Journal of Mammary Gland Biology and Neoplasia, Vol. 7, No. 3, July 2002 (© 2002)

Evolution of the Mammary Gland Defense System and the Ontogeny of the Immune System

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A decisive event in the evolution of mammals from synapsid reptiles was the modification of ventral thoracic–abdominal epidermal glands to form the mammary gland. The natural selection events that drove the process may have been the provision of certain immunological agents in dermal secretions of those nascent mammals. This is mirrored by similar innate immune factors in mammalian sebum and in protherian and eutherian milks. On the basis of studies of existing mammalian orders, it is evident that immune agents in milk such as immunoglobulins, iron-binding proteins, lysozyme, oligosaccharides, and leukocytes compensate for developmental delays in early postnatal production of antimicrobial factors. At least in human milk, anti-inflammatory and immunomodulating agents also evolved to provide different types of protection for the offspring. In addition, investigations reveal that the types or concentrations of immunological agents in milk vary depending upon the type of placenta, lactation pattern, and environment of the species.

KEY WORDS: evolution; immunology; mammals; mammary gland; milk; placenta.

INTRODUCTION

The basic axioms of biological evolution (1,2) are widely accepted. 1) All species descended from a common origin. 2) As a result of multiplication, populations of a species increase. 3) Because of environmental pressures, biological selection takes place. 4) Genetically well-adapted individuals of a species compete successfully for newly created environmental niches. 5) Because of natural selection and saturation of environmental sites, the overall genomic composition of a species stabilizes. Thus, evolutionary success is determined by reproductive capacity and the ability to cope with the environment long enough to reproduce and assure the survival of healthy offspring.

In some instances, adaptation occurs because existing proteins assume new functions. Otherwise, changes in structures of proteins and hence of their genes are required. The genetic changes (3) are 1) single nucleotide polymorphisms, 2) addition of DNA sequences to the ends of ancestral genes, 3) fusion of copies of preexisting DNA sequences arranged differently (exon shuffling), 4) fusion of functional genes to form new multifunctional complexes, 5) gene duplications to generate families of proteins, 6) gene duplication and tandem fusion, 7) gene duplication followed by independent mutations and 8) utilization during translation of a hitherto unused or noncoding open reading frame.

EVOLUTION OF MAMMALIAN IMMUNE SYSTEMS

Systemic and Mucosal Immunity

Defense systems in animals also evolved by natural selection and genetic mutations. Indeed, there is

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Abbreviations used: Gastrointestinal, GI; immunoglobulin, Ig; interleukin, IL; millions of years ago, mya; platelet activating factor, PAF.

1083-3021/02/0700-0277/0 © 2002 Plenum Publishing Corporation

a continuous struggle between microbial pathogens and metazoan defenses. In keeping with Darwinian principles, microbial pathogens exert selection pressure upon host defenses. If successful, the immune system provides sufficient protection for the host to survive long enough to reproduce.

Defense systems in members of Class Mammalia (mammals) are complex (4). The innate, constitutively expressed agents include iron-binding proteins, such as transferrin, lysozyme, complement components, acute phase reactants that rise during inflammation, interferons, cytokines, low-molecular-weight antimicrobial peptides, antiadherence oligosaccharides, inflammatory leukocytes, and natural killer cells that are lymphocytes that lack antigen recognition molecules. These agents are immediately available for defense.

Mammalian defense systems are also characterized by two types of lymphocytes that produce antigen recognition molecules. The first are thymic-independent lymphocytes (B cells) that express immunoglobulins on their surfaces and transform into antibody producing, secreting cells. The second are thymic-dependent lymphocytes (T cells) that express a different type of antigen recognition molecule (the T cell antigen receptor or TcR) that belongs to the same family as immunoglobulins. T cells are divided into two major subpopulations. $CD4^+$ T cells, or helper T cells, aid in the genesis of specific immune responses; $CD8^+$ T cells suppress the development of specific immune responses and are cytotoxic. Helper T cells are further divided into those that produce cytokines that lead to cellular immunity (Th1 cells) and those that produce cytokines that aid the development of humoral immunity (Th2 cells).

The production, differentiation, modulation, activation, and functions of the cells of the immune system are controlled by intricate genetic–molecular systems. For example, the production of the wide diversity of antigen recognition molecules by T cell and B cell occurs by sorting and recombination of several genes. This allows mammals to contend with a very large number of microbial pathogens and to adapt to new pathogens in the environment.

In addition, a large part of the mammalian immune system evolved to protect against pathogens at proximal sites of contact, the skin and mucosa. Mucosal immunity includes iron-binding proteins, lysozyme, cytokines, low-molecular-weight peptides, oligosaccharides, and immunoglobulins.

Although basic structures of immune systems are similar in all mammals, there are qualitative or quantitative differences between species. These differences narrow in closely related species. However, evolutionary convergence may account for certain similarities between species that are not closely related.

