REVIEW ARTICLE

Immunologic Defense Mechanism in Human Milk

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ABSTRACT Breast feeding has been intensively campained throughout the country in the last decade, which is tahought to be responsible for the decline of the incidence of gastroenteristis. One of the most important advantages of breast feeding is the immunologic properties of breast milk. The importance of breast milk in the protection of many infections of the newly born infants, who are naturally immunologically dificient, is well-established. Breast milk contains humoral as well as cellular immunity components, each component has its elements. Although it is possible to describe the role of each components, it is generally thought that the protective properties of human milk against infections in neonates and young babies are the results of interactions of many factors rather than the effect of solely each factor. The role of humoral (immunoglobulins) and cellular (macrophages, lymphocytes, and other cellular elements) immunity of human milk, especially its protective properties, is discussed in this article. **[Paediatr Indones 1995; 35:53-64]**

Introduction

During the last decade breast feeding is intensively campaigned throughout the country. The convincing result is indicated by the declining incidence of gastroenteritis among infants and babies.

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Other factors for the decline of the incidence of gastroenteritis include the improvement of sanitation, safe water supply, health education, personal hygiene, and better housing.

Breast feeding, however, is unique from the nutritional, physiological, and immunological points of view. It provides protection for the infants. Numerous contents of the human milk account for this protection, e.g., lysozyme, bifidus

factors, B-12 binding-protein,[1] lactoperoxidase, lactoferrin, interferon,[2] and complement;[3] however, emphasis has been weighed on the importance of the protective effects of humoral and cellular immunity.

We believe that the protection is a result of interaction of all factors involved rather than the effect of solely each factor. The immunologic aspects of human milk have been widely studied, especially the protective effect to the infants against infection.

The purpose of this article is to provide available information on the existence, the functional characteristics, and the potential role of the immunologic components of the human milk in the infants immunity system.

Humoral Immunity of Human Milk

Components of humoral immunity in human milk

All classes of immunoglobulins are found in human milk.[3,4] These immunoglobulins are IgG, IgM, IgA, IgE and IgD. Human colostrum and milk collected at different times after the onset of lactation from 20 subjects indicated no significant change in the individual level of IgG over 180 days of sequential testing. The levels of IgG immunoglobulins range from 1.4 to 4.9 mg/g of protein at different intervals after the onset of lactation. On the other hand highest levels of IgM and IgA in the colostrum and milk are observed during the first three to four days post-partum. The IgM levels range from 2 the 30 mg/g protein and IgA levels fro 22 to 35 mg/g protein. A 3 to 4 fold d cline in the levels of IgM and IgA immu noglobulins are demonstrated in mil samples collected 15 to 180 day post-partum.[5] Another study finds tha in early colostrum as much as 20 to 4 mg/ml of IgA is detected. After the first to 4 days there is a drop to values at th level of 1 mg/ml, but the increase in mil production may then compensate for th fall in immunoglobulin concentration.[6]

Another study indicates that IgG an IgM are found in human milk at a lo level with a daily output in mature mil of about 100 mg IgG and 70 mg IgM. It i possible that milk IgG and IgM are transferred from serum, but at least, a portio of IgG and IgM may be locally produced as suggested by investigations in human and rats. IgG-4 has been proposed as being produced in the human mammary gland.[1] The levels of IgG and IgM are much lower than the level of IgA, with less than 3 percent of serum value for IgG and 10 percent for IgM.[7] The slightly higher figure for IgM than for IgG may indicate a partly different mechanism for the production of the two, possibly including some local production of IgM .[8]

IgE and IgD have also been measured in colostrum and milk. Using radioimmunoassay techniques, colostrum is found to contain concentrations of 0.5 to 0.6 IU/ml IgE in 41% of samples and less in the remainder.

