Update on the Bioactive Agents Involved in the Immune Properties of Breast Milk

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Abstract

Background: Breast milk (BM) is considered the optimal source of nutrients for the infant. However, less is known regarding the agents involved in the protection elicited by BM against intestinal and respiratory infections. Although specific IgA antibodies have been extensively described, there is a lack of research regarding the non-specific immune effectors of BM that may exert a protective action. Among them, oligosaccharides of BM have recently demonstrated an outstanding role in the defensive properties of BM against different bacteria, viruses and even toxins.

Aim: The aim of this study was to review the current knowledge regarding the bioactive agents that elicit the immune properties of BM. Afterwards an update on the latest findings with respect to the non-specific immune effectors of BM will be conducted.

Methods: Scientific literature was reviewed using the pubmed.gov web browser. The terms searched were: IgA antibodies, probiotic factors, lactoferrin, lysozyme, mucin, lactadherin, fibronectin, complement factors, lipids, leukocytes, anti-inflammatory, antioxidant and immunomodulatory agents, oligosaccharides and glycoconjugates of BM. The publications included in this search ranged from the earliest phases of the investigation in BM to the most recent discoveries, in order to show the historical background of each finding and how biological functions have been attributed to each agent subsequently.

Results: BM contains a wide range of agents that may play a role in the protection of breastfed babies against different conditions. An outstanding role has been attributed to oligosaccharides of BM in the defense against rotavirus and norovirus, common agents of gastroenteritis in infants.

Conclusions: BM constitutes an unmatched supply of nutritional and essential protective substances for the infant. The effectors of the immune properties of BM are multiple, thus human milk is a unique biological fluid and cannot be replaced by artificial formulas. More studies are required in order to define precisely the agents responsible for the protection elicited by BM and how do they confer this action.

INTRODUCTION

The discovery of the agents of BM responsible for their immune properties has been a slow process. The earliest references of the existence of certain structures in breast milk (BM) dates back us to the microscopic observations of van Leeuwenhoek in the last years of the XVII century. In 1837 Alfred Donné described globules and granular corpuscles that were finally identified as leukocytes in 1968. Paul von Ehrlich reported in 1891 the first evidence that BM could confer an immune protection to the infant based on his observations in animal models. But it was not until the first half of the XX century when Woodbury and Grulee observed that the incidence and severity of intestinal infections was lower in breastfed vs. formula fed infants. The different susceptibility to the acquisition of infections in infants regarding the source of feeding was also observed in relation to respiratory infections afterwards [1]. Furthermore, BM has demonstrated its protective property in a particularly vulnerable group of infants: the preterm newborns. The maturation delay of their immune system, their digestive and respiratory medical problems and the requirement of invasive procedures make them especially susceptible to the acquisition of opportunistic infections. Winberg et al. were the first to report that the risk of sepsis decreases in breastfed preterm newborns...
The immune system of BM, its features and most of its components have been uncovered in the last four decades. The objective of this study was to thoroughly review what we currently know about the agents responsible for the immune properties of BM, and how was the process of investigation followed to arrive at such knowledge. Especial emphasis has been put on the oligosaccharides and glycoconjugates of BM that have recently demonstrated to play a prominent role among the non-specific immune effectors of BM.

**BIOACTIVE AGENTS OF BREAST MILK**

**Secretory antibodies**

After the discovery of the secretory IgA (sIgA) by Hanson in 1961 this antibody has been considered the main protective agent of BM. It is integrated by two identical monomers of IgA formed by light λ chains bond by a 75 KDa polypeptide called binding chain, in contrast to immunoglobulins of human serum where κ light chains prevail. Dimeric IgA would suffer modifications that would transform it in an in situ secretory IgA.

The discovery of IgA antibodies in BM with specificity against unusual pathogens and the study of their origin have suggested that the trigger events for their production could be previous maternal infections [4]. The pathogens stimulate mononuclear cells of Peyer plaques of the small intestine or other lymphoid centers and, in consequence, cytokines are released inducing lymphocytes B IgM positive to convert into B cells IgA positive or to complete their final differentiation to IgA secretory cells. These cells would migrate through lymphatic intestinal channels, the thoracic duct and the vascular circulation, by means of lactogenic hormones and other factors, to the mammary gland and the bronchial tree. Apparently this differentiation to plasmatic cells would occur after their arrival and, with this configuration they would remain in the lamina propria of the mammary gland. Dimers of IgA produced by those plasmatic cells would convert into secretory IgA at that moment and bind to specific receptors on the basolateral membranes of the epithelium. The resulting receptor-IgA dimer would be transported to the apical edge of the cell, where the original intracytoplasmatic portion of the receptor would be removed and the resulting molecule, the secretory IgA, secreted to milk.

