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The Immune System in Human Milk and the Developing Infant

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ABSTRACT

The concept of the immune system in human milk emerged in the 1970s from clinical and laboratory observations made between the late 18th through the mid-20th centuries. The discovery of living leukocytes in human milk in 1970 was the final link to the chain of evidence that culminated in the concept. The concept was later expanded to include not only antimicrobial but also anti-inflammatory and immunoregulatory agents. These agents evolved to compensate for developmental delays in the immune system during infancy. Indeed, that explains the defense by human milk against common infectious diseases in infancy, necrotizing enterocolitis in preterm infants, and immune-mediated disorders such as Crohn's disease in later childhood. These diverse evolutionary outcomes underscore the superiority of human milk for the nutrition of human infants. Finally, other components of the immune system in human milk and their fate and functions in the developing infant may well be discovered in the near future.

INTRODUCTION

MANY ASPECTS OF THE IMMUNE SYSTEM in human milk and its effects upon the recipient infants are widely appreciated, but the genesis and scope of the discoveries are not well known. Although most of the discoveries occurred during the last half century, the framework for those discoveries was set long before then. Moreover, those basic findings, as well as more recent scientific discoveries, depended upon basic immunological sciences and technologies. However, clinical clues derived from case studies or epidemiological studies often also set the stage for the investigations of the immune system in human milk. The risk of breastfed infants to common enteric infections raised the issue whether there were antimicrobial agents in human milk, the lack of inflammatory reactions during the protection lead to question whether that was due in part to anti-inflammatory agents in human milk, and the prolonged protection against certain diseases long after weaning led to search for immunoregulatory agents in human milk.

In this review, I will comment upon not only those clinical clues but also upon other observations that suggested the possibility of an immune system in human milk. Moreover, the importance and limitations of the laboratory methods that permitted the discoveries, the immune status of the recipient infant, the apparent effects of the components of the system upon the infant, and how those effects are viewed in the context of biological evolution will be discussed. Finally, I will suggest certain

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aspects of the immunobiology of human milk that are yet to be discovered.

CONCEPT OF IMMUNE SYSTEM IN HUMAN MILK

The development of a "new" field depends a few salient discoveries sometimes by obscure individuals. Gregor Johann Mendel[1] and Albert Einstein[2] for their discoveries in genetics and physics, respectively, come to mind. After pioneering studies are independently verified, other scientists are attracted to the new ideas and begin to communicate with founders of the field and others of like interests. In time, meetings are organized to share new findings and to seek collaboration. Consequently, research societies often develop and such societies may form the basis of research journals in the field. The length of time required for the development of each field varies, however, a great deal. In general, basic sciences develop quickly, whereas others develop more slowly, particularly if they require inputs from a number of basic disciplines. The biology of human milk is an example of the latter.

Furthermore, studies of the immune system in human milk were impeded because of the following. (1) It was difficult to visualize that human milk had immunological properties. Indeed, nutritionists were understandably preoccupied with its nutritional nature. With a few important exceptions, immunologists were focused upon systemic and mucosal immunology. (2) Investigative concepts and tools, sometimes that would have to specifically adapted to human milk, needed to be developed. (3) Certain arbitrary distinctions between nutrients and immune agents had to be questioned and finally set aside. (4) Animal models that might have shed some light on these matters were either inappropriate (nonprimate mammals) or unwieldy, expensive, and ethically problematical (nonhuman primates). (5) Some methods that would be acceptable in nonhuman animal models were ethically precluded in human infants because of their invasiveness.

Despite these obstacles, a few luminaries in basic immunology made certain discoveries that set the stage for future research and the realization of an extensive immune system in human milk. The scientists and their discoveries were as follows.

