Human Breast Milk: Current Concepts of Immunology and Infectious Diseases

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This is a review of the immunologic activities and protective benefits of human breast milk against infection. It details important concepts about the developing immunity of infants, bioactive factors and antiinflammatory properties of breast milk, intestinal microflora in infants, probiotics and prebiotics, and the dynamic interactive effects of breast milk on the developing infant. Studies documenting the protective effect of breast milk against various infectious diseases in infants are presented, including respiratory infections, diarrhea, otitis media, and infections in premature infants. Data are provided supporting the current recommendations of 6-months duration of exclusive breastfeeding for all infants in the United States and 12 months worldwide.

National statistics have shown increasing breastfeeding rates for the United States from 1975 through 1995, with rates remaining relatively high into 2004.[1,2] Data from 2004, the National Immunization Survey, reported national breastfeeding rates of 70.3% (CI ±0.9) for ever breastfeeding, 36.2% (CI ±0.9) breastfeeding continuing at 6 months, 38.5% (CI ±1.0) exclusive breastfeeding at 3 months, and 14.1% (CI ±0.7) exclusive breastfeeding at 6 months.[1] These numbers are comparable to reported rates from the Mothers' Survey, Ross Products Division of Abbott, for 2004: 64.7% of mothers breastfeeding in the hospital; 31.9% breastfeeding at 6 months; with 41.7% of mothers reporting exclusive breastfeeding in the hospital; and 17.4% exclusive breastfeeding at 6 months.[3]

Although the increasing trends are positive, the reported rates remain below the Healthy People 2010 goals. These goals are a set of 467 public health objectives promulgated by the Surgeon General of the United States, which recommend increasing the proportion of mothers who breastfeed to 75% at birth, 50% at 6 months, and 25% continuing breastfeeding until 12 months.[4] The rates are also well below the recommended 6-month duration of exclusive breastfeeding for all infants and mothers in the United States, put forth by the American Academy of Pediatrics (AAP), the American College of Obstetricians and Gynecologists, and the American Academy of Family Physicians.[5-7] The Section on Breastfeeding of the AAP has clearly outlined their recommendations for breastfeeding with over 200 references to studies documenting the health benefits to the child, mother, and community, in support of those recommendations.[8]

The intention of this review was to discuss important concepts related to the role breastfeeding plays in the normal development of the infant's immune system and the protection afforded the infant against infectious diseases during infancy and childhood, while the infant's immune system is still maturing. The discussion should provide ample evidence to support the current recommendations for 6 months of exclusive breastfeeding for all infants, help all health care providers adequately inform families of the real immune benefits of breastfeeding, and strongly support and advocate for breastfeeding in their day-to-day care of children.

Important Concepts Related to the Immunologic Significance of Human Milk

Any discussion of the immunologic significance of human milk will necessarily require the consideration

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of the infant's immune system, the maternal immune system, and the interaction between the two. Various immunologic concepts and models, such as innate and adaptive immunity, mucosal immunity, inflammatory and antiinflammatory responses, active versus passive immunity, dose–response relationships, and the dynamic nature of acute immune responses need to be considered.

Physicians certainly recognize neonates and infants as being immunologically immature and at increased risk for infection with common infections like otitis media, upper respiratory tract infections, or gastroenteritis, and serious infections such as sepsis or meningitis. Despite extensive advances in nutrition, hygiene, antiinfective therapy, and medical care for infants and children, infections remain a major cause of childhood morbidity and mortality in developed and developing countries. Although there are numerous contributing factors to neonates' and infants' predisposition to infection, there are clear deficits in various aspects of the infant's immune system that are a major cause of this increased susceptibility to infection. The recognition that the increased risk of infection in newborns, infants, and children is directly related to the infant's developing immune system demands a greater understanding of the immunologic benefits contributed by human breast milk.

Innate Immunity

The innate immune system forms the early defense against infection, acting within minutes of exposure to pathogenic microorganisms, by reacting as a preformed nonspecific response. Components of this system include the mucosal and epithelial cell barriers along with air, fluid, or mucus flow along these surfaces. It also involves the binding of pathogens by various substances to prevent entry or colonization as well as chemical inactivation or disruption of infectious agents due to such factors as low pH, enzymes, peptides, proteins, and fatty acids. Innate immunity entails the competition of potential pathogens with normal flora inhabiting the local host site. It also includes the activity of phagocytes, within tissues and along mucosal surfaces, which recognize broad classes of pathogens and cause complement activation. One example of this local innate immunity is the way collectins (surfactant proteins A and D) act on the epithelial surface of the lung alveoli to bind microbes leading to aggregation, opsonization, and increased clearance of organisms by alveolar macrophages.[9] The innate immune system is active primarily at the local level or the site of initial infection, which is most often the mucosa and epithelium.

The adaptive immune response is activated along with the innate defense system, but the response develops more slowly. Phagocytes play a role in both the innate response (local phagocytosis and destruction of the pathogen) and the adaptive response by cytokine secretion that stimulates recruitment of antigen-specific T- and B-cells to the site of infection. These effector cells attack the specific pathogen and generate memory cells that can prevent reinfection on exposure to the same organism. Adaptive immunity involves both cell-mediated responses involving T-cells, cytokines, and specifically activated effector cells as well as humoral immune responses including B-cells, plasma cells, and secreted immunoglobulins. Since it is antigen-specific, the adaptive immune response occurs later (usually after 96 hours) and can differentiate between closely related pathogens (antigens), through their interactions with antigen receptors on T- and B-cells. The capability of the adaptive immune response to recognize and react against thousands of specific antigens is dependent on T- and B-cell receptor expression and binding. Antigen receptor specificity and diversity result from both rearrangement of multiple gene segments encoding for the antigen-binding site as well as clonal expansion of specific T- and B-cells in peripheral lymphoid organs.

Within breast milk there are a number of factors that one could consider as acting as part of the infant's innate immune system. This was reviewed at a symposium on "Innate Immunity and Human Milk" as part of the Experimental Biology meeting in April, 2004.[10] Newburg referred to intrinsic components of milk or partially digested products of human milk, which have local antipathogenic effects that supplement the infant's innate immunity. This includes substances that function as prebiotics (substances that enhance the growth of probiotics or beneficial microflora),[11] free fatty acids (FFA), monoglycerides,[12] antimicrobial peptides,[13] and human milk glycans, which bind diarrheal pathogens.[14] In addition to these, there are other factors within breast milk that support or act in concert with the infant's innate immune system including bifidus factor, lysozyme, lactoperoxidase, lactoferrin, lipoprotein lipase, and even epidermal growth factor, which may stimulate the maturation of the gastrointestinal epithelium as a barrier. Newburg also proposed that some factors in milk, which may have

no demonstrated immunologic effect when tested alone, may have measurable effects in vivo after digestion or in combination with other factors in breast milk or in the intestine.

The Infant's Developing Immune System

In its simplest conceptualization, the immune system protects us against potential pathogens within our environment. It must have the capacity to distinguish foreign non-self antigens from "self." It must be capable of recognizing microorganisms and tumor cells and developing a protective immune response against them. It must also respond with immunologic tolerance against our own tissues, as well as foods and other related antigens. The immune system includes the "primary" organs, bone marrow, and thymus, where the T- and B-cells are produced and develop. The "secondary" organs include lymph nodes, spleen, and mucosa-associated lymphoid tissue (MALT), where mature T- and B-cells encounter and respond to antigens. Other distinct compartments such as peritoneum, genitourinary mucosa, pleura, and skin can also be the site of first contact between antigens and cells. It is in these "secondary" compartments that antigen-specific T- and B-cells are activated, resulting in the clonal expansion of lymphocytes bearing receptors with the most avidity for antigens and in the maturation of the immune response. The resulting immunity involves both the innate and the adaptive immune responses.

As with all mammals, human infants are born immature and require a period of maturation to reach the level of adult function. This is also true for each of the different organ systems of the human infant, each one maturing at different rates. The ongoing development of the infant's immune system will be addressed in the sections on developmental immune deficiencies and the mucosal immune system.

Main Arms of the Immune System

The four main arms of the immune system are as follows: (1) phagocytes and their secreted cytokines and interferons; (2) cell-mediated immunity composed of T-cells, natural killer cells (NK), and secreted proteins that stimulate, inhibit, and regulate the immune response such as cytokines and interferons; (3) humoral immunity including B-cells, plasma cells, and immunoglobulins; and (4) the complement cascade. Although considered separately, there are extensive and complex interactions among the four arms to form a coordinated and effective immune response against almost any human pathogen. The characteristics of the clinical disease experienced by an individual in response to a specific infectious agent are determined by the complex interactions between the pathogen, with its particular virulence factors, and the host's timely, effective, and controlled response to eradicate the infecting organism.

The most important host mechanisms against *viral pathogens* are specific neutralizing antibodies against viral surface proteins, specific CD8+ cytotoxic T-cell response, and production of interferons that disrupt viral replication. Other defense mechanisms that may play a role in protection against viral infection include NK cell activity against infected host cells, antibody-dependent cellular cytotoxicity (ADCC), and the direct cytotoxic effect of certain cytokines (like tumor necrosis factor-α (TNF-α)) on infected host cells.

Primary host defense mechanisms against *bacteria* on the skin and mucous membrane surfaces involve the integrity of the mechanical barrier, defensins, secretory immunoglobulin A, complement, other antimicrobial molecules, and circulating polymorphonuclear leukocytes (PMNs), which have migrated from the blood to the site of tissue invasion by bacteria. Important mechanisms against systemically invasive *bacteria* are phagocytes, complement and specific antibodies which enhance the bacteriolysis and opsonization effects of complement.

Although the host defenses against *fungi* are less clear overall, phagocytes and cell-mediated immunity play significant roles in protection against invasive fungal disease. Depending on the particular fungi involved, different components of the immune system may be more active, and phagocytosis may be more important in defending against *Aspergillus*, while cell-mediated immunity is more important against *Candida*.

Even less well understood are the defense mechanisms against *parasites* and against the different forms or stages in the parasitic lifecycle. Specific antibodies against parasitic antigens in different stages are important, along with an allergic-type (T2) cytokine response by CD4+ (helper) T-cells and activities of unique effector cells, mast cells, and eosinophils, in combating human parasitic infections.

There are numerous factors that contribute to the increased susceptibility to infection seen in neonates, infants, and children. The most important of these include factors that facilitate the host exposure to

TABLE 1. 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 trans-endothelial 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-alpha, Il-2, IL-4, IL-10
Diminished natural killer (NK) cell cytolytic activity (matures by 6 months)
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)
IgG_1 and IgG_3 production is limited (matures at 1–2 years of age)
IgG_2 and IgG_4 production is delayed (matures at 3–7 years of age)
B-lymphocytes and immunoglobulins:
Serum IgA levels are low (less than adult levels through 6–8 years of age)
Deficient opsonization by immunoglobulins
Poor response to T-cell independent antigens (polysaccharides) (matures at 2–3 years of age)
Complement cascade:
Decreased function in both the classical and the alternative pathways
Insufficient amounts of C5a

infectious agents through different mechanisms of transmission (damaged barriers, direct contact with fluids, and fomites, etc.) and the immaturity and/or ineffectiveness of their immune system. Development of immunity and susceptibility of infants and children at different ages to infection has been studied extensively. Deficiency of specific components and immune responses are characteristic of the developing infant and these deficiencies may be more severe in the premature infant or in infants who are physiologically or pathologically stressed. In considering how breast milk is of particular immunologic benefit to the developing infant, it is important to review these developmental defects in the infant (Table 1).

