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Defense Agents in Milk

A. Defense Agents in Human Milk

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I. Introduction

A. Factors with Nonnutritive Functions in Human Milk

During the past three decades, there has been a growing realization that breast-feeding not only provides the nutritional requirements of the infant, but also supplies a host of defense factors for the protection of the recipient and/or the mammary gland. The study of this remarkable defense system in human milk has been difficult, however, because of its biochemical complexities, the small concentrations of certain very potent agents in human milk, the need to develop special methods to quantify certain factors because of their particular forms in human milk, the compartmentalization of some of the agents, and the dynamic effects of the length of lactation and other maternal factors upon the concentrations or functions of the components of the system. In this chapter, we summarize the current information concerning the molecular forms, concentrations in milk during the several phases of lactation, biological activities, fate in the recipient infant, and *in vivo* functions of the defense agents in human milk.

B. General Characteristics of Defense Agents in Human Milk

The defense agents in human milk, though biochemically diverse, share certain features: (1) there is often an inverse relationship between the

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production of these factors in the mammary gland and their production by the infant during the same time frames of lactation and postnatal development. As lactation progresses, the concentrations of many of the factors in human milk fall. Concomitantly, the production at mucosal sites of those very factors rises in the developing infant; (2) most components of the immunologic system in human milk are produced throughout lactation and during gradual weaning; (3) the factors are usually common to other mucosal sites; (4) they are adapted to resist digestion in the gastrointestinal tract of the recipient infant; (5) they protect by noninflammatory mechanisms; and (6) the agents act synergistically with each other or with defense agents produced by the recipient. Representative examples of soluble defense agents are listed in Table I.

II. Types of Defense Agents in Human Milk

A. Direct-Acting Antimicrobial Agents

1. Oligosaccharides–Glycoconjugates

These agents include monosialogangliosides that are receptor analogues for heat-labile toxins produced by *Vibrio cholerae* and *Escherichiae coli* (Holmgren *et al.*, 1981), fucose-containing oligosaccharides that inhibit the hemagglutinin activity of the classical strain of *V. cholerae* (Holmgren *et al.*, 1983), fucosylated oligosaccharides that protect against heat-stable enterotoxin of *E. coli* (Newburg *et al.*, 1990), mannose-containing high-molecular-weight glycoproteins that block the binding of the El Tor strain of *V. cholerae* (Holmgren *et al.*, 1981), and glycoproteins and glycolipids that interfere with the binding of CFA/II fimbrae on enterotoxigenic *E. coli* (Holmgren *et al.*, 1987). The inhibition of toxin binding appears to be associated with acidic glycolipids that contain sialic acid (gangliosides). Although the total quantities of gangliosides in human and bovine milk are similar, the relative frequency of each type of ganglioside composition is distinct. Monosialo-ganglioside 3 predominates in human milk (about 74% of total gangliosides), but not in bovine milk (Laegreid *et al.*, 1986a,b), and the enterotoxin receptor ganglioside, GM1, as measured by a highly sensitive immunostaining method, is 10-fold greater in human than bovine milk (Laegreid *et al.*, 1986a). In that respect, GM1 inhibits the enterotoxins of *E. coli* and *V. cholerae* (Laegreid and Otnaess, 1987).

Oligosaccharides in human milk also interfere with the attachment of *Haemophilus influenzae* and *Streptococcous pneumoniae* (Andersson *et al.*, 1986). In particular, GlcNAc (β1–3)Gal-disaccharide subunits block the attachment of *S. pneumoniae* to respiratory epithelium. It is anticipated that carbohydrate side chains on a number of whey proteins in human milk will be found to function as receptor analogues.

TABLE I
Representative Soluble Defense Agents in Human Milk

	Representative function
Anti-infectious agent	
Oligosaccharides–glycoconjugates	Inhibit binding of bacterial pathogens and toxins to epithelium
Lactoferrin	Decrease multiplication of siderophilic bacteria/fungi by Fe^{3+} chelation
Lysozyme	Disrupts peptidoglycans of cell walls on susceptible bacteria
Secretory IgA	Antibodies inhibit adherence of pathogens to epithelium; neutralize toxins
Mucin	Inhibits rotavirus
Lipids	Disrupt enveloped viruses
Anti-inflammatory agents	
Uric acid, ascorbate, α-tocopherol, β-carotene	Antioxidants
Prostaglandins	Cytoprotective
Cortisol, lactoferrin, EGF	Epithelial growth factors
Platelet-activating factor–acetylhydrolase	Degrades PAF
Immunomodulators	
Interleukin-1β	Activates T cells/monocytes
Interleukin-6	Aids terminal differentiation of IgA-producing cells
Tumor necrosis factor-α	Upregulates production of secretory component.
	Activates T cells/monocytes