Macromolecular structures: leukocytes and milk fat globules

In the late 18th century, Thonius Philips (Anton) van Leeuwenhoek[3] first observed single cells, bacteria, sperm, and blood flow through small blood vessels with a single-lensed microscope that magnified up to 275×. He also described bodies in milk from several mammals (Box 1), but their charateristics were unclear. Alfred Donné,[4] the first to develop photomicroscopy, observed bodies in human milk. Since the specimens were unstained, their identities were undetermined. They were probably milk fat globules, which were later to be found to comprise part of the immune system in human milk, and some of the corpuscles de Donné in nonhuman milk were found to be cells by 1868.[5] But the basic properties of cells in human milk were unknown until my colleague, Wayne Smith, and I discovered in 1986 that they were living neutrophils, macrophages, and lymphocytes.[6]

Antibodies

Possible evidence of the transfer of specific immunity by murine milk was reported in the late 19th century by Paul Ehrlich (Box 1).[7,8] By the 1950s, antibodies against many enteric bacteria and viruses were found in human milk (Table 1). The physical nature of antibodies, the immunoglobulins, was determined in the mid-20th century. The dominant immunoglobulin

Box 1. Some Methods That Aided in Discovering the Immune System in Human Milk

1. Microscopy—living leukocytes in human milk
2. Bacterial and viral cultures—lysozyme and antibacterial peptides, fatty acids, and monogycerides
3. Protein electrophoresis—lactoferrin, secretory IgA
4. Chromatography—oligosaccharides
5. Antigen–antibody reactions—many proteins such as lactoferrin, lysozyme, secretory IgA
6. Monoclonal antibodies—cytokines in human milk
7. Flow cytometry—leukocyte populations and subpopulation and activated leukocytes
8. Aminoacid sequencing—low molecular weight antimicrobial peptides

Table 1. Antimicrobial Proteins in Human Milk

Agents	*Functions*
Lactoferrin	Blocks multiplication of siderophiles by chelating Fe +++ Kills enteric bacteria, *C. albicans* by lactoferricin
Secretory IgA	Interferes with attachment of microbial pathogens to epithelium; neutralizes bacterial toxins
Lysozyme	Digests peptidoglycans from cell walls of certain bacteria
Alpha-lactalbumin	Kills *Streptococcus pneumoniae*
Lactadhedrin	Blocks binding of rotavirus to intestinal epithelium
MUC1	Blocks binding of S-fimbriated *E. coli* to intestinal epithelium
C3	Precursor of opsonins, C3b and C3bi.
Fibronectin	Opsonin
CCL28	Kills *C. albicans* and certain bacteria
MIF	Aids in killing *Mycobacterium tuberculosis*
Defensins	Inhibit HIV-1 replication and disrupts *E. coli*

in human milk, secretory IgA, was found by Lars Å. Hanson[9] in 1961 by using immunoelectrophoresis.

Lysozyme

Alexander Fleming[10] observed in 1922 that tears, nasal secretions, and a number of other biological fluids lysed certain staphylococci *in vitro*. Two years later, Jules Bordet,[11] the immunologist who discovered complement (alexine) 2 decades before then, found that human milk had similar bacteriolytic properties (Table 1).[12] Many years later, the lytic agent, lysozyme, was found to be a low molecular weight enzyme that hydrolyzed β-1,4 linkages between N-acetylmuramic acid and 2-acetyamino-2-deoxy-d-glucose residues.[13]

Lactoferrin

The iron-binding protein, lactoferrin, was found in 1951 by electrophoretic studies of human milk[14] (Box 1). The protein was characterized several years later.[15] Its antibacterial property due to iron chelation (Table 1) was discovered shortly thereafter.[16]

Bacterial growth factors

Paul György[17] reported in 1961 that human milk, but not cow's milk, enhanced the growth of Bifidobacilli, one of the dominant bacteria found in the lower intestine of breastfed infants. Thus, it was suggested that protective bioactive factors in addition to direct-acting antimicrobial agents were in human milk.

Concept of an immune system in human milk emerges

When the afore-mentioned discoveries and other developments in the field were considered, the concept of an immunological system in human milk emerged.[18] The finding of living leukocytes in human milk and the realization that their morphology and functions were somewhat different than their precursors in human blood was the key. Indeed, an organized group of diverse leukocytes strongly suggested an immune system. In addition, the antimicrobial agents in human milk that were known at that time—secretory IgA, lysozyme, lactoferrin, and oligosaccharides—were present in higher concentrations that those found in serum and their features were different from the main defense agents in human blood.