Developmental Immune Deficiencies

Phagocytes. The effective functioning of the phagocytic arm of the immune system is dependent on adequate numbers of cells, the cells' ability to "sense" or be alerted to the presence of an infecting agent along with their ability to migrate to the site of infection (chemotaxis), and the cellular activity of ingesting and killing microorganisms (phagocytosis). Antibodies, complement, and cytokines play essential roles in the various stages of chemotaxis and phagocytosis. Neutrophils and monocytes are the primary phagocytic cells and are produced in the bone marrow. Neutrophils circulate in the bloodstream for roughly 24 hours, unless they are attracted to and migrate to a site of infection. Monocytes migrate from the circulation to tissue sites where they develop into specialized "tissue" macrophages, functioning there for 2 to 3 months.

The number of circulating neutrophils is higher in neonates than adults, but there is limited reserve capacity to produce additional phagocytic cells in response to an active infection.[15] Depletion of available neutrophils in newborns with sepsis is associated with increased mortality.[16] The cause of this depletion is undetermined, as increased numbers of immature neutrophils and increased levels of colony-stimulating factors are measurable in the blood of these neonates. The limited number of neutrophils reaching the site of infection directly contributes to a neonate's susceptibility to infection at different sites.[17]

Chemotaxis of neutrophils depends on chemical attractants produced by phagocytic immune cells that arrive first at the site of infection, the presence of adhesion molecules on the surface of neutrophils to allow binding to endothelial cells, and the cytoskeletal changes in the neutrophils that allow trans-endothelial migration out of blood vessels. Interleukin 8 (IL-8), the receptor for the C5a fragment of complement, and fibronectin all contribute to neutrophilic chemotaxis, and deficiencies in each of these have been described in infants.[18-20] The ability of neutrophils to be motile in the newborn has been described as abnormal due to membrane defects[21,22] and inadequate cytoskeletal changes, which limit trans-endothelial migration of neutrophils.[23] Selectins and integrins are important adhesion molecules. L-selectin appears to be down-regulated in term neonates, which may be aggravated in acute bacterial infection.[24] These abnormalities may contribute, additively, to inadequate numbers of neutrophils reaching the site of infection.

Neutrophil cytotoxicity in normal neonates seems similar to that in adults,[25] but production of hydroxyl radicals for killing pathogens may be reduced.[26] Neutrophil killing function appears to be decreased in "stressed" neonates[27] and one suggested mechanism for this deficiency is inadequate amounts of bacteri-

cidal permeability-increasing protein in the neutrophils of neonates, especially during Gram-negative sepsis.[28] Additionally, abnormal neutrophil function may be secondary to deficiencies in opsonizing factors, such as antibodies, complement, and fibronectin, and not strictly the result of abnormal neutrophil function. Satwani and coworkers demonstrated several aspects of dysregulated immunoregulatory function and cytokine gene expression in cord blood monocytes as another example of the immature, inefficient immune response in neonates.[29] To date, attempts to counteract these deficiencies in granulocyte response with granulocyte colony-stimulating factor (G-CSF) and granulocyte-monocyte colony-stimulating factor (GM-CSF) have resulted in an increased number of neutrophils in the blood, but not improvement in survival of neonates with infection.[30-32]

In summary, the primary deficiencies related to phagocytic function in neonates are due to inadequate numbers of neutrophils reaching the site of infection, insufficient reserve production of phagocytic cells during active, severe infection, and probably various abnormal immunostimulatory or immunoregulatory processes that contribute to a decrease in infants' phagocytic function.

Cell-mediated Immunity. T-lymphocytes function in the regulation of antigen-specific immune response, both helping and suppressing specific activities. Helper T-lymphocytes secrete cytokines that serve as the primary messages for this regulation and cytotoxic T-lymphocytes act by killing cells that express foreign antigens. Mature T-lymphocytes recognize antigen specifically through antigen binding to surface T-cell receptor. Unlike B-cells that can respond to soluble-free antigen, the T-cell receptor binds antigen bound to a self-major histocompatibility molecule expressed on the surface of an antigen-presenting cell.

There are increased absolute numbers of T-lymphocytes in cord blood (mean number in newborns 3100/μL) as compared with older children (mean number = 2500/μL) or adults (mean number = 1400/μL). Although the absolute number of T-lymphocytes decreases after the neonatal period, the percentage of T-lymphocytes increases within the total number of lymphocytes.[33] The proliferative response of neonatal T-lymphocytes is normal to mitogens such as phytohemagglutinin and alloantigens.[34] There is a decreased ability to form memory cells, however.[35] The cord blood contains large numbers of naïve T-lymphocytes (CD45RA+ cells) compared with memory T-lymphocytes (CD45RO+ cells).[36] As the immune system matures and is continuously exposed to antigens, an increased proportion of memory T-cells are formed. By 7 years of age, there are approximately 60% naïve T-lymphocytes. This percent continues to decline with ongoing exposure to antigens and development of memory T-cells along with the involution of the thymus through adolescence into adulthood.[37,38]

Neonatal T-lymphocytes, which predominately express CD45RA+, produce less interferon-γ (IFN-γ), interleukin-2 (IL-2), interleukin-4 (IL-4), interleukin-10 (IL-10), and TNF-α than adult T-lymphocytes produced after stimulation.[37-39] Decreased interleukin-3 (IL-3) production and gene expression has also been reported. Although GM-CSF and G-CSF are produced by a variety of other cells besides T-lymphocytes, they are present in decreased amounts in neonates.[30,40] The decreased cytokine production is certainly a function of the limited numbers of "memory" T-lymphocytes (CD4+, CD45RO+, and CD8+ CD45RO+ cells). There is also decreased cytotoxic activity of CD8+ lymphocytes in the newborn.[41] The predominant deficiencies of neonatal T-lymphocytes are related to their "immaturity," including decreased production of cytokines; poor cytotoxic activity; limited proliferation in response to antigens; poor contribution to antibody production and isotype switching by B-cells; and inadequate stimulation of phagocytic activity.

NK cells and cytolytic T-lymphocytes kill infected cells via proteins named perforin and enzymes named granzymes. Perforin creates pores in the cell membrane and granzymes enter through these pores to induce apoptosis of the targeted cells.[42] NK cells recognize tumor cells or virally infected cells through expression of tumor or virus antigen on the host cell surface. NK cells also can mediate ADCC killing cells coated with antibody. NK cells of infants have decreased cytotoxic activity and decreased ADCC, which continues through approximately 6 months of life.[43,44] There are a number of studies linking deficiencies of NK cell activity and ADCC in newborns to increased susceptibility to herpes simplex virus (HSV)[45-47] and human immunodeficiency virus (HIV) infection in preterm infants and newborns.[48] The diminished T-lymphocyte cytolytic activity and decreased IFN-γ production contribute to an increased susceptibility to viral infections in general and to other intracellular pathogens such as *Listeria* and *Toxoplasma gondii*.[38]

B-Lymphocytes and Immunoglobulins. B-lymphocytes contribute to pathogen-specific immunity through the production of antibodies to specific antigens including bacteria, free virus, parasites, and tumor cells. Immunoglobulins on the surface of B-cells bind to antigens, which leads to the formation of plasma cells and the secretion of antibodies. Antibodies function either alone through neutralization or with complement and phagocytes to inactivate infectious organisms.

The amount and repertoire of actively produced immunoglobulin G (IgG) antibodies by the fetus and infant is clearly deficient. This is in large part because antigen-exposed memory T-cells have not yet been generated that are necessary for IgG production and isotype switching. Transplacental transfer of IgG from the mother to the infant only partially corrects this deficiency. This transfer is a selective process, such that only IgG crosses the placenta and only certain IgG subclasses are included.[49,50] The majority of the transfer of IgG occurs in the third trimester. These passively acquired antibodies decrease rapidly after birth to a nadir level around 3 months postnatal age.

The overall amount of serum IgG in full-term infants at birth is equal to or slightly greater than IgG levels in the mother because of the active transport across the placenta.[51] The passively acquired antibodies from the mother contribute to a decreased risk of infection in the full-term infant in comparison to preterm (28 to 35 weeks gestational age) and extremely premature infants (less than 28 weeks gestational age). In parallel with the natural decline in maternal IgG in the infant's serum, due to the degradation half-life (approximately 30 days) of immunoglobulin, the infant begins to actively produce IgG antibody on exposure to antigens. Serum IgG levels in infants reach approximately 60% of adult levels by 1 year of age, but the complete antibody response, to a range of antigens equal to that of an adult, is not achieved until 4 to 5 years of age. This is due to deficient production of IgG_2, the primary antibody made against encapsulated organisms.

Premature infants have very low levels of IgG antibody, but the mean concentration increases with increasing gestational age. The mean concentrations of IgG in infants have been reported as ~60 mg/dL at 25 to 28 weeks of gestation, ~104 mg/dL at 29 to 32 weeks of gestation, and over 400 mg/dL after 38 weeks gestational age.[52,53] The passively acquired maternal antibodies against specific antigens are important for protection against some common pathogens in the neonatal period: herpes simplex virus, varicella-zoster virus, and group B streptococcus.[54] The fact that immunoglobulin M (IgM) does not cross the placenta leaves neonates susceptible to Gram-negative organisms, some of which require IgM and complement for opsonization.[55] Interventions to increase the immunoglobulin levels of infants via immunization of mothers or passive antibody infusions using intravenous immunoglobulin for the infant against specific infections (eg, group B streptococcus) have had limited success.

B-lymphocytes are produced in the bone marrow throughout life, and they differentiate in response to various cytokines such as stem cell factor, IL-1, IL-3, IL-6, and G-CSF.[56] Neonatal B-cells produce primarily IgM and limited amounts of IgA and IgG. IgM production can occur in the fetus in response to an intrauterine infection.[57] However, the IgG subclass production matures slowly, reaching 60% of adult levels for IgG_1 and IgG_3 at 1 year of age, and 60% of adult levels for IgG_2 and IgG_4 at 3 to 7 years of age.[49] IgG_2 production begins to develop at about 2 years of age. Secretory immunoglobulin A (sIgA) is a functioning part of innate mucosal immunity even in utero as demonstrated by increases in sIgA with congenital viral infections.[58] Systemic IgA is deficient in infants and children and may not be adequately produced until 5 to 8 years of age. The capability of B-lymphocytes to secrete all isotypes begins to mature between 2 and 5 years of age.

Early on, there is a good antibody response with IgG_1 to protein antigens such as diphtheria-pertussis-tetanus or poliovirus antigens due to infection or immunization. Both preterm infants and full-term infants seem to respond equally well to protein antigens after 2 months of life.[59-61] Usually within the first few days of life, full-term infants can begin to produce protective antibody responses to certain infectious agents, initially with IgM and then IgG.[62] The level of antibody production is still less than adult levels and this is probably due to limited activation of B-cells by T-lymphocytes. The response to thymus-independent antigens, such as polysaccharides of *Haemophilus influenzae* or *Streptococcus pneumoniae*, matures at about 2 to 3 years of age. This is the reason the unconjugated *H. influenzae* type b polysaccharide vaccine and the unconjugated Pneumovax vaccine stimulate poor IgG_2 antibody production in children less than 18

months of age, while their protein–polysaccharide-conjugated counterpart vaccines stimulate good IgG_1 antibody production as early as 2 months of age. The primary deficits in an infant's developing immune system relative to B-lymphocytes and immunoglobulins include (1) deficient amounts and repertoire-specificity of actively produced antibodies; (2) slow maturation of the antibody response to specific groups of antigens (polysaccharides); and (3) limited T-lymphocyte stimulation of B-cell antibody production and isotype switching. Surprisingly, administration of intravenous immune globulin does not decrease mortality in infants with suspected or subsequently proven neonatal infection.[63]

Complement System. The complement system is a cascade of enzymatically activated proteins yielding molecules that function immunologically. Two pathways, classic and alternate, function to activate complement. Both pathways induce the formation of C3b, which functions as an opsonin and acts to cleave C5 into C5a and C5b. C5a functions as a chemoattractant and C5b is part of the "membrane-attack complex" (C5b, C6, C7, C9) of the classical pathway. Part of the cascade is activated by antibody–antigen complexes in the "classical pathway." In the alternate pathway, activation of the cascade occurs by direct binding of components of complement to microorganisms. There are deficiencies in complement activation in both pathways in fetuses and neonates.[64] The measured levels of components C8 and C9 are low at all gestational ages.[65] The concentrations of most complement proteins except C5 and C7 are lower than in adults until 18 months of age.[65-67] The functional deficits in complement formation are not well understood. There is evidence that complement activation deficits contribute to susceptibility to *Escherichia coli* and type III group B Streptococcus,[68,69] but no interventions have been identified to correct these deficiencies.

