Levels in milk are 10 to 100 times higher than in serum. Levels in cow milk are very low, that is, a tenth of the level in mature human milk (0.03 mg/dL). Later in life, the human intestinal tract's subepithelial plasma cells secrete IgA. The intestinal secretion of sIgA does not occur in the neonatal period but increases between 4 to 12 months of life.

Discussion continues as to whether any antibodies are absorbed from the intestinal tract, although probably 10% are absorbed. Almost 75% of ingested IgA from milk survives passage through the intestinal tract and is excreted in the feces. All immunoglobulin classes have been identified in the feces.[230] A large body of evidence demonstrates the activity of the immunoglobulins, especially IgA, at the mucosal level of the GI and respiratory tracts. These antibodies provide local intestinal protection against microorganisms, which may infect the mucosa or enter the body through the gut or respiratory tract.

Other Bioactive Factors

BIFIDUS FACTOR

It is well established that the predominant bacteria found in breastfed infants are bifid bacteria. Bifid bacteria are gram-positive, nonmotile, anaerobic bacilli. Many observers have shown the striking difference between the flora of the guts of breastfed and bottle-fed infants. György[100] demonstrated the presence of a specific factor in colostrum and milk that supported the growth of *Lactobacillus bifidus*. Bifidus factor has been characterized as a dialyzable, nitrogen-containing carbohydrate that contains no amino acid.

In vitro studies by Beerens et al[17] showed the presence of a specific growth factor for *Bifidobacterium bifidum* in human milk, which they called BB. Other milks, including cow milk, sheep milk, pig milk, and infant formulas, did not promote the growth of this species but did show some activity

TABLE 5-3 Antibodies in Human Milk

Factor	Shown, In Vitro, to Be Active Against:	Assay	Effect of Heat
Secretory IgA	Enteroviruses		
	Poliovirus types 1, 2, 3	ELISA, NA, Precipitin	Stable at 56° C for 30 min; some loss (0%-30%)
	Coxsackievirus types A_9, B_3, B_5	NA	Stable at 62.5° C for 30 min; destroyed by boiling
	Echovirus types 6 and 9	NA	
	Herpesvirus		
	CMV	ELISA, IFA, NA	
	Herpes simplex virus	NA	
	HIV		
	Semliki Forest virus	IFA	
	Respiratory syncytial virus	IFA	
	Rubella	IFA, HAI	
	Reovirus type 3	ELISA, NA	
	Rotavirus		
	Measles		
	Norovirus		
	Escherichia coli (EIEC, EAEC, EPEC)		
	Shigella		
	Salmonella		
	Campylobacter		
	Vibrio cholerae		
	Haemophilus influenzae type b		
	Streptococcus pneumoniae		
	Clostridium difficile		
	Clostridium botulinum (toxin B16S)		
	Clostridium perfringens enterotoxin A		
	Klebsiella pneumoniae		
	Streptococcus group B, type III		
	Listeria monocytogenes		
	Staphylococcus aureus		
	Staphylococcal toxic shock syndrome toxin-1		
	Staphylococcal enterotoxin C		
	Helicobacter pylori		
	Entamoeba histolytica		
	Strongyloides		
	Giardia		
	Candida albicans		
IgM, IgG	CMV		Stable at 56° C for 30 min; IgG decreased by a third at 62.5° C for 30 min
	Respiratory syncytial virus		
	Rotavirus		
	Rubella		
IgE	Parvovirus B19	ELISA	

CMV, Cytomegalovirus; *EAEC,* enteroadherent *E. coli*; *EIEC,* enteroinvasive E.coli; *ELISA,* enzyme-linked immunosorbent assay; *EPEC,* enteropathogenic E.coli; *HAI,* hemagglutination inhibition; *HIV,* human immunodeficiency virus; *IFA,* immunofluorescent assay; *NA,* neutralizing assay.

supporting *B. infantis* and *B. longum*. This growth factor was found to be stable when the milk was frozen, heated, freeze-dried, and stored for 3 months. Growth-promoting factors were present for the six strains studied, which varied in their resistance to physical change. Because all these factors were active in vitro, they did not require the presence of intestinal enzymes for activation. It has not been possible to show the presence of this growth factor in other mammalian milks; thus it may contribute to the implantation and persistence of *B. bifidum* in a breastfed infant's intestine.

Lactobacillus has been described as one of a number of probiotic bacteria, which provide an immune protective benefit to their host. *Lactobacillus* reportedly stimulates antibody production and improves phagocytosis by blood leukocytes.[135,215] The use of probiotic bacteria has reportedly produced benefits in a variety of situations associated with infections. The addition of such bacteria to formula is another example of trying to make formula better by making it more like breast milk. Hatakka et al[112] examined the possible effect of adding probiotic bacteria to formula on the occurrence of infection in children attending day care. They reported modest reductions in the number of children with complicated respiratory infections or lower respiratory tract infections as well as the number of children receiving antibiotics for a respiratory infection in the group of children receiving formula supplemented with *Lactobacillus rhamnosus* GG compared with children receiving unsupplemented formula.

Resistance Factor

It was well known in the preantibiotic era that human milk protects human infants throughout lactation against staphylococcal infection. György[100] identified the presence of an "antistaphylococcal factor" in experiments with young mice that had been stressed with staphylococci. This factor, with no demonstrable direct antibiotic properties, was termed resistance factor and described as nondialyzable, thermostable, and part of the free fatty acid part of the phosphide fraction, probably C18:2, but distinct from linoleic acid.

Lysozyme

Human milk contains a nonspecific antimicrobial factor, lysozyme, which is a thermostable, acid-stable enzyme. This enzyme is a 130-amino-acid-containing glycoprotein that can hydrolyze the 1-4 linkage between N-acetylglucosamine and N-acetylmuramic acid in bacterial cell walls. It is found in large concentrations in the stools of breastfed infants and not in stools of formula-fed infants; thus it is thought to influence the flora of the intestinal tract.

Goldman et al[92] describe an initial fall in lysozyme levels from 85 to 90 mg/mL to 25 mg/mL at 2 to 4 weeks and then an increase during 6 months to 250 mg/mL (see Figure 5-5). Lysozyme levels show an increase over time during lactation; this finding is more apparent in Indian women than in those of the Western world. Reddy et al[229] studied the levels of lysozyme in well-nourished and poorly nourished women in India and found no difference between them (Table 5-4). As shown in this study, lysozyme levels increase during lactation. Levels in human milk are 300 times the level in cow milk. Lysozyme is bacteriostatic against Enterobacteriaceae and gram-positive bacteria.[219] It is secreted by neutrophils and some macrophages and is present in many body secretions in the adult.

In a study of immunologic components in human milk in the second year of lactation, Goldman et al[89] reported that concentrations of lysozyme, lactoferrin, and total and sIgA were similar to those in uninterrupted lactation and in gradual weaning at 6 to 9 months. sIgA antibodies to *E. coli* were also produced during the second year. The authors state that "this supports the idea that the enteromammary lymphocyte traffic pathway, which leads to the development of lymphoid cells in the mammary gland that produce IgA antibodies to enteric organisms, operates throughout lactation."[89] When cow milk formula is added to human milk, it reduces the effect of lysozyme; however, powdered human milk fortifier (Enfamil) did not inhibit the antiinfective properties.[141]

Lactoferrin

Lactoferrin is an iron-binding protein closely related to the serum iron transport protein, transferrin, and is part of the larger transferrin protein family. Lactoferrin is found in mucosal secretions (tears, saliva, vaginal fluids, urine, nasal and bronchial secretions, bile, GI fluids) and notably in milk and colostrum. A bacteriostatic effect of lactoferrin is well established for a wide range of microorganisms, including gram-positive and gram-negative aerobes, anaerobes, viruses, parasites, and fungi. The original proposed mechanism of action for its bacteriostatic effect was depriving the microorganism of iron. A second antibacterial action involving direct action with bacterial surfaces; binding negatively charged molecules (lipoteichoic acid) on the surface of gram-positive bacteria neutralizing the surface charge allowing the action of other antibacterial factors like lysozyme or binding lipid A on gram-negative bacteria, releasing the lipid, producing damage to the cell membrane. Another antibacterial action is binding bacterial adhesions blocking

TABLE 5-4 Antibacterial Factors in Colostrum and Mature Milk in Well-Nourished and Undernourished Indian Women

Group	Hemoglobin (g/dL)	Serum Albumin (g/dL)	Immunoglobulins (mg/dL)			Lysozyme (mg/dL)	Lactoferrin (mg/dL)
			IgA	IgG	IgM		
Colostrum (1 to 5 days)							
Well-nourished women	11.5 ± 0.37	2.49 ± 0.065	335.9 ± 37.39 (17)*	5.9 ± 1.58 (17)	17.1 ± 4.29 (17)	14.2 ± 2.11 (15)	420 ± 49.0 (28)
Undernourished women	11.3 ± 0.60	2.10 ± 0.081	374.3 ± 42.13 (10)	5.3 ± 2.30 (10)	15.3 ± 2.50 (10)	16.4 ± 2.39 (21)	520 ± 69.0 (19)
Mature milk (1 to 6 months)							
Well-nourished women	12.8 ± 0.43	3.39 ± 0.120	119.6 ± 7.85 (12)	2.9 ± 0.92 (12)	2.9 ± 0.92 (12)	24.8 ± 3.41 (10)	250 ± 65.0 (17)
Undernourished women	12.6 ± 0.56	3.47 ± 0.130	118.1 ± 16.2 (10)	5.8 ± 3.41 (10)	5.8 ± 3.41 (10)	23.3 ± 3.53 (23)	270 ± 92.0 (13)

From Reddy V, Bhaskaram C, Raghuramula N et al: Antimicrobial factors in human milk, *Acta Paediatr Scand* 66:229, 1977.
*Figures in parentheses indicate number of samples analyzed.

host cell interaction.[94] Lactoferrin can kill *Candida albicans* and *C. krusei* by changing the permeability of the fungal cell surface. Lactoferrin now is considered a multifunctional, immunoregulatory protein.

The biologic role of lactoferrin has been reviewed in several studies.[163,164,198,241] They point out that lactoferrin reversibly binds two ferric ions and that its affinity for iron is 300 times greater than that of transferrin, retaining iron down to a pH of 3. Human lactoferrin is strongly basic. Lactoferrin is normally unsaturated with iron,[35] and it is usually less than 10% saturated with iron in human milk.[72,241] Oral iron therapy for an infant can interfere with the bacteriostatic action of lactoferrin, which depends on its unsaturated state for some portion of its bacteriostatic function. Reddy et al[229] showed that giving iron to the mother did not interfere with the saturation of lactoferrin in the milk or thus its potential bacteriostatic effect. Protein energy malnutrition, rather than iron supplies, influences lactoferrin synthesis in the mammary gland. Malnourished but non–iron-deficient mothers are lactoferrin deficient.