The scope and other special qualities of the system were unknown at the time, but certain questions flowed logically from the concept. (1) What were its purposes? Was the protection for the recipient infant, the mammary gland, or both? (2) Did it just protect against infectious diseases? (3) Were the *in vivo* activities of the defense agents the same as their *in vitro* activities or their functions at other biological sites? (4) Did the concentrations or functions of the agents change as lactation proceded? This was found to be the case within a few years. (5) Could one learn about the nature and effects of the immune system in human milk by studying other mammals? If so, which ones were appropriate? (6) If the system protected the infant, what was lacking in the infant that needed aug-

Box 2. General Features of Antimicrobial Agents in Human Milk

1. Biochemically heterogeneous
2. Common to and adapted to function at mucosal sites
3. Functions somewhat redundant
4. Multifunctional-synergistic-interactive
5. Production often inversely related to that of the recipient
6. Resistant to digestion by gastrointestinal enzymes
7. Protect by noninflammatory mechanisms
8. Not well represented in other mammalian milks except from species closely related to our own

mentation? Was there a reciprocal relationship between the immune system in human milk and the immune status of the recipient infant? If so, how did the reciprocation evolve?[19,20] How were the processes controlled? These questions and their answers gradually emerged but are not completely resolved.

One of the first advances in our understanding of the immune system in human milk that eminated from that central concept was that even though the agents were biochemically heterogeneous, they shared certain features (Box 2). Those same features were later found to be present in other members of that immune system that were subsequently discovered.

Later on, it was discovered that fatty acids and monoglycerides generated by enzymatic digestion of lipids in human milk protected against enveloped viruses, intestinal parasites such as Giardia lamblia and Entameoba histolytica, and certain bacteria (Box 1).[21,22] Protective agents produced by partial digestion of the components of human milk such as lactoferricin from lactoferrin were also discovered.[23,24]

Furthermore, by the 1970s, some of the differences in the enteric flora in breastfed compared to nonbreastfed infants were found to be due to selective bacterial growth factors in human milk.[25] As previously mentioned, that was a presage of other discoveries of bioactive molecules in human milk.

Expansion of concept—anti-inflammatory agents in human milk

The protection by antimicrobial agents in human milk by noninflammatory mechanisms led to a realization that the defense system in human milk might also be composed of anti-inflammatory agents. A number of other pieces of evidence pointed in that direction. (1) The protection by breastfeeding against enteric infections was not accompanied by symptoms or obvious clinical signs of inflammation in the gastrointestinal tract. A review of the literature indicated that the known inflammatory agents or their precursors were absent in human milk.[26] Furthermore, many anti-inflammatory agents had already been found in human milk, but their importance to the recipient infant was not emphasized until 1986.[26]

Concept encompasses immunoregulatory agents in human milk

The discovery of immunoregulatory agents in human milk was triggerred by the realization that breastfeeding enhanced the development of certain aspects of the immune system of infants and had long-term protective effects against certain chronic diseases due to inflammatory-immunologically mediated processes. Infants fed human milk had increased concentrations of secretory IgA in the urine,[27,28] increased formation of serum IgG antibodies after systemic immunization,[29] and increased levels of interferon in the aftermath of respiratory syncytial virus infections.[30] In addition, long-term protection was found against certain leukemias and certain lymphomas[31] and Crohn's disease.[32]

Further indications of possible immunoregulators in human milk came from flow cytyometric studies that demonstrated that leukocytes in human milk bore the phenotypic markers of activation[33,34] and from *in vitro* functional studies that showed that macrophages[35] and T cells[36] in human milk were more motile than their counterparts in blood. Furthermore, human milk was found to activate human blood T cells,[36] neutrophils,[33] and blood monocytes,[37] and the enhancement of monocyte motility was to a great extent blocked by antibodies to human tumor necrosis factor-alpha (TNF-α).[37] That led to the immunochemical demonstration of TNF-α in human milk,[38] and subsequently, to the discovery of many other immunoregulatory agents in human milk.[39]

MORE RECENT DISCOVERIES

The last few decades have been replete with many new discoveries concening the immune system in human milk. Some of the highlights are as follows.