In addition, there is recent evidence that human milk interferes with the binding of human immunodeficiency virus envelope antigen gp120 to CD4 molecules on T cells (Newburg *et al.*, 1992). The physical characteristics of the inhibitor were consistent with mucins, sulfated proteins, glycoproteins, or glycoaminoglycans.

Some data from animal models suggest that the oligosaccharides and glycoconjugates in human milk protect *in vivo* (Cleary *et al.*, 1983; Otnaess *et al.*, 1982), but there is little information from human studies that pertains to this question (Glass *et al.*, 1983).

2. *Proteins*

Many whey proteins in human milk have direct antimicrobial properties. The principal ones are as follows.

a. Lactoferrin. Lactoferrin, a member of the transferrin family of iron-binding glycoproteins, is the dominant whey protein in human milk. Lactoferrin, a single-chain glycoprotein with 703 amino acids, has an M_r of 79 kDa and two globular lobes, both of which display a site that binds ferric iron (Anderson *et al.*, 1987). Except for a 20-kDa fragment of lactoferrin that immunologically cross-reacts with bovine β-lactoglobulin (Monti *et al.*, 1989), the vast majority of lactoferrin in human milk consists of intact molecules. Over 90% of the lactoferrin in human milk is in the form of apolactoferrin (i.e., does not contain ferric iron) (Fransson and Lonnerdal, 1980). This is highly advantageous, since apolactoferrin competes with siderophilic bacteria for ferric iron (Arnold *et al.*, 1977; Bullen *et al.*, 1972; Spik *et al.*, 1978; Stephens *et al.*, 1980; Stuart *et al.*, 1984) and thus disrupts the proliferation of those microbial pathogens. The epithelial growth-promoting activities of lactoferrin in human milk may also be advantageous to the defense of the recipient infant (Nichols *et al.*, 1987). The mean concentration of lactoferrin in human colostrum as measured by electro-immunodiffusion is between 5 and 6 mg/ml (Goldblum *et al.*, 1982). The concentration at 4 weeks falls to about 2 mg/ml (Goldblum *et al.*, 1981; Goldman *et al.*, 1982). Afterwards, the concentration of lactoferrin averages about 1 mg/ml (Goldman *et al.*, 1982).

In keeping with its resistance to proteolysis (Brines and Brock, 1983; Samson *et al.*, 1980; Spik and Montreuil, 1966), a number of groups have reported that the excretion of lactoferrin in the stools is higher in human milk- than in cow's milk-fed infants (Butte *et al.*, 1984; Davidson and Lonnerdal, 1985, 1987; Spik *et al.*, 1982). The total daily secretion of lactoferrin in human milk during the first 4 months of lactation has been investigated by a test-weighing procedure and immunologic assays. The approximate mean intake of milk lactoferrin per day in healthy full-term infants was reported to be 260 mg per kilogram per day at 1 month and 125 mg per kilogram per day by 4 months (Butte *et al.*, 1984). The amount of lactoferrin excreted in the stools of low birth weight infants fed human milk appears to be about 185 times that in infants fed a cow's milk formula (Schanler *et al.*, 1986). However, this estimate is probably too high because of the presence of immunoreactive fragments of that protein (Goldman *et al.*, 1990).