The numerous qualitative and quantitative deficiencies in a neonate's or infant's developing immune system are well documented. The extent to which each individual defect contributes to susceptibility to infection is unclear. It is more likely that some of the deficits are additive, resulting in a generalized increased susceptibility, and others are very specific, leading to susceptibility to a particular pathogen or group of pathogens.

The Mucosal Immune System

The mucosal epithelia of the gastrointestinal, upper and lower respiratory, and reproductive tracts cover a surface area estimated at over 200 times the surface area of the skin. These surfaces are especially vulnerable to infection due to the thin permeable barriers they present. The mucosal surface has many physiologic functions including gas exchange (in the lungs), food absorption (in the gut), sensory detection (in the eyes, nose, and mouth), and reproduction (in the uterus and vagina). The most important function of the collective mucosal surfaces is immunologic: protection against microorganisms, foreign proteins, and chemicals, and immune tolerance to many harmless environmental and dietary antigens.[70] It has been postulated that some 90% of microorganisms infecting humans cross the mucosa. This is particularly true in children less than 5 years of age who explore the world with their mouths. During these first years of life, when the infant is immediately and continuously exposed to numerous, previously "unseen" microorganisms, the infant's systemic and mucosal immune systems are still developing in response to this onslaught of antigens. Breast milk provides a number of bioactive factors during this crucial period to supplement the immune protection at the mucosal level and others that are immune modulating or growth stimulating, contributing to the development of the infant's immune system and mucosal barriers.

The mucosal immune system is composed of innate mechanisms of protection, which act in concert with adaptive immune mechanisms. Some of the innate mechanisms acting at the mucosal surfaces include enzymes, chemicals, acidity or pH, mucus, immunoglobulins, and indigenous flora, which limit infection. The intestinal epithelium functions as a barrier, limiting the entry of microorganisms from the lumen into the interior of the host. Enterocytes, goblet cells, and enterochromaffin cells are identifiable as early as 8 weeks of gestational age, at about the same time tight junctions between epithelial cells are evident, enhancing the barrier effect of the epithelium.[71] Mucus production is another innate mechanism of defense, blocking adherence of pathogens to epithelial cells. Expression of the *muc2* gene is detectable as early as 12 weeks gestational age.[72] Around the same time Paneth cells appear in intestinal crypts. These cells have the capability of producing various antimicrobial molecules including α-defensin, lysozyme, and TNF-

α.[73] The secretory immunoglobulins, sIgA and IgM, act predominantly, without inflammation, by blocking the colonization and entry of pathogenic organisms, and also by facilitating phagocytosis.

The MALT is located in well-defined compartments adjacent to the mucosal surfaces: tonsils and adenoids of Waldeyer's ring at the back of the mouth, Peyer's patches in the small intestine and appendix (gut-associated lymphoid tissue), and isolated B-cell follicles in the distal large intestine. The overlying follicle-associated epithelium of the gut contains specialized epithelial cells called "M"-cells. M-cells (membrane, multi-fenestrated, or microfold cells) lack a surface glycocalyx and are adapted to interact directly with antigens within the gut lumen. The M-cells endocytose or phagocytose molecules and particles on their surface. These materials are transported in vesicles to the basal cell membrane and released into the extracellular space in a process known as transcytosis. Lymphocytes and antigen-presenting cells are present at the basal surface of M-cells and function to process and present antigen. B-cells are located in large numbers within the submucosal aggregates of lymphoid tissue where they respond to the presented antigens. Activated follicular lymphocytes then migrate via the lymphatics into the thoracic duct and from there into the blood. These lymphocytes circulate in the blood to migrate back to mucosal tissues (primarily the same ones from which they originated) where they locate in the lamina propria and now function as mature effector cells. As part of this process, these lymphocytes increase their receptor avidity for antigen and are stimulated to proliferate. However, T-cells not expressing T-cell receptors with increased avidity are not stimulated to expand. This directed migration to specific sites occurs because of specific cytokines and adhesion molecules. As an example, the colon and salivary glands express a chemokine CCL28 (mucosal epithelial chemokine), whereas cells in the small intestine express a different chemokine CCL25 thymus-expressed chemokine (TECK), which contributes to the site-specific migration. T-lymphocytes that home to the skin express cutaneous lymphocyte antigen (an adhesion molecule) and respond to a combination of different chemokines.[74] This leads to a focused immune response to a specific repertoire of antigens localized to that same environment.[75] The lactating mammary glands in the mother are an integral part of MALT. Activated lymphocytes and antibodies in breast milk are the result of antigenic stimulation of MALT in both the gut and the respiratory mucosa. The mother's mature, more quickly activated, and effective immune response is capable of reacting to microorganisms to which she and the infant are exposed, putting activated cells and antibodies into the breast milk that can directly protect the infant against those pathogens.[76] This is one of the best examples of how breast milk benefits the infant, through the specific immunologic interaction of the mother's and the infant's immune systems. It is also an important reason for continuing breastfeeding when the infant or the mother has a suspected or proven infection. The efficacy of this protective mechanism is well documented in epidemiologic studies in environments with both poor and improved sanitary conditions.[77]

It is particularly important to note that mucosal immunity also undergoes a period of postnatal development. Although MALT is evident at birth in Peyer's patches and tonsils, the germinal centers within the lymphoid follicles do not develop until several weeks after birth.[78] MALT is activated by the postnatal exposure of the mucosal surfaces to numerous antigens. There are few immunoglobulin-producing intestinal plasma cells present in the first week or two of life.[78] After 2 to 4 weeks of age, the number of IgM- and IgA-producing cells in the intestine increase. From approximately 1 to 12 months of age, the IgA-producing cells predominate. The immaturity seen in the systemic immune system of the infant is also present in the mucosal immune system. Plasma cells, the immunoglobulin-producing cells in the blood, migrate to mucosal surfaces. Immunoglobulin-secreting cells in the lamina propria of neonates are very low at birth, but increase in number, especially during the first month of life, and this continues throughout the first year.[79]

By adulthood there are very large numbers of immunoglobulin-producing cells located in the intestinal lamina propria. It has been estimated that there are approximately 10^{10} cells per meter of adult intestine.[78] These immunoglobulin cells produce monomeric IgA. IgA is transported through epithelial cells to the mucosal lumen via an epithelial glycoprotein, the membrane secretory component (SC). The SC binds two IgA molecules forming a dimer on its "secretion" at the mucosal surface. Both sIgA and IgM (always a pentamer) contain the polypeptide J-chain and are transported by this same mechanism.[75] A portion of the SC remains bound to the sIgA and pentameric

IgM, which contributes to their protection against proteolysis. Secretory IgA antibodies are especially stable in saliva and feces.[80] Similarly, there is a tremendous amount of sIgA production and storage in the mammary glands, accounting for the large amounts of sIgA found in breast milk.[81] These biologically stable sIgA and IgM, transferred to the infant via breast milk, play an important role in the innate mucosal immune protection of the infant. These secretory antibodies can block the adherence and entry of microorganisms and cause inactivation, neutralization, or agglutination of viruses. Secretory IgA and IgM in human milk are active against a litany of viruses including enteroviruses, herpesviruses, respiratory syncytial virus, rubella, reovirus, and rotavirus. Many bacteria are targeted by sIgA in human milk, including *E. coli*, *Shigella*, *Salmonella*, *Campylobacter*, *Vibrio cholerae*, *H. influenzae*, *S. pneumoniae*, *Clostridium difficile*, and *C. botulinum*, *Klebsiella pneumoniae*, as well as the parasite *Giardia* and the fungus, *Candida albicans*.[76] It has also been reported that free SC in breast milk can bind to enterotoxigenic *E. coli* (ETEC),[82] pneumococcal surface protein A (SpsA),[83]and *C. difficile* toxin A,[84] which may provide additional specific protection for the infant.

Separate from the immunoglobulins, there are a number of other bioactive factors contained in breast milk that act primarily at the mucosal level.[85] These include lactoferrin, lysozyme, casein, oligosaccharides, glycoconjugates, and lipids. Lactoferrin has a high affinity for iron, which may limit the available iron required by microorganisms for growth. Lactoferrin has separate bactericidal and antiviral properties as well.[86] Partially hydrolyzed lactoferrin seems to block adsorption or penetration of specific viruses, such as herpes simplex virus, cytomegalovirus, and even HIV.[87] Lactoferrin can interfere with the adhesion of enteral pathogens ETEC[82] and *Shigella flexneri*.[88] Lactoferrin may also increase the growth of probiotic intestinal bacterial. Lysozyme, which seems to act by lysing bacteria, maintains high concentrations throughout lactation.[89] Casein inhibits the adherence of microorganisms to mucosal and epithelial cells (eg, *Helicobacter pylori, S. pneumoniae, H. influenzae*). A fragment of proteolysis of k-casein promotes the growth of *Bifidobacterium bifidium*, an important organism in the infant's microflora and a recognized probiotic bacterium.[89] Glycoconjugates and oligosaccharides function as ligands, binding to bacteria, toxins, and viruses, blocking the ability of these harmful organisms to bind to the infant's epithelial cells.[90,91] Mucin-1, lacadherin, and a glycosaminoglycan are specifically identified antimicrobial components in the milk-fat globule membrane. Digested components of the milk-fat globule, FFA, and monoglycerides can act via lysis of enveloped viruses, bacteria, fungi, and protozoa.[92] Lauric and linoleic acids, specific fatty acids that constitute a large fraction of the total fatty acids in human milk, are produced during lipolysis in the stomach and have documented effects against a variety of microorganisms.[85]

There are also immune modulating agents within breast milk, especially cytokines and growth factors, which can act at the level of the mucosa. IL-10 and IFN-γ act to modulate epithelial barrier integrity.[93] Transforming growth factor-α (TGF-α) and epidermal growth factor (EGF) are believed to increase barrier development.[94] Hormones, another group of bioactive factors in breast milk, may also act on mucosal development, but their specific effects have not been elucidated.[85]

There are many additional factors present in breast milk which have as yet unexplained functions and benefits to the infant. Many of these have the potential for activity at the level of the mucosa as well as the potential to act systemically. Some of these might include specific cells, nutrients, vitamins, nucleotides, enzymes, and soluble molecules with receptor-like structures (eg, soluble CD14 (sCD14), soluble toll-like receptor 2 (sTLR2)),[95,96] some of which will be considered in the section on bioactive factors in breast milk.