Enteromammary gland pathway

The enteromammary gland pathway that culminates in the formation of secretory IgA antibodies has been carefully investigated. (1) Foreign antigens taken up by M cells in Peyer's patches located in the distal ileum bind to local IgM^+ B cells. (2) Cytokines produced by local T cells as a result of antigen stimulation induce the B cells to switch from IgM to IgA. (3) Those isotype-switched B cells sequentially migrate into local intestinal lymphatics, mesenteric lymph nodes, the cysternia chylia, the thoracic duct, the blood, and the mammary gland. (4) Those B cells transform into plasma cells that remain in the lamina propria of the mammary gland. Those mammary gland plasma cells produce IgA dimers that are specifically directed against the antigens that initiated the transformation of IgM^+ B cells in the small intestine. (5) The IgA dimers then bind to polymeric immunoglobulin receptors on basolateral membranes of the mammary epithelium. (6) The resultant receptor–ligand complex is transported to the apical side where intracytoplasmic part of receptor is cleaved, and the remaining molecule, secretory IgA, is secreted through the luminal surface of the epithelium.

The mechanism for the attraction of IgA^+ B cells to the mammary gland was, however, poorly understood until recently. CCL28 is upregulated in mammary epithelium during lactation.[40] This provides the chemoattractant mechanism for IgA^+ B cells, which are $CCR10^+$, to exit the microcirculation in the mammary gland and move toward CCL28 on mammary epithelium.[40] The rest of the sequence of secretory IgA formation then occurs.

Antimicrobial agents

Oligosaccharides and glycoconjugates. Many oligosaccharides and glycoconjugates produced by glycosyltransferases in the mammary gland were discovered in human milk and found to be receptor analogues for enteric bacterial pathogens that interfere with the binding of those pathogens to epithelial cells.[41,42] Their prebiotic effects have also been emphasized.[43]

α-Lactalbumin. α-Lactalbumin is expressed only in the lactating mammary gland. α-Lactalbumin is not only part of lactose synthesase but also kills *Streptococcus pneumoniae in vitro*.[44] Furthermore, multimeric α-lactalbumin kills tumor cells *in vitro* by inducing apoptosis..[45]

MUC1. MUC1, the most abundant mucin in human milk, is found in the fluid phase and in milk fat globules.[46] This large, heavily glycosylated protein inhibits the binding of bacteria such as fimbriated *Escherichia coli*.

Lactadhedrin. Lactadherin is also found in the fluid phase and on milk fat globules in human milk.[47] That glycoprotein protects against rotavirus infections in experimental mice.[48]

Macrophage migration inhibitory factor (MIF). MIF is also found in the fluid phase and milk fat globules in human milk.[49] MIF, a pro-in-

Table 2. Anti-inflammatory Agents in Human Milk

Categories	*Examples*
Cytoprotectives	Prostaglandins E2, F2α
Epithelial growth factors	EGF, lactoferrin, polyamines
Maturational factors	Cortisol
Enzymes that degrade inflammatory mediators	PAF-acetylhydrolase
Binders of enzymes	α1-antichymotrypsin
Binders of substrates of enzymes	Lysozyme to elastin
Binders of toxins	Lactoferrin to lipid A of LPS
Modulators of inflammatory leukocytes	IL-10, TGF-β1
Antioxidants	Uric acid, β-carotene, ascorbate

Table 3. Cytokines in Human Milk

Types	*Examples*
T-cell production augmentation	IL-7
Cellular immunity enhancement	Interferon-γ, TNF-α, IL-12, & IL-18
Humoral immunity enhancement	TGF-β2, IL-4, IL-10
Macrophage stimulation	IL-β1, IL-6, IL-6, MIF
Chemokine activities	IL-8, RANTES, MIP-1, CCL28
Interferon-inducible proteins	IP-10 & MIG
Anti-inflammatory actions	TGF-β1, IL-10
Growth stimulation	EGF, M-CSF, G-CSF, erythrpoietin

flammatory cytokine, also upregulates TLR-4[50] and aids in killing *Mycobacterium tuberculosis* in human macrophages.[51]