Immunity in Newborns/Modification by Maternal Agents

Developmental delays in the immune system are found in all mammals that have been investigated. Those developmental delays involve mucosal or systemic immunity, and their exact patterns vary according to the mammalian species. For example in human infants, developmental delays in the production of IgG, secretory IgA, lysozyme, lactoferrin, memory T cells, interferon-γ, IL-1β, IL-10, and certain other cytokines have been found (reviewed in Ref. 5). Developmental delays in the immune system of young mammals would seem inconsistent with evolutionary principles, since a breach in defense would increase the likelihood of infections. However, the deficits are offset by transfer of protective agents during fetal life via the placenta and during postnatal life by milk. For example, non-breast-fed calves or piglets usually develop enteric and systemic infections, whereas suckling calves do not (reviewed in Ref. 6). Also, non-breast-fed human infants are more prone to enteric and respiratory infections with common viral and bacterial pathogens (reviewed in Refs. 5,7). These infections appear to be due to a lack of defense agents supplied by breast-feeding.

In keeping with the above, defense agents generated by the mammary gland have been investigated in many species. Comprehensive investigations in humans suggest that the immunological composition of mammalian milk is complex. In that respect, human milk contains a host of direct-acting antimicrobial agents, anti-inflammatory factors, immunomodulating agents, and living leukocytes (reviewed in Refs. 5,7,8) (Tables I–III), many of which are developmentally delayed in the recipient infant (5,7,8).

Although the susceptibility of immature mammals to infections and the protection afforded to them by breast-feeding are well documented, little attention has been given to the evolutionary aspects of these findings. How did the immune functions of the mammary gland develop and was that development coupled with repression of key parts of the immune system in the offspring? What were the advantages of those evolutionary events? Although it is impossible to entirely re-create the evolution of these intertwined

Table I. Major Types of Direct-Acting Antimicrobial Agents in Human Milk

Agents	Functions
Glycoproteins	
Secretory IgA	Antibodies neutralize or prevent adherence of microbial pathogens and their toxins
Lactoferrin	Kills or inhibits reproduction of certain bacteria, fungi, and viruses
Lysozyme	Lyses peptidoglycans of cell walls of certain bacteria
Mucins (MUC 1)	Prevent adherence of certain microbial pathogens
Lactadhedrin	Prevents adherence of certain rotavirus
Oligosaccharides and glycoconjugates	Interfere with attachment of certain pathogens and toxins to mucosa
Products of lipid hydrolysis	Lyse enveloped viruses, certain bacteria, and parasites

processes, some clues are found by considering the nature of mammalian precursors and by examining the immunological composition of milk and the immune status of the young of mammals of the subclass prototheria and the infraclasses metatheria and eutheria.

MAMMALIAN PRECURSORS/INFRACLASS PROTOTHERIA

Paleontological evidence, morphological studies, and comparisons of genes from living species (molecular clock approach) (9,10) suggest that vertebrates evolved from deuterostome ancestors approximately 500–600 million years ago (mya) and developed in a gradual manner, where many innovations were retained in their descendants (11). Mammals evolved from members of the reptilian order therapsida in the subclass synapsida some 150 mya, or as recent allometric and other morphological evidence from a skull of a small mammaliaform taxon, *Hadrocodium*, suggest, perhaps some 45 million years earlier (12). Mammalian prototypes retained the opening in the skull behind the ocular orbit found in synapsid reptiles and modified two articulation bones in the jaw to form the malleus and incus of the middle ear. Another important innovation that defined mammals was the mammary gland. Modified epidermal glands on the ventral part of the adult female's thorax/abdomen apparently produced postpartum secretions that were advantageous to the newborn infant (13). The ventral location of the mammary gland would have been favored by face-to-face interactions between mother and infant. Pheromones may have also played a role in attracting newborns to those dermal secretions.

Squamate reptiles such as the three-toed skink, *Chalcides chalcides*, are viviparous and have a specialized chorioallantoic epithiochorial placenta (14). *Chalcides chalcides* also expresses the *Hbeta58* gene involved in the development of the mammalian placenta (15). This may be an example of convergent evolution. Otherwise, the evidence indicates that the mammary gland appeared before the placenta.

Monotremes (from the subclass prototheria), the echidna (*Tachyglossus aculealus*), and the duck-billed platypus (*Ornithorhynchus anaticus*) closely resemble reptiles from which mammals emerged (16). Their reptilian features include microchromosomes, filiform sperm, egg-laying, and bones in the pectoral girdle found only in fossils of therapsids. These primitive mammals have mammary glands, but no placenta (16). Moreover, the platypus has no organized nipple (16). The milk simply flows onto the ventral fur of the mother, as expected in primitive mammals.

