

Neonatal Tolerance under Breastfeeding Influence: The Presence of Allergen and Transforming Growth Factor- β in Breast Milk Protects the Progeny from Allergic Asthma

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Once the umbilical cord has been cut, immunologists have often looked at the neonate as an entity that develops on its own. 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. 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. Here, we will present and discuss our data showing how breast milk can actively shape the immune response of the progeny, particularly in the context of allergic disease. Indeed, our data obtained in a mouse model suggest that the protection attributed to breastfeeding toward asthma development might rely on immune tolerance induction. For this to occur, the mother mice needed to be exposed to the allergen by aerosol or oral route during the lactation period, which resulted into the transfer of the allergen to breast milk. The presence of the allergen together with transforming growth factor- β in breast milk was necessary and sufficient to induce the development of regulatory T lymphocytes in the progeny and their protection from asthma development. If confirmed in human beings, this study may suggest new strategies for asthma prevention such as deliberate exposure of mother to allergens during breastfeeding and qualitative modification of artificial milks. (*J Pediatr* 2010;156:S16-20).

Although some controversy exists, many epidemiologic studies have shown a protective effect of breastfeeding on asthma regardless of whether mothers were allergic.¹⁻⁸ However, breast milk factors that are responsible for this protective effect have not yet been clearly identified. The more widely accepted hypotheses are (1) the prevention of respiratory infections, such as respiratory syncytial virus, that predispose to wheezing; (2) the promotion of gut colonization by protective bacteria such as lactobacilli and bifidobacteria; and (3) the presence of the immunosuppressive cytokine, transforming growth factor- β (TGF- β), in breast milk.⁵ As discussed in depth in this supplement by Dr Pentilla, TGF- β is considered as a key immunodulatory factor in breast milk. As a matter of fact, in the particular context of allergic disease, epidemiologic studies have shown a correlation between levels of TGF- β in breast milk and protection against wheeze and atopic dermatitis in breastfed children,^{9,10} and animal studies have demonstrated that TGF- β is able to prevent intestinal mucosa inflammation¹¹ and to prevent allergy in allergic-prone rats.¹²

Asthma is a chronic lung inflammatory disease that results from an inappropriate Th2 response against innocuous airborne antigens. For disease development, allergen encounter is necessary for both the sensitization step and for appearance of symptoms in sensitized persons.¹³ Accordingly, prevention of symptoms in already sensitized patients is based on allergen avoidance. For primary prevention, allergen avoidance has also been proposed,¹⁴⁻¹⁶ and several allergen avoidance trials involving young children were conducted and focused on environmental control measures targeting a reduction in indoor allergen concentrations. Although allergen avoidance resulted in reduced symptoms in sensitized children, there was no convincing evidence that sensitization itself was reduced.¹⁴⁻¹⁶ In striking contrast, sensitization was actually increased in some studies when allergen exposure was decreased.¹⁷⁻²¹ In those studies, no information was given regarding the way of infant feeding.

Hypothesis

We formulated the hypothesis that breastfeeding could afford protection against asthma through tolerance induction. Immune tolerance induction requires both the presence of the antigen and its presentation in a tolerogenic environment. Breast milk could in some instances meet these criteria and thereby afford protection. Thus if the mother is exposed to some environmental allergens, she could transfer these allergens to her child through breast milk as described for dietary antigens. In addition, the presence of immunomodulatory factors in maternal milk would allow tolerance induction toward the breast-milk-transferred allergen.

IL	Interleukin
TGF- β	Transforming growth factor- β

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The Model

We have assessed this hypothesis in a murine model.³ Lactating mice were exposed to aerosols of a model allergen, ovalbumin, until weaning. Aerosols were given during 20 minutes every other days, and pups were kept away during the mother's exposure to assess only the transfer of the antigen through their mother (**Figure 1**). When adults, the offspring were submitted to a classical protocol of asthma induction. For sensitization, the mice were injected twice intraperitoneally with ovalbumin adsorbed on alum. To recruit Th2 cells in the lungs and induce airway disease inflammation, mice were then challenged with ovalbumin aerosols. Asthma was assessed by the analysis of the following variables: airway hyperreactivity, bronchoalveolar lavage cellularity, lung histology, serum levels of ovalbumin-specific immunoglobulin E and lung Th2 cytokine secretion.