The concentration of lactoferrin is high in colostrum—600 mg/dL—then progressively declines over the next 5 months of lactation, leveling at about 180 mg/dL. Breast milk also contains small amounts of transferrin (10 to 15 mg/mL). Lactoferrin is 10% to 15% of the total protein content of human milk.[163] Lactoferrin is resistant to proteolysis, especially in its iron-saturated form. Intact lactoferrin is detectable in the stool of infants, with higher proportions of lactoferrin measurable in the stool of premature infants.[56] Both intact lactoferrin and fragments have been detected in the urine of premature infants, although absorption is less likely in full-term infants.[107] The absorption of iron from breast milk is directly enhanced by lactoferrin.[164]

Many bacteria require iron for normal growth, and one bacteriostatic effect of lactoferrin has been ascribed to its iron-binding action. In neutrophils, lactoferrin within neutrophilic granules tightly binds iron, but neutrophils with excessive iron are inefficient at destroying bacteria. Lactoferrin does not limit the growth of all microorganisms; *Helicobacter pylori* and *Neisseria, Treponema,* and *Shigella* species all have receptors for lactoferrin, directly binding iron and allowing adequate growth.

Some evidence supports various other proposed mechanisms of action for lactoferrin's antimicrobial effect. Lactoferrin has been shown to limit the formation of biofilms by specific organisms, inhibit adhesion to host cells by other organisms and to directly bind to viral particles of herpes simplex virus, HIV, and adenovirus. A proteolytic action of lactoferrin appears to inactivate virulence factors of some organisms. Separately, lactoferrin binds directly to glycosaminoglycans (GAGs) and integrins interrupting the binding of various viruses (herpes simplex virus, HIV, adenovirus, CMV, hepatitis B virus [HBV]) to host cells. Pepsin hydrolysate products of lactoferrin (B or H) may exert a direct bactericidal effect by binding to lipopolysaccharide of gram-negative organisms and disrupting bacterial membranes.[263] Lactoferrin may cause an increased release of cytokines by cells including antiinflammatory cytokines such as IL-10.[50,157] Others have shown that lactoferrin suppresses the release of IL-1, IL-2, IL-6, IL-8, and TNF-α, all

proinflammatory cytokines, which would be more of an immune-modulating effect.[157] Other investigators using a recombinant human lactoferrin (talactoferrin) demonstrated evidence of lactoferrin causing increased maturation of DCs[252] and talactoferrin causing the recruitment and activation of neutrophils and macrophages [233] as other examples of how lactoferrin affects the innate immune protection of the growing infant. Several other effects have been proposed for lactoferrin, including inhibition of hydroxyl radical formation, decreasing local cell damage; lipopolysaccharide binding, also leading to a diminished inflammatory response; and DNA binding, affecting transcription and possibly regulation of the production of cell products.[198] Activation of natural killer (NK) cells, modulation of complement activity, and blocking of adhesion of enterotoxigenic *E. coli* and *Shigella flexneri*[98] are other proposed actions of lactoferrin.

A specific region of lactoferrin, near the N terminus of the molecule, is strongly basic and is reported to mediate some of lactoferrin's antimicrobial activity. "Lactoferricins," small peptides containing this basic region, produced by proteolytic cleavage reportedly bind to lipopolysaccharide, leading to disruption of the bacterial cell wall and cytoplasmic membrane.[263]

In another area of immune protection, lactoferrin may limit cancer development.[157] The proposed mechanisms of its anticancer effects includes increasing NK cell cytotoxicity, increased production of IL-18 and inhibition of angiogenesis, augmented apoptosis of cancer cells and initiation of cell cycle arrest in growing tumor cells.[157]

The multiple roles and proposed mechanisms of action of lactoferrin in breastfed infants continue to be more specifically elucidated.

Interferon

Colostral cells in culture have been shown to be stimulated to secrete an interferon-like substance with strong antiviral activity up to 150 National Institutes of Health units/mL.[219] This property has not yet been identified in the supernatant of colostrum or milk. Interferon-γ has been produced by T cells from human milk when stimulated in vitro.[219] The T cells isolated from human milk were the CD45RO phenotype and have been identified as a source of interferon. Srivastava et al[254] have measured low levels of interferon-γ in not only colostrum but also transitional and mature milk. They postulated that the low level of interferon-γ (0.7 to 2 pg/mL) might be adequate to protect against infection without hyperactivation of T cells. Interferon is produced by NK cells and by T cells, phenotypically Thy0 and Thy1. It can cause increased expression of major histocompatibility complex molecules, increase macrophage function, inhibit IgE and IL-10 production, and produce antitumor and antiviral activity. The exact role of interferon-γ in breast milk has not been delineated.

Complement

The C3 and C4 components of complement, known for their ability to fuse bacteria bound to a specific antibody, are present in colostrum in low concentrations compared with their levels in serum. IgG and IgM activate complement. C3 proactivator has been described, and IgA and IgE have been identified as stimulating the system. Activated C3 has opsonic, anaphylactic, and chemotactic properties and is important for the lysis of bacteria bound to a specific antibody. No functional role for complement in breast milk has been identified.

Vitamin B_{12}-Binding protein

Unsaturated vitamin B_{12}-binding protein of high molecular weight has been found in very high levels in human milk and in the meconium and stools of breastfed infants compared with its levels in infant formulas and infants who are formula fed. The protein binding renders the vitamin B_{12} unavailable for bacterial growth of *E. coli* and *Bacteroides*.[99]

Glycans and Oligosaccharides

Glycans are complex carbohydrate structures attached to various other structures (a lactose moiety, a lipid component, peptides, proteins, or aminoglycans) that are present in large amounts in human milk.[192] They include glycoproteins, glycolipids (gangliosides), glycosaminoglycans, mucins, and oligosaccharides. Oligosaccharides are composed of a basic core structure derived from glucose, galactose, or N-acetylglucosamine and are linked to a variety of terminal fucose linkages or sialic acid linkages to create numerous different compounds. Oligosaccharides compose the major portion of glycoconjugates in milk and are present in the milk-fat globule membrane and in skim milk.[188,192] Gangliosides are glycolipids found in the plasma membrane of cells, especially in cells in the gray matter of the brain. More specifically, gangliosides are glycosphingolipids that contain sialic acid, hexoses, or hexose amines as the carbohydrate component and ceramide as the lipid component of the molecule. The predominant gangliosides in human milk are GM1, GM2, GM3, and GD3, as reported by Newburg.[190] A diverse abundance of these complex carbohydrates are synthesized by the many glycosyltransferases contained in the mammary gland. Mucin and lactadherin are two glycoproteins included in this group

that have antimicrobial effects.[238] Some of these carbohydrate molecules are structurally similar to glycans on the surface of small intestine epithelial cells that act as receptors for microorganisms. One proposed mechanism for the antimicrobial effect of these soluble substances is direct binding with the potential pathogenic organisms.[189,190] Schroten et al[247] proposed that mucins contained in the human milk fat-globule membrane can block bacterial adhesion throughout the intestine after studying the adhesion of S-fimbriated *E. coli* to buccal epithelial cells.

Gangliosides appear to be responsible for blocking the activity of heat-labile enterotoxin from *E. coli* and the toxin from *Vibrio cholerae* in rat intestinal loop preparations.[207] Another toxin from *Campylobacter jejuni*, with similar binding specificity, also seems to be inhibited by GM1.[151,235] Globotriaosylceramide, another glycolipid in human milk, is the natural cell surface receptor for the toxin from *Shigella dysenteriae* and verotoxin released by enterohemorrhagic *E. coli*.[191] The proposed mechanism of action of these glycolipids is that by binding to the toxin they form a stable complex that prevents the toxin from binding to the appropriate receptors on intestinal cells; however, Crane et al[48] proposed from their studies that the oligosaccharide binds to the toxin receptor to block the action of the heat-stable enterotoxin of *E. coli*. Human milk gangliosides may be important in protecting infants against toxin-induced diarrhea, but this has not been specifically demonstrated in vivo in controlled trials.[191,207] Evidence exists that human milk glycans inhibit a broad range of pathogens (Table 5-5).[188-193] Newberg et al[191] document the constitutive expression of various fucosylated glycans in human milk and secretions and present "typical" concentrations of these active agents in human milk from the literature. Their secretion is related to the "secretor" and Lewis genes, which control the individual differences in expression of Lewis blood group types.

Chaturvedi et al[44] have recently examined the survival of oligosaccharides from human milk in infants' intestines. They demonstrated that the concentrations of oligosaccharides were higher in the infants' feces than in mothers' milk and higher in feces than urine. The profile of oligosaccharides found in the infants was similar to that found in their mothers' milk. The formula-fed infants had lower concentrations of oligosaccharides and the profiles of the oligosaccharides were different from those found in the breastfed infants. The oligosaccharides remained intact passing through the intestine. A small percentage are absorbed and excreted intact in the urine. The oligosaccharides were available at these sites to block intestinal and urinary pathogens. Two other groups of researchers have documented variation of the composition of glycans in human milk over the first 4 months of lactation[47] and variations in the composition of glycans in diverse populations.[64] Therefore a diverse repertoire of glycans are present in large amounts in human milk, which persist intact in the intestine and reach the urine, and have demonstrated inhibitory effects on a variety of pathogens. These components constitute a major contribution of human milk to innate immunity at the level of an infant's gut.

TABLE 5-5 Nonimmunoglobulin Antipathogen Factors in Human Milk

Antipathogen	Pathogen
Ganglioside GM_1	Cholera toxin
	Labile toxin of *Escherichia coli*
	Toxin of *Campylobacter jejuni*
Globotriaosylceramide	*Shigella* toxin I
	Shigalike toxin of *E. coli*
GM3	Enteropathogenic *E. coli*
Fatty acids	Enveloped viruses
	Giardia lamblia
Chondroitin sulfate	HIV
Sulfatide	HIV
Glycoprotein (mucin)	Inhibition: rotavirus in vitro and in vivo
Glycoprotein (mucin, glycosaminoglycan)	HIV
Lactadherin	Rotavirus
Mucin	Adherence: S-fimbriated *E. coli*
MUC 1	Poxviruses, HIV
Glycoprotein (mannosylated)	*E. coli* intestinal adherence
Large macromolecule	Respiratory syncytial virus
Macromolecule-associated glycans	Norovirus, *P. aeruginosa*
Oligosaccharides	Adherence: *Streptococcus pneumoniae* and *Haemophilus influenzae*, enteropathogenic *E. coli*
	Listeria monocytogenes
Fucosylated oligosaccharide	Adherence, invasion, *C. jejuni*, stabie toxin of *E. coli* stable toxin in vivo, *Vibrio cholera*
Sialyllactose	Cholera toxin, *E. coli*, *P. aeruginosa*, influenza virus
	Aspergillus fumigates, Polyomavirus, *Helicobacter pylori*

Modified from Newburg DS, Ruiz-Palacios GM, Morrow AL: Milk glycans protect infants against enteric pathogens, *Ann Rev Nutr* 25:37-58, 2005.

GM, Granulocyte-macrophage; *HIV,* human immunodeficiency virus.