Table II. Major Categories of Anti-Inflammatory Agents in Human Milk

Categories	Examples
Cytoprotectives	Prostaglandins E2, F2α
Epithelial growth factors	EGF, lactoferrin
Maturational factors	Cortisol
Enzymes that degrade mediators	PAF-acetylhydrolase
Binders of enzymes	α1-antichymotrypsin
Binders of substrates	Lysozyme
Modulators of leukocytes	IL-6, IL-10, and other cytokines
Antioxidants	Uric acid, α-tocopherol, β-carotene, ascorbate

Table III. Examples of Immunomodulating Agents in Human Milk

Agents	Possible in vivo functions
Secretory IgA anti-idiotypic antibodies	Epitope binding sites on antibodies mimic foreign antigens
Nucleotides	Increase NK-cell activity
Prolactin	Aids in T cell development and activation
Cytokines	
M-CSF	Aids monocyte maturation
TNF-α	Th1 cytokine. Activates macrophages
IL-6	Enhances IgA production
IL-8	Chemotactic factor for PMNs
IL-10	Th2 cytokine. Inhibits production of pro-inflammatory cytokines
TGF-β	Anti-inflammatory cytokine

The primordial mammary gland may have secreted agents such as fatty acids, oligosaccharides, lysozyme and iron-binding proteins that protected the infant from the bacterial flora of the mother's skin. The fatty acids may have been similar to those found in human sebum (17). Also, lysozyme, which is phylogenetically ancient (3), is produced by mammalian apocrine glands (18). Furthermore, melanotransferrin, an iron-binding protein found in human sweat glands, has a 40% homology with lactoferrin, a protective iron-binding protein found in many mammalian milks (19).

Milk produced by monotremes displays certain immunological agents found in milk from eutherian mammals, including lysozyme (20), transferrin (20,21), and the oligosaccharide difucosyl-lactose (22) that interferes with the attachment of *Campylobacter jejuni* (23) or *E. coli* stable toxin (24) to epithelial cells.

INFRACLASS METATHERIA

Metatherian mammals (marsupials) diverged from eutherian species some 145 mya. The marsupial placenta is a noninvasive yolk sac. Since no trophoblast develops, fetal and maternal tissues are not closely connected. Given the limitations of a yolk-sac placenta, the period of gestation in marsupials is brief compared to eutherian mammals. Consequently, the newborn marsupial is altricial. However, the arms and legs are developed well enough to permit the newborn to journey to the teat of the mammary gland. The newborn locks onto the teat and suckles for 7–29 weeks, depending upon the marsupial species (reviewed in Ref. 6).

The basic structures of marsupial immunoglobulins are similar to those found in eutherian mammals. However, major changes in the composition and isotype repertoire of immunoglobulins occurred after the evolutionary separation of mammals from reptiles and prior to the evolutionary separation of marsupials and placental mammals (25). These changes are also found in immunoglobulins transferred from the mammalian mother to the offspring.

A small placental transfer of IgG occurs in tammar wallabies (26). Otherwise, there is no evidence of placental transfer of IgG in metatherians. Instead, IgG is transferred from the mother to the infant via colostrum and milk (27,28). *Macropus robustus* is of particular interest, since the transfer of IgG via milk in that type of kangaroo is greatly accentuated a few days before weaning when encounters with microbial pathogens by the infant will increase significantly (29).

More recently, it has been found that secretory IgA, transferrin, and IgG are in colostrum, and transferrin and IgG are in milk produced by brushtail possums (*Trichosurus vulpecula*) (30). Milk from the brushtail possum contains a lysozyme that is similar to the one in human milk (31). In addition, milk produced by certain marsupial species contains concentrations of oligosaccharides (up to 100 g/L) that exceed those found in eutherian mammals (32).

Leukocytes are also in milk produced by the tammar wallaby (*Macropus eugenii*) (33). In that marsupial, neutrophils predominate while the infant is attached to the teat. Afterwards, macrophages dominate. The mechanism for the switch is unknown.

INFRACLASS EUTHERIA

Eutherian mammals have a complete placenta. Molecular dating suggests that the earliest eutherian mammals appeared some 145 mya (34). Through natural selection, many different groups of mammals developed, and many species within each group emerged. The principal radiation of eutherian species occurred 65 mya or earlier (35,36).

Bayesian analysis of molecular genetic evidence from existing species suggests that the primary branching events in eutherian history that gave rise to the superordinal clades Afrotheria (aardvarks, elephants, elephant shrews, hyraxes, manatees, tenrecs), Xenartha (anteaters, armadillos, sloths), Euarchontoglires (rabbits, rodents, primates), and Laurasiatheria (carnivores, cows, horses, whales) coincided with the breakup of the giant southern continent Gondwana into Africa and South America. Most eutherian orders became extinct, but other orders survived. For example, primates appeared about 65 mya or earlier, the earliest hominids (the genus *Australopithecus*) diverged from other primates about 5–6 mya (37), our more direct *Homo* ancestors arose about 2.5 mya (38), and *Homo sapiens* emerged some 100,000–250,000 years ago (39–43).