In conclusion, the enteromammary and bronchomammary routes could be the way how immune protection mediated by secretory IgA against specific intestinal and respiratory pathogens is conferred to breastfed infants. It is relevant because the antibodies of the intestinal and respiratory mucosa and the repertoire of binding immunoglobulins are not produced in an optimal way during early infancy. However, the temporal difference existing from the maternal exposure to a new pathogen and the appearance of secretory IgA in her milk is enough for the pathogen to initiate the infection in the infant if he is exposed. This is why it was suggested that there might be other protecting agents in BM that could explain the clinical differences observed.

**Probiotic factors**

In 1905 Tissier et al. documented the different composition of the intestinal microbiota in infants in relation to the diet. They observed Lactobacillus bifidus (currently known as Bifidobacterium bifidum) and Escherichia coli in fecal samples from breastfed and formula fed infants, respectively.

Bifidobacterium sp. converts lactose into acetic and lactic acid and this, together with the low buffer capacity of BM, makes the pH of the distal intestine of breastfed infants lower than 5 and prevents from the colonization by many pathogens. Moreover, the strains of Lactobacillus GG trigger the production of specific antibodies against rotavirus [5] and cooperate in the recovery of this infection. These observations supported the statement that BM could mediate the protection of the lactating infant against certain intestinal pathogens through the contribution of promoting factors for the colonization of Bifidobacterium bifidum.

In the last decades the use of molecular biology methods has allowed the demonstration that BM is not a sterile fluid, since multiple groups of bacteria have been identified in it. Lactobacillus, Streptococcus and Enterococcus spp. are the predominant bacterial groups found in human milk. They are even found in samples from extremely preterm gestations [6]. It has been reported that the variations of the intestinal microbiota from the paradigmatic composition of a healthy breastfed infant have been associated to an increased risk for immune and inflammatory conditions such as allergic disorders [7] and, more recently reported, celiac disease and obesity [8,9].

**Inhibitors of the metabolism of pathogens**

Lactoferrin is a single chain glycoprotein with two globular components that contains a binding place to iron. It has recognized antimicrobial properties. One of its fragments (the lactoferricin) is able to destroy certain microorganisms damaging their external membranes, using Ca²⁺, Mg²⁺ and Fe³⁺. Approximately 90% of lactoferrin of BM is able to bind to certain bacteria and fungi and prevent their proliferation [10]. Moreover the promotion of the epithelial growth that lactoferrin initiates contributes to the defense of the lactating infant.

K-casein is a highly glycosilated protein with charged residues of syalic acid. It has demonstrated its capacity to block the binding of certain bacteria to the mucosa, such as Helicobacter pylori, due to its structural analogy with the receptors.

Very few studies have targeted the potential activity of α-lactalbumin. Three polypeptide fragments, consequence of its digestion in the intestinal tract, participate in the protection against Escherichia coli, Klebsiella pneumoniae, Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus sp. and Candida albicans [11].

Haptocorrin or B12 binding protein inhibits the growth of certain bacteria in the mammary gland and encourages the transport of vitamin B12 from the circulation to human milk [12].

The folate binding protein concentrates folate of BM and facilitates its capture by receptors in mucosa, preventing its use by the intestinal pathogens [13].
Enzymes
Lysozyme is an enzyme comprised by 15 KDa single chain protein fragments. It has the property to lyse bacteria with susceptible binding points, such as the β-1,4 bindings between the N-acetylneuraminic acid and the residues of 2-acetylamino-2-deoxy-D-glucose in their cell walls [14].

Peroxidase of human milk derives from leukocytes of milk and acts as a mileoperoxidase. It catalyzes the oxidation of thiocyanate ions to synthesize products with bacteriostatic activity [15] (Table 1).

Mucin
Mucin of human milk is a high molecular weight (1.000 to 10.000 KDa) glycoprotein. It has demonstrated a protective effect against the binding of the S’fimbria of Escherichia coli to epithelial cells [16]. It has also demonstrated the capacity to prevent the infection by rotavirus by the hydrolysis of the N-acetylneuraminic acid in an experimental model in mice [17].

Lactadherin
MFGE-8 (Milk fat globule-8) is a 46 KDa sialylated glycoprotein associated to mucin. In the analysis of breastfed infants exposed to the rotavirus infection, it has been described a higher amount of lactadherin in those who remained asymptomatic vs. those affected by the infection [18].