IgD is found in all samples in concentrations of 2 to 2000 μg/100 ml. Plasma levels are poorly correlated. The findings suggest possible local mammary production rather than positive transfer. Whether IgE or IgD antibodies in breast milk have similar specifities for antigens as IgA antibodies in milk remains unanswered.[2]

Compared with other immunoglobulins, IgA, especially secretory IgA (sIgA), is highest in colostrum, and although their levels fall over the next 4 weeks, substantial levels are maintained throughout the first year, during gradual weaning between 6 and 9 months and in fact during partial breast feeding in the second year of life.[2]

Precursor cells of plasma cells producing IgA are believed to be B-lymphocytes which receive antigen induced stimulatory "first signals" in Peyer's patches, mesenteric lymph nodes, (gut associated lymphoid tissue, GALT), and other mucosa-associated lymphoid tissue such as tonsils and bronchial lymph nodes (bronchus-associated lymphoid tissue, BALT).[6]

Entero-mammary pathway and broncho-mammary pathway migration of the most stimulated B-cells start from the lymphoid tissue through lymph and peripheral blood to various secretory tissue including mammary glands. They may receive signals for further differentiation in mesenteric lymph-nodes and spleen. Terminal differentiation to plasma cells at secretory sites are induced by "second signals" that are largely antigen dependent at least in the gut and to some extent in the bronchus. IgA in human milk exists in the form of sIgA, which consists of a dimer of IgA with two additional polypetide chains: the secretory component (SC) and the junction (J)-chain, which gives its specific characteristics.[1,8,9]

This composite molecule is more stable then serum IgA since it is more resistant to pH changes and enzyme attack.[3,8] There is no evidence that maternal antibodies are absorbed from the intestinal tract of the newborn infants. It seems more likely that the action of the antibodies in human milk is limited to the alimentary tract of the neonate. Besides the resistance to enzymatic and acid digestion, antibodies in colostrum would be expected to have an enhanced survival in the alimentary tract of the neonate because of the presence of inhibitor of trypsin in human colostrum for the first five days after delivery, and colostral IgA binds to trypsin and to a lesser extent to chymotrypsin, whereas serum IgA does not.[3]

The stable structure of SC-J-IgA-dimer makes the sIgA more resistant. Research has proven however, that some 20-80% of sIgA passes the gut undegraded.[1,6,10] After the start of breastfeeding sIgA rapidly increased in the feces whereas IgG and IgM were consistently low.[10]

It is possible that much of the sIgA unaccounted for stool sIgA is bound to fecal bacteria. Loss of sIgA may be due to the activity of bacterial IgA protease and reductase in the intestine. However, it should be noted that Fab of sIgA is still active, for instance in neutralizing polio virus.[6]

The potential role of immunoglobulin

Human milk contains immunoglobulins especially sIgA with the activity against a wide range of bacterial, viral, parasitic and food antigens. It is striking that the milk sIgA immunoglobulins seem to re-

flect intestinal exposure of the mother to the corresponding antigens. As the result of the entero-mammary pathway, a mother's milk carries IgA immunoglobulins against the microbes she is exposed to in the intestine. Human milk regularly contains antibodies to a wide variety of *E. coli O, K* and pili antigens, *Shigella, Salmonella O* antigens, *Klebsiela pili*, streptococci, poliovirus, cocksackie and ECHO viruses.[1-3,8]

There is also evidence that broncho-mammary pathway exists, with the appearance of milk sIgA immunoglobulins against syncitial virus after pulmonary infection of the mother.

It is originally expected that the antibodies in human milk would appear in the circulation of the breastfed infant in order they will get the maternal immunity; however, only trace or no transfer of milk antibodies to the blood of the breastfed infants can be demonstrated.[1]

sIgA immunoglobulins have a function on mucosal membranes, not in tissues. Since the IgG and IgM antibodies of milk are presumably similar to serum antibodies, they should have the same biological functions. The main functions of sIgA immunoglobulins are:

1. to bind antigens and microbes. The most important function in host defense is presumably to bind bacteria, preventing them from attaching to mucosal membranes.[1,8]
2. to prevent bacterial adhesion represented by antibody against bacterial adhesions such as pili and fimbria, may mediate such anti adherence activity.[1]
3. sIgA immunoglobulins can also neutralize toxins and viruses.[1]
4. to prevent internalization of attacking virus.[1]

Breast feeding may be important in protecting the mucosal surfaces of the infant, not only by providing a source of sIgA antibodies, but possibly also by increasing the rate of mucosal IgA maturation by the infant.[11]

Monomeric IgA and IgM, on the other hand, neutralize infectivity after the virus has entered the cell. The portion of sIgA that is reported to be degraded in the intestine may partly remain protective against viruses, since it is also known that the antigen binding fragments of sIgA can neutralize polio virus.

