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Breast milk: immunosurveillance in infancy

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ABSTRACT

Human breast milk is unique and a natural source of nutrition. However, it also helps to protect against various types of disease, not only infective but also immunological diseases. The wide variety of molecules in milk is responsible for its varied role for the newborn infant. Various breast milk proteins, contribute for its immunological, nutritional as well as its antimicrobial role. The naive immune system, intestinal mucosa and other organs of the neonate are also developed by various cellular factors. Breast milk protects not only during the neonatal period but also beyond it. By educating the neonatal immune system it also protects against the development of diseases later in life.

1. Introduction

In utero the fetus is in a highly protected in germ free environment without exposure to external antigen. Even though immunological defences exist in the new born, they are immature as the immunological development starts in the embryo, continues during fetal life, exists in immature form in newborn and is completed several years after birth.

The neonatal immune system differs from that of an adult. The 'immunosuppressed' state of the fetus is essential during gestation to avoid immunological reactions that would result in termination of pregnancy. This is reflected by an inappropriate chemical barrier[1], frail mucosal barrier[2], immaturity of T and B lymphocytes, poor T lymphocyte response to mitogens, reduced cytotoxic response, inadequate cytokine synthesis, marked deficiency of antibody production, reduced neutrophil, complement and natural killer cell activity[3]. The World Health Organisation and the American Academy of Pediatrics recommend exclusive breast feeding for six months as it provides optimum nutrition to the developing infant. Apart from a nutritional point of view, breast feeding maintains the maternal-fetal immunological link after birth. It also favours transmittance of immunocompetence from mother to her infant and is considered to be the central contributing factor for the immune defense system of the neonate[1].

Supportive data shows the benefit of breast feeding in preventing gastrointestinal and also respiratory diseases in not only developing countries but also developed countries[4]. It has also been shown to give protection

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against urinary tract infections and otitis media[5,6]. Breast milk protects the infant against infections as well as future growth of allergic diseases[7]. Conclusions drawn from a systemic review also reveal that breast feeding protects infants from the development of atopic diseases even if there is a family history[8]. Epidemiological studies have shown a reduced incidence of immune-mediated diseases including celiac disease, inflammatory bowel disease, type 1 diabetes mellitus, rheumatoid arthritis, asthma, eczema, necrotizing enterocolitis and multiple sclerosis in individuals who have been breast fed[9,10].

2. Compounds with immunological properties in human milk

Human milk is tailored for the infant's requirements. It compensates the relative lack of host defense by giving significant quantity of both nonspecific as well as pathogen-specific secretory immunoglobulin A (sIgA). Antibodies were the first bioactive components that were recognized in human milk. The mother's previous exposure to infectious agent results in these antibodies. Other factors in human milk also provide passive protection through immunological, hormonal, enzymatic and trophic activity[11]. During the early period of lactation, certain cells of the innate system like leukocytes and macrophages exert a modulatory effect on the neonatal immunity[12]. The following immuno-modulatory compounds include immunoglobulin G, immunoglobulin M, isoforms of immunoglobulins (sIgA), nucleotides, polyunsaturated fatty acids (PUFAs), specific amino acids (taurine, polyamines), monoglycerides, linoleic acid, cytokines and chemokines, soluble receptors [CD14, soluble Toll-like receptor (TLR) 2], antibacterial proteins/peptides, prebiotics and oligosaccharides that are found in breast milk[13].

3. Anti-microbial properties of human milk

Breast milk has a variety of antimicrobial substances that function against several viruses, bacteria, and protozoa.