Results

Breast-feeding by Ovalbumin-Exposed Mothers Prevents Asthma in the Progeny

All the assessed variables of allergic airway disease were decreased by more than 50% in mice breastfed by ovalbumin-exposed mothers as compared with mice breastfed by unexposed mothers.³ In addition, there was no evidence of a shift of the immune response toward Th1 phenotype. Protection was also observed in BALB/c mice breastfed by mothers that had been exposed to ovalbumin through the intranasal route ruling out that the protection observed in pups breastfed by ovalbumin aerosol exposed mothers was due to the absorption of the antigen by the pups licking the skin of their mothers. In addition, oral administration of the antigen to the mother did also confer protection suggesting that our observation could be extended to dietary antigens. Antigen-specificity was demonstrated by experiments in which mice breastfed by ovalbumin-exposed mothers were sensitized and challenged with an unrelated antigen, the *Leishmania* LACK antigen.²² In that case, no protection was observed.

Protection in Mice Breastfed by Ovalbumin-Exposed Mothers Does Not Require the Presence of Immunoglobulins in Breast Milk

Antigen-specific protection could result from the transfer of immunoglobulins or the antigen from the mother to the newborn through breast milk. To address this issue, wild type newborns were breast-fed by mothers that were exposed or not to the antigen and whose breast milk was devoid in immunoglobulins. For this purpose, we use either B cell-deficient or B and T lymphocyte-deficient recombination activating gene-2 knockout foster-mothers. In both cases, the levels of inhibition of asthma were similar to those observed in mice breastfed by wild type mothers exposed to ovalbumin.³ Therefore tolerance did not require the transfer of immunoglobulins from the mother to the newborn.

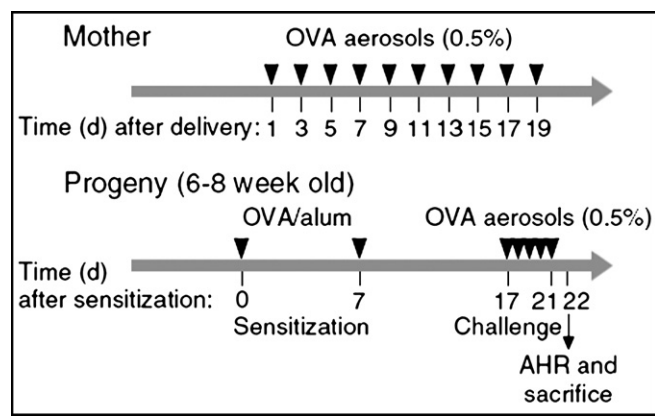


Figure 1. Experimental protocol. Lactating mothers were exposed or not to 0.5 % ovalbumin aerosols during 20 min every other day from delivery until weaning. During aerosol exposure, pups were kept away from their mother. When 6-8 wk-old, offspring was sensitized with two injections of ovalbumin in Alum, and challenged daily for 5 days with ovalbumin aerosols. Mice were analyzed one day after the last aerosol.

Protection in Mice Breastfed by Ovalbumin-Exposed Mothers Requires the Presence of the Antigen and TGF- β in Breast Milk

We next looked for the presence of ovalbumin in milk of ovalbumin-exposed mothers. Western blotting analysis with anti-ovalbumin mAbs showed the presence of both intact and degraded ovalbumin protein. Ovalbumin concentration in milk of ovalbumin-exposed mothers was in the same range as what is described for dietary antigens in human milk,²³ that is, 180 ± 20 ng/mL. Knowing that daily milk consumption by newborn mice is around 500 μ L at day 10, mice breastfed by ovalbumin-exposed mothers received about 100 ng of ovalbumin daily, thus 10 ng per gram. In adults, oral tolerance is usually achieved at doses in the range of the milligram per gram. In addition, oral tolerance is reported to be hard to induce in the neonate (see discussion below). This prompted us to check whether there was a cofactor in breast milk favoring tolerance induction.