Fibronectin
The fibronectin is a high molecular weight molecule. It is contained in the colostrum in a concentration of 13.4 mg/l [19]. Fibronectin cooperates in the capture of many different types of particles by phagocyte mononuclear cells in vitro. However, the in vivo effects of this opsonin are scarcely known.

Complement
The components of the classic and alternative pathways of the complement are present in BM. But their concentration, except from C3, is extremely low suggesting an absent or irrelevant biological functionality [20].

Lipids
Fatty acids and monoglycerides, generated by the digestion of more complex lipid substrates of BM inhibit the infectivity of encapsulated virus as Herpes simplex virus type 1, measles, vesicular stomatitis, Visna, Dengue virus, Cytomegalovirus, virus of the Forest of Semliki and Coronavirus [21] in vitro. They also deal with intestinal parasites as Giardia lamblia and Entamoeba histolytica [22].

Leukocytes
In contrast to B cells that arrive trough the enteromammary and bronchomammary pathways to the mammary gland and convert into plasmatic cells that remain in their epithelial cells,

Table 1: Function of the non-specific immune effectors of BM.

<table>
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<tr>
<th>Agents</th>
<th>Action</th>
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<td>Probiotic factors</td>
<td>Prevent the intestinal colonization by many pathogens</td>
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<td>Cooperate in the recovery of viral intestinal infections</td>
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<td>Decrease the risk for immune and inflammatory conditions</td>
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<td>Inhibitors of the metabolism</td>
<td>Lactoferrin prevents the proliferation of bacteria and fungi damaging</td>
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<td>of pathogens</td>
<td>their external membranes and promotes the epithelial growth</td>
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<td>K-casein blocks the binding of Helicobacter pylori to its receptors in</td>
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<td>mucosa</td>
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<td>α-lactalbumin protects against Escherichia coli, Klebsiella pneumoniae,</td>
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<td>and Candida albicans</td>
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<td>Haptocorrin inhibits the growth of bacteria in the mammary gland and</td>
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<td>promotes the transport of vitamin B12 from the circulation to human</td>
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<td>Folate binding protein prevents the use of folate by the intestinal</td>
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<td>pathogens</td>
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<td>Lyses the the N-acetylneuraminic acid of rotavirus</td>
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<td>Lactadherin</td>
<td>Associated to lower risk of rotavirus infection in vivo</td>
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<td>Fibronectin</td>
<td>Opsonine of phagocyte mononuclear cells</td>
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<td>Lipids</td>
<td>Inhibit the infectivity of encapsulated virus and intestinal parasites</td>
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<td>Anti-inflammatory and</td>
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<td>antioxidant agents</td>
<td>Antioxidant agents: α-tocopherol and β-carotene</td>
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<td>Enzymes that degrade intermediaries of inflammation: PAF-acetylhydrolase</td>
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<td>protects against the development of necrotizing enterocolitis</td>
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<td>Anti-inflammatory cytokines</td>
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<td>syncytial respiratory virus</td>
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<td>Glycans: oligosaccharides and</td>
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<td>glycoconjugates</td>
<td>receptors in intestinal and bronchial host cells</td>
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<td>Systemic effects: Participate in the interactions protein-carbohydrate</td>
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<td>(selectins, platelet-neutrophil, galectins, siglec5 and DC-sign)</td>
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leukocytes cross the mammary epithelium and mix with the lactic secretion. The highest concentrations of leukocytes in BM are in colostrum, 1-3x10^7/ml [23], being the percentage distribution of neutrophils, macrophages and lymphocytes approximately 80, 15 and 4%, respectively [24].

It is likely that leukocytes of BM provide a remarkable protection against the infection of the mammary gland at an early stage, which is particularly important at the beginning of breastfeeding when breasts are not completely emptied after each intake.

Neutrophils and macrophages of BM are phagocyte cells, since their stimulation allows the processing and presentation of antigens to T cells [25]. It has been observed that after their exposure to chemotactic agents, neutrophils of BM in contrast to those in blood do not increase their adherence, polarity, direct migration [26] or elasticity [27]. Macrophages of human colostrum, unlike those in blood, release high concentrations of secretory IgA during the phagocytosis [28], they also could synthesize lysozime, factors of complement, lactoferrin and of secretory IgA during the phagocytosis [28], they also could synthesize lysozime, factors of complement, lactoferrin and prostanilagin E₂ [29].

Lymphocytes: The proportion of T and B cells in the earliest lactic secretion is of 83% vs. 6%, respectively. CD4⁺ (helper) and CD8⁺ (cytotoxic and suppressor) T cells are present in human milk [30]. They contain the isoform CD45 (CD45 RO) associated to the immune memory. CD45 RO⁺ cells are the main source of cytokines in BM and characteristically produce IFN-alfa [31] and other cytokines. They have shown an in vitro response against different viral antigens. However, the role of T cells of BM in vivo is not completely known.