3.1. Immunoglobulins

The defensive role of sIgA which is present at very high concentration in the colostrum (~10 g/L) and in mature milk (~1 g/L) is well known. The IgA_2 is resistant to acidic pH of the stomach and to the digestion by enteric enzymes and bacterial proteases[14]. The immunoglobulin G and immunoglobulin M are present at a low concentration[15]. The transfer of highly specific protection from the mother to the infant is because of the entero-broncho mammary link of IgA with B lymphocytes. When the nursing mother is exposed to antigenic stimulus from environmental pathogens, M cells of Peyer's patches in the gut-associated lymphoid tissue or tracheobronchial tree mucosa take up and acquire the antigen to B cells. Plasma cells become active to produce IgA on the basolateral side of the mammary epithelial cell. The IgA attaches to the poly immunoglobulin receptor, the complex traverses the mammary epithelial cell, then cleaved by protease on the apical side as dimeric sIgA and secreted from the apex of acinar cells into the milk. During pregnancy and lactation, because of hormonal stimuli, IgA B lymphocytes colonize mammaryglands and produce specific secretory IgA that may bind to pathogen and prevent infection[16]. The antimicrobial effects of IgA antibodies are related both to immune exclusion, by inhibition of epithelial adherence and penetration or microbial agglutination and neutralization, and immune elimination, by phagocytosis and cytotoxicity[9]. However, the time that elapse between exposure of a mother (and infant) to a novel antigen and protection of the infant by sIgA in the milk makes this mechanism of protection incomplete at best. Also HIV-specific IgA in human milk from HIV-infected mothers do not show a protective role; on the contrary, specific IgA antibodies may be associated with an enhanced transmission of the infection[17,18].

3.2. Lactoferrin

As a proteolysis-resistant, iron binding glycoprotein, lactoferrin is the dominant whey protein. Its protective effect may be linked to competition with siderophilic bacteria and fungi for ferric iron and to the epithelial growth-promoting activity[19,20]. Lactoferrin limits the growth of bacteria and fungi by competing for essential iron. It modulates relocation, and activation of antigen presenting cells like macrophages[21-23]. The action of lactoferrin is also helped by certain soluble mediators like cytokines, chemokines and other effector molecules. Also epithelial growth promoting actions have been linked with lactoferrin[4]. It has been shown to relieve symptoms and increase the suppression of *Helicobacter pylori* in the stomach[24]. Lactoferrin has been shown to ameliorate rotaviral gastroenteritis by interfering with the early phases of infection and also slow up growth of colorectal adenoma[25]. Studies have shown that lactoferrin inhibits the attachment of enteropathogenic *Escherichia coli* (*E. coli*) to intestinal cells by mediating the serine protease activity of lactoferrin[26,27]. Degradation of the protein structures of

enteropathogenic *E. coli* that are needed for the attachment and invasion of the bacteria, results in infection block.

3.3. Lysozyme

As a key factor of human milk, Lysozyme is able of degrading the outer cell wall of Gram-positive bacteria by hydrolyzing β-1,4 linkages of N-acetylmuramic acid and 2-acetylamino-2-deoxy-D-glucose residues[28]. In a synergetic action with lactoferrin it has been shown to kill Gram negative bacteria *in vitro*[29]. It binds to the lipopolysaccharide and removes it from the outer cell membrane of bacteria. Lactoferrin allows lysozyme to enter, damage the inner proteoglycan matrix of the membrane, and kill the microorganism.

3.4. Lactoperoxidase

Lactoperoxidase in the presence of hydrogen peroxide (formed in small quantities by cells), catalyzes the oxidation of thiocyanate (part of saliva), forming hypothiocyanate, which can kill both Gram-positive and Gram-negative bacteria[30,31]. Thus, lactoperoxidase in human milk may contribute to the defense against infection already in the mouth and upper gastrointestinal tract.

3.5. κ Casein and α lactalbumin

κ Casein, a minor subunit of casein is a glycoprotein with sialic acid residues. It has been shown to inhibit the adhesion of *Helicobacter pylori* to human gastric mucosa[32]. κ Casein has been shown to prevent the attachment of bacteria to the mucosal lining by acting as a receptor analogue[33]. Oligosaccharide structures on the glycans of these glycoproteins act as decoys for similar surface-exposed carbohydrate structures on the gastric mucosa, thereby inhibiting adhesion. Recently three polypeptide fragments from α lactalbumin were found to have antimicrobial activities against *E. coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Staphylococcus epidermis*, *Streptococci*, and *Candida albicans*[34].