There are two other important aspects to the innate immune function in mucosal surfaces, especially active in the gastrointestinal tract: toll-like receptors (TLRs) and the interaction between indigenous bacterial flora and the intestine in developing the T-helper cell response. These gut-associated immune mechanisms have been reviewed by Forchielli and Walker.[97] TLRs are transmembrane receptors which can detect and discriminate among an extensive variety of pathogens and produce differential immune responses accordingly. Pathogen-associated molecular patterns (PAMPs) are a conserved feature in the pattern of molecules expressed by specific pathogens and commensal organisms that are unique to the bacteria. These PAMPs are recognizable by TLRs: TLR2 recognizes bacterial lipoproteins and peptidoglycan; TLR3 identifies double-stranded DNA; and TLR4

recognizes lipopolysaccharide. Of the 10 TLRs identified in humans, some have identified ligands to which they bind and others are still being investigated. Toll-like receptors have been identified on numerous cells within the gastrointestinal tract such as intestinal epithelial cells and dendritic cells. The expression of TLRs on intestinal epithelial cells appears to be influenced by gut flora and local immune response. It now appears from a variety of studies that these pattern recognition receptors in the gastrointestinal tract function in the interaction between the host and the intestinal flora, "priming" or influencing the host's immune response. This is what is meant by "cross-talk" between the indigenous intestinal flora and the body's immune responses. Recognition of specific bacterial antigens by intestinal epithelial cell TLRs activates different intracellular signal pathways that lead to different T-lymphocyte immune responses. It has been proposed that the ongoing immune stimulation due to the bacterial flora in the gut "programs" the host for different T-helper cell responses: TH1-like, TH2-like, and TH3-like. Th1-like response is recognized as delayed-type hypersensitivity or cellular immunity. It is characterized by the secretion of cytokines: IL-2, IL-12, and γ-interferon. The Th2-like response is primarily related to humoral immunity, antibody production, and IgE responses. It is associated with the secretion of interleukins: IL-4, IL-5, and IL-6. The TH3-like response is associated with oral tolerance and antiinflammatory effects and with the release of IL-10 and transforming growth factor-β (TGF-β). The theoretical ideal is some balance of the host's ability to respond to different stimuli and situations with an appropriately regulated T-lymphocyte response to effect protection without excessive inflammation or damage to the host. The theoretical disadvantage of an imbalanced (unregulated) response could be reaction against "normal" food proteins with an allergic-like response (TH2 excess) or an inflammatory response against self-antigens (autoimmune reaction—TH1 excess) causing disease such as inflammatory bowel disease.[97,98] Intense debate and research are exploring these theories and looking for additional proof for them. The effect of breast milk on the infant's indigenous flora (microflora), especially during the first year of life while the systemic and mucosal immune systems are maturing, takes on new importance relative to these new concepts and the idea that the mucosal immune system development depends and is determined by the microorganisms present.

Infant Microflora, Probiotics and Prebiotics

Probiotics are defined as live microorganisms that are ingested to change the indigenous microflora to produce a health benefit in the host. Prebiotics are substances that produce a change in the colonic environment to increase the growth of bacteria that stimulates the host's intestinal defenses. Common probiotics include *Lactobacillus rhamnosus GG, Bifidobacteria infantis, Streptococcus thermophilus, Bacillus subtilis, Saccharomyces boulardii,* and *Bifidobacteria bifidus*, although there are many more, some of which are available in commercial products.[99] Prebiotics are generally considered nondigestible oligosaccharides that undergo fermentation in the colon producing a lower pH and increased amounts of small-chain fatty acids (SCFA). Galacto-oligosaccharides and inulin-type fructans are food additives that have been tested as prebiotics. There are a number of proposed mechanisms of beneficial probiotic action: competition with pathogenic microorganisms for intestinal colonization, strengthened tight junctions (improving the barrier effect), production of antimicrobial bacteriocidins, increased mucus production, stimulating peristalsis, increased production of beneficial nutrients (arginine, glutamine, SCFA), increased secretion of sIgA, and "cross-talk"—the interaction between intestinal cells and bacterial microflora of the gut influencing the development of the mucosal immune system. In addition to the obvious effect of stimulating the growth of beneficial commensal bacteria in the gut, prebiotics can have a variety of other more direct beneficial effects on the intestine. These include serving as nutrients for "fermenting" bacteria that produce abundant SCFA (acetic, butyric, lactic, and propionic acid) and decreasing the intraluminal pH,[100] blocking the adherence of pathogens,[101] and stimulating the production of certain cytokines (IL-10 and γ-interferon).[102]

Microbial colonization of the neonatal intestinal tract begins during birth with maternal flora being the first source of colonizing organisms. Numerous factors can influence what organisms colonize the infant including gestational age, mode of delivery, ingestion of breast milk or formula, initiation of solid foods, the route of delivery of food, the time of onset of feeding, exposure to other microbes through contact (with mother, family, animals, hospital staff, etc.), antibiotics, illness,

etc.[103] The indigenous flora of breastfed infants includes *Lactobacillus bifidus* and *Bifidobacterium spp*., making up over 95% of the flora, with the remaining culturable bacteria including *Streptococcus*, *Bacteroides, Clostridium, Micrococcus, Enterococcus*, *E. coli*, and other less common organisms.[99] Bifid bacteria have been shown to be inhibitory to the growth of various pathogenic bacteria: *Staphylococcus aureus, Shigella, Salmonella, V. cholerae*, *E. coli, Campylobacter,* and rotavirus.[104] The intestinal flora of formula-fed infants contains only 40 to 60% bifidobacteria and higher percentages of Gram-negative coliform bacteria and *Bacteroides,* as well as other organisms such as *Clostridium*, *Enterobacter*, and *Enterococcus* than that of breastfed infants.[99]

Prebiotics present in human milk are primarily oligosaccharides, but proteins, peptides, and nucleotides in breast milk also contribute to the growth of lactobacillus and bifidobacteria in the infant's gut. Oligosaccharides are one of the four main components (lactose, lipids, oligosaccharides, protein) of human breast milk and the third in terms of quantity. They are highest in quantity in colostrum and decrease to approximately 12 to 14 g/L in mature milk. Cow's milk and infant formulas contain less than 1 g/L of oligosaccharides.[99] Various proteins and peptides in human milk have both antimicrobial and separate bifidogenic effects. Casein, α-lactalbumin, and lactoferrin are the best examples of bifidobacteria-promoting proteins in human milk. There is some evidence that nucleotides may also increase the growth of bifidobacteria.[99]

The importance of the intestinal microflora to the infant's developing immune system is discussed above in the section on the Mucosal Immune System. A number of studies have suggested a role between intestinal microflora and the development of necrotizing enterocolitis (NEC) in premature and very low birth weight (VLBW) infants.[105,106] Gewolb and coworkers identified low percentages of Bifidobacterium and Lactobacillus in the stool of VLBW infants during the first month of life and suggested this may be a risk factor for infection in these infants.[107] Several articles have examined the use of probiotics and the occurrence of NEC in premature or VLBW infants.[108-110] These studies demonstrated a lower incidence of NEC in the infants receiving the probiotics. There was no increased risk of sepsis due to the probiotic organisms or other noted complications in the treatment groups, although the studies were not explicitly set up to look for such rare events as sepsis due to the probiotic organisms. Given the many potentially influential variables (early versus late feeding, continuous versus intermittent bolus feeds, fortified human milk versus premature formula, etc.) and the many possible confounding variables (small for gestational age, hyaline membrane disease, infection, etc.) related to the occurrence of NEC, it will require several large carefully designed controlled trials to study the potential benefit of probiotics in preventing NEC.[111] Others researchers have examined the addition of probiotics or prebiotics to infant nutrition and the effect on the intestinal microbiota and measures of the infant's immune response.[112,113] A large clinical trial of probiotics in 585 preterm infants in Italy showed no differences in the occurrence of urinary tract infection, NEC, or sepsis between the control and probiotic group. However, the event rate was low in the control group and the probiotics were not begun until the second week of life.[114] Another report examined the effect of probiotics (*L. rhamnosus GG*) added to formula and the occurrence of infections in children attending daycare. There were small reductions in the number of children with lower respiratory tract infections or receiving antibiotic for respiratory infections in the probiotic-supplemented group.[115] Although the study of the potential benefits probiotics and prebiotics as additives to formula or infant feeding is of interest, it is still another example of trying to make formula more like breast milk.

Bioactive Factors in Human Breast Milk

Human breast milk is the ideal nutrition for human infants. The constant frenzy of formula companies including one more additive in their formula (polyunsaturated fatty acids, nucleotides, oligosaccharides) to make it better than the rest and more like breast milk is one more proof that breast milk remains the gold standard for infant nutrition. There are numerous "bioactive factors" contained in breast milk that contribute many of the beneficial effects of breastfeeding. A review of bioactive factors in breast milk has been recently completed by Margit Hamosh.[85] These factors provide immune benefits to the infant through a variety of mechanisms, most of which have been discussed above, including direct or indirect antimicrobial activity, stimulating immune function development, modulating immune function, antiinflammatory effects, and enhancing growth and development of tissues of the infant. Many components are multifunc-

tional and interact with other factors to produce their effects or work dynamically with the infant's immune system to produce the beneficial effects of breast milk. The benefits to the infant are more than the sum of the bioactive factors contained in human breast milk. Bioactive factors can be categorized according to their functions, their mechanism of action, or their chemical nature; these various components are present in human breast milk in differing amounts during different phases of lactation.[116]

Proteins, as a major nutrient group, contain a number of important bioactive factors including immunoglobulins, lactoferrin, lysozyme, α-lactalbumin, and casein. The specific immunoglobulins in breast milk (predominantly sIgA, and less IgM, IgG) function by directly binding to specific microbial antigens, blocking binding and adhesion, enhancing phagocytosis, modulating local immune function, and contributing to the infant's immune system development. Lactoferrin functions via iron chelation (limiting siderophilic bacterial growth), blocking adsorption/penetration of viruses and adhesion of bacteria, contributing to intestinal cell growth and repair (maintaining an effective barrier), and decreasing production of interleukins-1, -2, -6, and TNF-α from monocytes (immune modulation). Lysozyme causes bacterial cell wall lysis, binds endotoxin (limiting its effect), increases IgA production, and contributes to macrophage activation (immunomodulatory effects). Lactalbumin carries calcium, is an essential part of the enzyme complex that synthesizes lactose, and promotes the growth of bifidobacterium. After modification in the intestine, an altered lactalbumin called "human α-lactalbumin made lethal to tumor cells" seems to function by contributing to apoptosis of malignant cells (immune modulating and immune protective).[117] Casein inhibits adhesion of various bacteria in different epithelial sites and promotes the growth of *Bifidobacterium*.

Carbohydrates in breast milk include lactose and oligosaccharides as the major components and glycoconjugates. They primarily function as important nutrients for energy production. The oligosaccharides act as prebiotics stimulating growth of *Lactobacillus* and *Bifidobacterium* and by binding microbial antigens. The glycoconjugates bind specific bacterial (*V. cholerae*) and viral ligands (rotavirus).

Lipid, the third major nutrient and energy source in breast milk, includes triglycerides, long-chain polyunsaturated fatty acids (LC-PUFA), and FFAs. FFAs and monoglycerides, digestive products of triglycerides, have a lytic effect on various viruses. FFAs also have an antiprotozoan effect, specifically against Giardia.

Vitamins A, C, and E, in addition to their nutrient effects, have antiinflammatory effects due to oxygen radical scavenging. Various enzymes in human milk also have dual functions, in addition to breaking down nutrients into usable products: bile salt associated lipase activity releases FFA, which has antimicrobial activity; catalase has antiinflammatory effects due to degradation of H_2O_2; and glutathione peroxidase decreases inflammation by preventing lipid peroxidation.