Other antibacterial and antiviral properties of milk give an additional support in mucosal protection. Human milk contains lysozyme which has enzyme activity against the cell wall of gram positive bacteria, B-12 binding protein, and bifidus factors. The complement factors HC3 and HC4 are found in human milk as well as components of the alternative pathway.

Bacteria normally colonize the gut of human newborn during the first few days of life. *E coli* strains are able to colonize the intestine even when the infant is breast fed with milk rich in sIgA immunoglobulins against the O and K antigens of *E. coli* strains. *Shigella* and ente- roviruses are usually transient and do not cause symptoms if the infants are breast fed.

Although breast feeding is protective against cholera, it does not prevent intestinal colonization by *V. cholerae*. It may be that the volume of the intestinal content is too large for milk antibodies and other defense factors to eliminate the microorganisms.[1]

Little is known about the protective role of separate factors in clinical or community studies. An interesting evidence of cholera in breastfed infants showed a relation between protection and the content of mother's milk sIgA immunoglobulins against *V. cholerae* O antigen and enterotoxin. A report has suggested that the protection of breast feeding against campylobacter diarrhea is related to the level in mothers milk of sIgA immunoglobulins. There has been much interest in the possibility that the milk sIgA immunoglobulins against rotavirus can prevent rotavirus induced diarrhea. Another study indicated that eventhough exclusive breast feeding appeared to protect infants against severe rotavirus diarrhea, breast feeding alone conferred no overall protection during the first 2 years of life, suggesting that breast feeding temporarily postponed rather than prevented this outcome.[12]

Secretory IgA has a protective effect against nasopharyngeal colonization of non typeable *Hemophylus influenzae*. A study from Japan indicated that the level of human milk anti P6 secretory IgA antibody was inversely related to frequency of isolation of the organism. Prevention of colonization was most evident during breast feeding.[13]

Antiparasitic effect of human milk is believed to be the results of interaction of many factors including immunoglobulins, phagocytes, and milk lipids.[14] A mechanism by which antitrophozoite IgG, sIgA and phagocytic cells interact to promote parasite clearance has been suggested. In human, sIgA antibody against *Giardia lamblia* and *Entamoeba histolytica* are present in milk of mothers from endemic area. In vitro experiment shows that *Giardia lamblia* is rapidly killed by exposure to human milk and that this giardicidal effect does not depend on sIgA.

The bile salt stimulated lipase, which is secreted in human milk was suggested to be the responsible killing factor.[2] No giardiacidal effect of immunoglobulins in human milk was supported by the study of Waterspiel et al which indicated that the amount of anti-Giardia sIgA in human milk was associated with prevention of symptoms of diarrhea due to Giardia, but not with acquisition of the organism.[15]

Human milk and polio vaccination

Human milk can neutralize polio virus, and it is noted early that breast feeding may interfere with oral vaccination with live poliovirus vaccine.[1,9]

The fact that live poliovirus is not effective if given close to a meal of breast milk indicates that the antiviral antibodies of milk are efficient in neutralization.[8]

Other studies have not confirmed about this finding. It may be due to the very vide variation of milk sIgA antibody titers against poliovirus.

Milk antibody response to vaccination

Vaccination of the mothers may interfere the response of milk antibodies. Decrease of milk sIgA antibody levels against poliovirus has been noted after oral vaccination with live poliovirus vaccine.[7] The explanation for this decrease

of sIgA antibodies after intestinal exposure to live poliovirus is unknown. In contrast, parenteral vaccination with whole-cell cholera vaccine boosted the milk sIgA response. Furthermore, subcutaneous or intranasal vaccination with live attenuated rubella virus will usually give small but consistent milk sIgA responses.