Other authors propose that the gangliosides GD3 and GM3 may play an immunomodulatory role early in lactation by affecting DCs decreasing the production of interleukins (IL-10 and IL-12) and suppressing the expression of various cluster designation (CD) markers and major histocompatability complex class II on DCs.[26]

Interleukins

Interleukins (ILs) are considered a "subgroup" of cytokines.[161] Originally, when cytokines were first hypothesized, it was thought that they were primarily produced by leukocytes and acted on other leukocytes, and therefore they could be called ILs. Although much of their effect is on lymphocyte activation and differentiation, it is now known that ILs act on and are produced by a variety of cells.[91]

Goldman et al[91] identified IL-1β, IL-6, IL-8, and IL-10 in breast milk (Table 5-6). Srivastava et al[254] reported measuring moderate amounts of IL-6, IL-8, and IL-10 in the different stages of breast milk. Very low amounts of IL-1β were detected, especially in comparison with the amount of IL-1 receptor antagonist (RA), which presumably could block the activity of the small amount of IL-1. Hawkes et al[113] reported on the amount of cytokines in breast milk over the first 12 weeks of lactation. The proposed "proinflammatory" cytokines, IL-1β, IL-6, and TNF-α, were present in only 7 of 36 mothers who donated samples at each point throughout the study. A broad range of concentrations of each of these cytokines was seen during the course of the study. The "antiinflammatory" cytokines, transforming growth factor (TGF) -α_1 and TGF-β_2, were present in significant amounts in all samples. IL-2 has also been reported in breast milk in 81% of the mothers tested, with milk (aqueous) levels correlating with plasma IL-2 levels. IL-2 was constitutively produced from 57% of milk cell samples and IL-2 production was markedly increased by stimulation of the cells with Con A.[29]

TABLE 5-6 Bioactivity and Concentrations of Cytokines in Human Milk

Agents	Bioactivity in Milk	Concentrations*
IL-1β	±	1130 ± 478
IL-6	+	151 ± 89
IL-7	?	79-100 ± 19†
IL-8	?	3684 ± 2910
IL-10	+	3400 ± 3800
TNF-α	+	620 ± 183
G-CSF	?	~358
M-CSF	+	17,120
Interferon-γ	?	?
EGF	+	~200,000
TGF-α	+	~2200-7200
TGF-β_2	+	130 ± 108

From Goldman AS, Chheda S, Garofalo R, Schmalstieg FC: Cytokines in human milk properties and potential effects upon the mammary gland and the neonate, *J Mammary Gland Biol Neoplasia* 1:251, 1996.

*The concentrations of these agents were determined by enzyme-linked immunosorbent assay (ELISA) except for IL-1β and EGF by radioimmunoassay. Concentrations are expressed as pg/mL except for M-CSF (U/mL).

†From Ngom PT, Collinson AC, Pido-Lopez J et al: Improved thymic function in exclusively breastfed infants is associated with higher interleukin 7 concentrations in their mothers' breastmilk, *Am J Clin Nutr* 80:722-728, 2004.

CSF, Colony-stimulating factor; *EGF*, epidermal growth factor; *G*, granulocyte; *IL*, interleukin; *M*, macrophage; *TGF*, transforming growth factor; *TNF*, tumor necrosis factor.

IL-6 has been identified in breast milk by other investigators, especially in the first 2 days of life.[209,234] The authors suggest that IL-6 in human milk may augment the newborn's immune functions before the body can begin full production of cytokines. Specifically, this is accomplished by increasing antibody production, especially IgA; enhancing phagocytosis; activating T cells; and increasing α_1-antitrypsin production by mononuclear phagocytes. IL-7 is a chemokine known to improve thymic output in animals and appears related to the proliferation and survival of T cells in all stages of development.[194] Ngom et al[194] have described improved thymic function in exclusively breastfed infants associated with higher IL-7 concentrations in the mother's breast milk. The breast milk of Gambian mothers contained variable levels of IL-7, but the geometric mean levels were higher in the first 8 weeks postpartum in mothers whose infants were born in the "harvest-season" (January to June) compared with those mothers whose infants were born in the "hungry-season." The authors postulate that IL-7 in breast milk enhances T-cell proliferation and survival and overall thymic development in the infant, leading to long-term benefits in protection from infection.

IL-8 is a chemokine capable of attracting and activating neutrophils and attracting $CD45RA^+$ T cells. IL-8 is produced by mammary epithelial cells.[209] Srivastava et al[254] also detected messenger ribonucleic acid (mRNA) for IL-8, suggesting that cells in breast milk were capable of producing IL-8. The exact function of IL-8 in breast milk remains to be elucidated.

IL-10 is thought to have antiinflammatory effects, including decreasing the production of interferon-γ, IL-12, and other proinflammatory cytokines. It has been reported to enhance IgA, IgG, and IgM synthesis.

IL-18 has been identified in colostrum, early milk, and mature milk with the highest levels occurring in colostrum and in association with preterm deliveries and complications of pregnancy in the mothers.[260] The levels of IL-18 were correlated with soluble Fas ligand in colostrum. IL-18 was detected by immunohistochemical staining in actively secreting epithelial cells in a lactating breast. IL-18 has been shown to be produced by intestinal epithelial cells and activated macrophages. It leads to the production of other chemokines (GM-CSF, IL-2, TNF-α). It induces the expression of Fas ligand on lymphocytes. The authors suggested that IL-18 present in colostrum may play a role in stimulating a systemic T_H1 response and causing NK cell and macrophage activation in neonates.

The interaction and the direct effect of these ILs in breast milk must be clarified. The amount of T cells bearing markers of recent activation is increased in human milk compared with the results in peripheral blood of adults. Wirt et al[276] have described a marked shift from virginal to antigen-primed (memory) T cells in human milk, which suggests certain functional capacities for these cells. The phenotypic pattern of T cells may result from T-cell-activating substances or selective homing of T cells to the breast. These activated T-cell populations are transferred to the infant through breast milk along with a variety of ILs at a time when infants are capable of only limited production of ILs. A complex interaction of ILs and cells in human milk and at the mucosal level may provide antimicrobial and antiinflammatory benefits to the infant.

Cytokines

Of the many bioactive substances that have been identified in human milk, cytokines are some of the most recently identified and investigated agents, although their existence has long been suspected in attempts to explain certain immunologic and protective effects of breast milk on infants. More than 40 cytokines have been described,[166] and more than 10 of these have been identified in human milk.[91,254] Cytokines are small proteins or glycoproteins that, through binding to receptors on immune and nonimmune cells, produce a broad range of effects (many still unidentified) through autocrine, paracrine, and endocrine actions. Cytokines are produced predominantly by immune cells and function in complex associations with other cytokines to stimulate and control the development and normal functioning of the immune system. The nomenclature and abbreviations used are complicated and confusing. Newer systems of classification have been established according to which cells produce them or what their general functions are[161] or based on the relative position of their cysteine residues or their receptor types (CCR, CXCR, CX3CR).[166] Box 5-3 provides a simplified list with abbreviations.

BOX 5-3. Nomenclature and Abbreviations for Various Cytokines

Interferon alpha, beta, gamma	IFN-α,-β, -γ
Granulocyte colony-stimulating factor	G-CSF
Macrophage colony-stimulating factor	M-CSF factor
Stem cell factor	SCF
Interleukins 1, 2, 4, 6, 8, 10	IL-1, -2, -4, -6, -8, -10
Interleukin 1 beta	IL-1β
Interleukin 1 receptor antagonist	IL-1RA
Soluble interleukin 2 receptor	sIL-2R
Transforming growth factor $beta_2$	TGF-β_2
Tumor necrosis factor alpha	TNF-α
Transforming growth factor alpha	TGF-α
Macrophage inflammatory protein	MIP
Regulated on activation, normal T cell expressed and secreted	RANTES
Epidermal growth factor	EGF
Growth-regulated oncogene	GRO
Monocyte chemoattractant protein 1	MCP-1
Leukocyte inhibitory factor	LIF

Little evidence demonstrates specific in vivo activity of the different cytokines. Based on general information on the function and interaction of the particular cytokines, as well as consideration of as yet unexplained effects of breast milk, proposed functions of the cytokines include initiation of development of host defense, stimulation of host defenses, prevention of autoimmunity, antiinflammatory effects in the upper respiratory tract and GI tract, and stimulation of the development of the digestive system, especially the mucosal immune system of the alimentary tract and the proximal respiratory tract. The maternal breast may respond to feedback stimulation or suppression by secreted cytokines, influencing the growth, differentiation, and secretory function of the breast. As shown in other situations, cytokines may enhance receptor expression on cells in the respiratory and GI tracts for major histocompatibility complex molecules or immunoglobulins. Various cell types in the mucosal immune system may be activated or attracted to specific sites in the GI tract by the action of cytokines.

Beyond these proposed beneficial effects of cytokines, newer studies are identifying specific

immunologic and protective roles for different cytokines in developing infants. For example, extensive work has been done on epidermal growth factor (EGF) and other growth factors (HB-EGF, G-CSF, EPO, and EPO-like growth factors) have been studied relative to their role in preventing necrotizing enterocolitis (NEC) and gut homeostasis.[183] A number of potential roles for EGF in gut homeostasis have been proposed and studied, including intestinal development, proliferation and adaptive response to damage, repair and regeneration and diminishing inflammatory responses to various stimuli. TGF-β has been studied for its role in initiating and stimulating IgA production early on in infancy.[200]

The actual measurement of cytokines in breast milk has been complicated by a number of factors, including different assays used (bioassays, enzyme-linked immunosorbent assay [ELISA], radioimmunoassay), binding to proteins, their existence in monomeric or polymeric forms,[8] the presence of antagonists, and their varying presence in colostrum, early milk, or mature milk. Goldman et al[91] reported on the bioactivity and concentration of cytokines in breast milk from their own work and that of others (see Table 5-6). Srivastava et al[254] obtained some conflicting results using different assays in colostrum, early milk, and mature milk. They confirmed the presence of M-CSF throughout lactation, as well as TGF-β_1 and -β_2, IL-1RA, GRO-α, MCP-1, RANTES, and IL-8, but reported insignificant amounts of GM-CSF, stem cell factor, LIF, MIP-1α, IL-2, IL-4, IL-11, IL-12, IL-13, IL-15, sIL-2R, and IFN-α (see Box 5-3 for nomenclature). Srivastava et al[254] also used reverse transcriptase polymerase chain reaction to measure the production of cytokine mRNA by cells in breast milk. They reported the presence of mRNA for MCP-1, IL-8, TGF-β_1, TGF-β_2, M-CSF, IL-6, and IL-1β, which may be another source of these cytokines in breast milk. Hawkes et al[113] demonstrated that human milk cells from lactating women at 5 weeks postpartum are capable of active cytokine production in vitro (IL-1β, IL-6, TNF-α) with and without exposure to lipopolysaccharide. Continued cytokine production by human milk cells is another explanation for the variable amounts of cytokines identified in breast milk and is further evidence that the cells are capable of responding to an infectious stimulus.

In their investigations of the possible antiinflammatory effects of breast milk, Buescher and Malinowska[32] examined milk for the presence of soluble receptors and cytokine antagonists. They demonstrated soluble intercellular adhesion molecule 1, soluble vascular cell adhesion molecule 1, and soluble E-selectin in colostrum and at lower levels in mature milk, as well as high levels of soluble TNF-α receptor I (sTNF-αRI), sTNF-αRII, and IL-1RA. Also, they identified that most TNF-α did not exist "free" in breast milk but was associated with TNF receptors. The in vivo significance of these findings remains to be assessed.