The evolutionary proximity of existing mammals has been reconstructed from cladistic, phenotypic, and genotypic analyses of current mammalian species. For example, genomes of humans and chimpanzees (*Pan troglodytes* and *Pan paniscus*) are remarkably similar (44–46), whereas far greater differences in genomic and phenotypic features are found between *Homo sapiens*, other primates, and animals in other mammalian orders (44–46).

Some evolutionary relationships between the immunological activities of the mammary gland have

Table IV. Placental and Colostral Transfer of IgG

Type of placenta	Mammalian species	Placental transfer	Colostral transfer
Epitheliochorial	Horses, pigs, and lemurs	−	++++
Syndesmochorial	Artiodactyla mammals such as cows, sheep, and goats	−	++++
Endotheliochorial	Carnivores such as dogs and cats	±	+++
Yolk Sac	Rodents, guinea pigs, and rabbits	++++	±
Hemochorial	Great apes and humans	++++	+

been investigated. Some defense agents in mammalian milks are remarkably conserved. Two examples are the antigenic similarities between the carboxyterminal, intracytoplasmic regions of the MUC 1 mucins found in milks from many mammalian species (47) and structural similarities in lysozymes and α-lactalbumins in mammalian milks (3,48). In contrast, some immunological functions of the mammary gland are not as highly conserved in certain mammalian species. Because of exposures to dissimilar environmental microorganisms, one might anticipate that certain immune responses by different orders of mammals diverged as a consequence of natural selection. Furthermore, because of major distinctions in the rate and degree of motor development, the type and extent of exposure to microorganisms by young infants of those species vary considerably. Thus, it would be predicted that immunological adaptations to these different environments are also reflected in the immunological composition of milk produced by those species.

In general, the greater the evolutionary distance, the more dissimilar is the immunological composition of milks from those species. A well-studied example is the qualitative and quantitative immunological differences between human and cow milk. These include the quantities and types of antimicrobial oligosaccharides (49,50) and the concentrations of immunoglobulin isotypes (6,51–54), lactoferrin (51,52,55), lysozyme (51,52,56), lactoperoxidase (57,58), and milk mucin MUC 1 (reviewed in Ref. 59). A more detailed analysis of the types of immunological agents that are found in milks produced by eutherian species follows.

Antimicrobial Agents

Immunoglobulins

In eutherian mammals, the evolutionary relationships between type of placenta and the function of the mammary gland dictates the types of immunoglobulins that are transferred from the mother to the offspring by placental blood or by colostrum or milk (reviewed in Ref. 6) (Table IV).

A discussion of the types of eutherian placentas may be helpful before considering the transfer of immunoglobulins. In epitheliochorial placentas, fetal chorionic epithelium is in direct contact with uterine epithelium. That type of placenta is found in horses, pigs, bottle-nosed dolphins, and some primitive primates such as lemurs. In many artiodactyla mammals such as cows, sheep, and goats, the placenta is syndesmochorial. In that type of placenta, a uterine epithelium is absent. Consequently, chorionic epithelium directly contacts other uterine tissues. Carnivores such as seals, cats, and canines have an endotheliochorial placenta (60) characterized by chorionic epithelium that directly contacts capillary endothelium of the maternal uterus. In contrast, rodents, rabbits (lagomorphs), and guinea pigs (hystricomorphs) have a yolk-sac type of placenta. Finally, advanced primates including the hominoids have a hemochorial placenta that allows maternal blood to directly contact the placental trophoblast.

IgG is effectively transferred via hemochorial and yolk sac placentas. Much less transport occurs via the endotheliochorial placenta. Little if any IgG transfer occurs through epitheliochorial or syndesmochorial placentas (Table IV). In species where there is little or no placental transfer of IgG, IgG is transferred to the nursing infant via ingested colostrum, and this ingested maternal IgG is absorbed into the systemic circulation of the recipient.

In most mammals that transfer IgG via colostrum rather than the placenta, the dominant immunoglobulin isotype in milk after the colostral phase is usually secretory IgA (IgA dimers or trimers complexed to part of the sacrificial polyimmunoglobulin receptor that is termed secretory component). However, ruminants are an exception. Although secretory IgA and IgM are present (6,61), the dominant immunoglobulin in milk from ruminants is IgG1 (6). In mammals that display an effective placental transfer

of IgG, the dominant immunoglobulin in colostrum and milk is secretory IgA. In contrast to IgG transmitted by colostrum, secretory IgA antibodies remain at mucosal sites where they protect against microbial pathogens and their products.

Immunoglobulin patterns in two mammalian species deserve special mention. The concentration of IgD is ~100-fold greater in rat milk than in serum (62). The IgD does not appear to be absorbed into the systemic circulation. The biological effects of this ingested IgD in the gastrointestinal (GI) tract of the developing rat pup are unknown. The second unusual situation is the transfer of IgE in bovine colostrum to the calf's systemic circulation (63).