There is a higher presence of cytotoxic and suppressor phenotype (CD8⁺) in BM compared to blood as it is usually observed in mucous membranes. However, there is reduced number of B cells in BM as a consequence of their final conversion to plasmatic cells.

ANTI-INFLAMMATORY AND ANTIOXIDANT AGENTS

Promoting factors of epithelial and mucous membranes growth

Hormones and promoting factors of epithelial growth include epithelial growth factor, cortisol, lactoferrin and polyamines. They could affect not only growth but differentiation and replacement of epithelial cells, including the intestinal tract, thus limiting the penetration of free antigens and pathogen microorganisms [32].

Antioxidant agents

Components similar to the ascorbic acid [33], α-tocopherol and β-carotene are the main antioxidants of BM. It has been demonstrated that blood levels of these are higher in breastfed than in formula fed infants.

Enzymes that degrade intermediaries of inflammation

Certain enzymes of BM degrade inflammatory intermediaries that could damage the intestinal tract. Platelet activating factor (PAF) participates in the development of the necrotizing enterocolitis. The endogenous production of PAF-acetylhydrolase is delayed in the development; however human milk contains this enzyme [34] providing protection against the inflammatory process that culminates in this severe condition of the newborn.

Anti-inflammatory cytokines

Studies in animal models with genetic deficiencies suggest that anti-inflammatory cytokines of BM could play a role in the prevention of certain disturbances mediated by inflammatory processes. Homozygous for the null gen TGF-β1 mice show spontaneous multifocal infiltrations of macrophages and T cells in several organs: lungs, heart and saliva glands [35]. Moreover, there are experimental evidences that the effects of the deficiency of the tumoral growth factor β₁ (TGF-β1) are delayed with the ingestion of cytokines from murine milk [36].

IMMUNOMODULATORY FACTORS

Certain observations have provided the basis to raise the existence of immunomodulatory factors in human milk. First of all, epidemiological analysis suggest that breastfed infants are at lower risk to suffer type 1 diabetes [37], lymphoma [38] or Crohn disease [39] in later years. Additionally, the high levels of certain immune factors found in breastfed infants suggest that BM induces their production. It is the case of IFN-α in response to the infection by sincytial respiratory virus [40]. The third line of research has been the discovery that all leukocytes of human milk are activated.

In addition to TNFα other cytokines such as interleukin (IL)-1β, IL-6, INF-alfa, IL-8, β growth factor (TGF-β), granulocyte stimulating factor (G-CSF), macrophage stimulating factor (M-CSF) and IL-10 have been identified in BM. However, the extent of their effects in the infant is not completely known. Other agents of BM to which an immunomodulatory effect has been described include β-casomorphines, prolactine, antiidiopatic antibodies and nutrients such as the α-tocopherol, as well as a variety of nucleotides that increase NK (natural killer) cells, macrophages and the activity of T-helper cells [41].

GLYCANs: OLIGOSACCHARIDES AND GLYCOCONJUGATES

The glycans are present in BM as either free oligosaccharides or glycoconjugates, such as glycoproteins, glycopeptides, glycosaminoglycans or mucins. In the last years, oligosaccharides and glycoconjugates of BM have gained an increasing interest as agents of human milk with protective properties. By means of their ability to inhibit the binding of the pathogens to their specific receptors in intestinal and bronchial host cells they comprise an, up to this moment, underestimated mechanism that offers an efficacious protection to the lactating infant.

Structural diversity

Even though the lactose of human milk was isolated in 1633, it was not until 1933 when the existence of a carbohydrate fraction of milk different to lactose was described, and in 1950 when glycans of BM were first described [42]. Currently, there is believed to be hundreds and even thousands of oligosaccharides, since new molecules are continuously being recognized.
Most of the oligosaccharides of BM are structures that result from the addition of monosaccharides to the molecule of lactose by specific glycosyltransferases of the mammary gland [43]. The combination of D-glucose (Glc), D-galactose (Gal) and N-acetylglucosamine (GlcNac) by means of four types of terminal bindings of L-fucose (Fuc) and three types of syalic acid and N-acetylenuraminic acid (NeuAc) generates a wide variety of components. They mainly coincide in the content of lactose in its reductive terminal region and fucose or syalic acid in the non-reductive terminal region. Based on its chemical composition, oligosaccharides of BM could be classified in: (a) basic oligosaccharides that represent the fundamental structures for the synthesis of more complex oligosaccharides comprised by Glc, Gal and GlcNac; (b) fucosyl-oligosaccharides: that result from the addition of this basic structure to one or more molecules of Fuc; (c) sialyl-oligosaccharides: that result of the addition of the basic structure to one or more molecules of NeuAc and (d) sialyllfucosyl-oligosaccharides: that contain both Fuc and NeuAc [44]. Glycoconjugates could associate this basic structure to a protein (glycoproteins or glycopeptides), lipid (glycolipids) or carbohydrate (glycosaminglycans).