Given the complex interaction and regulation of cytokine production and cytokines' relation to coordinated inflammatory and antiinflammatory responses in tissues, one should assume that the interaction of cytokines in breast milk and the effect of cytokines, cytokine receptors (soluble and expressed on various cell types), and cytokine antagonists on the infant will be equally complex. A new methodology, antibody-based protein arrays has been applied to identify cytokines in human milk.[148] Kverka et al[148] analyzed colostrums and milk samples from the first 4 days postpartum using two different arrays capable of detecting 42 and 79 cytokines. Three cytokines (EGF, IL-8/CXCL8, GRO/CXCL1-3) were detected in all of the tested samples. Nineteen cytokines were present in more than 50% of the samples. An additional 32 cytokines were identified in human milk for the first time. The concentration of cytokines varied in the different women and varied over time. Continued investigation with this and other assays will be essential to understanding the significance and specific effects of these substances in breast milk.

Nucleotides

Nucleotides, nucleosides, nucleic acids, and related metabolic products are essential to many biologic processes. Although they are not essential nutrients because they can be synthesized endogenously and recovered from in vivo "salvage" sources, their presence in the diet may carry significant benefits under various conditions (i.e., "conditionally essential").[42,184,266] In situations of disease, stress, rapid growth, or limited dietary intake, supplementation of the diet with nucleotides may decrease energy expenditure to synthesize or salvage nucleotides, which optimizes the host response to these adverse situations.

Nucleotides exist in relatively large amounts in human milk, 15% to 20% of the nonprotein nitrogen, suggesting that they have some nutritional significance, although no clinical syndromes have been associated with nucleotide deficiency to date. Nucleotides are present in the natural milk of different species in varying amounts and composition. The nucleotide content and composition of bovine milk are particularly less and different from human milk. Infant formulas supplemented with nucleotides contain roughly the same amounts of nucleotides as

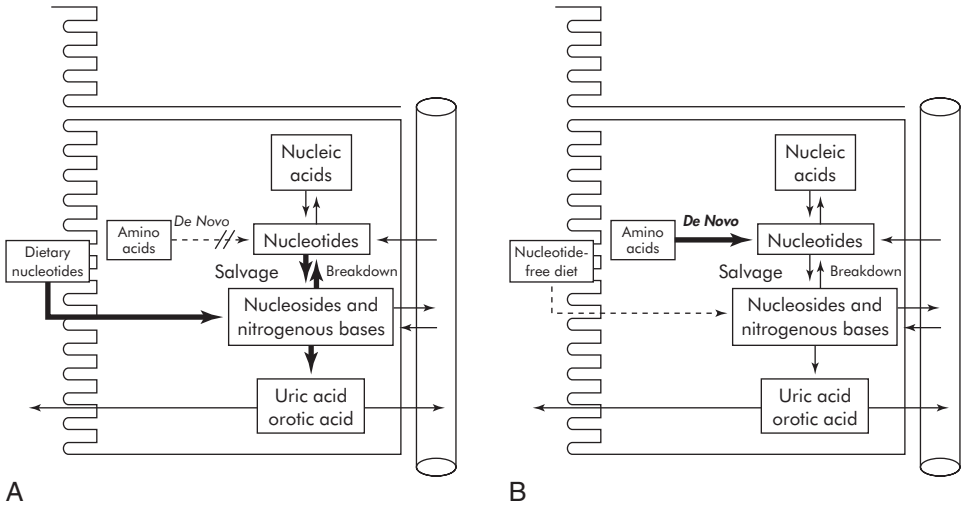

Figure 5-11. Metabolic regulation of cellular nucleotide pools in presence and absence of nucleotide in diet. **A,** Effect of dietary nucleotide activating salvage pathway. **B,** De novo nucleotide synthesis is enhanced with nucleotide-free diet. (From Quan R, Barness LA: Do infants need nucleotide supplemented formula for optimal nutrition? *J Pediatr* 11:429, 1990.)

human milk, from 20 to 70 mg/L.[39,40,154] Unsupplemented formulas contain less and different amounts of nucleotides.

Mammalian cells contain a large variety of nucleotides and related products, which have many metabolic functions, including the following[40-42]:

1. Energy metabolism: adenosine triphosphate is a major form of available cellular energy.
2. Nucleic acid precursors: the monomeric units for RNA and DNA are present.
3. Physiologic mediators: cyclic adenosine monophosphate and cyclic guanosine monophosphate serve as "messengers" for cellular processes; adenosine diphosphate is necessary for platelet aggregation; and adenosine has been shown to affect vasodilatation.
4. Related products function as coenzymes in metabolic pathways: nicotinamide-adenine dinucleotide, flavin adenine dinucleotide, and coenzyme A.
5. Related products function as intermediate carrying molecules in synthetic reactions: uridine diphosphate glucose in glycogen synthesis and guanosine diphosphate mannose, guanosine diphosphate-fucose,uridinediphosphate-galactose, and cytidine monophosphate sialic acid in glycoprotein synthesis.
6. Allosteric effectors: the intracellular concentrations of nucleotides influence the progression of certain steps of metabolic pathways.
7. Cellular agonists: extracellular nucleotides influence intracellular signal transduction (e.g., cyclic adenosine monophosphate and inositol-calcium pathway).

Nucleotide concentrations in cells and tissues are maintained by de novo synthesis and salvage from intermediary metabolism and diet (Figure 5-11).[224] Nucleosides are the predominant product absorbed in the small intestine. Nucleosides are probably transported by passive diffusion and a carrier-mediated process; purines and pyrimidines are transported by passive diffusion at high concentrations and by a sodium-dependent active mechanism at low concentrations (Figure 5-12).[224] The digestion and absorption of nucleotides, nucleosides, and pyrimidines and purines also involve polymeric and monomeric nucleotides and other adducts (nucleosides in a biologically active moiety).

In early reports on the nucleotide and nucleoside content of milk, various methods of measurement were used, and the amounts were described as either the monomeric fraction of nucleotides or the total RNA. Leach et al,[154] recognizing the complex nature of digestion and absorption of nucleotides and related products, attempted to measure the total potentially available nucleosides (TPANs) in human milk using solid-phase extraction, high-performance liquid chromatography analysis, and enzymatic hydrolysis of the various fractions. They analyzed breast milk samples at various stages throughout lactation (colostrum; transitional, early, and late mature milk) from 100 European women and 11 American women. They used an aqueous TPAN-fortified solution containing ribonucleosides, 5′-mononucleotides, polymeric RNA, and nucleoside-containing adducts to estimate the accuracy of their process.

The mean ranges of TPAN values were similar for European women from different countries and American women, although broad ranges were seen and the composition of individual nucleotides

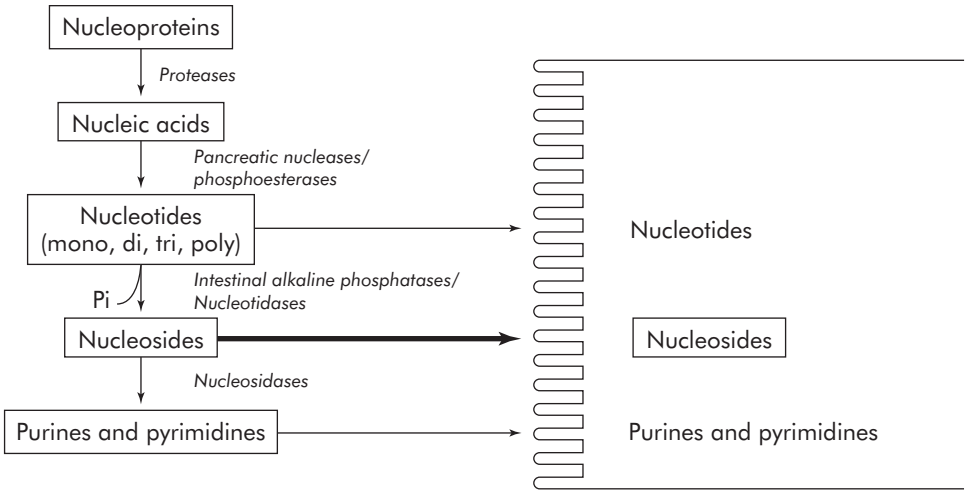

Figure 5-12. Digestion and absorptiion of nucleic acids and their relational products. (From Quan R, Barness LA: Do infants need nucleotide supplemented formula for optimal nutrition? *Pediatr Gastroenterol Nutr* 11(4):429, 1990.)

varied.[154] The mean TPAN value was lowest in colostrum but did not show a consistent upward or downward trend in transitional, early, or late mature milk. The mean ranges of TPAN values were 82 to 164 mmol/L for colostrum, 144 to 210 mmol/L for transitional milk, 172 to 402 mmol/L for early mature milk, and 156 to 259 mmol/L for late mature milk (Table 5-7). Monomeric and polymeric nucleotides were the predominant forms of TPAN in pooled samples. Cytidine, guanosine, and adenosine were found mainly in these fractions, whereas uridine was found primarily as free nucleotide and adduct (Table 5-8). The methods used recovered 90% to 95% of the true TPAN values compared with the TPAN-fortified solution, although the uridine and guanosine content was underestimated. Tressler et al[264] measured the TPAN in pooled breast milk samples from Asian women demonstrating average levels in colostrum, transitional milk, and mature milk and found it to be similar to the levels in European and American women.

Leach et al[154] concluded that their process of estimating TPANs, including sequential enzymatic hydrolyses, and measuring the entire nucleotide fraction provides a reasonable estimate of the in vivo process and the nucleotides available to the infant from human milk.

Proposed effects of dietary nucleotides include effects on the immune system, iron absorption, intestinal flora, plasma lipoproteins, and growth of intestinal and hepatic cells. Effects on the immune system, related to nucleotide supplementation to the diet, have mainly been reported from animal studies and include increased mortality rate from graft-versus-host disease, improved delayed-type cutaneous hypersensitivity and alloantigen-induced lymphoproliferation, reversal of malnutrition and starvation-induced immunosuppression, increased resistance to challenge with *S. aureus* and *C. albicans*, and enhanced T-cell maturation and function.[218] Spleen cells of mice fed a nucleotide-free diet produce lower levels of IL-2, express lower levels of IL-2 receptors, and have decreased NK cell activity and macrophage activity.[40,41] Presumably these nucleotide-associated changes are related to T-helper/inducer cells and the initial phases of antigen processing and lymphocyte proliferation.[41,42,266]

In vitro and in vivo experiments have documented that ingested nucleotides increased iron absorption, perhaps affecting xanthine oxidase.[224] Although in vitro studies showed that added nucleotides enhanced the growth of bifidobacteria, conflicting results have been obtained on the influence of dietary nucleotides on the fecal flora of infants receiving breast milk or nucleotide-supplemented formula.[11,224] Clinical studies in infants receiving nucleotide-supplemented formula demonstrated increased high-density lipoprotein cholesterol, lower very-low-density lipoprotein cholesterol, increased long-chain polyunsaturated fatty acids, and changes in red blood cell membrane phospholipid composition.[11] Supplementation studies in animals have shown enhanced GI tract growth and maturation, improved intestinal repair after diarrhea, stimulation of hepatic growth, and augmented recovery from hepatectomy.[218]

A recent review discusses the effects of dietary nucleotides on the immune system and protection against infection reported in studies in the literature.[244] Carver et al[41] compared infants receiving breast milk to those receiving commercially available infant formula and formula supplemented with nucleotides at a level of 32 mg/L. At 2 and

TABLE 5-7 Nucleotide and Total Potentially Available Nucleoside (TPAN) in Pooled Human Milk by Stage of Lactation (μmol/L)*

	Uridine	Cytidine	Guanosine	Adenosine	TPAN
Colostrum					
Site 1	27	84	22	20	153
Site 2	21	33	15	13	82
Site 3	30	82	26	26	164
Site 4	24	84	20	22	150
Mean	26	71	21	21	137
Transitional milk					
Site 1	23	82	22	19	146
Site 2	33	76	19	17	144
Site 3	37	84	43	42	206
Site 4	36	100	36	38	210
Mean	32	86	30	29	177
Early mature milk					
Site 1	30	86	28	28	172
Site 2	50	79	23	21	173
Site 3	44	96	36	37	214
Site 4	67	146	91	97	402
Mean	48	102	45	46	240
Late mature milk					
Site 1	36	73	22	25	156
Site 2	58	106	29	27	219
Site 3	49	81	20	24	173
Site 4	45	124	40	49	259
Mean	47	96	28	31	202
Grand mean	38	88	31	32	189
SD	13	24	18	20	70
Range	21-67	33-146	19-92	13-97	84-402
American pool†	37	70	30	24	161

From Leach JL, Baxter JH, Molitor BE et al: Total potentially available nucleosides of human milk by stage of lactation, *Am J Clin Nutr* 61:1224, 1995.