Do evolutionary relationships found in studies of immunoglobulins that operate in systemic and mucosal immunity pertain to the immunoglobulins in milks from various orders and species of eutherian mammals? Investigations of the types and quantities of immunoglobulins in mammalian milk shed some light on this issue (Table V). The most prominent immunoglobulin in milk produced by the closely related caprine and bovine species is IgG. The major immunoglobulin isotype in the milk of mammals such as swine, canines, cats, and harbour seals (Phoca vitulina) is also IgG. In contrast, the immunoglobulins of human milk are closest to the chimpanzee and gorilla, less similar to other primates, and even more different from milks produced by more distantly related mammals. In that respect, secretory IgA is the dominant immunoglobulin in milk produced by humans and chimpanzees (51,52,64).

The evolutionary proximity of antibodies in milk from humans and closely related primates is also demonstrated in an antigen-binding specificity of the antibodies. Antibodies against the mammalian α-galactosyl epitope that cross-reacts with enteric bacteria that display similar epitopes are common in humans, apes, and Old World monkeys, but are undetected in New World monkeys, prosimians, and other types of mammals (65) that are more distantly related to the genus *Homo*. Secretory IgA antibodies with that specificity are present in human milk (66). Thus, evolutionary relationships of mammalian species hold with respect to these immune functions of the mammary gland.

Table V. Immunoglobulin Isotypes in Mammalian Colostrum and Milk

Species	IgG	IgM	IgA Monomer	Secretory IgA	IgD	IgE
Human	+	+	−	++++	+	−
Cow	++++	++	−	+	?	+
Pig	++++	+	+	+	?	?
Rat	+	?	?	+++	++	?
Seal	++	?	?	?	?	?

Transferrin Family of Iron-Binding Molecules

Prototherian and metatherian milks contain transferrins that are identical to serum transferrins. Transferrin is also present in colostrum and milk produced by some eutherian mammals such as the cow (55). In cow colostrum, the concentration of transferrin is somewhat greater than the concentration of the related iron-binding protein lactoferrin. In cow milk produced later in lactation, the concentrations of transferrin are much less than the concentrations of lactoferrin. In milk produced by most other eutherian mammals, transferrin is not detected, whereas lactoferrin is prominent.

There are, however, variations in the structure and concentrations of lactoferrin in mammalian milks. As with immunoglobulins, the amino acid composition, major antigenic determinants, glycosylation pattern, and concentrations of lactoferrin in milks produced by humans and rhesus monkeys were found to be quite similar (67). Lactoferrin has also been found bound to membranes of human milk fat globules (68). It is unclear whether this form of lactoferrin is present in other mammalian milks.

In addition to its nutritional role in iron transport, lactoferrin has many immunological functions. Some are due to its iron-binding property, while others involve other sites of the molecule. The bacteriostatic and fungistatic activities of lactoferrin are due to the ability of the apo-form of the molecule to chelate ferric iron from siderophilic microorganisms (69,70). In contrast, bactericidal and fungicidal properties of lactoferrin are due to its N-terminal peptide lactoferricin (71). The antimicrobial activities of human, murine, caprine, and bovine lactoferricins have been compared (72). Bovine lactoferricin was found to have the most potent in vitro effects upon *E. coli* ATCC 25922, *S. aureus* ATCC 25923, and *Candida* species. In addition, human lactoferrin inhibits certain common viral pathogens (73–76).

Th pluripotency of lactoferrin is also shown by its ability to enhance the growth of mucosal cells and interfere with the activation of the complement system (reviewed in Ref. 77). Finally, lactoferrin in milk may also exert certain systemic effects. Human preterm infants absorb whole lactoferrin and its fragments

derived from human milk and then excrete them in their urinary tract (78). These maternal moieties as well as absorbed oligosaccharides from human milk that are excreted into the urine (79) may aid the recipient to resist bacterial urinary tract infections.

Lysozyme

Lysozyme originated some 400–600 million years ago (3). Lysozyme that does not bind calcium is found in most mammalian milks. However, Ca^{++}-binding lysozymes are expressed in at least two mammalian orders, carnivores and perissodactyls (equines) (80).

Concentrations of lysozyme in milk vary from very low levels in bovine milk to concentrations of about 100–300 mg/L in human milk (51,52). In contrast to most other antimicrobial proteins, the daily production of lysozyme by the human mammary gland rises during the first 4 months of lactation (51,52). Similar quantitative studies of lysozyme production by other lactating mammals have not been carried out.

Lysozyme is an enzyme that cleaves the bond between *N*-acetylglucosamine and β1-4-*N*-acetylmuramic acid on cell walls of gram-positive bacteria (81). The enzyme acts synergistically with lactoferrin in milk from certain other mammalian species such as the human to kill gram-negative bacterial pathogens (82). In addition, lysozyme inhibits elastase, a hydrolytic enzyme that operates in inflammation, by binding to its substrate, elastin (83).

