Novel Insights into Human Lactation as a Driver of Infant Formula Development

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Abstract

Progress in research on human lactation and breast milk has advanced our knowledge about the significance of breast milk for the recipient infant and the effects of various components on long-term outcomes. Recent findings have expanded our knowledge in this area. Several growth factors and cytokines are present in breast milk and their capacity to persist in the infant gut and exert their activities is likely to affect maturation of immune function, possibly affecting the development of oral tolerance. A proper balance of polyunsaturated fatty acids (n-3/n-6 ratio) may also be of significance for allergy prevention in children, emphasizing the need for the mother to achieve a balance of these fatty acids in her diet. The recent findings that specific strains of bacteria are present in breast milk and act as probiotics in the early colonization of the infant gut and that human milk oligosaccharides are specific substrates for these probiotic strains may not only affect the defense against pathogens, but also affect energy utilization and development of obesity. Previously neglected milk fat globule membranes contain several components involved in protection against infection and may be an additional arm in the multifaceted shield that breastfed infants have developed against bacterial and viral antagonists. All these findings have implications for development of improved infant formulae.

Introduction

Lactation is a complex and very dynamic physiological process. Initially, very small volumes of milk (colostrum) are being produced, which then rapidly increase up to some 600–1,000 ml per day, with large individual differences. Towards the end of lactation, involution starts to occur and volumes decrease. During these periods, concentrations of many individual milk components change considerably, whereas others change only modestly or not at all. While part of this is due to the changing metabolic activity of the
mammary gland, it is highly likely that the alterations in milk volume and the changes in composition also meet the infants’ changing requirements and maturing metabolism. Our knowledge about lactation as a process and the components of breast milk and their bioactivities has rapidly increased. The ability of breast milk to provide both passive protection and to affect development of the infant’s mucosal and systemic immune responses is coupled to its contents of antimicrobial, anti-inflammatory and immunomodulatory activities. This knowledge now can be utilized as a guideline for improving the composition of infant formulae and their use, thereby hopefully improving nutrition, health and long-term outcomes of formula-fed infants.

**Growth Factors and Cytokines**

Human milk has been reported to contain several growth factors (epidermal growth factor, transforming growth factor-β – TGF-β, erythropoietin, insulin-like growth factors, etc.) and cytokines/adipokines (interleukin 1β – IL-1β, IL-2, IL-6, IL-7, IL-8, IL-10, etc.) [1–3]. In some cases, changes in concentrations during lactation have been described, but often only mean values for mature milk have been given. Recently, developmental patterns for some of these components have been characterized, but, more importantly, evidence for bioactivities in vivo has been provided. Cytokines work in networks and produce a cascade of effects that contribute to the regulation, development and function of the immune system (table 1). Several of these compounds are involved in immune responses of the intestinal epithelium. One example is TGF-β, which is present in high concentrations in colostrum and early milk, but also at biologically relevant concentrations in mature milk [1, 4]. TGF-β is known to affect cell growth and differentiation, but is also a potent immunoregulatory molecule [5]. It regulates differentiation, proliferation and

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<td>TGF-β</td>
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<td>Erythropoietin</td>
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Table 1. Growth factors and cytokines in breast milk
activation of macrophages, T cells, B cells, NK cells and dendritic cells, and thus plays important roles in tolerance, in prevention of autoimmunity and in anti-inflammatory processes. TGF-β knockout mice were shown to develop widespread tissue inflammation and die soon after weaning [6], demonstrating a critical role for TGF-β in immune modulation and inflammatory responses. It is likely that TGF-β in breast milk is particularly important at an early age, when production of endogenous TGF-β in the intestine is very low [4]. During this time, TGF-β can play an important role in instructing B cells to undergo class switching to IgA [7]. IgA, in turn, is important for protecting the epithelial surface of the intestine, and antigen-specific IgA can prevent adherence and penetration of bacterial and dietary antigens that can provoke inflammation. It is possible that TGF-β can promote IgA production in infants. Böttcher et al. [8] showed that IgA concentrations were correlated with TGF-β concentrations in breast milk, and may be important for induction of oral tolerance. This was supported by a study by Ogawa et al. [7] who showed that TGF-β in breast milk was associated with IgA production in infants. Further, a study by Rigotti et al. [9] suggests that TGF-β in breast milk is involved in the prevention of atopic disease in infants, which is supported by the finding of an inverse correlation between TGF-β in breast milk and wheezing in infants [10]. A recent systematic review of studies on the association between TGF-β in human milk and immunological outcomes in infants and young children showed that 67% of these studies showed a positive association between TGF-β and protection against allergy-related outcomes [5].