Nucleotides, nucleosides, nucleic acids, and related products constitute approximately 15 to 20% of the nonprotein nitrogen contained in breast milk. They are important in a number of cellular functions including energy metabolism (ATP), nucleic acid production (RNA, DNA), physiologic mediators (messengers cAMP, cGMP, and ADP), coenzymes in metabolic processes (NAD, CoA), carrier molecules in synthetic reactions (UDP, GDP, CMP), and signal transduction (cAMP). Nucleotides are not essential nutrients because they can be synthesized endogenously and recycled during metabolic processes. Nevertheless, they are important in the diet, especially in situations of increased demand and metabolic activity such as disease, infection, rapid growth, or other physiologic stresses.[118] Leach and coworkers described the total potentially available nucleosides (TPANs) as a concept of the amount of nucleosides available to the infant for use from human milk.[119] Leach and others measured the mean ranges of TPAN in breast milk in different populations of women.[119,120] These mean values are being used by formula companies to guide the addition of nucleotides to formula. In vitro and in vivo experiments suggest a variety of different roles for ingested nucleotides: increased iron absorption; increased growth of Bifidobacterium; improved growth, development, and repair of the gastrointestinal mucosa; and increased NK cell activity and IL-2 production. Several clinical studies in infants, primarily investigating nucleotide supplementation of formula, showed small benefits, with fewer episodes of diarrhea and higher plasma IgM and IgA levels in the supplemented groups.[121,122] In a 12-month-long, nonrandomized study of 311 infants, the nucleotide-supplemented formula group had fewer episodes of diarrhea and higher geometric mean titers of antibody against *H. influenzae* type b antigen and diphtheria toxoid after immunization with conjugated *H. influenzae* b and DTP vaccines than in the breastfed group

and the control group. Infants who breastfed for longer than 6 months had higher antibody production after oral poliovirus vaccination than did children who breastfed for less than 6 months and the two formula groups (supplemented and unsupplemented). The breastfed group varied dramatically in terms of the amount (differing patterns—exclusive, complete, partial) and duration of breastfeeding, while the nucleotide-supplemented group received a constant amount of supplemented nucleotides throughout the 12 months.[123] The proposed mechanisms of action, related to decreased diarrhea and improved antibody response, were enhanced mucosal immunity in the gut, more rapid repair of damaged mucosa (improved barrier integrity), and improved systemic immune response due to increased TPANs.

There are a number of agents present in human breast milk that are considered immune modulating factors. The majority of theses factors are cytokines, but soluble receptors of these cytokines are also present in breast milk. The list includes the interleukins-1, -3, -4, -5, -6, -8, -10, -12, γ-interferon, tumor necrosis factor-α, and TGF-β. TGF-α, IL-10, and the receptor for TNF-α are associated with antiinflammatory effects. The actual physiologic effects and function of each of these factors in the infant have not been elucidated.[124]

Hormones and growth factors are among the other bioactive components found in human breast milk. Some hormones may have a direct effect on the breast and milk production (insulin, corticosteroids, prolactin), while others may contribute to the growth, differentiation, and development of various tissues in the infant. The various growth factors (epidermal growth factor, nerve growth factor, insulin, TGF-α, and TGF-β, relaxin, insulin-like growth factor) primarily influence growth and development of the gastrointestinal tract, but may have some effect on glucose levels and systemic growth. The function of certain hormones in breast milk, such as erythropoietin, leptin, and melatonin, is speculative at this time.[85]

The list of bioactive factors contained in human breast milk is incomplete because investigators are still identifying new components (cathelicidin antimicrobial peptides).[125] The specific actions and contributions of each of these factors have yet to be determined, because of their involvement in the complex interactions and dynamic processes that have already been demonstrated to be part of the unique benefits of breast milk.

Antiinflammatory Properties of Breast Milk

Although not well understood, the antiinflammatory effects of breast milk are tremendously important in the overall immune protection of the infant. There is no doubt that inflammation plays a dominant role in the pathogenesis of many illnesses. Common examples include infection—sepsis or meningitis; allergy—allergic rhinitis or anaphylaxis; autoimmune disease—systemic lupus erythematosus or inflammatory bowel disease; and chronic diseases—diabetes mellitus or coronary artery disease. In each of these, there is evidence that the inflammatory reaction that leads to disease is misdirected, excessive, uncontrolled, poorly modulated, progressive, or chronic, over the course of the illness. The real benefit of breastfeeding is in the modulated and focused interaction of the many antimicrobial and antiinflammatory factors, contributing to the immune protection of the infant. Garofalo and Goldman have presented a review of this concept and provided an extensive list of the many factors with antiinflammatory activity in human milk.[93]

There are multiple lines of evidence supporting the antiinflammatory activity of human breast milk. There are limited amounts in human milk of the factors that make up several important systems that produce inflammation in the body including the coagulation system, the kallikrein-kininogen system, and the complement system. There are small numbers of several types of cellular effectors of inflammation (basophils, mast cells, eosinophils, and cytotoxic T-cells) contained in breast milk. Although there are proinflammatory cytokines in breast milk, there are also soluble receptors against those factors in milk. There is research evidence that soluble receptors (IL-1Ra, sTNF-α R1 and R2) compete with and/or bind to these cytokines (IL-1, TNF-α), limiting or blocking their inflammatory activity.[126,127] In vivo studies, in animal models, suggest that colostrum decreased the recruitment of neutrophils[128] and feeding with human milk led to decreased myeloperoxidase activity in a rat model with chemical colitis.[129]

Another mechanism of human milk's antiinflammatory action is through antioxidants. Antioxidants contained in human milk include α-tocopherol, β-carotene, ascorbic acid, and L-histidine, all of which scavenge oxygen radicals. These factors may work at both the mucosal level and systemically after absorp-

tion (α-tocopherol, β-carotene).[130] The enzymes catalase and glutathione peroxidase, as well as lactoferrin, have functional antioxidant properties, either degrading or limiting the production of oxygen radicals. Prostaglandins (PGE_1 and PGE_2) act by decreasing superoxide generation.[131] Antioxidant activity is present in both colostrum and to a less extent in mature milk. Inhibition of protease activity via the factors, α_1-antitrypsin, α_1-antichytrypsin, and elastase inhibitor, is present in colostrum and mature milk.[129] Platelet-activating-factor-acetylhydrolase (PAF-AH) is another enzyme present in human milk. PAF is known to cause gastrointestinal mucosal damage and has been associated with NEC in neonates. PAF-AH activity is low in the newborn infant compared with adults.[132] IL-10 is a recognized immune modulator, which has significant potential antiinflammatory effects by decreasing cell activation (macrophages, T-cells, NK cells) secondary to limiting cytokine synthesis, and by increasing B-cell production of IgG, IgA, and IgM. It is contained in large amounts in human milk and its activity has been demonstrated to be blocked by anti-IL-10 antibody.[133] TGF-β is a growth factor that limits production of proinflammatory cytokines (IL-1, IL-6, and TNF),[134] but it may also act by limiting white blood cell adhesion to endothelial cells or decreasing the production of nitric oxide by activated macrophages.

Other antiinflammatory properties of human milk are more indirect. Secretory IgA prevents the adherence of microorganisms to mucosal surfaces without activating the complement cascade. The blocked adherence of pathogens by sIgA and other bioactive factors (proteins lactoferrin, lysozyme, casein, oligosaccharides, and lipids) also limits systemic immune activation. All the factors that function as prebiotics and enhance the growth of *Lactobacillus* and *Bifidobacterium* limit the presence of pathogenic organisms and their potential inflammatory action in the gut. Growth factors (EGF, TGF-α, TGF-β) promote the growth, differentiation, and functional development of the gastrointestinal mucosa, improving its function as a barrier, without causing inflammation. Many of the bioactive factors in breast milk have multiple functions and complementary antimicrobial activities, and these functions and activities are effective against multiple types of organisms. This economy of function and activity is another indirect way of providing broad immune protection for the infant without resorting to excess inflammatory activation. Further investigation of the mechanisms of action of the many bioactive factors in breast milk and their interaction with the infant's developing mucosal and systemic immune systems will be necessary to fully understand and appreciate the benefits of human breast milk's antiinflammatory properties.

Dynamic Nature of the Immune Benefits of Breast Milk

Walker and Wagner and many other researchers have referred to the concept of dynamic changes or interactions or evolution of breast milk and the immune benefits it provides for the infant.[135,136] It is the dynamic nature of breast milk, with all its bioactive factors, and the interaction of the infant and maternal immune systems through breast milk that makes human breast milk the truly unique, incomparable, and ideal nutrition for human infants that it is.

First, breast milk evolves in terms of its volume, its biochemical composition, and its content of bioactive factors over the course of lactation. The ontogeny of human infant development is dependent on this evolution of breast milk to provide not only the required nutrients, but the immune protection, immune stimulation, and developmental modulation via the important components provided in the right quantities during the appropriate timeframes in the infant's growth, development, and ongoing adaptation to extrauterine life. The volume and composition of milk changes from the first stage, Lactogenesis I, with prepartum milk and colostrum, through the second stage, Lactogenesis II, with transitional milk (through 7 to 14 days postpartum), and mature milk.[116] Many factors affect the volume and composition of human milk: stage of lactation; parity; volume of milk production; infant feeding; maternal diet and energy status; and maternal health, illness, and stress.[137,138] The complexity of these evolving changes in the composition of breast milk is addressed in an entire book edited by Jensen, *Handbook of Milk Composition*.[139] Transitional milk varies considerably in its composition, between mothers, and even in the same mother from day to day through the first month of lactation. This is logical ontologically; each individual mother providing for the specific developmental needs of her individual child, which are rapidly evolving in the first month of life. Mature milk is more stable in its composition after about day 30.[140]

The composition of the various bioactive factors in breast milk also varies during lactation. As the infant's

mucosal immune system and systemic immune system develop, it has different needs for the various factors according to their function and effect on the infant. Secretory IgA and cells are at their highest levels in colostrum and transitional milk. Lactoferrin levels decline over the first 12 weeks of lactation, while lysozyme levels increase and both remain relatively constant in breast milk from 6 to 24 months of lactation.[116] The relative percentages of the individual nucleotides and the total potentially available nucleosides in human milk also change over time, from colostrum to mature milk.[119]

There is a dynamic and dramatic change in nutrient requirements due to the metabolic response to infection in the human host. There are multiple changes in the host metabolic response to an acute infection.[141] Numerous variables contribute to the host's actual metabolic response to infection. The state of growth and nutrition before the infection, immune system function, the severity, duration, and progression of the infection, and the ongoing nutritional intake of the individual are all important, as is the localization of the infection to specific organs. Gastrointestinal infections limit the availability and absorption of nutrients; hepatic infections alter carbohydrate and amino acid metabolism, and shock causes other metabolic derangements such as hypoxia, acidosis, and uncoupling of oxidative phosphorylation. One simple example of the benefits of breastfeeding during infection is the improved outcome for infants with diarrhea (without a need for fluid supplementation), when breastfeeding continues or early refeeding with breast milk is practiced.[142,143] There is ample evidence of the presence in breast milk of multiple factors (sIgA, glycans) against specific infectious agents that cause diarrhea in infants.[14,76] The metabolic response to acute infection requires increased amounts of carbohydrates and amino acids for energy production as well as nucleotides for activation of a cellular immune response.[144] Breast milk is the ideal source for adequate amounts of these readily available and absorbable nutrients during any infection, but especially infantile diarrhea.

Another aspect of the dynamic nature of the immune protection afforded infants via the bioactive factors in breast milk is that they act additively and synergistically.[12] Isaacs describes the synergistic effect of specific antiviral lipids and peptides against HSV. The inactivation of HSV is synergistic in that the components attack the pathogen at different points in its replication and require lower concentrations of the factors and less time to effectively inactivate the virus. The antimicrobial activity of breast milk is not measurable solely on the quantity of a specific factor or its apparent activity in vitro. As an example, one of the antiviral activities of lactoferrin is dependent on its proteolysis in vivo, releasing peptides with anti-HSV activity not found in vitro.[145]

The most important contribution to the dynamic nature of breast milk is the MALT system. When an infant and mother are exposed to a potential pathogen within their environment, the mother's mature immune system can react more quickly and effectively than the infant's. The contact of the pathogen with the mother's mucosa (respiratory, intestinal, or vaginal) leads to an immune response that can deposit additional cells and specific secretory IgA and cytokines into the breast milk for the infant. Additional nutrients (carbohydrates, amino acids, fats, and nucleosides) and micronutrients (vitamins, zinc, and selenium) are immediately available to the infant for its accelerated metabolic response to infection. This all occurs before the mother is perhaps even aware of her own exposure/infection or the potential risk to the infant through their mutual exposure.