Breast milk contains interleukin (IL)-10 and TGF- β that both exhibit immunosuppressive activities and favor tolerance induction.^{1,24-26} Mice breastfed by ovalbumin-exposed IL-10-deficient mothers were protected from allergic airway inflammation as efficiently than those breastfed by ovalbumin-exposed mothers, suggesting that protection could occur in the absence of IL-10 in milk. Because TGF- β -deficient mice die prematurely, we assessed the role of milk-borne TGF- β by injecting anti-TGF- β -neutralizing mAb to lactating mothers. Levels of TGF- β in breast milk were at the limit of detection in that case. We observed that the protection by ovalbumin-exposed mothers was totally abolished when TGF- β in breast milk was neutralized, demonstrating the key role of this factor for breast feeding-induced tolerance.

Protection in Mice Breastfed by Ovalbumin-Exposed Mothers is Mediated by CD4⁺ Regulatory T Lymphocytes and Requires TGF- β Signaling in T Lymphocytes

The protection observed in mice breastfed by ovalbumin-exposed mothers might rely on the deletion of ovalbumin-specific T lymphocytes, their unresponsiveness (also called *anergy*) or on the presence of T lymphocytes able to suppress immune response to ovalbumin, the so-called regulatory T lymphocyte. To assess whether regulatory T lymphocytes were present in tolerant mice, we isolated CD4⁺ T cells from tolerant mice, injected them in mice breastfed by unexposed mothers, and analyzed whether they were able to suppress asthma development. Animals injected with CD4⁺ T cells from mice breastfed by ovalbumin-exposed mothers exhibited reduced allergic airway inflammation, showing that a mechanism of active immune suppression by CD4⁺ T cells was responsible for breastfeeding-induced tolerance.³ Among the different subclasses of regulatory T lymphocytes, the one that express the CD25 molecule have recently received particular attention and were shown to be involved in oral tolerance in adult²⁷ and in the regulation of allergic disease.²⁸ Experiments with anti-CD25 mAb demonstrated that CD25⁺ regulatory T lymphocytes were not necessary for breastfeeding-induced protection.

Because we observed that TGF- β presence in breast milk was necessary for tolerance induction, we next assessed whether breastfeeding-induced protection required TGF- β signaling in T cells. Therefore we used TGF- β DNRII mutant mice in which T cells do not respond to TGF- β .²⁹ TGF- β DNRII newborns were breastfed by wild type mothers that were exposed or not to ovalbumin aerosols. Exposure of foster mothers to ovalbumin did not induce protection in TGF- β DNRII mutant mice, demonstrating that tolerance observed in mice breastfed by ovalbumin-exposed mothers were dependent on TGF- β signaling in T cells.³ We next investigated whether TGF- β was required for protection when mice were adults. Mice breastfed by ovalbumin-exposed mothers were treated with either anti-TGF- β or isotypic control mAb 1 day before sensitization, challenged with ovalbumin aerosols and analyzed for allergic airway inflammation. Neutralization of TGF- β before sensitization did not prevent breastfeeding-induced protection, demonstrating that TGF- β was no longer required once T_{reg} cells have already been induced during the neonatal period.

Discussion

We have demonstrated that an airborne antigen can be transferred from lactating mice to their progeny through breast milk, eventually resulting in antigen-specific tolerance and prevention of asthma (Figure 2). Breast milk contains dietary antigens,²³ but the presence of airborne antigen has not yet been assessed. Antigen distribution after aerosol administration has been previously assessed with radiolabeled iodine 125-bovine serum albumin or -ovalbumin.^{30,31} Both studies demonstrated that 2% to 4% of antigen was found in the lung