*Data from 100 individual samples collected at four sites and combined into 16 pooled samples (5 to 7 individual samples per site per stage of lactation). *Site 1*, Rouen and Mount Saint Aignau, France; *Site 2*, Mainz, Germany; *Site 3*, Bolzano, Italy; *Site 4*, Treviso, Italy.

†Pooled sample of milk collected from 11 American women between 2 and 4 months postpartum.

TABLE 5-8 Percentage of Total Potentially Available Nucleoside (TPAN) in Pooled Human Milk as Adducts, Polymeric Nucleotides, Monomeric Nucleotides, and Nucleosides*

	Uridine	Cytidine	Guanosine	Adenosine	TPAN
Polymeric nucleotides	19 ± 7	57 ± 12	59 ± 21	47 ± 11	48 ± 8
Monomeric nucleotides	36 ± 12	37 ± 13	34 ± 14	35 ± 10	36 ± 10
Nucleosides	18 ± 14	5 ± 5	1 ± 2	5 ± 4	8 ± 6
Adducts†	27 ± 12	1 ± 1	7 ± 15	13 ± 9	9 ± 4

From Leach JL, Baxter JH, Molitor BE et al: Total potentially available nucleosides of human milk by stage of lactation, *Am J Clin Nutr* 61:1224, 1995.

*$x \pm$ SD. Based on the mean of entire pool of human milk collected from 100 individuals at four stages of lactation at four sites.

†Adducts are of the form nucleoside-phosphate-phosphate-X, where X is a biologically relevant moiety (e.g., uridine diphosphate-galactose or nicotinamide-adenine dinucleotide).

4 months, NK cell activity and IL-2 production were higher in the breastfed and nucleotide-supplemented groups compared with those receiving formula without nucleotide supplements. Infections occurred infrequently in all groups, but slightly less in the breastfed group. No differences were noted in hematologic profiles and plasma chemistry values, and no toxicity or intolerance was associated with nucleotide supplementation. The sample size was small, marked variability was seen in the IL-2 measurements, and the differences noted at 4 months were less than at 2 months. Therefore the authors concluded that dietary nucleotides may contribute to improved immunity in breastfed infants.

Brunser et al[28] examined the effect of a nucleotide-supplemented formula on the incidence of diarrhea in 392 infants in Chile, studied through 6 months of age. Although the infants receiving the supplemented formula (20 mg/L) experienced less diarrhea, the difference in the duration of diarrhea was small. The numbers were too small to comment on the causative agents of diarrhea, although no apparent protection against any one agent was seen. The beneficial effect of nucleotides against diarrhea was proposed to be secondary to enhanced immune response to intestinal pathogens or improved intestinal integrity or a combination of both. In a larger study of 3243 infants younger than 6 months of age, the severity of the diarrhea (duration and number of bowel movements) as well as the incidence of diarrhea was lower in the nucleotide supplemented group.[152]

Two groups of premature infants fed either nucleotide supplemented (20 mg/L) or unsupplemented formula were followed, measuring the concentration of plasma immunoglobulins throughout the first 3 months of life.[184] IgG plasma concentrations were not different in the two groups during the study period. IgM plasma levels were higher in the nucleotide supplemented group at 20 to 30 days and 3 months of life, while IgA plasma levels were significantly higher at 3 months of age in the supplemented group.

Pickering et al[218] published a 12-month, controlled, randomized study of 311 infants to examine the effect of added nucleotides at levels comparable to human milk on infants' immune responses to various vaccine antigens; 103 nonrandomized infants received breast milk for at least 2 months and then either human milk or a standard infant formula. Another 208 infants were randomized to receive either a standard infant formula or one supplemented with nucleotides. The amount and actual nucleotide content added were based on TPANs, as measured by Leach et al,[154] equaling 72 mg/L. Overall growth and nutrition tolerance were similar in each group. The nucleotide group had significantly higher geometric mean titers of *H. influenzae* type b antibody and diphtheria antibody than the control group or the breastfed infants. No significant difference was seen between the nucleotide and control groups for the IgG response to oral poliovirus vaccine or tetanus. Infants who are breastfed for longer than 6 months had significantly higher antibody responses to oral poliovirus vaccine than children breastfed for less than 6 months or either of the two formula-fed groups. No significant differences were found between the different groups with respect to total IgG, IgA, or IgE. Differences were seen in the number of children who experienced at least one episode of diarrhea: the nucleotide group (4/27, 15%) versus the control group (13 of 32, 41%, $p < 0.05$) and the breastfed group (6 of 27, 22%). Notably, the breastfed group was heterogeneous relative to the amount of breast milk received and the duration of feeding, whereas the nucleotide group received supplementation for the entire 12 months.

Questions that remain concerning nucleotides and their proposed beneficial effects in an infant's diet include the following:

- What are the proven mechanisms of action of these proposed benefits?
- What form and concentration of nucleotides are necessary to effect these benefits?
- Is adequate information available to justify using nucleotides in infant formula in higher amounts and different compositions than are currently used?

Debate and research to answer these and other questions concerning nucleotides will continue.

Mucosal Immune System

A primary function of each of the body's different mucosal surfaces is immunologic. Each distinct mucosal surface has multiple other physiologic functions including gas exchange (in the lungs), nutrient absorption (in the gut), sensory detection (in the eyes, nose and mouth) and reproduction (in the uterus and vagina). The thin, permeable nature of these barrier mucosal surfaces, their large surface area and the constant exposure to microorganisms, foreign proteins, and chemicals predisposes the mucosal membranes to damage and infection. During the first year(s) of life, when the infant's immune system is developing and maturing, it is doing so on a systemic and a mucosal basis as well as involving both innate and adaptive immune mechanisms. That development must include the ability to respond to and protect against invasive pathogens at the same time as "tolerate" or "ignore" the multitude on commensal organisms that reside at these surfaces. During this early development, breast milk contains numerous bioactive factors that supplement the immune protection at the mucosal level while limiting

inflammation and contribute to the immune modulation and growth stimulation of infants' mucosal and systemic immune defenses.

The mucosal immune system involves both innate mechanisms and adaptive immune mechanisms functioning in concert. The development of the mucosal immune system occurs in the prenatal period and continues in the postnatal period. The functional mucosal barrier includes the action of enzymes, chemicals, acidity or pH, mucus, immune globulins and indigenous flora. In as early as 8 weeks of gestational age, researchers have identified changes in the intestinal barrier with the development of enterocytes, goblet cells and enterochromaffin cells along with evidence of development of tight junctions between the epithelial cells.[211,222] Mucus production, which can block adherence of pathogens to epithelial cells, demonstrates both pre- and postnatal development beginning with evidence of expression of the *muc2* gene as early as 12 weeks' gestational age.[34] This is approximately the same time that Paneth cells appear in intestinal crypts. These cells secrete various products, including α-defensin, lysozyme, secretory phospholipase A_2, and TNF-α, which contribute to protection from pathogens, stem cell protection within the epithelia layer and influence the selection and number of commensal organisms.[180,240] Secretory immune globulins sIgA and IgM act at the epithelial surface, largely without inflammation, by limiting adherence and transmigration and facilitating phagocytosis of potential pathogens.

Mucosal-Associated Lymphoid Tissue

The well-recognized MALT is present in localized areas beneath the mucosal surfaces: tonsils and adenoids in the nasopharynx and Peyer patches and isolated lymphoid follicles in the intestine. Overlying the isolated lymphoid follicles of the gut are specialized epithelial cells called M-cells. M-cells (membrane, microfold, or multifenestrated cells) come in direct contact with microorganisms and antigens due to a lack of a surface glycocalyx covering. These remarkable cells endocytose, phagocytose and transcytose molecules, and antigens from their luminal surface to their basal surface. Antigen-presenting cells and lymphocytes process the trancytosed molecules, presenting them to submucosal aggregates of lymphocytes. The activated lymphocytes that have responded to the specific presented antigens migrate via the lymphatics to the thoracic duct and into the blood. These lymphocytes circulate in the blood, until they return to mucosal tissues, predominantly the same ones they originated from, where they now function as effector lymphocytes in the lamina propria. This process of "directed migration" to specific sites occurs due to the influence of cytokines and adhesion molecules, such as chemokine CCL28 (mucosal epithelia chemokine) expressed in the colon and salivary glands and CCL25 (thymus-expressed chemokine) which effects the site-specific migration.[217] The immune response of lymphocytes in the submucosa and the subsequent directed migration to the same and other mucosal sites produces a focused response to a selected repertoire of antigens at those sites. The lactating mammary gland is an essential component of MALT. A mother's mature effective immune response to microorganisms in her and her infant's environment through this antigenic stimulation of MALT in the mother's gut and respiratory mucosa and the directed migration of cells to the breast results in activated lymphocytes and antibodies in the breast milk providing protection to the infant against those microorganisms. This is a well-recognized example of how breast milk can provide additional immune protection to the infant. It is also one of the reasons to continue breastfeeding when a mother or the infant have a possible infection.

The mucosal immune system undergoes significant postnatal development, in part due to the dramatic exposure of the mucosa to large numbers of microorganisms in early postnatal life. Peyer patches are rudimentary, and few immunoglobulin-producing intestinal plasma cells are present until several weeks after birth.[24] After several weeks, germinal centers within the lymphoid follicles develop, and the number of IgM- and IgA-producing cells in the intestine increase. Immunoglobulin-producing intestinal plasma cells (primarily IgA-producing cells) in the lamina propria increase in number from 1 to 12 months of age.[257] With normal maturation of the mucosal immune system, large numbers of immunoglobulin-producing cells locate in the intestinal lamina propria. The monomeric IgA produced by these plasma cells is transported through epithelial cells to the mucosal lumen. Attachment of an epithelial glycoprotein, the membrane secretory component to two IgA molecules leads to the formation of a dimeras; the sIgA molecule is "secreted" at the mucosal surface. IgM, in the form of a pentamer, contains a polypeptide J-chain and is transported by the same mechanism.[25] A portion of the secretory component remains attached to the sIgA and IgM, which protects these molecules against proteolysis and contributes to their stability. Large amounts of sIgA and IgM are produced, in a similar fashion, by the mammary glands and delivered to the infant via breast milk. The sIgA and IgM remain stable in saliva and feces[105] and provide specific protection by blocking adherence and entry and facilitating inactivation, neutralization, and agglutination of a wide variety of microorganisms.