In addition, there is a significant increment in the urinary excretion of intact and fragmented lactoferrin as a result of human milk feedings (Goldblum *et al.*, 1989; Goldman *et al.*, 1990; Prentice, 1987). Recent stable isotope studies suggest that the increment in urinary lactoferrin and its fragments is principally from ingested human milk lactoferrin (Hutchens *et al.*, 1991).

b. Lysozyme. Lysozyme, a 15-kDa single-chain protein, is found in relatively high concentrations in external secretions including human milk (Chandan *et al.*, 1964; Jolles and Jolles, 1967; Goldblum *et al.*, 1981; Goldman *et al.*, 1982, 1983a,b; Peitersen *et al.*, 1975). Lysozyme lyses susceptible bacteria by hydrolyzing β-1,4 linkages between *N*-acetylmuramic

acid and 2-acetylamino-2-deoxy-D-glucose residues in cell walls (Chipman and Sharon, 1969). The agent is relatively resistant to digestion by trypsin or denaturation due to acid. The mean concentration of lysozyme in colostrum as measured by electroimmunodiffusion is about 70 μg/ml (Goldblum *et al.*, 1981). The concentration drops to about 20 μg/ml at 1 month of lactation and then rises to a mean of 250 μg/ml by 6 months (Goldman *et al.*, 1982). The approximate mean intake of milk lysozyme per day in healthy full-term infants was reported to be 3 or 4 mg per kilogram per day at 1 month and 6 mg per kilogram per day by 4 months (Butte *et al.*, 1984).

Limited studies have been conducted concerning the fate of human milk lysozyme that is ingested by the infant. The amount of lysozyme excreted in the stools of low birth weight infants fed human milk is about eight times that found in infants fed a cow's milk formulation (Schanler *et al.*, 1986). There was no increment in the urinary excretion of this protein as a result of human milk feedings. Otherwise, the *in vivo* fate and functions of the agent remain to be determined.

c. Fibronectin. Significant amounts of fibronectin, a high-molecular-weight protein that facilitates the uptake of many types of particulates by mononuclear phagocytic cells, are present in human milk (mean concentrations in colostrum, 13.4 mg/liter) (Friss *et al.*, 1988). Structural analyses of fibronectin in human milk have not been reported. The *in vivo* effects of this broad spectrum opsonin in human milk are not known.

d. Complement components. Although there is evidence that the components of the classical and alternative pathways of complement are present in human milk, the concentrations of these components, except for C3, are exceptionally low (Ballow *et al.*, 1974; Nakajima *et al.*, 1977). The quantitation of these components has been limited to hemolytic assays. Additional studies with newer immunoassays have not been reported. It is also unclear whether the structures of these molecules are the same as those in human blood.

e. Immunoglobulins. The pattern of the concentrations of major immunoglobulin isotypes in human milk is quite different from those found in blood and interstitial fluids. The predominant immunoglobulin in human milk is IgA (Goldman and Goldblum, 1989). IgA is the dominant immunoglobulin in human milk, whereas IgG is the most common one in adult blood and interstitial fluids. The principal molecular forms of IgA in blood and milk are different. The main form in serum is a four-chain structure consisting of two identical heavy polypeptide chains (predominantly the α 1 isotype) and two identical light chains (either κ or λ) linked by disulfide bonds. In contrast to the IgA monomers, a polymeric form of IgA, secretory IgA, comprises over 95% of IgA in human milk (Goldman and Goldblum, 1989). This type of IgA consists of two identical IgA monomers united by a 15-kDa polypeptide called the joining or J chain

and complexed to a 75-kDa glycopeptide designated as secretory component (Mostov and Blobel, 1982; Mostov *et al.*, 1984; Solari and Kraehenbuhl, 1984). This form of IgA is assembled when dimeric IgA binds to the first domain of polymeric immunoglobulin receptors (Bakos *et al.*, 1991) on the basolateral surface membranes of epithelial cells. Before the assembled molecule is secreted, the intracellular part of the original receptor is cleaved so that the final molecule consists of the ectoplasmic portion (secretory component) and dimeric IgA. Secretory IgA is more resistant to proteolytic enzymes and therefore is more able to persist in the intestinal tract than other immunoglobulins.

Specific antibodies in human milk arise from a triggering of enteromammary (Allardyce *et al.*, 1974; Goldblum *et al.*, 1975; Roux *et al.*, 1977; Weiz-Carrington *et al.*, 1978) and bronchomammary (Fishaut *et al.*, 1981) immune pathways. In the case of the enteromammary pathway, the responsible immunogen is taken up by M cells on the surface of Peyer's patches. The immunogen is recognized by B cells which display specific IgM antibodies to the immunogen. The immunoglobulin isotype of the B cells is switched to IgA, and those B cells are then launched into a migration pathway that begins in the intestinal lymphatics and ends in the lamina propia of the mammary gland. Those B cells undergo terminal differentiation to become plasma cells that produce dimeric IgA antibodies that are specific for the very immunogens that originally triggered the pathway.