A role for TGF-β in neonatal immune function is supported by animal studies. Penttila et al. [11] showed that supplementation of rat milk formula with TGF-β resulted in downregulation of humoral and mast cell response to formula antigens and also directed the immune response away from inflammation even after weaning. Penttila [12] also showed that whey protein concentrate enriched in TGF-β added to formula could downregulate inflammatory responses in allergy-prone rats. Verhasselt et al. [13] showed in a lactating mouse model that airborne antigens are transferred into milk and that breastfeeding-induced tolerance relied on the presence of TGF-β during lactation. Thus, TGF-β may support immune priming to food antigens and induce oral tolerance.

We have investigated whether TGF-β2 in human milk and formula can resist proteolysis under conditions similar to those in the infant gut [14]. We found that the level of TGF-β2 in infant formula was variable and in some cases exceeded that of human milk samples. Digestion with pepsin at pH 2.0 or 3.5, followed by digestion with pancreatic enzymes substantially increased the immunodetectable TGF-β2 in human milk and formula. Additionally, the TGF-β2 in these digests was highly bioactive as measured in a cell-based assay. Thus, TGF-β2 present in some infant formulae and human milk continues to be immunodetectable and retains activity after in vitro digestion, strongly suggesting that TGF-β2 can survive in the infant gut and exert its biological activities.
Interestingly, growth factors/cytokines with proinflammatory activity, such as tumor necrosis factor-α, have soluble receptors in breast milk inhibiting their activity. It is possible that these factors may be of biological significance in the mammary gland, but need to be inactivated when reaching the developing infant gut.

**Essential Fatty Acids in Human Milk and Development of Allergy**

Addition of docosahexaenoic acid (DHA) and arachidonic acid to infant formulae has received special interest. DHA is important for brain development (usually assessed by visual acuity) in preterm infants, and as breast milk from most women is higher in DHA than infant formula, some manufacturers have supplemented their products for term infants with DHA. A recent study on increased DHA intake of infants leading to lower BMI [15] and a study showing a positive correlation between breast milk DHA and EPA levels with developmental scores [16] may strengthen the argument for DHA supplementation.

Recent findings on essential fatty acids in breast milk also suggest that a proper ratio of n-3 fatty acids to n-6 fatty acids may be important with regard to development of allergic disease. Levels of EPA (C20:5 n-3) and DHA (C22:4 n-3) as well as the total n-6/n-3 ratio were significantly lower in breast milk from mothers of allergic children as compared to those having non-allergic children [17]. In a follow-up study, women were supplemented with polyunsaturated fatty acids during pregnancy and lactation, and the prevalence of food allergy as well as IgE-mediated eczema was lower in the n-3-supplemented group compared to the placebo group [18]. Interestingly, Laiho et al. [19] found that women with allergic disease had lower concentrations of TGF-β2 in their milk. A positive association was found between polyunsaturated fatty acids and TGF-β2. It is therefore possible that the lower levels of TGF-β2 in the breast milk may interfere with the development of the mucosal immune system of the breastfed infant.

**Probiotics in Human Milk**

It has been well known that the gut microflora of breastfed infants is dominated by lactobacilli and bifidobacteria and is quite different from that of formula-fed infants. This has largely been believed to be due to fecal ‘contamination’ from the mother at delivery (which has made the differences less pronounced with increased sanitary measures) and to bioactive components stimulating the growth of beneficial bacteria and inhibiting the growth of pathogens. Recently, however, careful studies in which the breasts of lactating women have been cleaned rigorously have shown that live bifidobacteria
are found in breast milk [20–22] or that bacterial DNA signatures are present in breast milk cells [23]. Thus, the breastfed infant is essentially given oral doses of probiotics from birth on, and in every meal. This very early colonization may be very important, as it is known that it is difficult to alter a gut microflora that has already been established. Interestingly, lactating mothers with allergy had significantly lower concentrations of bifidobacteria in their breast milk than nonallergic mothers [24]. In addition, maternal allergy status had a significant effect on their infants’ fecal bifidobacteria.