CCL28. CCL28, a chemokine that is part of the terminus of the enteromammary gland pathway, kills *Candida albicans* and many Gram-positive and Gram-negative bacteria.[52] The killing is mediated by the 28 amino acid C-terminus of the molecule.[52]

Low molecular weight antimicrobial peptides. In addition to antimicrobial peptides generated by partial digestion of lactoferrin (Box 1), cysteine-rich, cationic low molecular weight peptides are in human milk including β-defensin-1[53] and α-defensins-1, -2, and -3.[54] β-defensin-1 disrupts *E. coli*;[53] α-defensins inhibit HIV-1 replication and may interfere with postpartum transmission of HIV-1.[54]

Anti-inflammatory agents in human milk

The known spectrum of anti-inflammatory agents and the scope of their effects continue to increase (Table 2). For example, lysozyme binds to elastin and therefore protects it from enzymatic degradation,[55] and lactoferrin binds to lipid A of LPS and thus prevents its inflammatory effects.[56] Their *in vivo* actions are still, however, poorly defined.

Table 4. Evolutionary Adaptations

Evolutionary adaptations	*Examples*
1. Direct compensation	Secretory IgA, lysozyme, lactoferrin, PAF-acetylhydrolase, cytokines
2. Do not directly compensate	Oligosaccharides and nucleotides
3. Initiate/enhance functions poorly expressed in the recipient	Antiidiotypic antibodies; many cytokines
4. Change the physiological state	Intestinal tract permeability
5. Anti-inflammatory effects of human milk	Anti-inflammatory agents in HM (see Table 2)
6. Agents in HM are immunoregulators	See Table 3
7. Enhanced survival of agents from HM	Secretory IgA, lysozyme, lactoferrin
8. Cells that produce HM antibodies originate in small intestines and bronchi	See Box 2
9. Defense agents in HM resist enzymatic digestion and are thus able to function in recipient's GI tract	Secretory IgA, lysozyme, lactoferrin oligosaccharides.
10. Production of defense agents by partial digestion of substrates in HM	Lactoferricin, β-casomorphin, fatty acids, monoglycerides
11. HM transmits certain micro-organisms and that leads to protective, asymptomatic infections	Immunization against cytomegalovirus
12. HM promotes growth of commensal bacteria	Enhanced growth of bifidobacteria and lactobacilli in the large intestine of the infant

Box 3. Evolutionary Outcomes: Immune System in Human Milk (HM) and Immune Status of the Recipient Infant

1. Some postnatal developmental delays in immune system compensated by those agents in HM.
2. Others are offset by dissimilar agents in human milk (HM).
3. Some agents in HM initiate or augment functions poorly expressed in the infant.
4. Some agents in HM alter physiological state of alimentary tract from one suited for fetal life to one appropriate for extrauterine life.
5. Defense agents in HM protect against pathogens without provoking inflammation.
6. Many agents in HM inhibit inflammation or are immunoregulators.
7. Cells that produce antibodies in HM originate in small intestines and bronchi.
8. Defense agents in HM are resistant to enzymatic digestion and are thus able to function in the recipient's gastrointestinal (GI) tract.
9. Certain defense agents are created by partial digestion of substrates in HM in the infant's GI tract.
10. When defense agents in HM interact with some pathogens, immune responses develop in the infant but no symptomatic infections occur.
11. Agents in HM augment growth of commensal enteric bacteria that protect against bacterial pathogens and conveys other benefits.

Immunoregulatory agents in human milk

A great number of cytokines that display diverse types of immunoregulatory activities including augmentation of cellular immunity, humoral immunity, macrophage activity, and anti-inflammatory effects have been recently recognized in human milk (Table 3). The precise *in vivo* effects of these agents upon the recipient infant are yet to be determined.