The concentrations of IgM are much lower in human milk than in serum (Jatsyk *et al.*, 1985; Mata and Wyatt, 1971). IgM molecules in blood and milk display a pentameric structure. However, in contrast to serum IgM, some human milk IgM is complexed to secretory component (Brandtzeg, 1974). In the few studies that have been published, the antibody specificities of human milk IgM are similar to those of the major immunoglobulin isotype in human milk, secretory IgA.

IgG, the principal immunoglobulin in human serum, is present in modest amounts in human milk (Jatsyk *et al.*, 1985; Keller *et al.*, 1983; Mata and Wyatt, 1971; McClelland *et al.*, 1978; Ogra and Ogra, 1978; Peitersen *et al.*, 1975). Each IgG subclass has been detected in human milk, but the relative proportion of IgG4 is higher in human milk than serum (Keller *et al.*, 1983). It has therefore been suggested that IgG4 may be produced in or specifically transported to the mammary gland. An alternate explanation is that the increased proportion of IgG4 is due to a more efficient exclusion of other IgG subclasses from human milk.

The quantity of IgD in human milk is lower than that in serum, but the decrease is proportionally less than is reported for IgG and IgM (Keller *et al.*, 1984). IgE, the principal type of antibodies responsible for immediate hypersensitivity reactions, is essentially absent in human milk (Underdown *et al.*, 1976).

There has been considerable interest in specific antibodies in human milk. Depending upon the precise question, it may be necessary to determine the fine specificity, avidity, isotypes, and quantities of antibodies in

milk. The determination of the fine specificity depends upon the use of highly specific antigens. Even then, immunologic cross-reactivity, particularly against common enteric microorganisms and food antigens, may occur. Because of the structure of secretory IgA, immunoassays that are designed to quantify IgA will also detect secretory IgA, but the resultant data may also reflect the presence of other molecular forms of IgA, such as monomeric or dimeric IgA, that are not complexed to secretory component. Secretory IgA antibodies may be distinguished from other types of IgA antibodies by using specific antibodies to secretory component in solid-phase immunoassays, although secretory IgM antibodies will also be detected.

Solid-phase immunoassays in which the capture antibody is directed against the α-chain of IgA and the antibody detector recognizes secretory component have been used to quantitate total secretory IgA in human milk (Goldblum *et al.*, 1981). The concentrations of secretory IgA in human milk were highest in colostrum (Goldblum *et al.*, 1981) and gradually declined to a plateau of about 1 mg/ml for the remainder of lactation (Goldman *et al.*, 1982). The approximate mean intake of secretory IgA per day in healthy full-term breast-fed infants was found to be 125 mg per kilogram per day at 1 month and ~75 mg per kilogram per day by 4 months (Butte *et al.*, 1984).

The fate of human milk secretory IgA fed to infants has been examined. In one study, the amount of secretory IgA excreted in the stools of low birth weight infants who were fed human milk was about 30 times that in infants fed a cow's milk formula (Schanler *et al.*, 1986). In addition, there was a significant increment in the urinary excretion of intact secretory IgA antibodies as a result of human milk feedings (Goldblum *et al.*, 1989). The origin of those antibodies in the urine of infants fed human milk is undetermined.

f. Mucins. Human milk mucins have recently been reported to be antimicrobial. Membrane mucins on human milk fat globules interfere with the binding of S-fimbriated *E. coli* (Schroten *et al.*, 1992) and human milk mucins defend against rotavirus (Yolken *et al.*, 1992), the most common cause of infectious enteritis in human infants (Kapikian *et al.*, 1981). The range of the antimicrobial effects of these compounds in human milk and their abilities to cooperate with other defense agents in milk are unclear.

B. Growth Promoters of Protective Microorganisms

In contrast to bovine milk, human milk contains a growth-promoting activity for *Lactobacillus bifidus* var. *Pennsylvania* (György *et al.*, 1974). It appears that this activity is responsible for the predominance of *Lactobacillus* in the bacterial flora of large intestines of breast-fed infants. Those bacteria produce acetic acid, which aids in suppressing the

multiplication of enteropathogens. The bifidus growth-factor activity is due to *N*-containing oligosaccharides (György *et al.*, 1974) and glycoproteins and glycopeptides (Bezkorovainy *et al.*, 1979; Nichols *et al.*, 1975). The bifidus growth-promoter activity associated with caseins may reside in the oligosaccharide moiety of those molecules (Bezkorovainy and Topouzian, 1981).