The addition of the residues of fucose depends of the action of fucosyltransferases in a genetically determined process: (a) the α 1,2-fucosyltransferase (FUT2) is present in 80% of the Caucasian individuals from N Europe and America, the so-called secretor individuals. BM from secreter individuals. BM from secretor women is characterized by the presence of 2-fucosylactose (Fuc α1,2-Gal β1,4-Glc), lacto-N-fucopentose I (Fuc α1,2-Gal β1,3-GlcNac β1,3-Gal β1,4-Glc) and more complex oligosaccharides that contain the residues Fuc α1,2-Gal β1,3-GlcNac; (b) the fucosyltransferase 3 (FUT3) is present in approximately 90% of the population, it binds Fuc residues by α 1,4 bindings to GlcNac residues from chains type 1 [45]. In BM from non-secretor women that own the FUT3 gene, the main fucosylated oligosaccharide is the lacto-N-fucopentose II (Gal β1,3-Fuc α1,4-GlcNac β1,3-Gal β1,4-Glc). In the case that both genes, FUT2 and FUT3 are present, the most common oligosaccharide would be lacto-N-difucohexose I (Fuc α1,2-Gal β1,3-Fuc α1,4-GlcNac β1,3-Gal β1,4-Glc). In the case of lack of both genes the main oligosaccharide would be the lacto-N-fucopentose III (Gal β1,4-Fuc α1,3-GlcNac β1,3-Gal β1,4-Glc). Apart from fucosyltransferases, several sialyltransferases could bind NeuAc in several positions to oligosaccharides of BM [46].

Concentration

There are many reports with respect to the total concentration of oligosaccharides of BM [47]. The data provided by different groups should be compared with precaution because there are different methods to separate the oligosaccharides of BM (ionic interchange chromatography, gel filtration or high performance liquid chromatography) which provide variable results. Additionally, the used method is not always specified and thus it is not possible to ascertain if all or only a part of lactose has been eliminated from the fraction of oligosaccharides and this widely modifies the results. Moreover, the protein residues could contaminate fractions with neutral or acid oligosaccharides.

Human milk contains 20 to 23 g/l oligosaccharides in colostrum and 12 to 14 g/l in mature milk. In such a way oligosaccharides represent the third component of BM after lactose and lipids, in contrast to cow’s milk in which only a small amount is identified [48]. The concentration of the different oligosaccharides of BM differs from one geographic group to another. Even though generally the main oligosaccharide is the lacto-N-trhealose (LNT, 0.5 to 1.5 g/l), that together with its monofucosylated derived products could involve 50 to 70% of the total of oligosaccharides of BM, in contrast to cow’s milk where sialyllactose prevails. Lacto-N-fucopentose I and II follow them. Sialyllactose is the most common of the sialylated components (NeuAc α 2,6 Lac and NeuAc α 2,3 Lac) of BM, approximately 1 g/l, followed by the isomers of the monosyalylated and disyalylated LNT.

Local effects of glycans

After the discovery of the oligosaccharides of BM it was stated that they could lack of functionality due to different reasons. It was thought that they were metabolic products result of an excess of function of the enzymes that participate in the synthesis of glycoconjugates. Moreover, the high amount found in fecal samples of breastfed infants led to the hypothesis that they were not digestible and might not have a nutritional role. Nonetheless, the fact that oligosaccharides of BM might play a biological role was plausible and thus several investigations were developed to ascertain the processes in which they could participate [49].

Oligosaccharides ingested by infants resist the acidity of the intestine, the degradation by the pancreatic enzymes and the action of the brush-border of the enterocytes. Intact arrive to the colon where they act as nutrients of the bacterial flora exerting a prebiotic effect [50].

Additionally, the oligosaccharides of BM and some glycoconjugates are synthesized by the same type of glycosyltransferases responsible for the synthesis of the glycans of the cell surfaces: the histo-blood group antigens (HBGAs). HBGAs are used by most of the intestinal and respiratory pathogens for the identification and binding to target cells as a critical step for their infectivity. Due to the structural analogy between both molecules, it was stated that oligosaccharides of BM could competitively act with cell receptors preventing the binding of certain pathogens and thus avoiding the development of infection. This would imply, in the case of intestinal pathogens, the inhibition of their ability to bind to enterocytes and the protection of the lactating infant from certain intestinal infections and, in the case of respiratory pathogens, the blockade of their binding to bronchial cells and thus the lung infection.