EVOLUTION OF IMMUNE SYSTEM IN HUMAN MILK AND THE DEVELOPING INFANT

Some 150–160 million years ago the first mammal developed from synapsid reptiles.[57] An important early innovation that defined mammals was the mammary gland. As first suggested by Darwin,[58] modified epidermal glands on the ventral part of the adult female's thorax/abdomen apparently produced secretions that were advantageous to the newborn infant.[19] The ventral location of the mammary gland would have advantageous to the infant because of face-to-face interactions between the mother and infant. Pheromones may have also played a role in attracting newborns to those dermal secretions.

The primordial mammary gland may have secreted defense agents such as fatty acids, oligosaccharides, lysozyme, and iron-binding proteins that protected the infant from the bacterial flora of the mother's skin.[19] Indeed, the constituents of rudimentary milk may have been similar to those found in human sebum and apocrine and sweat glands. Human sebum contains antibacterial fatty acids.[59] Mammalian apocrine glands contain lysozyme,[60] which is phylogenetically ancient.[61] Furthermore, melanotransferrin, an iron-binding protein found in human sweat glands, has a 40% homology with lactoferrin,[62] the protective iron-binding protein found in many mammalian milks.[20]

The striking advances in the knowledge of the immune system in human milk inevitably led to a consideration of the evolutionary relationships between that system and the immunological status of the immune system of the developing infant. A number of principles were adduced (Box 3, Table 4), one of which was based upon a reciprocal relationship between the immune factors produced by the mammary gland and those produced by the developing infant (Table 5).

Table 5. Immune Factors in Human Milk Whose Production Is Delayed in Recipient

Immune functions	*Representative agents*
Antimicrobial	SIgA
	Lactoferrin
	Lysozyme
Anti-inflammatory	IL-10
	PAF-acetylhydrolase
	Lactoferrin
	Lysozyme
Immunomodulatory	Memory T cells
	IL-4
	IL-10
	IL-12
	G-CSF
	TNF-α
	Interferon-γ
	RANTES

At first it seemed incongruous that developmental delays in the immune system would be consistent with biological evolution since the infant would be rendered more susceptible to infections. However, further analysis suggested that such developmental delays allowed considerable energy and nutrients to be diverted to the growth and development of other systems such as the central nervous system, skeleton, and skeletal muscles as long as the mother was providing the necessary defense agents through her milk. Indeed, mammalian mothers pick up the slack by providing defense agents that not only effectively defend against many pathogens, but they do so in a noninflammatory way. By preventing inflammation, the integrity of the gastrointestinal tract and the respiratory system is preserved to ensure normal nutrition and normal gas exchange, respectively. Furthermore, the immune system in human milk evolved to aid the switch from marked intestinal permeability, a physiological state suited for fetal life, to a much less permeable intestinal tract that helps to protect against enteric pathogens. These changes also permit the infant to become actively immune to environmental pathogens without displaying overt signs of infection or inflammation. Finally, the immune system provided by the human mammary gland decreases the risks to certain inflammatory immune-mediated diseases that appear long after weaning.

Although physicians are understandably most concerned about humans, different and equally interesting evolutionary adaptations in other mammalian species provide important insights as to why human milk is superior to milks from all other mammalian species for feeding human infants.[20]

CONCLUSIONS

Evidence concerning the immune system in human milk and its effects on the recipient infant began to be uncovered over 200 years ago, but the greatest acceleration in those research endeavors occurred in the past 5 decades once the concept of the system was established. Indeed, the concept of the immune system in human milk, the myriad components in the system, the dynamic changes in many components as lactation proceeds, the often parallel but reciprocal changes in the immune status of the recipient infant, and many of the short-term and long-term clinical benefits of the system upon the infant have become increasingly apparent during the last part of the 20th century and the beginning of the 21st century. As suggested in this brief, incomplete review, much more will be discovered in the near future as new ideas are formulated and new technologies are devised to test those hypotheses (Box 1). Among those questions are the extent of the genetic variations in the production of immune agents by the mammary gland as exemplified by very low and high producers of IL-10 in human milk,[63] the fate and molecular effects of the anti-inflammatory and immunoregulatory agents in human milk in the recipient infant, and the basis of the long-term protective effects of human milk.

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