C. Defense Agents Created from Partially Digested Substrates from Human Milk

Human milk may also protect by supplying defense agents from substrates that are partially digested in the recipient's alimentary tract. Fatty acids and monoglycerides produced from milk fats by bile salt-stimulated lipase or lipoprotein lipase in human milk or lingual/gastric lipase from the recipient are able to disrupt enveloped viruses (Issacs *et al.*, 1986; Stock and Francis, 1940; Thromar *et al.*, 1987; Welsh *et al.*, 1979; Welsh and May, 1979). These antiviral lipids may aid in preventing coronavirus infections of the intestinal tract (Resta *et al.*, 1985). They may also defend against intestinal parasites such as *Giardia lambdia* (Gillin *et al.*, 1983, 1985).

The second example of the generation of biologically active agents from enzymatic digestion of substrates in human milk is the production of β-casomorphins from ingested human casein (Brantl, 1985). These peptide fragments have not only opioid, but also immunostimulating effects (Berthou *et al.*, 1987; Parker *et al.*, 1984).

A 20-kDa fragment of lactoferrin has been described in human milk (Monti *et al.*, 1989), but its function is not known. Fragments of human lactoferrin have been demonstrated in the stools of human milk-fed infants, and the multiplicity of those fragments suggests that some apolactoferrin from human milk feedings is partially cleaved in the gastrointestinal tract of the recipient (Goldman *et al.*, 1990). Similar fragments of lactoferrin were demonstrated in the urine suggesting that they were from absorbed from the gastrointestinal tract and excreted into the urinary tract (Goldman *et al.*, 1990). The biologic activity of the fragments of lactoferrin in the excreta of the recipients is undetermined. It is undetermined whether one of those fragments is similar to the pepsin-derived fragment of lactoferrin, lactoferricidin-B, that is bactericidal (Bellamy *et al.*, 1993; Yamauchi *et al.*, 1993).

D. Leukocytes

Living white blood cells are present in human milk particularly during the first 3 or 4 months of lactation. The concentrations of these leukocytes are highest in the first 2–4 days of lactation and gradually decline during the next few months. Neutrophils and macrophages are the most prominent cells in human milk. It is necessary to employ special cytochemical stains

or immunologic markers to distinguish the neutrophils from the macrophages in human milk because their morphology is altered by the large amount of lipid vesicles in their cytoplasm (Smith and Goldman, 1968; Smith *et al.*, 1971).

Although human milk neutrophils are phagocytic, they are unable to adhere to common substrata, move as rapidly as neutrophils from venous blood, or respond to chemotactic agents (Thorpe *et al.*, 1986). Those features may be due to prior activation in that recent flow cytometry studies demonstrate that neutrophils in human milk display changes in their surface phenotypes (increased expression of CD11b, decreased expression of leukocyte adhesion molecule-1) that are found on activated neutrophils (Keeney *et al.*, 1992).

Macrophages in milk also appear to be activated. This is suggested from their morphology (Smith *et al.*, 1971), their surface phenotypic features (Keeney *et al.*, 1992), and their enhanced motility *in vitro* (Mushtaha *et al.*, 1989; Özkaragoz *et al.*, 1988). Human milk macrophages have also been found to produce toxic oxygen radicals (Tsuda *et al.*, 1984) and express class II gene products of the major histocompatibility complex (Wirt *et al.*, 1992). The *in vivo* functions of these leukocytes are not established.