Considerable research work has targeted the hypothesis that oligosaccharides and glycoconjugates of BM could act as decoy receptors that compete with the binding of certain bacterial and viral pathogens, and even certain toxins, to their specific receptors in the intestinal tract and the bronchial tree. In 1983 Parkikinen et al. demonstrated that the sialylated oligosaccharides of BM were able to inhibit the activity of the binding of the strains of *Escherichia coli* that caused neonatal meningitis and sepsis [50]. This is related with the specificity of binding of the *S* fimbria of certain strains of *Escherichia coli* and the sialylated galactosides. It could be speculated that human milk with high content of these epitopes could be responsible of a lower rate of infections by *Escherichia coli* in breastfed vs. formula fed infants. GM1 Gangliosides and monosyalilated glycosphingolipids (GlcNAC

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β(1,3), could also act as analogue receptors of thermolabile enterotoxins produced by *Vibrio cholerae* and *Campylobacter jejuni* [51]. Approximately, 70% of the cases of otitis media in neonates are caused by infections by *Streptococcus pneumoniae* and *Haemophilus influenzae*. In 1986, Andersson et al. demonstrated that the adhesion of these microorganisms to specific carbohydrate structures of the pharyngeal or oral epithelial cells were inhibited by BM, the subunits Gal-disaccharide seem to be responsible for this protective effect [52].

It is possible that certain oligosaccharides and glycoconjugates of BM could also display the property to interfere with the binding of certain virus to epithelial targets. A good example of this would be *Influenza A, B* and *C* virus that recognize NeuAc α 2,6-Lac and NeuAc α 2,3-Lac, both principal components of human milk. With regard to intestinal viruses, BM has demonstrated a protective property against the infections by rotavirus [53], the first cause of gastroenteritis in children. Furthermore, it has been recently described that rotavirus recognize histo-blood group antigens and the oligosaccharides of BM that interfere between the viral particles and the receptor [54] have been identified. Norovirus are a leading cause of viral intestinal infections in children. Effective vaccines against noroviruses are not yet available, enhancing the interest of the protection mechanisms elicited by BM. Indeed one of the most plausible settings where oligosaccharides could exert a protective action would be preventing from the intestinal infection by norovirus in lactating infants [55].

**Protection of the oligosaccharides of BM against norovirus infections**

There is increasing evidence regarding the protective activity of the oligosaccharides and glycoconjugates of human milk against the intestinal infections by norovirus in breastfed infants.

This hypothesis starts from the observation that a group of individuals show a natural resistance against the infections by these viruses: the non-secretor individuals. They lack of the *FUT2* gene that encodes for the α 1,2-fucosyltransferase. This led to the hypothesis that the epitope α 1,2-Fuc (antigen H type 1) is essential for the binding of norovirus to enterocytes [56]. Accordingly the antigen H type 1 is recognized as the main receptor of Norwalk virus (the prototype of norovirus) in the enterocytes. However, there are other receptors that bind specifically to different strains of norovirus making the interaction of norovirus with the host of a high level of complexity.

The different genetic endowment of the individuals establishes the type of glycans that they express in the surface of their enterocytes and are present in their biological fluids, such as BM and saliva. It is the case of non-secretor individuals that lack of the gene *FUT2* that encodes for α 1,2-Fuc epitopes. As virus Norwalk requires this receptor for their internalization, these individuals would not be susceptible to this virus. Moreover, the specificities of binding between other strains of norovirus and histo-blood group antigens have been described. In consequence, certain genes might determine the profile of susceptibility or resistance of each individual to the different strains of norovirus. Additionally, in the case that the genetic endowment and thus the profile of HBGAs on the enterocytes make an infant susceptible to a specific strain of norovirus, oligosaccharides of BM would offer a chance to avoid the intestinal infection. Due to the resemblance between the oligosaccharides of BM and the receptors in enterocytes, oligosaccharides would act as decoy receptors preventing the binding of norovirus to their receptors on intestinal cells and thus avoiding the infection. In other words oligosaccharides of BM could be effectors of the non-specific immunity of BM as the mother could confer a protection to her child by breastfeeding.