Distinct from the action of immunoglobulins, a large number of bioactive factors in breast milk act at the mucosal level to supplement the innate defenses.[103] These include lactoferrin, lysozyme, casein, oligosaccharides, glycoconjugates and lipids. Mucin-1, lactadherin, and a glycosaminoglycan are antimicrobial components, which are part of the milk-fat globule. Free-fatty acids and monoglycerides, digested components of the milk-fat globule, can cause lysis of enveloped viruses, bacteria, fungi, and protozoa. Lauric and linoleic acids, which constitute a large percentage of the FFE in human milk, are two such acids produced by lipolysis in the stomach.[104]

Additional factors contained in breast milk with demonstrated activity at the level of the mucosa include cytokines, hormones and growth factors. IL-10 and IFN-γ act by influencing epithelial barrier integrity.[74] Other factors that are considered to contribute to mucosal growth and development are TGF-α, EGF, and hormones (insulin and insulin-like growth factor).[56] Many other factors contained in breast milk have the potential for activity at the level of the mucosa, including nutrients, vitamins, nucleotides, enzymes, and soluble molecules with receptor-like structures (soluble CD14, soluble toll-like receptor 2)[150,155,267]

Toll-Like Receptors

Toll-like receptors (TLRs) and the complex interaction between indigenous bacterial flora and the intestine is an important aspect of research into the development of the mucosal immune system. Forchielli and Walker [69] have reviewed many of these immune mechanisms acting at the mucosal level. TLRs are transmembrane receptors (pattern recognition receptors) that are capable of detecting and discriminating among various groups of potential pathogens and initiate different immune responses to them. TLRs "recognize" pathogen-associated molecular patterns, conserved features in the pattern of molecules expressed by pathogens and commensal organisms. Specific TLRs recognize a particular repertoire of patterns: TLR2 identifies bacterial lipoproteins and peptidoglycan molecules; TLR3 recognizes double-stranded DNA and TLR4 identifies lipopolysaccharide. Ten TLRs are recognized in humans to date; some have identified legends (pathogen-associated molecular patterns from viruses, bacteria, and protozoa) to which they bind. TLRs are present on some epithelial cells, but are predominantly expressed on macrophages and DCs.[228] Intestinal epithelial cells are influenced by gut flora and local immune response to express specific TLRs. The recognition of specific antigens by epithelial cell TLRs stimulates different intracellular signal pathways that lead to different T-lymphocyte immune responses. It has been postulated that the ongoing immune stimulation elicited by the microbial flora in the gut "programs" the host to predominately express different T-helper cell responses: T_H1-like, T_H2-like and T_H3-like. This is referred to as "crosstalk" between the indigenous intestinal flora and the body's immune system. The T_H1-like response is described as delayed-type hypersensitivity or cellular immunity; characterized by the predominant release of IL-2, IL-12 and interferon-γ. The T_H2-like is primarily humoral immunity, antibody production (especially IgE) associated with ILs: IL-4, IL-5, and IL-6. The T_H3-like response is related to oral tolerance and antiinflammatory effects in association with the release of IL-10 and TGF-10. A theoretical "ideal" for this system is the ability of the host to respond to various stimuli with balanced protection against the microbial invasion without excessive inflammation or damage to the host. An imbalanced (or poorly regulated) response of this system could result in an allergic reaction against food proteins (T_H2 excess) or an autoimmune inflammatory response against self-antigens (T_H1 excess).[69]

Ongoing research continues to explore these molecular mechanisms, their potential contribution to allergy, autoimmune disease and normal immune function development within a fetus, infant, and young child. The role of breast milk in the development of the systemic and mucosal immune systems takes on new significance when considering these concepts and mechanisms. This is especially true when examining the role of breast milk in adding to the innate and adaptive immune response at the level of the mucosa, the effect breast milk has on the respiratory and intestinal microbiota, the purported antiinflammatory effects of breast milk, and the proposed influence of breast milk on the maturational development of the intestine. Vorbach, Capecchi, and Penninger[270] postulated that the mammary gland evolved from a protective immune gland as part of the innate immune system. They present a list of various protective molecules that are part of both mucosal secretions and human milk. They discuss how specific nutritional factors in human milk have dual functions: nutritional and protective. This highlights the dual role of the breast as a nutritional and immune organ and should stimulate further research into the breast's role in innate immunity as a component of the mucosal immune system.

Microbiota, Probiotics, and Prebiotics

Investigation into the microbial colonization of the intestinal tract has exploded. Much of this investigation has been driven by new molecular techniques

involving the analysis of ribosomal RNA sequences of microbes that might not have been identified by traditional culture techniques. The diversity of the microbiota (all the microbes which colonize the GI tract) can be viewed from different perspectives based on the technical methods used.[255] Probiotics have been broadly defined as microorganisms that can exist within a host while affording benefits for the organisms and the host. Prebiotics are substances that (through different mechanisms) increase the growth and survival of probiotic bacteria within the host. Commonly recognized probiotic bacteria are *Lactobacillus rhamnosus GG, Bifidobacteria infantis, Streptococcus thermophilus, Bacillus subtilis*, *Saccharomyces boulardii,* and *Bifidobacteria bifidus.* Many more organisms are considered to be probiotic, some of which are commercially available.[178] Prebiotics are predominantly nondigestible oligosaccharides that ferment within the colon changing the ambient pH and producing small-chain fatty acids. Breast milk with its significant composition of oligosaccharides functions as a prebiotic source for an infant, facilitating the growth of bifidobacteria and lactobacilli.[52,249] Ongoing research is exploring the potentially mutually beneficial relationship between the microbes and the host with particular attention to nutrition (the availability of nutrients, energy sources, and synthesis of vitamins as influenced by the microbes), the developing GI tract (including angiogenesis and mucosal barrier repair),[75,186,210] the maturation of mucosal immunity,[140,216] both the innate system[119] and adaptive system,[170] and the bioavailability and metabolism of drugs and chemicals in the GI tract.[57,121] Specific proposed mechanisms of how probiotic bacteria and prebiotic substances contribute to an infant's developing immune system include competition with pathogenic bacteria for colonization, strengthening the tight junctions to enhance the mucosal barrier, producing antimicrobial bacteriocidins, stimulating mucus production, stimulating peristalsis, influencing the secretion of sIgA, stimulating the crosstalk interaction between intestinal cells and colonizing bacteria to affect the mucosal immune development, and increasing the production of certain cytokines (IL-10 and interferon-γ).[75,140,214,216]

Gastrointestinal Commensal Organisms

The microbial colonization of an infant's intestinal tract begins at birth, with organisms from the maternal flora being the first colonizers. Numerous additional factors directly influence the composition of the intestinal microbiota in early infancy, including gestational age, ingestion of breast milk or formula, initiation of solid foods, mode of delivery, the route of delivery of food, the time of onset of feeding, exposure to other microbes through contact (with family, animals, persons from other environments), antibiotics, and intercurrent or chronic illness.[66,215] The predominant flora of breastfed infants are *Lactobacillus bifidus* and *Bifidobacterium* spp., which constitute up to 95% of the culturable organisms. The remaining minority of bacteria include *Streptococcus, Bacteroides, Clostridium, Micrococcus, Enterococcus*, *E. coli,* and other uncommon organisms in small numbers.[178,186,282] *Lactobacillus bifidus* metabolizes milk saccharides, producing large amounts of acetic acid, lactic acid, and some formic and succinic acids, which create the low pH of the stool of breastfed infants. *L. bifidus* also produces short-chain fatty acids in the course of colonization. Large numbers of bifidobacteria can lower the pH of the intestine, which limits the growth of some pathogens such as *E. coli, Bacteroides,* and staphylococci. The flora of bifid bacteria is inhibitory to certain pathogenic bacteria. Substantial clinical evidence is available to demonstrate protection against intestinal infections from *S.aureus, Shigella, Salmonella, Vibrio cholerae, E. coli,* rotavirus, *Campylobacter,* and protozoa.[81] Two facilitory actions of breast milk are apparent. The first encourages the growth of *L. bifidus* and thus crowds out the growth of other bacteria. In the second, the number of pathogens is also kept low by the direct action of lysozyme and lactoferrin. When the number of pathogenic bacteria is kept low, immune antibodies can keep the growth of potentially pathogenic bacteria under control and limit the invasion of bacteria through the gut wall into the bloodstream.

The intestinal flora of formula-fed infants is made up of predominantly gram-negative bacteria, especially coliform organisms, *Bacteroides,* and including *Clostridium, Enterobacter,* and *Enterococcus*.[282]

Studies have demonstrated that potentially four distinctly different "microhabitats" for microflora within the GI lumen, within the mucus layer, separately within crypt mucus, and directly on the surface of the intestinal epithelium. The significance of the microhabitats and the effect of specific microorganisms has yet to be determined.[75] At weaning, the facultative anaerobes decline in number and obligate anaerobes *(Bacteroides)* become the predominant organisms in the intestine. Preterm infants are colonized with different types and numbers of bacteria from full term infants. The environment of neonatal intensive care units (NICUs), including incubators, widespread use of antibiotics and parenteral nutrition, and illness influence the microbial colonization. The short- and long-term effects of the different and changing GI microbiota are a concern.[186,206] This is particularly true when

one considers the contributing factors or causes for sepsis, NEC, chronic lung disease, or poor neurologic outcome in NICUs.

The question of a causal role of intestinal microflora and the development of NEC in premature and very-low-birth-weight (VLBW) infants has been proposed.[123,174] Gewolb et al[79] suggested that a low percentage of *Bifidobacterium* and *Lactobacillus* in the stool of VLBW infants within the first month of life is a risk factor for infection. Some studies on the use of probiotics and the occurrence of NEC have demonstrated a lower incidence of NEC in infants receiving probiotics.[19,124,162] Here again, the use of mothers' breast milk for premature infants and VLBW infants decreases the risk to the infant of sepsis, NEC, and infection-related events.[73,245] In a controlled prospective study of high-risk, low-birth-weight infants in India using donor human milk, significantly fewer infections and no major infections were found in the group receiving human milk, although the control infants experienced diarrhea, pneumonia, septicemia, and meningitis.[185]

Effectiveness of Human Milk in Controlling Infection

The properties of human milk do appear to control or limit infections in infants. Hundreds of articles[168,185] have been written about the protective effect of breastfeeding, including the recent Agency for Healthcare Research and Quality publication on Breastfeeding and Maternal and Infant Health Outcomes in Developed Countries from 2007. [126] Using evidence-based analyses, the report documents the decreased risk for acute otitis media, GI infections, and lower respiratory tract diseases in breastfed infants in developed countries.[126]

Protection Against Bacterial Infection

Breast milk IgA has antitoxin activity against enterotoxins of *E. coli* and *V. cholerae* that may be significant in preventing infantile diarrhea. Antibodies against O antigen of some of the most common serotypes of *E. coli* were found in high titer in breast milk samples collected from healthy mothers in Sweden. The infants who had consumed reasonable amounts of breast milk with high titers of *E. coli* antibodies had antibodies in their stool.[106] Protection against cholera in breastfed children by antibodies in breast milk was studied by Glass et al.[81] A prospective study in Bangladesh showed cholera antibody levels to vary in the colostrum and milk. The correlation among colonization, disease, and milk antibodies led the authors to conclude that breast milk antibodies against cholera do not protect children from colonization with *V. cholerae*, but they do protect against disease.