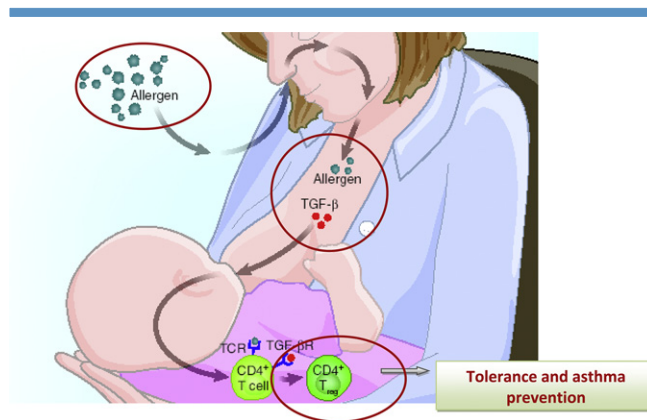


Figure 2. Breastfeeding induced tolerance. Allergens inhaled by lactating mouse are present in breast milk. Furthermore, the allergen ingested by nursing offspring is accompanied by TGF- β ; together, allergen and TGF- β are sufficient to induce T-cell differentiation. The resulting CD4⁺ regulatory T cells provide offspring with allergen-specific protection in a mouse model of human asthma. TCR, T cell receptor; Treg, regulatory T cell; TGF- β R, TGF- β receptor. Adapted from *Nature Medicine*.⁴³

and 65% to 80% in the digestive tract 1 to 2 hours after aerosol exposure. Therefore, although some airborne antigens penetrate into the distal alveoli, the bulk of inhaled antigen is found in the gut. Indeed, inhaled antigens are either trapped in the nasal passage and swallowed or deposited to the lung and cleared via the mucociliary escalator to the digestive tract. Therefore the presence of airborne ovalbumin in milk most likely results from the transfer of ovalbumin from the airways to the mammary gland mainly through the gut and for a small proportion through the alveolar-capillary barrier of the lung.^{32,33} As a matter of fact, we also observed protection when the antigen was given orally to the mother, suggesting that breast feeding-induced tolerance also operates for dietary allergens.

Even though the oral administration of an antigen to adult rodents results in tolerance induction,²⁷ inducing oral tolerance in neonates is far more difficult.³⁴⁻³⁷ Early studies pioneered by Medawar have suggested that neonates are immunologically immature and prone to tolerance induction. Thus neonates injected at birth with allogeneic splenocytes become tolerant and accept transplants from an allogeneic donor when they are adult (reviewed in reference 38).³⁸ In contrast with these studies, several authors have more recently shown that systemic neonatal exposure to antigen can prime T-cell responses, depending on the amount of antigen given and the adjuvant used (reviewed in reference 34). For oral antigen administration, tolerance is even more difficult to achieve in neonates than in adults.^{34,35,37,39,40} For example, oral administration of myelin basic protein to rat neonates increased susceptibility to experimental autoimmune encephalomyelitis.³⁵ Likewise, oral administration of ovalbumin to mouse neonates increased delayed type hypersensitivity and antibody responses in mice.^{37,39} In addition, neonates are biased for Th2 responses as compared with adults.³⁴ Altogether these observations are in apparent

contrast with our data showing that the transfer of an antigen from the mother to the newborn via the milk induces tolerance toward a Th2-mediated disease. Breast-feeding-induced tolerance probably relies on the chronic administration of an antigen at low dose, a setting known to promote regulatory T-cell development and tolerance induction.⁴¹⁻⁴³ In addition, we observed that the presence of milk-borne TGF- β was crucial for tolerance induction. This probably reflects the necessity for an exogenous source of TGF- β in neonates for tolerance induction given the fact that the endogenous gut TGF- β synthesis is defective in the neonate.²⁵ Ongoing studies are assessing what is the impact of the immune status of the mother on tolerance induction.

Conclusion

Epidemiologic studies on the relationship between breast-feeding and the development of allergic diseases have reached conflicting results. However, maternal airborne allergen exposure and antigen content in milk were not recorded in these studies. Our work may confer a rationale for new epidemiologic studies assessing the presence of airborne antigens in human milk and the prevalence of allergic diseases in children breast-fed by mothers exposed to airborne allergens. If our observations are confirmed in human beings, we might propose the deliberate exposure of lactating mothers to allergens to prevent asthma development. We also highlighted the necessity of TGF- β presence in breast milk for tolerance induction toward antigen transmitted to the pups. These observations might also have implications for artificial milk manufacturing. In a broader context, we provided new insights into the mechanisms underlying tolerance induction in neonates and pinpoint maternal influence through breast milk antigen transfer in the presence of TGF-beta as a critical factor in this process. ■

Author Disclosures

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