Lymphocytes are also found consistently in human milk. About 80% of them are T cells (Wirt *et al.*, 1992). There is controversy concerning the relative frequencies of the major subsets of T cells in human milk and some of the differences in the results from different studies may be due to methodologic variables. The proportions of $CD3^+CD4^+$ and $CD3^+CD8^+$ in unfractionated human milk leukocytes examined by immunofluorescent microscopy were similar to those in human blood (Keller *et al.*, 1986), whereas the proportions of CD^+CD8^+ in human milk leukocytes that were separated by density gradient centrifugation and examined by flow cytometry were higher than those in peripheral blood (Richie *et al.*, 1982). Recently, unfractionated human milk leukocytes were found by flow cytometry to have a higher relative frequency of $CD3^+CD8^+$ than those in peripheral blood (Wirt *et al.*, 1992). The cytotoxic responses of these cells are poor (Kohl *et al.*, 1978, 1980), but the cells are capable of generating certain lymphokines, including interferon-γ and monocyte chemotactic factor Emodi and Just, 1974; Keller *et al.*, 1981; Lawton *et al.*, 1979). In that respect, essentially all T cells in human milk bear the phenotypic marker of memory T cells (leukocyte common antigen isoform, CD45RO) (Bertotto *et al.*, 1990; Wirt *et al.*, 1992), and that type of T cell is the principal producer of interferon-γ (Berlotto *et al.*, 1990; Sanders *et al.*, 1988).

The *in vivo* role of T cells in human milk is uncertain, but it is of considerable interest that very small numbers of memory T cells are detected in blood in infancy (Hayward *et al.*, 1989). Thus, it may be possible that maternal memory T cells in milk compensate for the developmental delay in their production in the infant. There is evidence from experimental animal studies that milk lymphocytes enter tissues of the neonate (Head *et al.*, 1977; Jain *et al.*, 1989; Schnorr and Pearson, 1984; Weiler *et*

al., 1983), but that has not been demonstrated in humans. In that regard, comparisons between the phenotypic expression of CD45RO on T cells in the blood of breast-fed and nonbreast-fed infants will be of interest. In addition, further studies are in order of the pattern of antigens to which these T cells respond (Parmeley *et al.*, 1976) and the repertoire of the T cell antigen receptors of those cells in human milk.

E. Anti-inflammatory Agents

One of the extraordinary features of the protection afforded by human milk is the virtual absence of clinical signs of inflammation during the gastrointestinal infections. This may be due in part to the more rapid elimination or neutralization of microbial pathogens in the lumen of the gastrointestinal tract by defense agents from human milk, but other features of human milk suggest that this is not the sole explanation. Phlogistic agents and the systems that give rise to them are poorly represented in human milk. In addition, human milk contains a host of anti-inflammatory agents (Goldman *et al.*, 1986; Goldman *et al.*, 1989b), some that double as antimicrobial factors (lactoferrin, secretory IgA, and lysozyme). The major classes of these anti-inflammatory agents in human milk include factors that promote the growth of epithelium, such as cortisol (Kulski *et al.*, 1981), epithelial growth factor (Carpenter, 1980), polyamines (Romain *et al.*, 1992), and lactoferrin (Nichols *et al.*, 1987), antioxidants (ascorbate-like compound, uric acid, β-carotene), and agents that inhibit nonoxidative inflammatory systems such as prostaglandins (Nen *et al.*, 1988) and platelet-activating factor acetylhydrolase (Furukawa *et al.*, 1992). Like the antimicrobial factors, these factors are well adapted to operate in the hostile environment of the alimentary tract.

The nature of the antioxidants in human milk has recently been investigated (Buescher and McIlheran, 1992). It was found that the peaks of antioxidant activity in colostrum were due to an ascorbate-like compound and uric acid. In addition, two other antioxidants in human milk, α-tocopherol (Chapell *et al.*, 1985; Ostrea *et al.*, 1986) and β-carotene (Ostrea *et al.*, 1986), are absorbed into the circulation where they may have systemic effects. In that regard, serum vitamin E concentrations rise in breast-fed infants from a mean of 0.3 mg/ml at birth to ~0.9 mg/ml on the fourth day of life. Otherwise, there is little information concerning the *in vivo* fate and functions of the anti-inflammatory agents in human milk.

F. Immunostimulating Agents

If human milk stimulates certain defense systems in the infant, one might predict that the effects might lead to long-lasting resistance. Supporting epidemiologic evidence for that premise has been mounting for several years. The incidence of juvenile diabetes mellitus (Mayer *et al.*, 1988) and Crohn's disease (Koletsko *et al.*, 1989) appears to be less among children

who have been breast-fed during infancy. In addition, a recent retrospective analysis suggests that breast-feeding lessens the risk from lymphomas (Davis *et al.*, 1988). In each study, considerable reliance has been placed upon the abilities of mothers to recall the type and duration of feeding given to their offspring; yet, recall of events that transpired many years beforehand may be suspect. Undoubtedly, prospective studies of the possible long-term protective role of human milk will be required to further explore the possible long-term benefits of human milk.