A recent study assessed mature milk and serum samples from 108 mothers for specific IgA to norovirus GI.4-2006 b and for their blocking activity on the binding of norovirus virus-like particles to fucosyltransferase positive and negative saliva. It was demonstrated that BM inhibits norovirus GI.4-2006b virus-like particles binding to receptors in saliva, and specific IgA antibodies to norovirus are only partly responsible for this activity. The FUT2 status of the receptor seems to be a strong predictor of this effect. But more studies are required to ascertain the participation of HBGAs in the protection against norovirus infections elicited by BM [57].

There are three prerequisites that oligosaccharides must meet to allow this to have an effect *in vivo*: (a) a constitutive expression, they would be synthesized in BM regardless the history of exposure of the mother to certain pathogens; (b) resistance to the intestinal digestion that would allow them to reach the site of infection; (c) correlation between the presence of these glycans in BM and a lower risk of disease by certain pathogens in breastfed infants.

**Constitutive expression:** Secretor and non-secretor individuals are defined by the presence or absence of the *FUT2* gene that encodes for the activity of the α 1,2-fucosyltransferase and, in consequence, the presence of glycans with α 1,2 bindings in their BM and specific HBGAs receptors in their cells. This genetic variation has been classified in terms of homozygous secretor individuals (that own high levels of glycans with α 1,2 bindings), heterozygous secretor individuals (with medium levels of glycans with α 1,2 bindings) and non-secretor individuals (that do not contain glycans with α 1,2 bindings) [58]. However, it has been observed that the profile of oligosaccharides of BM changes during breastfeeding regardless of the genetic endowment. It is the case of the non-secretor individuals that after approximately six months of breastfeeding are able to secrete BM containing oligosaccharides with α 1,2 bindings. Moreover, secretor individuals produce milk containing α 1,2-fucosylligoligosaccharides during their lactation, but the absolute and relative amount of these with respect to other type of oligosaccharides (α 1,3/4-fucosyloligosaccharides) changes along the different stages of the breastfeeding. The relation between α 1,2 and α 1,3/4-fucosyloligosaccharides declines exponentially, from factor of five during the first month to an approximate relation 1:1 at the end of the first year [59]. This suggests a bigger complexity than currently known in the production of oligosaccharides of BM and a likely intervention of other agents different to α 1, 2, 3 or 4 fucosyltransferases in their synthesis, such as *FUT1*, 5, 6, 7 and probably 9.

**Resistance to digestive enzymes:** It is necessary that glycans of BM remain unaltered by their passage through the intestinal tract to the site of infection in order to allow a biologically relevant
function. There are some evidences that they remain intact and are able to act in the intestinal and respiratory tract. First, several oligosaccharides remain unaltered because their bindings are completely different than those found in the nutrients that we assimilate, hindering the digestion by the intestinal enzymes. Additionally, the protein components of these structures could develop their function in regions of the intestinal and respiratory tract where the proteolysis enzymes are not released. The time of transit of the digested BM through the intestinal tract is so fast, within a few hours, minimizing the chance for their destruction. Finally, the buffer capacity of BM would help to protect the components that are labile to acids such as the antiproteases.

Clinical efficacy of the protection of glycans: The third requirement to state that oligosaccharides could act as effectors of the non-specific immunity of the BM is the existence of a clinical correlation between the ingestion of certain oligosaccharides and the risk of intestinal infections. Provided that specific oligosaccharides and glycoconjugates have demonstrated their ability to inhibit certain pathogens in their evaluation in vitro, the demonstration of their clinical relevance is fundamental because it is possible that they might not be effective in the complex matrix of the intestinal content and even their effect quantitatively insignificant in the context of other protective components of BM.

The variation of the expression of the glycans of BM provides the chance to evaluate the effectiveness of these inhibitors of intestinal pathogens in the population [60, 61]. A prospective study followed 93 mother and child couples from their birth up to two year-old. The data of the infants’ diet and the appearance of diarrhea weekly were registered. Additionally, a sample of BM was collected in the first five weeks postpartum and the content of oligosaccharides was analyzed. Results showed that children that developed a diarrhea associated to the stable toxin of Escherichia coli were ingesting milk with a ratio of oligosaccharides with bindings α 1,2 and α 1,3/4 lower than those who were not affected. Moreover, the diarrhea by Campylobacter sp. occurred in a lower rate in those children whose mothers secreted milk containing a higher content of 2'-FL (a fucosyloligosaccharide with bindings α 1,2). Furthermore, the diarrhea by norovirus affected in a lesser extent those whose mothers produced milk with higher levels of lactose N-difucohexose I (LDFH-I), an α 1,2-fucosyloigosaccharide [62].