Salmonella infection was similarly studied by France et al[71] to evaluate the immunologic mechanisms in host colostrum and milk specific for salmonellae. Vigorous responses of colostral and milk cells against these organisms and nonspecific opsonizing capacity of the aqueous phase of colostrum and milk were demonstrated.

Gothefors et al[95] showed that *E. coli* isolated from stools of breastfed infants differed from strains found in formula-fed infants in two respects. First, *E. coli* strains were more sensitive to the bactericidal effect of human serum. Second, and more often, spontaneously agglutinated bacteria from other sites, such as the prepuce or periurethral area, were less sensitive in breastfed infants. These findings support the theory that breast milk favors proliferation of mutant strains, which have decreased virulence. This mutation of bacterial strains is another way breastfeeding may protect against infection.

It has been suggested that "milk immunization" is a dynamic process because a mother's milk has been found to contain antibody to virtually all her infant's strains of intestinal bacteria. The mother exposed to the infant's microorganisms through either the breast or the gut responds immunologically to those microorganisms and thus directly provides protection for her immunologically immature infant.

The orderly review of data on the presence of antibodies in human milk has produced a substantial list of affected organisms. In addition to *E. coli*, antibodies to *Bacteroides fragilis, Clostridium tetani, Haemophilus pertussis, Diplococcus pneumoniae, Corynebacterium diphtheriae, Salmonella, Shigella, Chlamydia trachomatis, V. cholerae, S. aureus,* and several strains of *Streptococcus* (Table 5-9) have been identified. Noguera-Obenza and Cleary[196] have summarized the contribution of sIgA in breast milk in protecting infants from bacterial enteritis.

A study in Oslo by Hanson[106] of an outbreak of severe diarrhea caused by *E. coli* strain 0111 showed that six severely ill children were formula fed. Two infants who were breastfed had *E. coli* strain 0111 in their stools but showed few symptoms. Their mothers had no detectable antibodies for strain 0111 in their milk, which would suggest that other factors in human milk protect the infant from serious illness when no antibodies are in the milk. Hanson[106] also reported the results of another study in which after colonization with a specific strain of *E. coli*, mothers had large numbers of lymphoid cells in their milk with antibodies to that *E. coli*. The mothers' serum showed no such response. This supports

TABLE 5-9 Nonantibody, Antibacterial Protective Factors in Human Milk

Factors	Proposed Mechanisms of Action	Organisms Affected	Effect of Heat
Bifidus factor	Inhibits replication of certain bacteria in GI tract by causing proliferation of lactobacilli	Enterobacteriaceae, including shigellae, salmonellae, and some *E. coli*	Stable to boiling
Complement components	Opsonic, chemotactic, and bacteriolytic activity	*E. coli*	Destroyed by heating at 56° C for 30 min
Lysozyme	With IgA, peroxide, or ascorbate, causes lysis of bacteria	*E. coli* Salmonellae	Some loss (0%-23%) at 62.5° C for 30 min; essentially destroyed by boiling for 15 min
Lactoferrin (nutrient binders)	Binds ferric iron	*E. coli* *Candida albicans*	Two thirds destroyed at 62.5° C for 30 min
Lactoperoxidase	Oxidizes bacteria	*E. coli* *Salmonella typhimurium*	Presumably destroyed by boiling
Nonantibody proteins: receptor-like glycolipid or glycoprotein	Inhibit bacterial adherence	*Vibrio cholerae*	Stable to boiling for 15 min
Gangliosides (GM1-like)	Interfere with attachment of enterotoxin to GM1 cell membrane ganglioside receptors	*E. coli* and *V. cholerae* enterotoxins	Stable to boiling
Nonlactose carbohydrate factors	Prevent action of stable toxin	*E. coli* stable toxin	Stable at 85° C for 30 min
Milk cells (macrophages, polymorphonuclear leukocytes, B- and T-lymphocytes)	By phagocytosis and killing: *E. coli*, *S. aureus*, *S. enteritidis* By sensitized lymphocytes: *E. coli* By phagocytosis: *C. albicans* lymphocyte stimulation by *E. coli* K antigen		Destroyed at 62.5° C for 30 min

Modified from May JT: Antimicrobial properties and microbial contaminants of breast milk: an update, *Aust Paediatr J* 20:265, 1984; and Pickering LK, Kohl S: Human milk humoral immunity and infant defense mechanisms. In Howell RR, Morriss RH Jr, Pickering LK, editors: *Human Milk and Infant Nutrition and Health*, Springfield, Ill, 1986, Charles C Thomas.

the concept that antigen-triggered lymphoid cells from Peyer patches seek out lymphoid-rich tissue, producing IgA in the mammary gland. The mother is immunized in the gut at the same time as her milk. It has also been shown that *E. coli* enteritis can be cured by feeding human milk.

Schlesinger and Covelli[246] studied possible cell-mediated immunity in breastfed infants. They showed that tuberculin-positive nursing mothers had reactive T cells in their colostrum and early milk. Furthermore, 8 of 13 infants nursed by tuberculin-positive mothers had tuberculin-reactive peripheral blood T cells after 4 weeks. Cord blood had no such activity. No clinical or research data suggesting a protective effect of this apparently induced tuberculin reactivity in infants are available.

Protection Against Viral Infection

Protection against viruses has been the subject of similar studies. Breast milk contains antibodies against poliovirus, coxsackievirus, echovirus, enterovirus, influenza virus, reovirus, RSV, rotavirus, and rhinovirus.[187,237] It has been confirmed that human milk inhibits the growth of these viruses in tissue culture. Nonspecific substances in human milk are active against arbovirus and murine leukemia virus, according to work by Fieldsteel.[67]

A high degree of antiviral activity against Japanese B encephalitis virus as well as the two leukemia viruses has been found in human milk. The factor was found in the fat fraction and was not destroyed

TABLE 5-10 Nonantibody, Antiviral, and Antiprotozoan Factors in Human Milk

Factors	Proposed Mechanisms of Action	Organisms Affected	Effect of Heat
Lipids (unsaturated fatty acids and monoglycerides)	Inactivate lipid-enveloped virus	Herpes simplex	Stable to boiling for 30 min
		Semliki Forest virus	
		Influenza	
		Ross River virus	
Macromolecules	Inhibit attachment and penetration	Herpes simplex	Most stable at 56° C for 30 min
		Coxsackievirus B_4	Destroyed by boiling for 30 min
		CMV	
		Rotavirus	
α_2-Macroglobulin protein	Inhibits hemagglutinin activity	Influenza	Stable to boiling for 15 min
		Parainfluenza	
α_1-Antitrypsin	Trypsin-dependent inhibition	Rotavirus	Stable to boiling for 10 min
Bile salt-stimulated lipase	May generate fatty acids and monoglycerides that inactivate organisms	*Giardia lamblia*	
		Entamoeba histolytica	
Nonlipase macromolecule	Unknown	*G. lamblia*	
Milk cells	Induced interferon by virus or phytohemagglutinin; induced lymphokine by phytohemagglutinin; induced cytokine by herpes simplex virus; lymphocyte stimulation by rubella, CMV, herpevirus, measles, mumps		Destroyed at 62.5° C for 30 min

Modified from May JT: Antimicrobial properties and microbial contaminants of breast milk: an update, *Aust Paediatr J* 20:265, 1984; and Pickering LK, Kohl S: Human milk humoral immunity and infant defense mechanisms. In Howell RR, Morriss RH Jr, Pickering LK, editors: *Human Milk and Infant Nutrition and Health*, Springfield, III, 1986, Thomas.

by extended heating, which distinguishes it from antibodies. May[168] believes the nonimmunoglobulin macromolecule antiviral activity in human milk is caused by specific fatty acids and monoglycerides (Table 5-10). It is important to recognize other factors besides immmunoglobulins are contained in breast milk that can play a role in protection of the breastfeeding infant from viral infections.[102,104]

Specimens of human colostrum have been found to contain neutralizing activity against RSV. RSV has become a major threat in infancy and is the most common reason for hospitalization in infancy in some developed countries. It has a high mortality rate. Epidemics have occurred in special care nurseries. Statistically significant data collected by Downham et al[58] showed that, compared with uninfected control subjects who were breastfed (46 of 167), few breastfed babies (8 of 115) were among the infants hospitalized for RSV infection.

Fishaut et al[68] studied the immune response to RSV prospectively in 26 nursing mothers during several months. Antiviral IgM and IgG were rarely found in colostrum or milk. RSV-specific IgA, however, was identified in 40% to 75% of specimens.

TABLE 5-11 Antiprotozoan Factors in Human Milk

Factor	Organisms Affected (in vitro)	Effect of Heat
Bile salt–stimulated lipase	*Giardia lamblia*	Destroyed at 62.5° C for 1 min
	Entamoeba histolytica	
	Trichomonas vaginalis	
Nonimmunoglobulin, nonlipase macromolecule	*G. lamblia*	Stable to boiling for 20 min

Modified from May JT: Antimicrobial properties and microbial contaminants of breast milk: an update, *Aust Paediatr J* 20:265, 1984.

Two mothers with the disease had specific IgG, IgM, and IgA antibody in serum and nasopharyngeal secretions, but only IgA was found in their milk. This confirms that IgA antibody to specific respiratory tract pathogens is present in the products of lactation. Because RSV appears to replicate

only in the respiratory tract, the authors suggest that viral-specific antibody activity in the mammary gland may be derived from the bronchiole-associated lymphoid tissue.

Antiprotozoan Factors

In human milk, bile salt-stimulated lipase has been found to be the major factor inactivating protozoans (Table 5-11).[168] The mechanism by which lipase acts is not known, although it may generate fatty acids and monoglycerides, which inactivate enveloped bacteria, viruses, or protozoa. A non-immunoglobulin, nonlipase, heat-stable factor has been identified in human milk that can inactivate *Giardia lamblia*.