There are very few maternal infectious contraindications to breastfeeding. Specifically these would include special situations of maternal infection that constitute a significant risk of infection to the infant strictly *through* breast milk rather than maternal–infant contact or mutual exposure from the environment.[146] In particular, breastfeeding is not recommended for women with confirmed infection with HIV-1 and -2, human T-lymphocyte virus-I, West Nile virus, or cytomegalovirus when the infant is premature or very low birth weight. For certain diagnosed maternal infections, it is appropriate to withhold breast milk until the mother has received 24 hours of effective treatment, as in *H. influenzae* type b, *Neisseria gonorrhea*, *S. aureus*, or group B *Streptococcus.* In the case of infection with the spirochetes *Treponema pallidum* (syphilis) and *Borrelia burgdorferi* a longer interval may be appropriate. Confirmed local infection of the mother's breasts with HSV-1 or HSV-2, Varicella virus, Vaccinia virus (smallpox vaccine), *S. aureus*, or *Mycobacterium tuberculosis* is another reason to avoid breast milk feeding. Otherwise, maternal fever, nonspecific viral infections, and undiagnosed maternal infections are not contraindications to breastfeeding as long as the mother is physically able to breastfeed. Continuing to provide the infant with

TABLE 2. 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 given infrequently
	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 has never ingested any human milk		

breast milk will also provide the infant with additional protective factors at the time the infant needs them the most.

Evidence of Protection Against Infectious Diseases from Breastfeeding

Definitions and Concepts

In addition to known emotional and psychological benefits to both mothers and babies, there are clear immunologic advantages of breastfeeding over formula feeding. Immunologic benefits of breastfeeding can be measured in terms of mortality and risk of infection in breastfed infants compared with non-breastfed infants.

In evaluating the validity of studies assessing the protective effects of breastfeeding, one of the most important factors to consider is the definition of breastfeeding. That is, do the authors define breastfeeding as only exclusive breastfeeding, or do they include any breast milk ingested by the infant? Studies that break down feeding categories into ever-breastfed versus never-breastfed may be able to show long-term protective effects of human milk; including infants who received only a small amount of breast milk may actually dilute the demonstrated protective effect.

In 1988, the *Interagency Group for Action on Breastfeeding* (IGAB) developed a set of standardized terms to describe breastfeeding behavior, summarized by Labbok and Krasovek.[147] The schema divides breastfeeding into the two main categories of full and partial (Table 2). Full breastfeeding is further subdivided into exclusive and almost exclusive. Exclusive breastfeeding literally denotes that the infant ingests no other solids or liquids, while almost exclusive breastfeeding acknowledges that small amounts of substances such as vitamins, water, juice, or tea may be given to the infant at infrequent intervals. Partial breastfeeding includes three levels of feeding: high, medium, and low. This breakdown is not clearly defined; the authors state that some have described these categories as "nearly all feeds are breastfeeds, about half are breastfeeds, almost none are breastfeeds," or alternatively by percentage of feeds that are breastfeeds, with 80% described as high, 20 to 80% medium, and less than 20% low. Another designation, token breastfeeding, refers to breastfeeding which is for comfort only and not for nutritive purposes. Other common terms used are "any breastfeeding," which includes full, partial, or token versus "never breastfed," indicating that the identified child never received any breast milk via any mechanism of delivery. IGAB's framework presents additional parameters: time postpartum or child's age; frequency of breastfeeding; intervals; duration; artificial nipples or other devices; type, timing, and amount of other feedings; expression of breast milk and later use; and other influences. Using IGAB's schema and framework, breastfeeding behavior at a given point in time can be described in detail. These distinctions are important to ensure that data interpretation regarding breastfeeding's impact on the health of infants and children is accurate and interstudy comparisons are appropriate.[147]

The specific definitions of breastfeeding inherently address the concept that there is a potential relationship between the "dose" or amount of human milk ingested over time and the potential benefit received. Investigating dose–response relationships implies more objective quantification of the amount of human milk ingested. In premature infants this is sometimes easier, as precise measurement of breast milk, often given by gavage feeds, is

possible. In full-term infants the percentage of feeds at the breast versus bottle may be the best that can be recorded.

Critical review of studies on breastfeeding and infection require that a variety of potential confounding variables be considered. Factors such as level of maternal education and socioeconomic status can have an effect on the amount of breastfeeding (frequency and duration) as well as access to medical care. The presence of siblings and/or daycare contact clearly affects the risk of maternal and infant infection by the increased exposure to infectious agents. Passive exposure to environmental tobacco smoke has been shown to damage the respiratory mucosa and increase children's susceptibility to infection.[148] In studies of preterm infants, additional confounding factors including gestational age and/or birth weight, dexamethasone exposure, multiple birth, obstetrical and other infant risk factors all impact the infant's susceptibility to infectious and noninfectious causes of morbidity and mortality.

The actual method of data collection can be an important influence on actual outcome measures. For example, studies collecting data through home visits may subtly influence mothers' reports of type of feeding. In addition, particularly in developing nations, home visits may include not only data collection, but also education on hygiene practices which could affect infection rates. Mail questionnaires relying on maternal reports of illness and exclusivity of breastfeeding are subject to recall bias.

The concept of *reverse causality* refers to the possibility that the type of feeding might change in response to an illness rather than the illness being a result of a particular feeding practice. In an attempt to avoid reverse causality many studies link infectious episodes with previously reported feeding practices, rather than the feeding practice at the time of diagnosis or hospitalization. This approach eliminates the possible influence of the illness itself changing the feeding practice just before report.

Heinig's rigorous review of a large group of studies from industrialized nations evaluating the effect of duration and exclusivity of breastfeeding on infant health discussed many of the above issues.[77] A subsequent systematic review by Kramer and Kakuma in 2004 critically examined the breastfeeding issues of optimal duration and exclusivity in both developed and developing nations. These two articles provide invaluable insight into the study of breastfeeding, its methodology, confounding factors, and outcomes.[149]

Mortality Data

Infants in the United States in general have a lower risk of mortality, especially when compared with developing nations; however, the US is ranked 27th among developed nations and there continue to be significant racial and ethnic disparities in infant mortality. Black infants have almost twice the infant mortality rates of white infants, with socioeconomic status also affecting infant mortality risk.[150] Breastfed infants have a lower risk of death, but unfortunately breastfeeding rates have been found to be lower in blacks, younger mothers, less educated women, and those from lower socioeconomic groups.[150] Chen and Rogan reviewed data from the 1988 National Maternal and Infant Health Survey for over 1000 postneonatal deaths and almost 8000 control cases. They demonstrated that ever-breastfed infants had 0.79 times the risk of dying (CI 0.67-0.93) compared with never breastfed babies.[151] This study evaluated deaths between 28 to 365 days, excluding those resulting from congenital anomalies and malignant tumors, but including infectious etiologies, injuries, sudden infant death syndrome, and other nonclassifiable causes. Moreover, longer duration of breastfeeding was associated with a lower mortality risk; 3 months or more of breastfeeding revealed an odds ratio of 0.62. This was less than the OR for both the never or the ever-breastfed groups. They estimated that 720 postneonatal deaths could have been prevented that year in the United States alone if all children had been breastfed.[151]

A very large multicenter study examining 9424 infants between 6 weeks and 6 months of age in Ghana, India, and Peru demonstrated that exclusively or predominantly breastfed infants had a significantly lower risk of death from diarrhea and acute respiratory illness in comparison to nonbreastfed or partially breastfed infants. Of note, the investigators controlled for maternal age and education, water source, place of defecation, family size, sleeping space, and infant gender and birth order.[152]

A prospective, observational study in the slums of Dhaka, Bangladesh revealed that partial or no breastfeeding was associated with a 2.23-fold higher risk of death in infancy; deaths attributable to acute respiratory tract infection were 2.40 times more likely, while

deaths from diarrhea were 3.94 times more likely than in exclusively breastfed infants.[153]

Diarrheal Disease

General Background. Worldwide, breastfeeding is a major protective factor against diarrheal illnesses, which cause approximately 2.2 million deaths per year in children under 5 years of age in developing nations.[154] Multiple mechanisms of protection against gastrointestinal illnesses are provided by human milk. Growth factors, such as EGF, may help to induce more rapid maturation of the intestinal epithelium leading to decreased permeability to pathogens. The presence of sIgA prevents attachment of enteropathogens. Secretory IgA specific to many pathogens has been found in human milk: *E. coli, Shigella, Salmonella, H. influenzae, S. pneumoniae*, Rotavirus, respiratory syncytial virus, poliovirus, influenza virus, Giardia, and *C. albicans*, among others.[76] Oligosaccharides inhibit pathogen binding to host cell ligands; they also selectively stimulate the growth of beneficial bacteria in the infant's gut.[155] Certain of these glycans have been shown to be active against specific pathogens, such as ETEC, enteropathogenic *E. coli* (EPEC), *S. pneumoniae, Listeria monocytogenes,* rotavirus, and influenza virus.[156] Lactoferrin has broad antimicrobial properties including disruption of the bacterial outer membrane.[155] While the presence of nucleotides is known to be crucial to cognitive development, they are also critical substrates for cellular growth in intestinal regeneration and protection against diarrhea.[123]

Developed Nations. The protective effect of breastfeeding has been shown in studies in many developed nations. In the United States, Scariati and coworkers evaluated data gathered through the Infant Feeding Practices Study, using a series of mail questionnaires to collect information prospectively about infant feeding practices and health status from the time of pregnancy until 1 year of age. This sample was not completely representative: as compared with a nationally representative sample of mothers participating in the National Maternal and Infant Health Survey, mothers in this study were more likely to be in middle or upper income groups, more likely to be older, married, and white, and less likely to smoke or drink alcohol. Infection in a given month was linked to feeding method for the preceding month to rule out reverse causality. Infants who were exclusively fed formula had an 80% increase in the risk of developing diarrhea over those who were exclusively breastfed ($P < 0.001$).[157]

A longitudinal study conducted in the United States, involving weekly phone interviews and daily symptom logs, demonstrated that the incidence of diarrheal illness in the first year of life for breastfed infants was half that for formula-fed babies. In this study by Dewey and coworkers, the formula-fed group included infants whose mothers had decided prenatally not to breastfeed, as well as those who had stopped breastfeeding before 3 months of age. This inclusion of infants who were breastfed at all up through 2 months of age could have diminished the risk of infection in the formula-fed group; however, the persistent evidence of protection strengthens the outcome and conclusions from this study.[158]

A Canadian study by Beaudry and coworkers of 776 first-born infants utilized a mail questionnaire at 6 months of age; since this method relied on maternal recall, illnesses may have been underreported but this would be equally likely for both feeding groups. The investigators here included both exclusively breastfed infants as well as partially breastfed infants in the breastfed group. Again there was support of the protective benefit of breastfeeding with the incidence density for gastrointestinal illnesses being 47% lower in breastfed than formula-fed infants [incidence density ratio (IDR) = 0.53; 95% CI 0.27-1.04].[159]

A large cluster-randomized trial in the republic of Belarus enrolled over 17,000 mother–infant pairs intending to breastfeed, with over 96% of these dyads completing the 12-month follow-up. The overall goal of the study was to determine if an experimental breastfeeding promotion intervention affected the duration and exclusivity of breastfeeding; secondary outcome measures included the occurrence of gastrointestinal illnesses, respiratory infections, and atopic dermatitis or eczema. Within the control group a large proportion of breastfeeding occurred with 60% of mothers still breastfeeding to some extent 3 months after the infant's birth. The experimental intervention group noted a positive effect on the main outcome measure of duration and exclusivity of breastfeeding. In this group, 78% of infants were still breastfeeding at 3 months of age. The proportion of mothers exclusively breastfeeding was 7 times higher at 3 months (43.3% versus 6.4%, $P < 0.001$) and 12 times higher at 6 months (7.9% versus 0.6%, $P < 0.01$) in the experimental group. The authors detected a significant reduction in the incidence of gastrointestinal infection

from 3 to 6 months of age in the 6-month exclusively breastfed group (adjusted incidence density ratio: 0.35 95% CI 0.13-0.96), but no significant differences in infant respiratory illness. Of note, there was a relatively low incidence of infections in all infants in the Belarus study, which the authors attributed to prolonged obligatory maternity leave (3 years), absence of infant daycare, and the presence of breastfeeding in both the control and the experimental groups. Moreover, they noted that maternal hospital stays of 6 to 7 days following routine vaginal delivery are standard and may help establish good breastfeeding practices.[160,161]