Recent findings also suggest that the subspecies of probiotic bacteria such as bifidobacteria may also be important. Sela et al. [25] completed the genome sequence of *Bifidobacterium longum* subsp. *infantis* and found that it reflects a competitive nutrient utilization strategy targeting milk-borne molecules that otherwise lack nutritive value to the neonate. Thus, this specific subspecies may be uniquely adapted to utilize milk oligosaccharides. Interestingly, *B. longum* subspecies *infantis* and *longum* were found in all breast milk samples in a study by Guiemonde et al. [22], whereas other biotypes were less abundant. Thus, it is possible that probiotics provided in breast milk are perfectly matched to the substrates (oligosaccharides) present in breast milk. This, in turn, may have important implications for infant formulae – what probiotic strain(s) should preferably be used, and what substrates (oligosaccharides) should be present? To date, the strains that are being used commercially in formula differ from those found in the feces of breastfed infants, and the prebiotic oligosaccharides differ considerably from the complex and dynamic mixture found in breast milk [22].

The establishment of an appropriate gut microflora, possibly initiated by the lactating mother through her milk, or by feeding formula with specific probiotic strains, may have significance beyond that of discouraging pathogens. The recent findings of ‘crosstalk’ between the microbiota and the host leading to effects on energy metabolism in the small intestine are very interesting and thought provoking. Bäckhed et al. [26] suggested that the gut microflora facilitates the hydrolysis of nondigestible oligosaccharides to easily absorbed monosaccharides and the activation of lipoprotein lipase by their interaction with the intestinal epithelium. Together, this leads to increased glucose absorption and storage of fatty acids as triglycerides, which increases weight gain. For example, increased numbers of *Bacteroides* in the gut microbiota were demonstrated to increase energy stores and obesity in experimental animals [27]. Interestingly, Kalliomäki et al. [28] recently found that bifidobacteria were present in higher numbers in children maintaining normal weight than in children becoming overweight. Being overweight was instead associated with a greater number of *Staphylococcus aureus*. The authors suggest that high numbers of bifidobacteria and low numbers of *S. aureus* protect against overweight and development of obesity, which may be supported by recent meta-analyses showing that breastfed infants are 13–22% less likely to become overweight or obese in childhood and that breastfeed-
Lönnerdal

ing is inversely associated with the risk of overweight [29, 30]. Thus, it is feasible that specific probiotic strains in breast milk not only facilitate colonization of the gut with beneficial bacteria and deter pathogens, but that they also modulate energy metabolism which in turn can affect development of obesity.

**Milk Fat Globule Membrane Proteins and Defense against Infections**

The protein fraction of the membranes surrounding the fat globules in human milk is quantitatively minor [31], but may be of significance in the defense against infections. Several of these proteins, such as lactadherin, butyrophilin, xanthine oxidase, alkaline phosphatase, etc., have been shown to have antimicrobial activity in vitro. Human milk mucin components were able to bind to various rotavirus strains and prevent replication and the ability was correlated to lactadherin [32]. Further, the content of lactadherin in breast milk was shown to be negatively correlated to symptomatic rotavirus infection in Mexican infants [33].

Infant milk formula, however, is made from skim milk powder and whey protein concentrate and consequently does not contain any milk fat globule membrane (MFGM). Recently, milk fractions enriched in MFGM have become available on a large scale commercially, and may therefore be added to infant formulae in the future. Some bovine proteins in the MFGM have been demonstrated to have broad activities against pathogens and a bovine whey protein concentrate enriched in MFGM may therefore help to prevent diarrhea of bacterial and viral origin [34]. This protein fraction contains several bioactive components including mucin (MUC1), lactadherin, folate-binding protein, lactoferrin, sialic acid, sphingomyelin, and gangliosides [34]. A bovine milk fraction containing MUC1 has been shown to inhibit hemagglutination of *Vibrio cholerae* and *Escherichia coli* [35]. In addition, purified mucin, a MFGM constituent, was demonstrated to decrease the adherence of *Yersinia enterolytica* to intestinal membranes [36]. The MFGM fraction has also been found to reduce rotavirus in vitro [37]. Sphingolipids, particularly gangliosides, have been shown to inhibit enterotoxins both in vitro and in vivo [38]. Infant formula with added sphingolipids (gangliosides) has been shown to reduce *E. coli* counts in the stool, and to increase beneficial bifidobacteria [39].