Antiinflammatory Properties

Human milk protects against many intestinal and respiratory pathogens with minimal evidence of inflammation. Goldman et al[90] hypothesize that human milk is poor in initiators and mediators of inflammation but rich in antiinflammatory agents. Several major biochemical pathways of inflammation, including the coagulation system, the fibrinolytic system, and complement, are poorly represented in human milk. Box 5-4 outlines the

BOX 5-4. Antiinflammatory Features of Human Milk

Paucity of initiators and mediators	
Foreign antigens	
IgG antibodies	
Complement system	
Fibrinolytic system	
Coagulation system	
Kallikrein system	
Antiinflammatory agents	
Lactoferrin	Inhibits complement
Secretory IgA	Prevents bacterial adherence
	Inhibits neutrophil chemotaxis
	Limits antigen penetration
Lysozyme	Inhibits neutrophil chemotaxis, generation of toxic oxygen radicals
Catalase	Destroys hydrogen peroxide
α-Tocopherol	Scavengers of oxygen radicals
Cysteine	
Ascorbic acid	
Histaminase	Degrades histamine
Arylsulfatase	Degrades leukotrienes
α_1-Antichymotrypsin	Neutralizes enzymes that act in inflammation
α_1-Antitrypsin	
Prostaglandins (E_2, $F_2\alpha$)	Cytoprotective: inhibits neutrophil degranulation, lymphocyte activation
Pregnancy-associated α_2-glycoprotein	Inhibits lymphocyte blastogenesis
Oligosaccharides	Inhibits microbial attachment
Epidermal growth factors	Strengthens mucosal barriers
Special features of leukocytes	
No basophils, mast cells, eosinophils, or platelets	
T lymphocytes respond poorly to allogeneic cells	
Low natural killer cell activity or antibody-dependent cytotoxicity	
Poor response of neutrophils and macrophages to chemoattractants	

Modified from Goldman AS, Thorpe LW, Goldblum RM et al: Anti-inflammatory properties of human milk, *Acta Paediatr Scand* 75:689, 1986.

antiinflammatory properties of various constituents and the paucity of certain proinflammatory mediators in breast milk.

The interaction of factors in the milk with one another or with host defenses cannot be entirely predicted by examining each factor separately. When the decreased response of human milk leukocytes to chemoattractant peptides was demonstrated by Thorpe et al,[262] the failure of the response of human milk leukocytes was not caused by alterations in maternal peripheral blood leukocytes. This suggests that inhibitors are in the milk and that human milk leukocytes may be modified in the mammary gland to protect through noninflammatory mechanisms.[262] Only low numbers of basophils, mast dells, eosinophils, and cytotoxic T-cells are present in breast milk. Many other studies have documented the decreased function of milk polymorphonuclear leukocytes and macrophages in both colostrum and mature milk.[30]

The antioxidant properties of human colostrum were demonstrated by Buescher and McIlheran[31] using aqueous human colostrum on human PMNs. The colostrum significantly interfered with PMN oxygen metabolic and enzymatic activities that are important in the mediation of acute inflammation.

Antioxidants in breast milk can also contribute to the overall antiinflammatory effect of breast milk. Demonstrated antioxidants contained in breast milk include an ascorbate-like compound, uric acid, α-tocopherol, β-carotene, and L-histidine, all of which scavenge oxygen radicals. Blood levels of α-tocopherol and β-carotene are higher in breastfed than unsupplemented formula-fed infants. Catalase, glutathione peroxidase, and lactoferrin are functionally antioxidants. Antioxidant activity has been demonstrated in colostrum and at lower levels in mature human milk.

Additionally, specific cytokines that can exhibit antiinflammatory effects have also been identified in human colostrum and milk: TGF-β_1 and -β_2[195,239,259] and IL-10.[74] A cytokine antagonist, IL-1RA, and soluble receptors for TNF-α are also found in colostrum and milk.[32,254] Palkowetz et al[209] have reported that IL-1RA can decrease the action of IL-1β.

Both human colostrum and milk cause a diminished influx of polymorphonuclear cells to a local site of inflammation in two different in vivo models of inflammation in rats.[38,96,181]

The inflammatory response can be protective for the host at the same time as it can produce the symptoms of clinical illness. Breast milk contains a large variety of antimicrobial factors that exert their protective effect without causing significant inflammation (e.g., sIgA, oligosaccharides, lactoferrin, nucleotides). Many other cells and factors in breast milk participate in a complex interaction to both protect the infant and limit the potential damaging effects of an uncontrolled inflammatory response. Further study into the dynamic interplay of the many factors in breast milk with developing infants' mucosal barriers and immune systems is needed to fully understand the protective immune response and the antiinflammatory benefits of human milk.

Allergic Protective Properties

(See Chapter 17 on human milk as prophylaxis in allergy)

In discussing the allergic protective properties of human milk, it is difficult to identify specific protective factors that are proved to protect against allergy. It is equally difficult to discuss the proposed mechanisms of protection because the exact mechanism of "oral tolerance" remains theoretic and the relative importance of contributing factors to hypersensitivity must still be adequately defined. Some of the important variables concerning tolerance and sensitization are genetic background of the host, nature and dose of the antigen, frequency of exposure, timing (age) at first and subsequent exposures, immunologic status of the host, and route of exposure.

During the neonatal period the small intestine has increased permeability to macromolecules. Infants have more serum and secretory antibodies against dietary proteins than children or adults. Production of IgA in the intestinal tract is delayed until 6 weeks to 3 months of age. IgA in colostrum and breast milk prevents the absorption of foreign macromolecules when an infant's immune system is immature. Mucin, oligosaccharides, and other factors within breast milk may affect antigen presentation. Protein of breast milk is species specific and therefore nonallergic for human infants. No antibody response has been demonstrated to occur with human milk in infants. It has also been shown that macromolecules in breast milk are not absorbed.

Indirect evidence can be inferred from a demonstration of an infant's response to cow milk protein. Within 18 days of taking cow milk, the infant will begin to develop antibodies. Since the advent of prepared formulas, in which the protein has been denatured by heating and drying, the incidence of cow milk allergy has been considered to be 1%. The most reliable means of diagnosing cow milk allergy is by challenging with isolated cow milk protein. Although circulating antibodies and coproantibodies have been identified, these are not reliable techniques for a clinician involved in patient care.

The allergic syndromes that have been associated with cow milk allergy include gastroenteropathy, atopic dermatitis, allergic rhinitis, chronic pulmonary disease, asthma, eosinophilia, failure to

thrive, and sudden infant death syndrome, or cot death, which has in some cases been attributed to anaphylaxis to cow milk.[132,156] GI symptoms have received the greatest attention and include spitting-up, colic, diarrhea, blood in the stools, frank vomiting, weight loss, malabsorption, colitis, and failure to thrive. Cow milk has been associated with GI protein and blood loss. The diagnosis is best made by elimination of cow milk from diet and, when appropriate, challenge tests. Cutaneous testing is of little help. Cow milk allergy has been described in breastfed infants, and exclusive breastfeeding alone is not sufficient to protect an infant at high risk to become sensitized to cow milk proteins.[129] The incidence of cow milk allergy in exclusively breastfed infants has been estimated as 0.4% to 0.5% compared with the overall incidence ranging from 1.9% to 7.5% in population-based studies.[129]

Murray[182] showed the association of nasal secretion eosinophilia with infants freely fed cow milk or solid foods compared with eosinophilia in strictly breastfed infants. In infants receiving cow milk, 32% had high eosinophilic secretions, and only 11% of breastfed infants had eosinophils present in nasal secretions.

Not surprisingly, many different antigenic specificities are recognized when the colostrum or milk of one species is fed to or injected into another species. Cow milk is high on the list of food allergens, particularly in children. Sensitivity to cow milk is responsible for at least 20% of all pediatric allergic conditions, according to Gerrard.[78] Evidence indicates that IgA antibodies play an important role in confining food antigens to the gut. Food antigens given to bottle-fed infants before they can make their own IgA, and when they are deprived of that in human milk and the plasma cells, may be expected to be more readily absorbed.

Glaser[80] first made the association between the drop in breastfeeding and the rise in allergy. He pioneered the theory of prophylactic management of allergy. Allergy in infancy is associated with a familial history of atopic disease and elevated cord blood IgE levels. The introduction of "foreign" proteins to an infant's diet and even to the mother's diet in the breastfeeding dyad can lead to allergic symptoms in the infant. Exclusive breastfeeding does not protect high-risk children from allergic symptoms unless the mother also adheres closely to a restrictive diet that excludes common allergens.[5,12]

A large body of literature examines whether breastfeeding protects against atopic disease. In 1988, Kramer[148] defined 12 standards for methodology and the study of allergy and breastfeeding. The standards clarified the definitions of breastfeeding, measurable outcomes, and the diagnostic criteria for specific allergic syndromes, defined children at high risk for atopic disease, and addressed methods to decrease bias and control for confounding variables. Several recent large meta-analyses have been performed assessing the protective effect of breastfeeding against allergic rhinitis, atopic dermatitis, and asthma.[76,77,175] Exclusive breastfeeding during the first 3 months of life protected against allergic rhinitis (summary odds ratio 0.74; 95% confidence interval 0.54 to 1.01) with or without a family history of atopy.[175] Exclusive breastfeeding for at least 3 months was associated with lower rates of atopic dermatitis in children with a family history of atopy.[76] Exclusive breastfeeding in the first months of life was protective against asthma during childhood (odds ratio 0.70; 95% confidence interval 0.60 to 0.81).[77]

Chapter 17 discusses the prophylactic management of the potentially allergic infant.

Protection Against Chronic Disease in Childhood

The major elements in human milk related to the infant's immune system are direct-acting antimicrobial factors, antiinflammatory factors, and immunomodulating bioactive compounds.[108] Epidemiologic studies have produced compelling information that suggests that breastfeeding for 4 months or longer can provide some immunologic protection against some childhood-onset diseases.[85,91,169]

In 1991, Viirtanen et al[268] reported a prospective long-term study among children in Finland that showed a significantly lower incidence of type 1 diabetes in those at-risk children who had been breastfed for 4 months or longer. Other epidemiologic studies have demonstrated a decreased incidence of type 1 insulin-dependent diabetes mellitus in breastfed children.[20,169,197] These clinical observations have been supported in the laboratory by studies of diet control in diabetic mice. The isolation of a bovine albumin peptide as a possible trigger of type 1 insulin-dependent diabetes mellitus makes further study imperative.[136] Based on limited data, the recent Agency for Healthcare Research and Quality report cautiously concluded that breastfeeding for at least 3 months reduced the risk for type 1 diabetes compared with breastfeeding for less than 3 months. For type 2 diabetes, the same report concluded that breastfeeding in infancy produced a decreased risk compared with not breastfeeding.[126]

The review of the national perinatal collaborative study by Davis et al[54] showed a protective effect against development of childhood cancer by being breastfed for 4 months or longer for children followed for 10 years. The effect was greater for acute leukemia and lymphoma. The role of infant feeding practices showed a similar effect of breastfeeding as protective in postponing or decreasing

the occurrence of inflammatory bowel disease in childhood.[145,232] Greco et al[97] reported a decreased risk for celiac disease in breastfed infants. The AHRQ report concluded that an association exists between breastfeeding for at least 6 months and a decreased risk for developing acute lymphocytic leukemia and acute myelogenous leukemia.[126]

Maternal renal allografts have a better survival rate in individuals who were breastfed in infancy compared with those who were not breastfed.[37,143] The mechanism of these apparent long-term immunologic benefits remains unclear, although theories abound.[85] Given the potential for confounding factors and bias in large long-term studies, confirmation of these proposed benefits by additional carefully controlled trials is required.

Summary

An increasing amount of accumulated epidemiologic literature, utilizing improved methodology and statistics, demonstrates the protective benefits of human milk for infants. A large number of bioactive factors have been identified and measured in breast milk during the period of lactation. Additional research is needed to clarify the interactions and the mechanisms of action of the many bioactive factors in human milk and then correlate these immunomodulatory actions with specific protective benefits for the infant.

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