The lysozyme C gene gave rise after gene duplication 300–400 mya to a gene that codes for α-lactalbumin, a protein expressed only in the lactating mammary gland of nearly all species of mammals, binds to and accentuates the function of lactose synthase. It is of interest that three domains from the evolutionary descendant of lysozyme are antibacterial (84). Furthermore, a partially unfolded molecule of α-lactalbumin in conjunction with a C 18:1 fatty acid triggers apoptosis of cultured neoplastic cells (85). It is likely that the unfolding of the protein structure and the liberation of the fatty acid from milk lipid occurs in the stomach of the recipient infant (85).

Oligosaccharides

Many oligosaccharides in mammalian milk act as receptor analogues that block the binding of certain bacterial pathogens or their products to epithelial surfaces. In contrast to antibodies in milk that require antigenic stimulation for their formation, the production of oligosaccharides is antigen-independent.

The types and quantities of oligosaccharides vary in milks from different mammalian species (reviewed in depth in Ref. 32). A comparison between humans and elephants is also instructive (86). The concentration of oligosaccharides is three times higher in elephant milk than in human milk (86). Furthermore, oligosaccharides comprise about 40% of the carbohydrate content of elephant milk but only 10% of the carbohydrate content of human milk. Qualitative differences are also remarkable. *N*-Acetylneuraminic-acid-containing oligosaccharides comprise almost half of total oligosaccharides in elephant milk, whereas they comprise only about 30% of oligosaccharides in human milk. Most oligosaccharides in elephant milk are more fucosylated or sialylated than those in human milk. Moreover, high levels of 3′-galactosyllactose (up to 4 g/L) and lacto-*N*-neotetraose are in elephant milk, but are poorly represented in human milk. Conversely, human milk displays oligosaccharides that are not well represented in elephant milk (86).

Recently, milks from primates (human, gorilla, chimpanzee, bonobo, and orangutan), members of the Order Carnivora (black bear, grizzly bear, dog), a member of the Order Perissodactyla (black rhinoceros), a member of the Order Artiodactyla (the giraffe), a marsupial (the goodfellowtree kangaroo), a sirenian mammal (manatee), and a mammal in the Suborder Odontoceti (the bottle-nosed dolphin) were examined for 12 fucosylated neutral oligosaccharides (87). Remarkable differences were found between land and aquatic mammals. Very low concentrations of oligosaccharides were found in milks obtained from the aquatic mammals compared to milk from terrestrial mammals. In addition, qualitative differences have been found. Milk oligosaccharides from marine mammals are almost solely lactose, 2′-fucosyllactose, and 3′-fucosyllactose, whereas milk from terrestrial mammals also usually contains tetrasaccharides and hexasacccharides (87). These differences probably reflect adaptations to different microbial flora encountered in land and aqueous environments.

Leukocytes

Leukocytes including neutrophils, macrophages, T cells, and B cells have been found in colostrum and milk obtained from many eutherian mammals (reviewed in Ref. 6,88–91). In a few species such

as the pig, eosinophils have also been found (91). Neutrophils are the most prevalent leukocytes in colostrum. As they decline, macrophages and T cells are more strongly represented. All human milk leukocytes are activated (88,89). The activation status of leukocytes in other mammalian milks is unknown.

Little is known concerning the in vivo fate and actions of leukocytes in most mammalian milks, but in vitro studies and some in vivo observations suggest what their functions may be. For example, macrophages in human milk are highly motile, phagocytic, antigen-presenting cells, and T cells in human milk are motile and divide after exposure to specific antigens (reviewed in Ref. 92). In certain species such as sheep, there is evidence that milk mononuclear leukocytes take up residency in tissues of the small intestines and mesenteric lymph nodes of the recipient infant (reviewed in Ref. 6). That has not been proven to occur in primates, but these same types of leukocytes reside in mucosal sites of primates. Furthermore, T cells in human milk are $CD45RO^+$ as are intraepithelial T cells. Since $CD45RO^+$ T cells are developmentally delayed in the recipient infant (93), it seems likely that the milk T cells compensate for that developmental delay by entering those mucosal sites.

In contrast, human milk neutrophils, though phagocytic, have a reduced motility compared to their counterparts in blood or to macrophages in human milk (reviewed in Ref. 92). Therefore, there is little likelihood that they invade the alimentary tract of the recipient infant. It is more likely that they engulf bacterial enteropathogens such as *Shigella*, *Salmonella*, and *Yersinia* in the lumen of the alimentary tract, use elastase to degrade virulence proteins of those pathogens (94), and thus prevent the escape of those pathogens from phagolysosomes where they will be killed.

Anti-Inflammatory Agents

A great number of anti-inflammatory agents including antioxidants, epithelial growth promoters, enzymes that destroy inflammatory mediators, agents that bind enzymes or substrates that operate in inflammation, and anti-inflammatory cytokines have been discovered in human milk (reviewed in Ref. 7,8,77,95) (Table II). It is of interest that many of these agents are also nutrients, antimicrobial agents, or immunomodulators. That is further evidence of the pluripotency of defense agents in milk. Several antioxidants including ascorbic acid, α-tocopherol, and carotenoids, have been found in bovine milk (96). There is little information concerning the types of antioxidants or other anti-inflammatory agents in milk from other mammalian species.

