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Neonatal tolerance under breastfeeding influence

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Diseases due to defect in tolerance induction such as allergy, celiac disease, or Type 1 Diabetes develop mostly in childhood indicating the necessity of early intervention for primary prevention. Epidemiological studies report that breastfeeding could protect from these diseases. However, data are controversial and the mechanisms unclear. Experimental data suggest that breastfeeding-induced protection might rely on tolerance induction as long as some criteria are fulfilled. Thus, the tolerogenic potential of breast milk would depend on maternal exposure to common environmental and dietary antigens and the efficiency of antigen transfer across mammary epithelium. Induction of tolerance upon breast milk-mediated antigen transfer will also depend on the presence of immunomodulatory factors in breast milk and of its impact on neonatal gut and immune system maturation. The better understanding of maternal influence on tolerance induction through breastfeeding should allow the development of new strategies to prevent immune-mediated diseases.

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Introduction

For years, breast milk was considered mainly as a source of nutrients for the developing child. The extensive observations that breastfeeding affords protection toward infectious diseases and could reduce by more than the half the mortality rate because of common infections have added another key role to breastfeeding [1]. This protection relies in great part on the passive transfer through breast milk of high amounts of microbe-specific immunoglobulins that compensate for the deficiency of immunoglobulins synthesis during the first year of life [2]. In addition, epidemiological studies suggest, with controversies, that breastfeeding can protect from the development of immune-mediated diseases such as Type 1 Diabetes (T1D) [3,4], celiac disease [5], allergic disease [6•], and graft rejection [7,8]. Many hypotheses have

been formulated to understand the protection afforded by breastfeeding and to explain why results obtained can be so divergent. Since the pathogenesis of these immune-mediated diseases involves a defect in tolerance induction, one of the hypotheses that might be worth to look at is the induction of antigen-specific tolerance by breastfeeding. Breastfeeding might affect tolerance induction in infants because of both the possible transfer of antigens through breast milk and the presence of factors in breast milk that affect immune system maturation and responses.

Presence in breast milk of antigens involved in the pathogenesis of immune-mediated disease

Analysis of human milk has described the presence of intact human insulin [9], gliadin [10], food allergen such as peanut, ovalbumin or bovine beta-lactoglobulin [11] as well as soluble maternal semi-allogeneic HLA molecules and maternal cells [12]. As a matter of fact, these proteins are antigens involved in the pathogenesis of T1D, celiac disease, food allergy, and graft rejection. In addition, in an animal model, we described that respiratory allergens could be found in breast milk and thus could contribute to asthma prevention by breastfeeding [13•,14•]. This observation is supported by studies in rodent demonstrating that the great majority of the inhaled proteins are found in the digestive tract and thus could then follow the same path as dietary antigens to breast milk [15].

The amounts of dietary and self antigens in milk vary a lot from one mother to the other but are in the range of ng/ml [9,11]. Bottle-fed children will only be exposed to cow's milk antigens such as bovine beta-lactoglobulin and alpha-lactalbumin antigens in amounts that are about 10 000 times higher. In addition, before reaching the mammary gland, maternal dietary antigens will be handled by maternal digestive tract that can affect the generation of tolerogenic forms of the antigen [16,17]. This might be important for neonates as antigen handling in infant gut might be deficient due to their higher gastric pH and their lower secretion of pancreatic enzymes [18]. The factors that determine the levels of antigens found in breast milk are poorly defined. Maternal antigen exposure certainly affects antigen presence in breast milk [19]. Other studies have shown that increased mammary gland epithelium permeability, assessed by the ratio of sodium to potassium in breast milk, correlated with increased risk of atopy in breast-fed children from atopic mothers [20] but no study correlated mammary gland epithelium permeability to the levels of environmental antigens in breast milk.

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Evidence from animal studies that antigen transfer through breast milk can induce immune tolerance

Although studies demonstrated that tolerance induction by direct oral antigen administration in neonatal rodent was hard to achieve [21,22], others demonstrated that indirect administration through breast milk achieved tolerance induction at very low doses of antigen. Breast milk-mediated transfer of an antigen could prevent antigen-specific immune responses [13^{••},14[•],22–25] and allergic disease development in rodents [13^{••},14[•]]. In addition, physiological transfer of semi-allogeneic maternal HLA molecules during pregnancy and lactation favored the acceptance of semi-allogeneic heart, skin, or bone marrow transplant [26,27[•]]. In this latter case, the sole transfer of HLA antigen through breast milk was sufficient to prevent allogeneic reactions [27[•]].