3.6. Haptocorrin

In an unsaturated form in human milk this vitamin B_{12}-binding protein has be suggested to inhibit bacterial growth by tightly binding and withholding the vitamin from the bacteria[35]. However whether this is the inhibiting mechanism, how broad its antimicrobial activity is, and whether haptocorrin quantitatively contributes to the defense against infection in breastfed infants remain to be explored.

3.7. Mucin and lactadherin

Mucins are high-molecular-mass glycoproteins. The most commonly studued mechanism is a sialic acid moiety of mucin 1 interacting with the pathogen, thereby inhibiting the ability of the pathogen to bind to its infant host cell surface glycan receptor. Thus, mucin 1 plays a role in innate immune defense of the infant against invading microorganisms[36]. Lactadherin, a glycoprotein of the human milk far globule membrane, binds specifically to rota virus and inhibits its replication, thereby protecting the infants from symptomatic rotavirus infection[37]. Lactadherin has EGF1-EGF2 domains (epidermal growth factor homology) at the amino terminus and the C1 and C2 domains share homology with phosphatidyl serine domains on coagulation factors V and VIII[38,39]. Milk lactadherin is present in the intestines of breastfed infants before the tight junctions of the intestinal epithelium close and when fat complexes can cross the mucosa by bulk transport. Thus human milk lactadherin could gain access to the circulation of the neonate, where its strong anticoagulant effects would be mediated through modulating factors V and VIII activities and through microvesicle clearance.

3.8. Oligosaccharides and prebiotics

Prebiotics are nondigestible food ingredients, generally oligosaccharides, that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of intestinal bacteria, such as bifidobacteria and lactobacilli[40]. The nondigestible sialylated and fucosylated oligosaccharides, in human milk protect against infectious diarrhea by directly inhibiting the attachment of microbes to the mucosal surfaces[41,42]. Studies indicate that when oligofructose is consumed by children, there is increase in fecal bifidobacteria counts and reduction in fecal clostridia counts[43]. Additional benefits with prebiotics include improved bone health, reduced risk of colorectal cancer, boost in immunity, and improving satiety and controlling weight. Experimental models have shown that mixture of synthetic oligosaccharides (fructo oligosaccharides and galacto oligosaccharides) stimulate the response to influenza vaccination[44,45].

3.9. Fatty acids

When milk is consumed it mixes with lingual and gastric lipases, and the triglycerides are digested into free fatty acids and monoglycerides. These strongly stall enveloped viruses, some bacteria and protozoans[46,47]. Monoglycerides as well the fatty acids, linoleic and lauric acids exhibit

the strongest inhibition, acting as detergents on pathogen membranes. With the presence of free oleic acid, human milk lactalbumin is converted into an alternate conformation named HAMLET[48], which induces apoptosis in tumors, leading to remission of the tumor. PUFAs such as arachidonic acid (AA) and eicosapentaenoic acid (EPA), gamma-linoleic acid (GLA), and docosahexaenoic acid (DHA) have been shown to potently alter the functioning of immune cells. Diets rich in n-3 PUFA tend to inhibit excessive immune responses which are associated with chronic inflammatory diseases such as asthma and rheumatoid arthritis[49]. Riediger *et al.* have demonstrated that diets rich in n-6 PUFA promotes immune responses, leading to inflammation that affects chronic inflammatory diseases[49]. EPA and GLA have the capacity to replace AA ultimately as substrate for the synthesis of eicosanoids[24], which is the basis for its anti-inflammatory property. Literature shows that addition of n-3 PUFAs like EPA and GLA results in noticeable reduction in AA-derived eicosanoids and proinflammatory cytokines. EPA- and GLA-rich supplements have been used to attenuate inflammatory processes in various chronic and autoimmune diseases. Faber *et al.* have shown that AA, EPA, and DHA contents of human immune cells can be altered through oral administration of EPA and DHA[50], which alter their phagocytosis capability, T cell signaling, and antigen-presentation capability. Human milk of healthy mothers have an optimal ratio of n-3 and n-6 long chain PUFAs, which varies over the course of lactation[51].