Allergic protective properties of human milk

Protein of breast milk is species specific, and therefore non-allergic for the human infant. No antibody response has been demonstrated to occur with human milk in human infants. It has been shown that macromolecules in breast milk are not absorbed.[2] sIgA in colostrum and breast milk prevents the absorption of foreign macromolecules when the infant's immune system is immature.

The periods of relative IgA deficiency in infant of atopic parents resulting failure of antigen disposal resulted in development of allergic reactions, can be prevented by breast feeding and by allergen avoidance.[7]

This result is not surprising because human milk consistently contains sIgA antibodies against food such as cow's milk protein. It has been hypothesized that breast feeding protects allergic diseases through two major mechanisms:

1. Provision of immunoglobulin A (sIgA), which prevents infections and blocks absorption of intact allergens through the gut.
2. Avoidance of exposure at an early age to large amount of foreign protein present in cow's milk.[17]

Milk immunoglobulins in malnutrition

Malnutrition may interfere the immunoglobulins production. Evidence has accumulated although there is conflicting data about this issue. A study conducted by Miranda et al found decreased colostral levels of IgA, IgG and C4 in undernourished mothers,[7] while Gambian mothers on a low energy intake had a higher daily mammary output of IgG, IgM, C3 and C4 than well-nourished British mothers, whereas that of IgA and lactoferin were the same in both.

Underprivileged mothers in Ethiopia and Guatemala produce the same amount of milk sIgA daily as privileged control mothers.[1] Guatemalan mothers with chronic malnutrition show, as all mothers, wide variations of the levels of milk sIgA antibodies against *E. coli*, but they have lower levels than privileged Guatemalan mothers. This is not due to undernutrition, however, but rather to differences in exposure. A similar investigation of milk sIgA antibodies in these privileged and underprivileged women against salmonella and shigella somatic antigen shows that, although there are differences between the antibody concentration among the groups, there are no differences in daily output of the sIgA antibodies.

Stability of immunoglobulins

As discussed above sIgA is more resistant to proteolytic enzymes and the low pH of the stomach. Preservation of human milk at -20°C alters the levels of IgA, IgM, C3, C4, α–1 antitrypsin and sIgA antibodies to *E. coli* and lactoperoxidase. Boiling essentially destroy 100% of immunologic activity. sIgA and lysozyme activities drop by 20% with Holder pasteurization and by 65% at 65°C. IgG and IgM activities are markedly reduced by Holder pasteurization.[2]

Cellular Immunity of Human Milk

Components of cellular immunity in human milk

Many studies have suggested that the cellular constituents of human milk and colostrum consist of varying proportion of macrophages, T and B cells, other lymphocytes[14] and polymorphonuclear leukocytes (neutrophils).[6,14,18-20] All substances control microbial populations at mucosal surfaces.[20]

Macrophages

Milk macrophages comprise about 90% of the leukocytes.[2] Their average concentration in the immediate post partum period is about 2100/milliliter. These cells can be identified as large cells, 18 to 40 cm in size, contain globules, micelles, casein and other particulate material, and small cells, 8 to 18 cm in size.[19]

Lymphocytes

At most they represent 10% to 15% of cells in the colostrum. Highest lymphocytes counts are observed in the first 3-4 days of lactation and their number may range from 100 000 to 1 000 000 cells/milliliter. After the first few days the total lymphocytes counts range from 50.000 to 100.000 cells per milliliter. A significant proportion of lymphocytes in the colostrum and milk appear to be T cells and probably represent 50% to 70% of total lymphocyte in milk. However, unlike peripheral blood T cells which form E-rosettes only at 4°C, colostral and milk cells have the characteristic property of forming stable E-rosettes at 37°C.[19]

Colostral and milk lymphocytes bear surface phenotype associated with mature cells (CD3), CD4 (helper-inducer) and CD8 (suppressor-cytotoxic). The ratio of CD4 : CD8 positive cells is somewhat lower in the enriched fractions of colostrum and milk T cells than in peripheral blood cells. However, discrepancies among some studies exist concerning this issue. In some studies the ratio have been found to be similar in milk and peripheral blood when the whole cells populations of colostral T cells is employed On the other hand, other studies agree with a CD4 : CD8 ratio of less than 1.[16]

As lactation progresses, lymphocyte counts decrease steadily. By 6 months, T cells make up about 15%-20% of total lymphocytes. In contrast, peripheral blood T cells remain a constant proportion of total circulation lymphocytes pool.