Less evidence is available to document the effect of feeding human milk on the incidence of gastrointestinal viral infections, but it appears that breastfed infants do experience some advantages over formula-fed infants. A prospective study using maternal–infant pairs from a low-income clinic in Buffalo, NY examined rates of illness and microbiologic results of stool samples of infants during the winter rotavirus season. Infants were recruited that would be 6 to 9 months of age during the time of the study. Very few of them were in daycare. Infants were classified by feeding type at birth: exclusively breastfed, exclusively bottle fed, or a combination of the two. At 4 months a category was added to differentiate those who had been exclusively breastfed but were switched to exclusive formula feeds. Overall, breastfed infants had a lower attack rate for gastrointestinal illness with no identified pathogen (RR = 0.83, 95% CI 0.62-1.12) and those exclusively breastfed for at least 4 months had the lowest attack rate (RR = 0.29, 95% CI 0.24-0.83). There was not a protective effect for rotavirus infection except in those exclusively breastfed for 4 months (RR = 27, 95% CI 0.28-1.90).[162] Most notable, however, was the increased severity of symptoms in formula-fed infants. Severity was defined based on scales of number of loose stools and duration, episodes, and duration of emesis, body temperature, and degree of dehydration. A cumulative clinical score led to classification of severity of illness as mild, moderate, or severe. None of the severely ill infants were in the group breastfed at 4 months of age. In addition, seven of nine infants who received combined feedings were infected with rotavirus within 4 weeks of being partly weaned from breast milk. Due to routine surveillance of stools in this study, it is possible that earlier and milder cases of rotavirus were detected.[162] The authors further analyzed the data from this group of infants and noted that breastfed infants had a predominance of bifidobacteria in their stools; however, the infected bottle-fed infants had no detectable bifidobacteria in their stools.[163] While bifidobacteria are thought to limit the proliferation of pathogenic enteric bacteria, their role in decreasing viral infection is unclear.

Developing Nations. In developing nations the need for immune protection for infants and children is even more crucial given poor sanitation, low water quality, contaminated food sources, and other risks for infection. The protection afforded an infant by antibodies in his mother's milk is a reflection of her lifetime exposure to enteric pathogens[155] and in particular those endemic in her environment. More importantly, the mother's mucosal immunity and MALT will allow for antibody production to recent exposures much more rapidly than the infant's still-immature immune system can respond. In addition, the bioactive factors in human milk provide nonspecific protection against various diarrheal pathogens through common mechanisms of action.

A study in Bangladesh demonstrated a significant protective effect against ETEC in exclusively breastfed infants during the first year of life (RR = 0.51, 95% CI 0.28-0.96), but no protective effect during the second and third years of life. All breastfed infants were partially breastfed after 12 months rather than exclusively breastfed. In this study there were very small numbers of nonbreastfed infants: 2 cases and 10 controls in the under 1-year age group, and 11 cases and 624 controls in the 12- to 35-month age group. This same study found a greater protective effect against cholera infection due to breastfeeding. In infants under 12 months of age, the relative risk was 0.02, while during ages 12 to 35 months the relative risk was 0.27. This was a retrospective case-control study, where the groups were divided into partial breastfeeding (ie, any breastfeeding), exclusive breastfeeding, and no breastfeeding.[164] Another previously mentioned study from Bangladesh showed that deaths from diarrhea were almost four times more likely in non- or partially breastfed infants as in exclusively breastfed infants.[153]

An interesting study of almost 200 Mexican term infants correlated the amount of secretory IgA in a mother's milk with presence or absence of infection with Giardia in her infant as well as presence or absence of symptoms in infected infants. The mother–infant pairs were evaluated prospectively and followed

from birth through 18 months of age. Infants were followed weekly with stool cultures as well as field visits to determine type and frequency of feeding and symptoms; milk samples were collected weekly for the first month and monthly thereafter. There was no significant difference between sIgA concentration in milk fed to infected and noninfected infants. However, symptomatic infants received significantly lower concentrations of anti-Giardia IgA than infants who were infected but asymptomatic (mean log 3.73 $\pm$ 0.20), thus indicating a dose–response relationship between the specific protective factor and symptomatic infection.[165]

Another Mexican study looked at 98 infants followed prospectively from birth to 2 years of age. The infants were visited by a study nurse on a weekly basis and were seen in clinic if the infant developed diarrhea. Diarrhea was defined as at least three loose or watery stools for at least 1 day, ascertained by parents, and the study nurse and physician. Stool specimens were collected during acute and convalescent phases of illness. Milk was obtained from lactating mothers monthly, as well as when her child had diarrheal symptoms. In this study, breastfeeding was defined as any breastfeeding; there was also a postbreastfed group who had previously been breastfed, but had been completely weaned. Breastfed children remained free of diarrhea longer than nonbreastfed children (68% versus 26% by 3 months of age, and 48% versus 13% by 6 months of age; $P < 0.0005$). Infants less than 6 months of age who did not receive breast milk had a 2.3 times greater risk of having diarrhea versus breastfed infants (95% CI 1.4-3.9, $P < 0.03$). Once breastfeeding was discontinued, the protective effect was lost.[166]

The second part of the same study looked specifically at *Campylobacter jejuni* infections related to anti-*Campylobacter* antibody in human milk. The risk of *Campylobacter* was significantly greater in nonbreastfed than breastfed children (3.2, 95% CI 1.2-8.6; $P < 0.022$). Concentrations of secretory IgA to the glycine-extractable common antigen of *Campylobacter* were measured in maternal milk samples. Overall, sIgA concentrations were highest in colostrum, declined over the first month of lactation, and remained constant thereafter. The children who developed *Campylobacter* diarrhea while breastfeeding consumed milk that did not contain *Campylobacter*-specific IgA.[166]

Giardia lamblia is extremely common in infants and children in both developing and developed nations. A prospective study of 197 infants in Mexico found that lack of breastfeeding was a significant risk factor for *Giardia* infection (adjusted rate ratio 5.0; 95% CI 1.5-16.9 for no breastfeeding versus complete breastfeeding and 3.0 with a 95% CI 0.9-9.9 for partial versus complete breastfeeding) as well as symptomatic infection (none versus any breastfeeding, adjusted rate ratio 2.5; 95% CI 0.9-6.8). However, breastfeeding did not affect chronic carriage of the organism.[167] In Nicaragua, children of mothers who lacked anti-*Giardia* antibodies in their milk were three times as likely to be infected versus children of mothers with *Giardia*-specific antibody present in breast milk.[168]

In summary, the above articles represent generally large study populations, with 5 of the 12 containing data from developed nations and the remaining 7 from developing nations. The sample sizes ranged from 86 to 17,046 with a mean of 2732, a median of 252, and a mode of 197. There were some differences in the way breastfeeding was defined; some looked at exclusively breastfed versus exclusively formula fed, while others categorized the feeding into full, partial, or any breastfeeding. As mentioned above, lack of a consistent definition of breastfeeding sometimes hampers the ability to draw conclusions on the protective effect of breastfeeding. Most of the above studies looked at exclusive breast versus exclusive formula feedings. These comparisons generally reveal the most significant differences in outcome measures, and in the case of these studies on diarrheal disease, demonstrate the benefits of breast milk.

Respiratory Infections

Respiratory infections are a major source of morbidity and mortality in infancy, and breastfeeding has been shown to protect against a variety of respiratory pathogens. A meta-analysis of seven studies conducted in developed countries by Bachrach and coworkers evaluated rates of hospitalization for lower respiratory tract disease. These 7 were selected from 34 relevant studies meeting the inclusion criteria of a focus on only industrialized nations, healthy infants without other risk factors (eg, prematurity, low birth weight, or chronic illness), and comparison groups with a minimum of 2 months of exclusive breastfeeding or 9 months of any breastfeeding versus no breastfeeding. Specifically, four studies compared exclusive breastfeeding for at least 4 months with no

breastfeeding, another compared exclusive breastfeeding for 6 or more months with no breastfeeding, and two compared any breastfeeding for 9 or more months with no breastfeeding. This meta-analysis detected that the rate of severe respiratory illness resulting in hospitalization for formula-fed was three times higher than for the breastfed infants.[169]

Cesar and coworkers compared a group of 152 Brazilian infants hospitalized with physician-diagnosed pneumonia with 2391 controls in a population-based nested case–control study. Feeding groups categorized infants as receiving exclusive breast milk, breast milk and formula, or formula and other fluids; the data also were stratified based on feeding of other supplemental liquids (such as tea or juice) or solids. The study revealed that formula-fed infants were 17 times more likely to be admitted for pneumonia than exclusively breastfed infants; the calculated relative risk was 61 (19.0-195.5) for those less than 3 months old, and 10 (2.8-36.2) for those 3 months or older.[170]

Sinha and coworkers evaluated the effect of breastfeeding on the risk of neonatal respiratory infections. Within this large US cohort of 13,224 mother–infant pairs, there were 241 neonatal respiratory tract infections recorded. Case subjects were more likely to (1) be born during winter respiratory syncytial virus season; (2) have a sibling in the household; or (3) be socioeconomically at-risk. This latter category was defined as meeting one of the following criteria: enrollment in Medicaid program; maternal age <22; residing in a census tract with either a median income under $25,000 or more than one-third of the adult population not having a high-school diploma or its equivalent by age 25. Case patients also were less likely to be exclusively breastfed; the odds ratio of exclusive breastfeeding to exclusive formula feeding was 0.70 (95% CI 0.49-0.99).[171]

Similarly, a study by Beaudry and coworkers identifying any infection in an infant's first 6 months of life found that the crude incidence density for respiratory illnesses was 34% lower in breastfed versus formula-fed infants (IDR = 0.66; 95% CI 0.52-0.83).[159]

The protective effect of breastfeeding may be modulated by many factors, such as the presence of older siblings and/or attendance at daycare, which can influence the degree or frequency of exposure to infectious agents. Pettigrew and coworkers demonstrated this protective effect only in first-born breastfed infants. Their investigation was part of a larger prospective study of breastfeeding practices and mastitis in the United States. Telephone interviews were conducted at 3, 6, 9, and 12 weeks postpartum, or until breastfeeding ceased. At 6 months postpartum a questionnaire was mailed inquiring about illnesses which resulted in a visit to a health care provider (IRHP) within the preceding 30 days. For firstborn children, the likelihood of an IRHP decreased by 4% for each additional week of breastfeeding; the difference was not significant for those who had siblings in the household.[172] When stratified by infant gender, the protective effect of breastfeeding on risk of neonatal respiratory tract infection was only evident in girls (unadjusted OR 0.5, 95% CI 0.29-0.78 for exclusive breastfeeding).[171] The authors accounted for this finding due to male neonates having lower absolute and relative pulmonary flow rates and airways more susceptible to obstruction. Thus the findings would be consistent with what has long been known in neonatal intensive care nurseries that girls have higher survival rates than boys.