3.10. TLRs and soluble forms of CD14

Human breast milk contains high levels of soluble forms of CD14 and TLRs[52-54]. Soluble CD14 mediates TLR-4 binding to lipopolysaccharide, the pattern recognition molecule of Gram-negative bacteria in endothelial and epithelial cells thereby sensitizing the innate mucosal immune system to such bacteria[55]. Some receptors in cell-surface TLR complexes exist in soluble forms in milk and act as decoy receptors for microbial motifs. Other proteins found in milk, such as IL-10 and transforming growth factor β may modify TLR-mediated responses by regulating the expression of the various proteins in the TLR receptor complex. In this respect, IL-10 increases the expression of CD14 on monocytes but does not alter their expression of TLR-4[56,57].

3.11. Antimicrobial proteins and peptides

Antimicrobial proteins and peptides are key effectors of the innate immune response; they are expressed by circulating cells and epithelial cells and mediate their effect by disrupting membranes of the microorganisms. With broad spectrum of antibiotic activity they can modulate the composition of the intestinal microbiota and provide protection against environmental and pathogenic organisms. Important peptides are the cationic α- and β-defensins and the cathelicidins, which protect against bacterial colonization of gut, lung, and skin epithelia. α-Defensins are constitutively expressed in human neutrophils and Paneth cells of the small intestinal crypts; β-defensins are expressed in the epithelial cells of the gastrointestinal tract and cathelicidin is expressed by neutrophils and mast cells, as well as by differentiated epithelial cells in the colon and stomach and in Brunner's glands of the duodenum[58]. Lactose has been shown to induce the cathelicidin antimicrobial peptide gene, which leads to protection of the neonatal gut from pathogens and regulation of the microbiota of the infant[59]. Paneth cells secrete other antimicrobial peptides such as lysozyme and secretory phospholipase A_2. Animal studies have demonstrated that following ablation of the Paneth cell population, susceptibility to infection in animals increased[60]. The absence of lysozyme in the Paneth cell of preterm and term infant with necrotizing enterocolitis support a causative link between Paneth cell secretion of antimicrobial peptides and necrotizing enterocolitis[61].

4. Immune development and maturation properties of human milk

Human milk contains its own immune system and a wide range of soluble and cellular factors, which likely facilitate immune development and maturation in infants.

4.1. Leucocytes

Although viable cells are present in human milk, their concentration declines during lactation. The viable cells include granulocytes, macrophages and lymphocytes, which are predominantly T cells, progenitor cells and stem cells[62]. A large number of $CD8^+$ T cells are constantly present in human milk and may be important in the control of viral passage from mother to infant[63]. Recent work demonstrated that $CD14^+$ milk mononuclear cells in milk express human leukocyte antigen DR, CD86, CD83, and dendritic cell-specific ICAM-3-grabbing nonintegrin, suggesting that these cells are partially differentiated dendritic cells[64]. These cells are a source of some soluble factors found in milk such as soluble forms of CD14[52,53], osteoprotegerin[65], as well as several cytokines and chemokines[66]. Evidence shows that the neutrophils and macrophages in milk are phagocytic, and upon ingestion they induce a respiratory burst[67]. Also it has been shown that human milk leukocytes

are cells that have migrated from the gut- and bronchial-associated lymphoid tissue to the lactating mammary gland via the lymphatics and the circulation[66,68,69]. It now appears that such migrating cells also transport bacteria and their genetic material[70]. The role of these cells in the neonate is unknown, but they may represent an inoculum for the development of the microbiota and/or be way to educate the neonatal immune system[71]. Recently identification of microRNAs inside breast milk exosomes have also been shown to play a critical role in the development of the infant's immune system[72].