Presence of tolerogenic factors in breast milk TGF-beta

The low endogenous levels of TGF-beta in the neonatal intestine and the high amounts of TGF-beta found in breast milk illustrate nicely how maternal milk can compensate for neonates physiological deficiency [28]. The role of maternal milk TGF-beta in neonatal gut mucosa homeostasis was demonstrated by experiments performed by Penttila who showed that rat fed milk formula devoid of TGF-beta displayed intestinal inflammation [29] and developed an allergic response to bovine beta-lactoglobulin when on a susceptible background [30]. In addition, we found that the induction of tolerance upon antigen transfer through breast milk required the presence of TGF-beta in maternal milk [13^{••}]. However, we also found that TGF-beta was not required when the antigen was associated to specific IgG in milk. As discussed below, we believe that the intrinsic tolerogenic properties of milk borne IgG-antigen immune complexes render milk TGF-beta dispensable for tolerance induction.

Human studies support the role of milk TGF-beta in the protection from allergic diseases. Thus, TGF-beta content in milk was shown to correlate inversely with the risk of allergy development in breast-fed children [31,32]. The physiological factors that condition TGF-beta levels in milk are presently unknown. We found that TGF-beta levels in mouse milk were extremely constant whatever their genetic background, immune competence, or allergic status (Verhasselt, unpublished data). In human, TGF-beta in milk is much variable from one mother to the other [31,32]; interestingly, recent data demonstrated that milk TGF-beta levels could be modified maternal supplementation with probiotics [33,34].

Other cytokines

In addition to TGF-beta, other anti-inflammatory cytokines such as IL-10 and pro-inflammatory cytokines such

as TNF- α , IL-6, IL-1, IFN- γ , IL-4, IL-5, and IL-13 were found in human breast milk in variable amounts from one mother to the other [35]. Thus, the balance of these cytokines in breast milk will probably dictate the tolerogenic potential of breast milk. Interestingly, the presence of IL-7 was recently described in breast milk and its levels correlated with improved child thymus function [36].

Other factors

The ratio of n-6 to n-3 fatty acids in maternal diet during lactation was shown to influence the induction of oral tolerance in rats [25]. Osteopontin was also found recently in high amounts in maternal milk as compared to bovine and milk formula [37]. This molecule is involved not only in numerous biological processes such as bone remodeling but also immune regulation [38] and its role in breast milk induced tolerance has not been assessed yet. Another factor present in breast milk with pleiotropic actions including immune regulation is Vitamin A [39]. Its levels depend on maternal diet and its impact on oral tolerance induction in neonates remains to be assessed.

Impact of breastfeeding on factors involved in neonate immune system maturation

Gut microbiota

Gut microbiota is necessary for the development of oral tolerance as illustrated by defect in regulatory T cells induction and oral tolerance process in germ free mice [40–43]. In addition, there is evidence that some microbiota might be associated with more efficient tolerance than others [44^{••},45,46]. Recent studies have paid much attention to the use of lactobacilli and bifidobacteria as live microorganisms with possible benefit for health and in particular for allergy prevention [46].

The infant intestinal tract progresses from sterility at birth to extremely dense colonization at the end of the first year of life. By one year of age, the infants retain their uniqueness but have converged toward a profile characteristic of the adult gastro-intestinal tract. It is often stated that breastfeeding has a profound influence on gut microbiota and in particular is associated with the presence of bifidobacteria. A recent review of studies after 1980 brings nuance to this statement (see [47] and Table 1). Thus, in the studies realized after 1980, bifidobacteria seem to be found equally often in breast-fed and formula-fed infants and are the dominant bacteria found in children microbiota. This probably reflects modifications in the composition of formula in the past 30 years. Lactobacillus are also similarly represented with the exception of *L. rhamnosus*, which may be favored by breastfeeding. The main difference between breast-fed and bottle-fed children would be a more diverse microbiota and in particular, the presence of Bacteroides, Enterobacteriaceae, and Clostridia in bottle-fed children while Staphylococcus

Table 1**Gut microbiota of breast-fed as compared with formula-fed infants**

Bacterial group	Number of studies		
	Increased	No difference	Decreased
Bifidobacteria	7/27	20/27	–
Bacteroides	2/19	9/19	8/19
Clostridia	–	4/17	13/17
Lactobacilli	2/16	10/16	4/16
Enterobacteria	1/21	9/21	11/21
Enterococci	–	5/16	11/16
Staphylococci	5/12	7/12	–