Otitis Media

Studies have demonstrated that ear infections are not only less common in breastfed infants, but also less likely to become chronic. Beaudry and coworkers determined that the protective effect of breastfeeding against otitis media (OM) persisted even when adjusted for confounding variables or analyzed based on length of illness.[159]

One reason for the decreased incidence is purely mechanical; the Eustachian tube closes in breastfed infants while they are nursing, thereby preventing reflux of milk into the middle ear, which can lead to inflammation and subsequent blockage of the tube. Also, breastfed infants are typically held in a different position while feeding, which also makes them less prone to milk reflux than bottle-fed infants, who are more likely to be fed supine.

Dewey and coworkers found that the percentage of infants with one or more episodes of acute otitis media (AOM) before 1 year of age was 19% lower in breastfed versus formula-fed infants, and the percentage of infants with prolonged episodes (greater than 10 days) was 80% lower in breastfed versus formula-fed infants. Because breastfeeding has been shown to provide prolonged protection against OM, the inclusion of infants who were breastfed for short periods of time (less than 3 months) strengthens the evidence for protection.[158]

A prospective cohort study in upstate New York investigated the effect of feeding practices, parental smoking, and daycare attendance on the incidence of AOM, otitis media with effusion (OME), and colonization with middle-ear pathogens. The infants were evaluated frequently until 2 years of age, including monthly for the first 6 months. The investigators found that in the first 3 months of life, first episodes of AOM were increased significantly in infants fed only formula versus those fed only breast milk (RR 1.39, 95% CI 1.00-1.94). At 6 months infants who were formula fed had almost double the risk for both AOM (RR 1.82, 95% CI 1.15-2.90) and OME (RR 2.06, 95% CI 1.01-4.18) than exclusively breastfed infants. Rates of colonization with middle-ear pathogens such as *S. pneumoniae*, nontypable *H. influenza*, and *Moraxella catarrhalis* were higher in formula-fed versus exclusively breastfed infants at 3, 6, and 12 months of age; the rate differential was statistically significant ($P = 0.003$) at 6 months (54.3% versus 27.3%). Although daycare attendance by index case and sibling(s), parental smoking, and family history of OM were all evaluated in this study, a multivariate logistic regression demonstrated that formula-feeding remained the most consistent predictor of episodes of OM at 3, 6, and 12 months of age.[173]

Another prospective US study followed 1220 infants for the first year of life. Infants who were exclusively breastfed for 4 months or more had 50% fewer mean episodes of AOM than the exclusively formula-fed infants, and 40% fewer than breastfed infants supplemented before 4 months of age. The investigators controlled for marital status, socioeconomic status, parental education, family history of allergy, gender, number of siblings in the home, number of others sharing a bedroom with the infant, use of daycare, and maternal smoking. The rates of recurrent OM were also affected by the ingestion of breast milk. The rate of recurrent OM was 10% in infants exclusively breastfed for 6 months versus 20.5% in those not breastfed, or breastfed for less than 4 months.[174]

Aniansson and coworkers studied 400 children in Sweden to determine the effect of breastfeeding on OM. The frequency of AOM in breastfed infants was significantly lower than in nonbreastfed infants for each age group ($P < 0.05$). However this benefit did not continue across groups of children with siblings or with daycare attendance.[175] Scariati and coworkers also found an 80% increased risk of developing AOM in low-mixed ($P < 0.003$) or formula-only ($P < 0.001$) -fed infant groups as opposed to the breast-milk-only infant group.[157]

Urinary Tract Infections

Substances in breast milk such as secretory IgA or oligosaccharides may interfere with bacterial adhesion to urinary epithelium. The increased excretion in urine of lactoferrin, a noninflammatory antimicrobial constituent of breast milk, may also contribute to a decreased frequency of urinary tract infections (UTI) in breastfed infants.[77,176] Breastfeeding also has been shown to lower enteric bacterial flora counts and lead to *E. coli* of lower virulence. *E. coli* is well-recognized as one of the common pathogens responsible for UTIs.[176]

Ongoing exclusive breastfeeding has been shown to be associated with a significantly lower risk of UTI. A prospective case-control study in Sweden published in 2004 demonstrated that a longer duration of breastfeeding imparted a lower risk of UTI even after weaning. The impact of breastfeeding, as determined by Poisson regression analysis, demonstrated a hazard ratio of 2.30 (95% CI 1.56-3.39) for nonbreastfed as opposed to breastfed infants.[177]

An Italian case-control study categorized infants into one of three groups: exclusively breastfed; combined feedings of breast milk and formula; and exclusively formula-fed. In addition to limit reverse causality bias, another classification schema of ever-breastfed or never-breastfed was also utilized. The formula-fed infants had a five-fold higher risk for urinary tract infection than the breastfed infants. Breastfed infants had a relative risk of UTI of 0.38 (95% CI 0.22-0.65) when the dichotomous classification ever- or never-breastfed was used. When evaluated in terms of feeding group at the time of admission, the odds ratio for breastfed infants (both exclusively and combined with formula) was 0.18 (95% CI 0.09-0.36).[178]

Protection in Premature or Low Birth Weight Infants

As important as breastfeeding is to improving the immune status of healthy term infants, it is even more crucial to premature infants who have had inadequate time to obtain transplacentally acquired maternal antibodies in the third trimester and whose skin, respiratory, and gastrointestinal epithelium is even more immature. Sepsis, meningitis, and NEC are all major

causes of morbidity, mortality, and long-term sequelae in these vulnerable infants, but there is evidence that human milk can help protect against these illnesses.

Although the structure of the gastrointestinal system is fully developed by approximately 20 weeks of gestation, gastrointestinal function remains immature until late in the third trimester. Gastric acid and protective mucus levels are lower in preterm infants and intestinal permeability is increased, which may lead to invasion of bacteria from the gut into the bloodstream.[179] As discussed previously, human milk has been shown to enhance the maturation of the intestinal epithelium and promote colonization with less virulent strains of enteric bacterial flora.[176,179]

There are many important issues when discussing the nutritional support of premature infants. Differences arise in growth parameters when comparing infants fed premature formula versus human milk. It is unclear whether this may lead to long-term growth failure. In addition, whether adding fortifiers to breast milk has any deleterious effect on its immunologic activity deserves clarification. Discussion of these topics is beyond the scope of this review.

However, a meta-analysis of four small studies demonstrated that human-milk-fed infants were three times less likely to develop clinical NEC and four times less likely to have confirmed NEC.[180]

Schanler and coworkers in a controlled US trial investigated not only human milk versus formula, but also early versus late initiation of feeds and continuous versus bolus feeding regimens. The type of milk was determined by parental choice. If parents chose mother's milk, the milk was fortified; otherwise, the infants were fed preterm formula. Only infants fed >50 mL/kg/d were included in the study group. Their study revealed that human-milk-fed infants were discharged earlier (73 ± 19 versus 88 ± 47 days) despite slower growth parameters, and they experienced less NEC (1.6% versus 13%) and late-onset sepsis (31% versus 48%).[181]

Hylander and coworkers followed 212 consecutive VLBW infants in a US NICU who survived to receive enteral feeds. Characteristics of the human milk and formula groups were similar in terms of risk factors for infection for parameters such as gestational age and Apgar score. The breastfed infants showed a reduction in the odds of sepsis/meningitis (53%) as well as other infections (57%). There was also a higher rate of multiple infections in formula-fed infants.[182] el-Mohandes and coworkers demonstrated that the lower odds ratio for sepsis in human-milk-fed infants (0.4) was unrelated to the documented increased colonization with *E. coli* and *Enterococcus sp.*[183]

A randomized controlled trial assessing the benefits of nucleotide-enriched formula showed that human milk feeding was a statistically significant factor in decreasing serious adverse events both during initial hospitalization and on hospital readmissions; however, this study found no effect on the risk of occurrence of necrotizing enterocolitis or sepsis.[184]

An interesting finding was shown in a randomized, blinded study by Schanler and coworkers in 2005. Infants of less than 30 weeks gestational age whose mothers chose to breastfeed were randomly assigned to receive either pasteurized donor human milk or preterm formula if the supply of their own mothers' milk became insufficient; both human milks were fortified. Infants in the donor milk group failed to reveal a lower incidence in NEC, late-onset sepsis, or other infections, nor was there a difference in their length of stay or mortality rate. However, infants who only received their own mothers' milk had fewer episodes of NEC, late-onset sepsis, and other infections and experienced a shorter length of stay than either the donor-milk-fed infants or those fed premature formula (75 ± 37 versus 87 ± 53 versus 90 ± 37). Of note, 21% of infants in the donor milk group were switched to premature formula due to poor weight gain. Although there were no differences in terms of infant birth weight, gestational age, duration of mechanical ventilation, or achievement of full feeds, the three maternal groups were not comparable in all parameters. The mothers who provided a sufficient milk supply were older, more educated, more frequent nursery visitors, and practiced kangaroo care more often than those in either the donor milk group or the preterm formula group.[185] One caveat is that the donor milk was pasteurized, and it is known that heat can change the function of bioactive factors in human milk and hence the potential for immune benefits.

A small study of 39 infants investigated whether human milk feeding after discharge affected the subsequent occurrence of illness in premature infants. Infants who received mother's milk (± formula) after discharge had fewer days of upper respiratory tract infection than those who received only formula when evaluated at 1, 3, and 7 months after discharge; however, the difference in the groups at 1 year post-discharge was not significant.[186]

Dose–Response Relationship

A dose–response relationship has been noted such that the higher the proportion of an infant's feeds are from human milk, the lower the incidence of infection. In a study of over 7000 infants in the US, where monthly questionnaires were used to determine the extent of breastfeeding and the occurrence of infections during the previous month, there was a documented dose–response relationship between breastfeeding and both ear infections and diarrhea.[157]

Raisler and coworkers in another US study stratified infants by feeds: fully breastfed, mostly breastfed, equal breast milk and other foods, less breast milk than other foods, and no breast milk groups, were established. These data were obtained through the National Maternal and Infant Health Survey and specifically focused on high-risk groups; therefore, black and low birth weight infants were over-represented. Outcome measures included the number of illness visits to a health care provider and number of months of illness. Monthly, mothers were asked to report whether their infant had had any of the following seven symptoms or illnesses: diarrhea, cough or wheeze, ear infection, runny nose or cold, fever, vomiting, or pneumonia. Two scores were obtained: one indicating whether the infant had had any of the seven illnesses in a month, and another for whether the infant had each one of the seven illnesses in a given month. Fully breastfed infants had a lower odds ratio of diarrhea, cough or wheeze, vomiting, and lower mean ratios of illness months and sick baby medical visits. Full-, mostly, and half-breastfed infants without siblings had lower odds ratios of ear infections and other illnesses, but those with siblings did not.[187]

Two studies in the premature infant population also address the issue of a dose–response relationship. Furman and coworkers found that at least 50 mL/kg/d of human milk was necessary to show a decrease in the rate of sepsis in VLBW infants,[188] while Schanler and coworkers demonstrated that infants who received at least 50 mL/kg/d of milk had reduced rates of sepsis and NEC.[185]

Summary

Overall, the evidence for a protective effect of breast milk is unequivocal. With convincing data from both developed and developing nations, this information can be generalized to all populations and used to encourage both increased rates of breastfeeding as well as increased duration of nursing, especially in high-risk populations. Multiple studies directly support the concept of a positive dose–response relative to the amount of breast milk ingested and the benefit received. Evidence from specific studies supports exclusive breastfeeding through 6 months of age. There are also data from studies supporting the concept that any amount of breastfeeding can provide some immune protective benefits. Basic laboratory data document the importance of breast milk both supplementing the infant's mucosal and systemic immune systems during this period of developmental deficiency, as well as demonstrating the beneficial influence of breast milk on the mucosal environment and directly on the ongoing normal development of the infant's gastrointestinal tract and immune systems.

Clinicians can utilize this information to accurately and effectively communicate the existing knowledge about the benefits of breast milk to their patients and families, to discuss the advantages of breastfeeding specifically as it relates to each particular mother–infant dyad, and to provide ongoing support and encouragement to all breastfeeding mothers.

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