We have tested the concept of MFGM protein fractions having an effect on infectious diseases in Peruvian infants. The infants were given MFGM proteins in a milk-based meal twice daily for 6 months in a randomized controlled double-blind study [40]. Prevalence of diarrhea was significantly lower in the group given MFGM than in the group given the same type of meal with skim milk protein instead of MFGM. Although the exact constituents of MFGM having an inhibitory effect on diarrhea were not identified, it is quite possible
that addition of the MFGM fraction to infant formula may have an effect on infectious disease.

**Conclusions**

The breast milk constituents described above are all likely to affect several outcomes in the recipient infant, either individually or, more likely, in a synergistic fashion (fig. 1). Long-term outcomes include an ‘optimal’ gut microflora, enhanced resistance against infection, improved immune function, reduced allergy, and decreased obesity. Our increased knowledge about these breast milk components is also likely to result in new and improved infant formulae.

**References**

Lönnerdal


Human Lactation and Infant Formula Development


**Discussion**

**Dr. B. Koletzko:** You showed the fascinating results of your study on milk fat globule membrane effects on diarrhea, and you also showed the comparison of different components where mucins appeared to have beneficial effects. Would it not be easier to add just the active components to a dietetic product than the whole milk fat globule membrane? Secondly, I wish to voice my concerns as to what conclusions we can draw from association studies. The data of the Bäckhed study in mice, with very strong effects of bacterial colonization on body fat accumulation is impressive, but the physiology may be different from that in real humans. In germ-free mice, scavenging energy from undigested substrates reaching the colon by microbial fermentation is not achieved, which is considered a significant part of the overall energy balance. I am not sure we get the answer as to how important such effects on healthy humans are from association studies. If you find an association between the bacterial colonization patterns and obesity or non-obesity, one would not be sure whether this different bacterial colonization is the cause or consequence of differences in lifestyle and in diet in families who show obesity or no obesity. We clearly need other types of studies to address this question.

**Dr. Lönnerdal:** You bring up very good points. The first one is coming back to what we are addressing at this conference, which is what kind of drivers are behind the innovations that were taken. I agree with you that it would be very nice to study components of the MFGM. It’s just that the entire MFGM fraction is the only one commercially available; that is, you can buy it in hundreds of kilograms and therefore you have the possibility to do human intervention studies. We have too many in vitro studies that give suggestions but not much more. I am not aware of any company that has mucins in a purer form, in commercially viable quantities. It’s possible but I haven’t
seen it, and I haven’t seen human studies on such components, but I agree that it’s most likely the crucial component for the outcomes we looked at. Unfortunately, we didn’t have the resources to look at developmental outcomes where perhaps gangliosides and sphingomyelin could have an effect. Coming back to your other comment, I couldn’t agree more with you when it comes to associations like in the Kalliomäki study. I think such observations are only suggestive. I think it’s an area that needs more investigation; we need to look at the microbiota and its interaction with a lot of different factors. Mouse studies are easier because you can refine the hypotheses much more than you can in any type of observational human study. I think it is worthwhile coming back to where we started. We know today that the average daily intake of a formula-fed infant is about 1,000 ml per day, while the average intake of a breastfed infant is about 800 ml. Thus, you have an overabundance of energy intake in formula-fed infants. I think the Gordon group has a lot of things planned which at least would spur us into moving into that area. They have done studies on western style cafeteria diets where you also have an abundance of calories. How does that higher energy intake interact with the microbiota when it comes to development of obesity? This needs to be looked at from an energy point of view, and also from a lipid and carbohydrate perspective. I think that the subclasses of energy coming from various nutrients and the gut microbiota could be very interesting.

**Dr. Gibson:** To what degree can factors like TGF-β contribute to becoming obese or not?

**Dr. Lönnerdal:** Very difficult issue. When it comes to TGF-β, there may be clinical conditions that may shed some light on this. In the Verhasselt study, a knockout mouse model was used. There may be mutations in humans which haven’t really been pursued that much. Sometimes you can find mutations and follow-up animal studies with human studies. When it comes to the obesity issue, it’s a much more complex issue and we have to consider many things. Five years ago, we really didn’t think that the gut microbiota had much to do with obesity and what they have shown at least is that it certainly can affect both energy reutilization in the gut but more importantly the crosstalk between the products of energy metabolism by the microbes in the gut with the mucosa. I think this is something which needs further studies.