This table summarizes the results of studies comparing the gut microbiota of breast-fed and formula-fed infants. Adapted from [47].

tend to be more common in breast-fed infants [47]. Recent analysis of microbiota using DNA methodology could also identify the presence of an additional non-culturable bacteria group, *Ruminococcus*, in early infant gut microbiota of breast-fed children [48]. As a matter of fact, a non-culturable clostridia-related species could recapitulate in mice the coordinated maturation of T cell response induced by the whole mouse microbiota highlighting the probably up to date underestimated role of non-culturable bacteria in the maturation of the immune system [49]. New DNA-based methodology might thus reveal differences in microbiota in breast-fed children versus formula-fed children that were not accessible by classical culture methods and that might be important for immune system maturation and susceptibility to oral tolerance induction.

The effects of breastfeeding on the gut microbiota composition are attributed to the presence of breast milk components that affect bacterial growth and that are much less abundant or absent in formula milk. Oligosaccharides are unique to human milk and favor the growth of bifidobacteria. Anti-microbial human milk factors such as lactoferrin, lysozyme, and IgA will also prevent the growth of pathogenic microorganisms and allow commensal bacteria expansion. In addition, soluble receptors for pathogen such as CD14 and TLR2 were identified in maternal milk but not in formula milk and might affect bacterial colonization [44,50,51]. Finally, recent studies described the presence of bacteria in aseptically collected human breast milk [52,53] and animal studies suggested that these bacteria resulted from translocation from the mother's flora to the mammary gland in the perinatal period [53]. The relative importance of this path in infant gut colonization is presently unknown.

Diet

Antigenic constituents of food clearly exert a stimulatory effect on neonatal immune system as mice fed hydrolyzed milk proteins or parenterally fed mice or babies have reduced numbers of B and T cells [54]. The impact

of food antigens on immune system development was studied in detail by de Faria and co-workers [55]. These authors have observed that mice receiving a balanced amino acid-based protein free diet had a poorly developed gut-associated lymphoid tissue as well as signs of decreased systemic immunity. Furthermore, this group demonstrated that nasal tolerance was impaired in those mice [56]. Thus, diet might be an important driving force for immune system maturation upon introduction of solid food but also before weaning. Indeed, as discussed above, breast-fed children will receive minute amounts of numerous maternal dietary antigens, self and allogeneic antigens through breast milk while children fed with classical formula will receive huge amounts of only cow's milk antigens. Finally, in addition to its direct antigenic stimulatory effect, the diet can also influence the immune system by providing factors that influence microbiota development.

Impact of breastfeeding on factors involved in antigen transfer across the gut barrier

Gut permeability

Studies suggest that abnormal gut permeability might be involved in the pathogenesis of disease associated with defect in tolerance induction such as T1D, celiac disease, or food allergy [57–59]. The relatively high permeability of the intestine epithelium for macromolecules declines after birth, a process that is often called gut closure. The time of gut closure is species dependent and takes places in rodent at the time of weaning while already within the first postnatal days in humans. Colostrum and to a less extent maternal milk contain many biologically active substances that stimulate intestinal maturation [60]. Among these, Epidermal Growth Factor (EGF) is probably one of great importance [61]. EGF is present in high amount in colostrum and its level declines gradually during the first months of lactation. Interestingly, EGF in milk of pre-term neonates is much higher than those in milk of mothers with full-term infants and is absent from formula milk. Although the possible role of milk borne EGF in the prevention of necrotizing enterocolitis in premature babies is well documented [61], its role in the prevention of immune-mediated disease due to increased intestinal permeability needs to be studied. Other growth factors identified in breast milk are Erythropoietin, Insulin like Growth factor, hepatocyte growth factor, and basic fibroblast growth factors. These factors probably act in concert to induce intestinal growth by acting on different tissues (mucosa, submucosa, and muscle) of the intestine [60]. In addition to promoting intestinal growth and maturation, some milk factors probably also play an important role in maintaining gut integrity. In addition to EGF, breast milk TGF- β is a key factor in this function [62]. Another milk factor that might require attention is milk borne soluble TLR2 [51]. Indeed, this factor was recently identified as an important factor involved in intestinal barrier integrity [63].