Systemic effect of the glycans of BM

The fact that part of the oligosaccharides of BM is absorbed unaltered in the small intestine of the infant and appears in their urine [63] is a relevant observation that provides the basis for two hypotheses. First, oligosaccharides of BM could exert an action as analogue receptors for the urinary pathogens. Secondly, the presence of oligosaccharides in the urine could prove in an indirect way their presence in the systemic circulation. It is estimated a concentration of serum oligosaccharides of 100 to 200 mg/L based on the concentration of oligosaccharides in human milk, the average daily intake by the infant, his blood volume and the excreted amount in the urine.

If we assume that oligosaccharides of BM reach the systemic circulation we could state that they could participate in the interactions protein-carbohydrate in a systemic level. For instance, selectins participate in the reaction cell-cell of the immune system [64]. The P and E-selectin mediate the leukocyte deceleration of the activated endothelial cells and initiate the leukocyte extravasations to the site of inflammation. Moreover the P-selectin also participates in the production of the complexes platelet-neutrophil, a subpopulation of highly activated neutrophils responsible of the adhesion, phagocytosis and increased production of reactive species of oxygen [65]. Selectins bind to fucosylated and sialylated oligosaccharides [66], in such a way they are able to reduce the adhesion of the human leukocytes to activated endothelial cells, in contrast to neither fucosylated nor sialilated oligosaccharides that lack of these effects [67]. Moreover, the oligosaccharides inhibit the formation of the complexes platelet-neutrophil and reduce the subsequent neutrophilic activation [68].

Moreover, such findings raise the question if the immunomodulatory effects of the oligosaccharides of BM could imply a compromise for the immune system of the child or even a protection against exacerbated immune responses such as the necrotizing enterocolitis. It is well known that the incidence of necrotizing enterocolitis is up to an 85% lower in lactating infants compared to formula fed infants [69]. Although the pathogenic mechanisms of the necrotizing enterocolitis have not been completely understood, it seems that the own invasive leukocytes and the excessive production of reactive species of oxygen are responsible of the propagation of the disease after the trigger by an initial noxa [70]. The ability of oligosaccharides to inhibit the adhesion of leukocytes in the sites of inflammation and reduce the formation of complexes of highly activated platelet-neutrophil could explain the protection of breastfed infants against the necrotizing enterocolitis and other inflammatory conditions [71].

Due to their structural resemblance oligosaccharides of BM could interfere with other interactions of the type of protein-carbohydrate. Galectins, lectins that bind galactosides, join β-galactosides and glycans enriched with poli-N-acetyllactosamines and in this way regulate the cell growth, proliferation and apoptosis, as well as the complex matrix of interactions cell-cell [72]. The siglecs, Ig-like lectins that bind the terminal region of the syalic acid in the binding points α 2,3 or α 2,6 [73] and DC-SIGN, dendritic cell specific for the intercellular adhesion of the non-integinn collector of the molecule 3, could be another potential target of the oligosaccharides. They express in the dendrite cells of the intestine and other tissues and participate in the capture of different pathogens, including HIV, hepatitis C, Ebola, CMV, Dengue virus, Mycobacterium sp. and Candida albicans [74].

CONCLUSIONS

BM is the gold standard for infant’s nutrition during the first semester of life. However, it is recommended to maintain its intake beyond this age as additional benefits for the lactating child have been demonstrated. These are not only of nutritional, but anti-infectious, anti-inflammatory, antioxidant and immunomodulatory character. Breastfed infants have demonstrated a lower susceptibility to intestinal and respiratory infections. Moreover, benefits of BM have been observed even after the lactation has finished. Indeed, it has been associated
a lower risk to develop autoimmune and allergic diseases to breastfeeding in infancy.

There are numerous effectors of these properties. Specific secretory IgA antibodies to certain pathogens have been widely described, but non-specific immune agents and the properties they confer are less known. These are probiotic factors, inhibitors of the metabolism of pathogens, enzymes, mucin, lactadherin, fibronectin, complement factors, lipids, leukocytes, anti-inflammatory agents, immunomodulatory factors and oligosaccharides of human milk.

There is increasing interest with regard to the role of oligosaccharides of BM in the protection against certain infections, especially in the case of intestinal infections such as rotavirus and norovirus. They have demonstrated to act as decoy receptors that could interfere with viral particles preventing from their binding to receptors in enterocytes. More studies are required to define precisely this mechanism and to describe the systemic effects of oligosaccharides.

BM constitutes an unmatched supply of nutritional and essential protective substances for the infant. The effectors of the immune properties of BM are multiple, thus human milk is a unique biological fluid and cannot be replaced by artificial formulas.

REFERENCES