Colostral and milk lymphocyte manifest in vitro proliferative responses on stimulation with a number of mitogens and antigens. Several studies have shown a selectivity in lymphocyte stimulation responses in milk lymphocytes to various antigens when compared with the responses in autologous peripheral blood lymphocytes.

Antigens such as rubella virus stimulate T lymphocytes in secretory sites and milk as well as in systemic sites. In contrast, the *E. coli* K, antigen, whose exposure is limited to mucosal sites, produces stimulation of lymphoproliferative responses only in milk lymphocytes. These studies support the concept of selected T-cell subpopulations in the mammary gland, which may be derived from the antigen sensitized precursor T-cells from the bronchus and gut associated lymphoid tissue and thus resemble the activity patterns of secretory IgA in the colostrum and milk.[19]

B lymphocytes first studied in human milk was IgA producing B cells.[15] The observation to date indicate that IgA-bearing lymphocytes of bronchus associated (BALT) or gut associated lymphoid tissue (GALT) origin will preventially populate the mucosal lamina propria of the bronchial and intestinal mucosa after antigenic exposure in the mucosal sites of the respiratory and intestinal tracts, also occupy mucosal lamina propria of mammary gland.[19]

Specific antibody activity in milk has been demonstrated against essentially all organism introduced naturally via the mucosal portals of respiratory or intestinal tracts. Although the role of BALT and GALT in the appearance of microbial or dietary antigen specific antibody activity in the milk is well documented several questions concerning the distribution of antibody producing cells in the mammary glands and the milk remain to be answered. Immunofluorescence studies fail to demonstrate large number of immunoglobulin containing plasma cells.

One investigation failed to induce differentiation of colostral cells into immunoglobulin containing cells in vitro after stimulation with pokeweed mitogen, phytohemaglutinin, or Epstein-Barr virus. Experiments in rats have suggested that the mammary glands accumulate levels of B-cells that are only about 25% to 36% of the level in small intestine, and the magnitude of B-cells migration to the mammary gland may not be sufficient to maintain these levels in the glands. Thus it has been proposed that clonal expansion of migrated IgA-B cells must take place locally in the mammary gland; however, studies have failed to demonstrate significant local proliferation of IgA-B cells in the rat mammary gland. Thus the possibility that B-cells bearing cytoplasmic IgA in the mammary gland are not derived from the same pool of precursors that provide IgA producing B-cells for the small intestine must be seriously considered.[19]

Other cellular elements

In vitro experiments show that colostral cells exhibit natural killer (NK) cytotoxicity, which is enhanced by interferon and interleukin 2. Colostral cells also elicit antibody dependent (ADCC) or lectin dependent cellular cytotoxic (LDCC) responses, although these responses are significantly lower compared to autologous peripheral blood cell.[19]

The Potential Role of Milk Cells

Macrophages and Phagocytes

These cells have been suggested:

1. as potential transport vehicles for the large quantities of immunoglobulins present in milk.
2. participate in antibody dependent cell mediated cytotoxicity of herpes simplex type 1 virus infected tissue.
3. involved in a variety of biosynthetic and excretory activities including production of lactoferin, lysozyme, complement, properdin factor B, epithelial growth factors, T lymphocytes suppressive factors and IgA-B cell helper factors.
4. regulation of T cell function
5. phagocytic activity against *Staphylococcus aureus, E. coli, C. albicans.*[20]

In addition to antibacterial function, there appears to be three possible antiviral functions associated with cells of colostrum and milk:[18,19]

1. secretion of interferon
2. direct phagocytosis
3. production of specific IgA molecule

The qualitative and quantitative aspects of phagocytic activity of macrophages and neutrophils in milk appear to be comparable, however, the phagocytic activity in cells in milk samples obtained from mothers who have delivered full-term infants appears to be significantly inferior to the activity observed in neutrophils or monocytes in autologous peripheral blood. On the other hand, the activity in monocytes and neutrophils in milk samples from mothers who have delivered preterm infants is quite similar to the activity in peripheral blood cells.