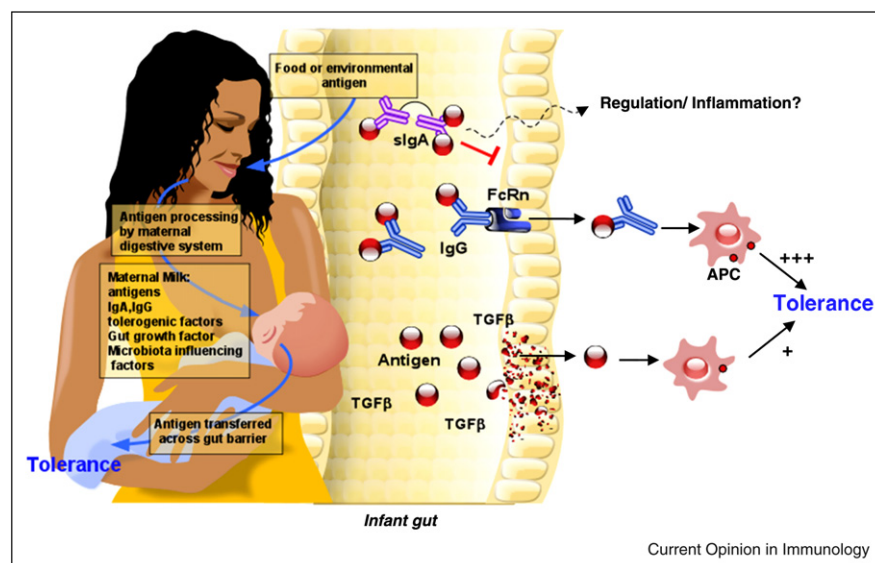
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Antigen association with maternal immunoglobulins

sIgA is the predominant Ig class found in human maternal milk and maternal milk IgA will compensate for the IgA deficiency of the infant. Their role in the prevention of respiratory and enteric infectious diseases in breast-fed children is well documented [2]. sIgA could also play a role in the regulation of the immune response to dietary antigen. Data in the adult describe that IgA can trap food antigens and thereby are responsible for immune exclusion of dietary antigens and their enhanced degradation by pancreatic enzymes [64]. IgA-antigen immune complexes could also play an immunoregulatory role. Indeed, very old data in rodent demonstrated that IgA-antigen immune complexes were responsible for the process of oral tolerance [65]. More recently, data in adult rodent indicated the possible inverse transport of IgA immune complexes into the peyer patches followed by the induction of regulatory immune responses in the MLN that would contribute to the maintenance of local homeostasis of the gastro-intestinal tract [66]. As a matter of fact, maternal dietary antigen such as ovalbumin, beta-lactoglobulin, and gliadin have been found in breast milk bound to IgA [10,67,68]. Moreover, epidemiological studies have reported an inverse relation between the levels of maternal milk IgA and the development of allergy [69,70]. However, recent data in mice and human, suggest that IgA-mediated transport of a dietary antigen from the

lumen to the mucosa can be associated with harmful reactions as it was recently described for gliadin in celiac patient [71^{••}]. Moreover, oral tolerance could be induced in adult mice deficient in IgA secretion [72] and, in a mouse model of neonatal oral tolerance induction through antigen transfer through breastfeeding, we found that IgA was not necessary for protection induction [13^{••},14[•]]. Thus, more data in humans will be necessary to firmly demonstrate if milk IgA can contribute to tolerance induction in infant.

Antigen in breast milk may also be found associated to maternal IgG. In a murine model, we found that OVA-sensitized mothers who are exposed to OVA during lactation present high levels of IgG-OVA immune complexes in breast milk [14[•]]. In humans, dietary or environmental antigen-IgG immune complexes might also be found in breast milk. Indeed, such immune complexes were found in sera of both healthy and atopic individuals [73,74] and a FcR in the mammary gland epithelium allows the transfer of IgG and IgG immune complexes from sera to the milk [75,76]. In our animal model, we found that milk IgG-OVA immune complexes induced a profound and long-lasting antigen-specific tolerance in the breast-fed mice. Interestingly, protection lasted longer and was of greater amplitude when antigen was bound to IgG in breast milk as compared to tolerance

Figure 1

Possible maternal influence on neonatal tolerance induction through breastfeeding. Before reaching milk, ingested airborne and dietary antigens are handled by maternal digestive system that could contribute to the generation of tolerogenic peptides. According to maternal antigen exposure and mammary gland permeability, various amounts of antigen will be found in breast milk. Maternal sensitization to the ingested allergen will dictate whether the transferred antigen will be found in milk free or complexed to antigen-specific IgA and IgG. The presence of IgA will trap antigen and prevent their transfer to the child while antigen bound to IgG will be very efficiently transferred across the gut barrier using the FcRn. Free antigen can also be transferred passively across gut barrier with a much less efficiency. Then antigen will be probably picked up by infant antigen presenting cell (APC) that will induce the generation of regulatory T cells. Maternal milk TGF-beta is a necessary cofactor for tolerance induction when antigen is not bound to maternal IgG. Maternal milk will also affect indirectly the capacity of the breast-fed child to mount tolerance because of its effect on gut microbiota and permeability.