A number of experimental observations also suggest that human milk provides active protection for the recipient infant. The production of IgA at mucosal sites may be enhanced by human milk (Goldblum *et al.*, 1989; Prentice, 1987; Schanler *et al.*, 1986; Stephens, 1986; Stephens *et al.*, 1984). Although in most of those studies it has been difficult to exclude the effect of passively transferred secretory IgA, in two investigations the excretion of IgA was increased in the urinary tract, a system removed from direct contact with human milk (Goldblum *et al.*, 1989; Prentice, 1987). Furthermore, in one report the urinary excretion of free secretory component was also remarkably increased in infants fed human milk (Goldblum *et al.*, 1989). The M_r of these proteins far exceeds the size of molecules filtered by glomeruli and neither secretory component nor secretory IgA are transported from the systemic circulation into external secretions by epithelial cells. It is, therefore, likely that human milk feedings stimulated the synthesis of secretory component by epithelial cells in the urinary tract and that this, in turn, enhanced the transport of secretory IgA into the urine of the infants. The components in human milk that may be responsible for such an enhancement are undetermined at this time.

The second type of evidence that the recipient infant's immune system is stimulated by breast-feeding is the increase in certain immune factors in the blood of breast-fed infants that cannot be accounted for by the levels of those factors in human milk. The response of breast-fed and nonbreast-fed infants to respiratory syncytial virus (RSV) infection was compared by measuring their serum interferon-α levels (Chiba *et al.*, 1987). The serum levels of interferon-α were strikingly increased in breast-fed infants in the first 2–4 weeks after RSV infection. Since there is little interferon-α in human milk, it seems likely that human milk is able to prime leukocytes in the host to produce that cytokine. In addition, there is evidence that the plasma concentrations of fibronectin are higher in breast-fed than nonbreast-fed infants (237 and 17/mg/liter, respectively) (Friss *et al.*, 1988). Since the amount of ingested fibronectin is not sufficient to account for the increment in plasma fibronectin that has been observed, it seems likely that human milk induces the synthesis of that opsonin in the infant.

The last piece of evidence comes from discoveries of immunomodulators in human milk. Human milk contains a high concentration of α-tocopherol, an agent which, in addition to its antioxidant effects, is known to stimulate the development of immunity (Bendich *et al.*, 1983, 1984, 1986; Tengerdy *et al.*, 1981). Several glycoproteins that orchestrate the development and functions of the immune system have been found in

human milk. These agents, termed cytokines, require only minute quantities to be bioactive. Moreover, there are many interrelationships between those agents. The cytokines and their concentrations (mean values unless otherwise indicated) in early human milk are (1) interleukin-1β (IL-1β) (~1130 pg/ml) (Munoz *et al.*, 1990), (2) IL-6 (~150 pg/ml) (Saito *et al.*, 1991; Rudloff *et al.*, 1993), (3) IL-8 (~3680 pg/ml) (Palkowetz *et al.*, 1994), (4) IL-10 (~3300 pg/ml) (Garofalo *et al.*, 1995), (5) tumor necrosis factor-α (~620 pg/ml) (Mushtaha *et al.*, 1989; Rudloff *et al.*, 1992), and (6) transforming growth factor-β (~130 pg/ml) (Noda *et al.*, 1984; Palkowetz *et al.*, 1994), granulocyte colony-stimulating factor (45–1554 pg/ml) (Gilmore *et al.*, 1994), and macrophage colony-stimulating factor (Hara *et al.*, 1995). The effects of these agents in human milk on the recipient infant are as yet unknown, but it is likely that they will influence the development of the defenses of the respiratory and alimentary tract.

III. Coda

Because of the heterogeneity and complexity of the immunologic system in human milk, definitive investigations of the molecular, quantitative, and functional features of the components of the system have often been incomplete. Although considerable progress has been made toward defining many aspects of the defense system in human milk, further research will be required to identify the entire system, unravel the molecular biology and mechanisms of production and secretion, and determine the discrete role of each component of the system in the defense of the mammary gland or the recipient infant. In the process, it will continue to be important to anticipate that the ultimate role of these defense agents may depend upon a multiplicity of interactions with other factors in the system found in milk or with defense agents or cells produced by the breast-fed infant.

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