Macrophage synthesis, C3, lizozyme, C4 and lactoferin

Complement C4 and C3 are present in human milk, but their concentrations are low as compared to those in human serum. It seems paradoxical that C4 and C3 are in milk since the immunoglobulins IgG and IgM which activate complement by binding C1 are present in only low concentrations.

Recently, an alternate pathway of complement activation, C3 proactivator has been described and it has been found that IgA and IgE stimulate this system. Activated C3 in human milk should be potentially important because of its known opsonic, anaphylatoxic and chemotactic properties.[3] However, the activity of colostral C3 in the recipient infants is undetermined.

Interleukin 1 (IL-1) which is produced by milk macrophages, is also present in mature milk.[16] The functional role of IL-1 has been suggested to activate T-cells.

The discovery of tumor necrosis factors alpha (TNF alpha) heralded the identification of other cytokines in human milk including IL-6 and transforming growth factor beta (TGF beta). The possible function of IL-6, TNF-alpha and TGF-beta are suggested to be enhancement of IgA production, enhancement of SC production and enhance- ment of isotype switching to IgA-B cells respectively.[21] Other monokines synthesized by macrophages include transferin and lysozyme.[4]

There is evidence that macrophages and neutrophils are activated in human milk. Macrophages in human milk are motile in two dimensional, subagarose system. When the movement of these cells was tested in a three dimensional system, the macrophages were found to be significantly more motile than their

counterparts in peripheral blood, the monocytes. The macrophages and neutrophils display an increased expression of CD 11 b and a decreased expression of L-selectin, as found with activated neutrophils.[21]

T and B cells

The function of colostral T cells in human milk is not fully understood. Some reports showed similarities in antigen responsiveness and production of various mediators, e.g., monocyte chemotactic factors and interferons. Cytotoxic lymphocytes have been shown in milk, as have cells mediating, though poorly, antibody dependent cellular cytotoxicity (ADCC). Colostral cells transformed by Epstein-Barr virus can produce IgM, IgA and IgG.[16]

Modest and conflicting evidence is available that suggest a possible transfer of maternal systemic cellular reactivity via the process of breast feeding to the suckling neonate. The transfer of maternal T cells reactivity to tuberculin protein from mother to the neonate has been observed via the process of breast feeding.[16,19]

The implication of these results on soluble mediators of cellular reactivity may be transferred passively to the neonate.

Survival of human milk leukocytes

Data obtained by Paxson et al demonstrated that survival of human milk leukocytes is best near body temperature and that human milk leukocytes should not be subjected to extreme cold or freezing temperature.[22]

When human milk is stored, however it has been shown that the cellular components do not tolerate heating to 63°C, cooling to -23° C, or lyophilization.[2]

Furthermore Paxson study also demonstrated that human milk leukocyte survival is best below a pH 0.8-8. Human milk can probably be placed directly into the stomach of preterm infants without concern that the acidic pH will produce detraction of the leukocyte.

The procedure of the study was to alternate feedings of preterm infants between commercial formula and human milk that also occur in the community. Whereas the infants receive cow's milk formula while being breastfed. Such procedure may produce mixing of human milk with a solution of varying osmolality and protein concentration. Result of the study indicated that mixture of human milk with non isotonic solutions was not detrimental to leukocyte survival or viability, since variations in osmolality and protein concentration has been known to produce no changes in retrieval or phagocytosis of leukocytes. The placement of human milk into glass containers markedly reduces retrieval of the white cells but phagocytosis is unaffected.

Transmission of disease via the milk

Several diseases can be transmitted via the human milk to the neonates. Human milk can contain hepatitis B virus, rubella virus, cytomegalovirus, and also HIV.[1] It has been suggested that infected monocytes are the vehicle of transmission.[2] Viruses are known to produce persistent infections in humans and to appear in milk.

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