Table 2**Possible factors involved in tolerance induction through breast milk**

Factors involved in oral tolerance induction	Breast milk factors possibly affecting tolerance induction in infants
Antigen exposure	Antigen present in breast milk: dietary antigens [11], self antigens [9], maternal allogeneic antigens [12], and respiratory antigens (at least in murine models) [13^{**},14[*]] <i>The presence of these antigens can induce antigen-specific tolerance but also drive neonatal immune system maturation</i> Maternal digestion of dietary antigens <i>The handling of antigens by maternal gut will affect the generation of tolerogenic forms of the antigens [16,17]</i> Maternal specific IgG and IgA <i>Milk antigen binding to IgG and/or IgA will affect antigen transfer across the gut barrier and their presentation to infant antigen presenting cells [13^{**},14[*],64]</i>
Tolerogenic molecules	Tolerogenic molecules: TGF-β [13^{**},30], IL-10 [35], Vitamin A, n-3 fatty acids [25], osteopontin [37], and antigen binding to maternal IgG [14[*]] Inflammatory molecules [35]: TNF-α, IL-6, IL-1, IFN-γ, IL-4, IL-5, and IL-13 <i>The balance between the tolerogenic and inflammatory mediators in milk will influence breast-fed child immune response to the transferred antigen</i>
Gut microbiota	Maternal IgA [2], lysosome, lactoferrin, oligosaccharide, nucleotides [1], soluble CD14 [50], soluble TLR2 [51], and bacteria in maternal milk [52,53] <i>The exposure of the infant to environmental bacteria and to maternal milk bacterial growth factor and anti-microbial molecules will shape infant microbiota and thereby immune system maturation</i>
Gut permeability	Growth factors [60]: EGF, Erythropoietin, Insulin like Growth factor, hepatocyte growth factor, and basic fibroblast growth Healing factors: EGF [61], TGF-β [62], and soluble TLR2 [63] <i>Milk gut growth factors and healing factors will stimulate infant gut maturation and barrier integrity which will affect antigen transfer across the gut barrier</i>

induced when antigen was found free in breast milk [14^{*}]. We further demonstrated that efficient tolerance induction by breast milk IgG–antigen immune complexes relies at least on a more efficient transfer of the antigen across the gut barrier because of a FcRn-mediated transport of antigen bound to IgG [14^{*},77]. The induction of immune tolerance upon transfer of antigen bound to IgG might be surprising according to the well-known inflammatory immune responses induced by IgG immune complexes in diseases such as erythematous lupus or post-streptococcus glomerulonephritis. In addition, Blumberg and co-workers showed in adult mice that FcRn-mediated transfer of IgG immune complexes resulted in the induction of effector immune responses [78,79]. This apparent discrepancy might rely on the fact that, in our model, we are dealing with neonates. In addition, our *in vitro* data suggest that milk borne immune complexes have intrinsic tolerogenic properties, as they were able to induce the differentiation of antigen-specific FoxP3⁺ T_{reg}. The high ratio of IgG/antigen of milk borne immune complexes [14^{*}] as well as milk antibodies glycosylation might be responsible for these tolerogenic properties [80–82].

Conclusions

Maternal milk is a complex and dynamic body fluid that provides nutrients, passive immunity, gut growth factors, and bioactive compounds that can actively shape newborn immune system development. Breast milk-mediated transfer of innocuous environmental and self

antigens can help in the education of the infant immune system toward tolerance induction. Each breast milk tolerogenic potential will be different from one mother to the other and will depend on maternal antigen exposure, her immune responses to the transferred antigen, mammary gland permeability, milk levels of gut growth factors, microbiota influencing factors, and tolerogenic molecules (Figure 1 and Table 2). A better understanding of how the levels of these compounds in breast milk are controlled and the identification of the key promoters of tolerance induction in neonates should help in the establishment of new strategies to prevent immune-mediated diseases.

Conflict of interest

The author declares no conflict of interest

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