Immunomodulating Agents

There is little information concerning immunomodulating agents in milk produced by most mammalian species. Epithelial growth factor, insulin-like growth factor, and transforming growth factor-β have been found in porcine milk (reviewed in Ref. 91). A much wider spectrum of immunomodulating agents has been found in human milk (Table III), including anti-idiotypic antibodies and a host of nucleotides and cytokines (reviewed in Ref. 7,8,95). They include pro-inflammatory, anti-inflammatory, growth promoting, and chemotactic cytokines. The in vivo fate and precise functions of these agents in the recipient infant remains uncertain, but long-term protective effects of breast-feeding in humans suggest that they profoundly regulate the infant's immune system (7,8,95). Otherwise, there is little information concerning cytokines or other innate immunomodulating agents in mammalian milks except for nucleosides and nucleotides in bovine milk (reviewed in Ref. 97). There is in vitro evidence that some immunomodulating agents are created by partial digestion of certain bovine milk proteins such as caseins but the physiological effects of those degradation products are undetermined (reviewed in Ref. 98).

EVOLUTIONARY ADVANTAGES

The change from intrauterine to extrauterine life in mammals is important in many ways. One aspect is the necessity to contend with a host of environmental pathogens that are encountered on the skin and mucous membranes in the face of developmental delays in the immune system. These developmental delays are, however, often offset by the immune system in milk of the species. Several overlapping evolutionary strategies have been recognized concerning the immune activities of the lactating mammary gland and the recipient infant (5,99) (Table VI). 1) Defense agents in milk directly compensate for developmental delays in those same agents in the recipient infant. 2) Defense agents in milk do not directly compensate for developmental delays in the production of those same agents in the recipient, but nevertheless confer a survival advantage. 3) Agents in milk initiate or enhance functions that are absent or poorly expressed in the recipient. 4) Agents in milk alter the

Table VI. Evolutionary Strategies Concerning Immune Functions of the Lactating Mammary Gland and Infant

Evolutionary strategy	Examples of agents in milk
Directly compensate for developmental delays	Secretory IgA, lactoferrin, lysozyme, cytokines, PAF-acetylhydrolase
Indirectly compensate for developmental delays	Oligosaccharides and nucleotides
Enhance poorly expressed functions	Cytokines, anti-idiotypic antibodies
Adapt the GI tract to extrauterine life	Epithelial growth factors
Prevent GI inflammation	PAF-acetylhydrolase, anti-oxidants, IL-10
Enhanced survival of defense agents	Innate resistance to digestion inhibitors of proteolysis
Produce defense agents by partial digestion of substrates in milk	Liberation of antimicrobial fatty acids and monoglycerides from triacyglycerols
Establish commensal bacterial flora	Lactobacillus growth factors

physiological state of the intestinal tract from one that is better adapted to intrauterine life to one that is better adapted to extrauterine life. 5) Certain agents in milk are anti-inflammatory. 6) Defense agents from milk have an enhanced survival in the recipient's GI tract. 7) Certain defense agents are created by partial digestion of substrates in milk in the gastrointestinal tract of the recipient infant. 8) Agents in milk augment the growth of commensal enteric bacteria.

These evolutionary strategies have been particularly well investigated in *Homo sapiens*. Examples of the strategies found in humans are as follows (Table VI). 1) Defense agents in human milk that directly compensate for developmental delays in those agents in the recipient infant include secretory IgA, lysozyme, lactoferrin, platelet activating factor-acetylhydrolase, and several cytokines. 2) Defense agents in human milk that do not directly compensate for developmental delays in those agents appear to include a host of oligosaccharides and nucleotides. 3) Agents in human milk that initiate or enhance functions that are poorly expressed in the recipient include antiidiotypic antibodies and many types of cytokines. 4) The decrease in the permeability of the intestinal tract during breast-feeding appears to be an example of the provision of agents in human milk that change the physiological state of the intestinal tract from one adapted to intrauterine life to one adapted to extrauterine life. 5) The paucity of clinical evidence of inflammation in the face of enteric infections is evidence of the anti-inflammatory effects of human milk upon the recipient. 6) The sixth evolutionary strategy is evidenced by the enhanced survival of defense agents from human milk such as secretory IgA, lactoferrin, and lysozyme throughout the recipient's GI tract. The enhanced survival of the factors may be due to intrinsic resistance to proteolysis as in the case of human secretory IgA (100) and lactoferrin (101), possible protection by coupling with soluble receptors, as shown with TNF-α in human milk (102), and developmental delays in the productions of gastric HCl and pancreatic proteases (103). 7) Examples of the prodution of defense agents by the partial digestion of substrates in milk are peptide fragments of lactoferrin (104) and β-casein (105) and the liberation of antimicrobial fatty acids and monoglycerides from human milk triacyglycerols by bile salt-stimulated lipase in milk or lipases in the recipient infant (106). 8) The eighth evolutionary outcome is an intersection between human and microbial biology. Human milk provides growth factors for commensal bacteria that predominate in large intestines of breast-fed infants. Enteric commensal bacteria also resist natural low-molecular-weight antimicrobial peptides (107). The bifidobacteria and lactobacilli in the large intestine produce organic acids that inhibit bacterial enteropathogens. Furthermore, commensals stimulate epithelial cells to produce defensins that kill certain bacterial pathogens (108).