**Dr. Gibson:** I am not disputing the bioactivity of TGF-β. I am wondering how we should design studies that compare formulas with different levels of TGF-β?

**Dr. Lönnerdal:** It is very difficult, but if we know that you can select a whey protein concentrate high in TGF-β which is commercially available, powdered infant formula with similar levels of TGF-β as you have in breast milk can be produced, and then you can have basically the identical product in liquid form with no TGF-β. This provides a possibility to do an intervention study.

**Dr. Ivarsson:** I have a comment about the possibilities of different study designs. The experimental design is the one that’s often mentioned, and it is of course very useful. However, I want to emphasize that we also have several different observational study designs – so far underutilized – that can increase knowledge. Among these, the prospective cohort study with long-term follow-up is the most demanding; however, it is necessary to get the final evidence.

**Dr. Lönnerdal:** I agree totally.

**Dr. Greer:** How do the specific probiotic bacteria get into the breast milk that is secreted by the mammary gland? I can accept the fact that maybe this is a migration of the bacteria up the mammary ducts from the nipple. But if anaerobic probiotic bacteria are translocating across the GI tract and into the blood stream and then into the mammary gland, this would be a very hostile, aerobic environment and a complicated process. Are we just talking about PCR evidence of bacteria in human milk without any viable organisms?
Dr. Lönnerdal: That's not my area of expertise. I think we need both a bacteriologist and a lactation physiologist to resolve this. I have been fascinated by the transfer of various things into breast milk for a long time. We did some studies in which we looked at the transfer of dietary antigens into breast milk, and you actually can find β-lactoglobulin from cow's milk in intact form in breast milk. Thus, fairly large cow's milk protein can first be absorbed from the diet, pass through several biological membranes in the small intestine, be transported through the body, and then through several membranes in the mammary gland and finally be secreted in intact form. I am not sure how that happens, but it does. When it comes to bacteria, they are even larger. In this case, I believe the work of those scientists that performed the bacterial analyses in breast milk; they took all the care they could to clean the breasts, etc. I don't know how much more you can do unless you do some biopsies perhaps and see what is actually inside the mammary gland, but I wouldn't recommend that.

Dr. Bier: What were the intervention period and the primary end point of this study?

Dr. Lönnerdal: The study was a 6-month intervention. We started when they were between 6 and 8 months old and we followed them for 6 months. The evaluation was therefore between 12 and 14 months of age. Primary outcomes were diarrheal disease and morbidity. We also had a very complete evaluation of nutritional status, which was another part of the study.

Dr. Bier: You showed us several different ways to measure diarrheal disease.

Dr. Lönnerdal: I presented incidence and prevalence data. We also analyzed the pathogens in the stool, and in some cases saw a significant effect of specific pathogens.

Dr. Bier: What was the primary variable?

Dr. Lönnerdal: Diarrhea prevalence.

Dr. Bodenstab: You mentioned rotavirus vaccination. There is a debate about its efficacy. Do you think it's important for future infant nutrition products to have anti-rotavirus functionality?

Dr. Lönnerdal: I can't respond to how efficient the vaccine is today, or how widely distributed it will be to the populations that we are looking at. I think both economic and social factors will determine this accessibility. I still think that during a transition period, dietary factors that can affect rotavirus may be important, but, like I said, in our study we had expected rotavirus to be a significant part, but it was not, so what we saw was a reduction in bacterial diarrhea and not rotaviral diarrhea.

Dr. Szajewska: I don't think there's been any debate regarding the use of the rotavirus vaccine. It's efficacious in preventing severe rotavirus gastroenteritis and hospitalization due to rotavirus gastroenteritis.

Dr. Haschke: I am coming back to the innovation process. You have shown a couple of molecules in relation to breast milk. What is your speculation, which components should formula milk include in 5–10 years from now?

Dr. Lönnerdal: There are certainly several components that I think are worthy exploring, TGF-β may be the easy one because it's not costly. It's there already, it has a physiological function like Dr. Gibson alluded to and therefore it can be tried. I think the right type of studies have not been done yet. I didn't talk today about lactoferrin which I think is very important and could be added, but there you have a significant economical factor, as it would be expensive. We have seen it in the past that if the formula is too expensive nobody will buy it, and then we haven't achieved much either. This needs to be looked at, and that's why I like this conference – we have an opportunity to discuss aspects that are driving innovation. It's not just about having an idea and trying to correlate things in vitro, we need to take it to the next step.