4.2. Cytokines

Human milk contains an array of cytokines and chemokines, such as interleukin (IL) 1, IL-1β, tumor necrosis factor α (TNF-α), IL-4, IL-5, IL-6, IL-8, IL-10, interferon γ (IFN-γ), macrophage colony-stimulating factor, and granulocyte-macrophage colony-stimulating factor, macrophage inflammatory protein and regulated upon activation normal T cell expressed and presumably secreted. The primary source of cytokines is the mammary gland. Leucocytes recovered from expressed human milk have been shown to be capable of producing a number of cytokines. Evidence generated shows that production of certain cytokines or expression of their cognate mRNAs by neonatal cells is either slightly (TNF-α), moderately (granulocyte-macrophage colony-stimulating factor), or markedly decreased (IL-3, IL-4, IL-5, IL-8, IL-10, IFN-γ) compared with adult T cells[73]. The cytokine deficiency may reflect, among others, the poor antibody response of the neonate to polysaccharide vaccines or capsulated bacteria such as group B streptococci, or neonatal T cells not proliferating *in vitro* as well as adult T cells do in response to anti CD2 or anti CD3 antibody stimulation. In addition, there is now strong clinical and experimental evidence that reduced production of colony stimulating factors results in immature myeloproliferative response in the newborn and in functional defects of effector neutrophils. Also cytokines in human milk can potentially interact with mucosal/lymphoid tisssues in the upper parts of the respiratory and alimentary tracts. Certain cytokines (IL-6 and TNF-α) have been implicated in the regulation of development and functions of the mammary gland, and others (IL-1 and IFN-γ) may influence the production of defense agents, sIgA or other cytokines by the mammary gland[74]. Certain chemokines which are potent activators of neutrophils have chemotactic activity for intestinal intraepithelial lymphocytes and play an important role in host defense against bacterial infections. Finally it has also been demonstrated that mice with a targeted disruption of the *IL-10* gene spontaneously develop a generalized enterocolitis at the time of weaning that is prevented by the parenteral administration of IL-10, suggesting its role in homeostasis of the neonatal intestinal barrier and in regulation of abnormal immune responses to foreign antigens[74].

4.3. Nucleotides

Nucleotides, nucleosides and nucleic acids in human milk have been shown to augment immune function in infants. Nucleotides are important in situations such as infection, or rapid growth where there is increased metabolic activity[75]. In some clinical studies, fewer episodes of diarrhea and these benefits have been attributed to the addition of nucleotides to infant formulas. The projected mechanisms of action include increased iron absorption, increased growth of *Bifidobacterium*, improved development, and repair of the gastrointestinal mucosa[25]. Also improved systemic immune responses like increased natural killer cell activity and IL-2 production have been included[76].

4.4. Antioxidant molecules

The anti inflammatory effects of many molecules in human milk attribute to the oxygen radical scavenging property. Molecules responsible for antioxidant capacity of breast milk are α-tocopherol, β-carotene, ascorbic acid and l-histidine.

4.5. Growth factors and hormones

Human milk contains hormones which include epidermal growth factor, insulin growth factor, and leptin that can modulate the immune system of the intestinal mucosa via the regulation of cytokine expression and other signaling pathways[76,77]. Adiponectin found in human milk suppress TNF-α production in intestinal epithelial cells and macrophages[78]. Adiponectin inhibits the proliferation of myelomonocytic progenitor cells and induces apoptosis; this may contribute to the anti-inflammatory effects of this adiponectin[79].

5. Tolerance and priming of immune system by human breast milk

Dietary antigens present in milk along with immunosuppressive cytokines (IL-10 and transforming growth factor β) help in promoting tolerance to dietary and microflora antigens[80]. Supporting data show that breast feeding tolerate infants to maternal histocompatibility antigens. Kidney transplants from a maternal donor were

shown to survive better if recipient had been breast fed by the mother[81]. Recently there is support evidence for the long chain fatty acids in milk to promote tolerance. Anti-idiotypic antibodies are naturally occurring antibodies with specificity against other autologous antibodies. In breast milk, these antibodies are proposed to have the capability of priming the infant's antibody response against the antigen the idiotype is directed against. Demonstration by animal studies have shown that relatively small amount of anti idiotypic antibody given in the neonatal period influences the immune system profoundly that the effects can still be detected two generations later[82].

6. Conclusion

Human milk is a complex mixture of various bioactive elements which have a profound influence on the immune status of the infant by providing protection and also facilitating its development, tolerance and inflammatory response. It also educates the immune, metabolic, and microflora system in the infant. The milk components comprise an innate immune system of human milk by which the mother protects her nursing infant. Thus the immunological factors of breast milk may contribute to the nutritive, bioactive, and functional role of milk.

Conflict of interest statement

We declare that we have no conflict of interest.

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