The effects of mammalian milks upon the development and function of the GI tract of the infant should also be considered in the context of biologic evolution. Two intertwined processes, natural selection and the slow stream of spontaneous mutations, drive biologic evolution. Positive selection occurs when individuals of a species gain a reproductive advantage or develop a superior adaptation to either a static or new environment. It has not been emphasized, however, that adaptation pertains not only to the adult of a species but also to the young. A host of developmental delays in the fetus and newborn infant are offset by maternal factors transferred via the placenta or amniotic fluid during intrauterine life and by milk during postnatal life. In addition, milk provides agents that either regulate the function or enhance the maturation of key mucosal structures during early postnatal life (reviewed in Ref. 99).

There may have been a number of evolutionary advantages to the maternal/infant relationships. Since developmental processes increase nutritional demands, when some of those events are deferred, spared energy/nutrients may be used for growth and development of other organs until such is required to successfully adapt to the environment or to begin

reproductive activities. In early infancy, the complete maturation of the immune system is deferred for several weeks in certain species to as long as 2 years in human infants. Those postnatal developmental delays are compensated by immune factors in milk. Spared energy/nutrients are diverted to growth and development of certain organs/systems such as the central nervous system, lungs, and skeletal–muscular tissues that will be required for the infant to begin to develop independence.

In addition, some developmental delays may be physiologically advantageous. For example, the increased permeability of the fetal GI tract permits amniotic fluid to be absorbed into the fetus (reviewed in Ref. 99). In certain mammals such as cows, pigs, dogs, and cats, newborns are deficient in serum IgG (reviewed in Refs. 6,54). The dominant colostral immunoglobulin in those species is IgG, and that maternal protein is quickly absorbed via specific receptors found in the GI tract of the newborn animal. The transferred IgG corrects the deficiency and protects the newborns against many types of infections. After the first few days of postnatal life, the concentrations of immunoglobulins decrease precipitously and little immunoglobulin is absorbed by the recipient. A protracted increased permeability in postnatal life is, however, a liability because of exposure to foreign agents. Natural selection would have thus favored the survival of newborns that were able to quickly strengthen their mucosal barriers. This came about by evolutionary innovations that culminated in the defense system produced by the mammary gland.

CODA

There are opportunities to further investigate the outcomes of evolutionary experimentation that began some 145 million years ago when certain therapsid reptiles first developed mammalian features and to track the elaborations caused by natural selection. In the process, the evolutionary connections between current and past mammalian species, as well as between existing mammals, may be better defined.

The questions are, however, more intriguing than originally thought, because evolutionary modifications are expressed not only in adult life but also in young mammals, and these modifications are variable and complex. Moreover, a significant part of this mosaic pattern of evolution is the interface between mammalian infants and their mothers. In that respect, the Swedish biologist Carolus Linnaeus named the class of animals to which we belong after an organ that is vestigial in males (109). Linnaeus was not an evolutionary biologist but the consummate taxonomist of his time. He could have singled out other features common to all mammals, but instead he chose to recognize the paramount importance of the mammary gland in mammalian biology.

The mammary gland may not have been the first step in mammalian evolution, but it was one of the key events. Some 100,000 years after our species appeared, we are beginning to appreciate that the mammary gland provides not only nutrition for young mammals but also a host of bioactive agents that protect the infant against many types of infections, inflammatory processes, and late-onset diseases that are immunologically mediated. In that respect, it is exceptionally important to recognize that the complex immune systems in mammalian milks are species-specific. The public health implications of breast-feeding are evident. Infant mammals regardless of the species are healthier when they are fed milk from their own species. Even so, just the surface of the immense body of molecular-genetic processes that produce and control the immunological relationships between the maternal-infant dyad of mammalian species has been examined. The understanding of this aspect of evolutionary biology has just begun.

ACKNOWLEDGMENTS

Susan Kovacevich assisted in the preparation of this paper. Lynn Burke from the Moody Memorial Library at the University of Texas Medical Branch obtained key references including the 1735 first edition of Linnaei's Systema naturae sive regna tria naturae systematice proposita per classes, ordines, genera, and species. I also thank the editor of this journal and the other reviewers for their helpful suggestions.

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