0031-3998/06/5905-0650
PEDIATRIC RESEARCH

Vol. 59, No. 5, 2006
Printed in U.S.A.

Effects of Transforming Growth Factor-Beta and Formula Feeding on Systemic Immune Responses to Dietary β-Lactoglobulin in Allergy-Prone Rats

IRMELI PENTTILA

Child Health Research Institute, North Adelaide, South Australia, Australia 5006; Department of Paediatrics, University of Adelaide, Adelaide, South Australia, Australia. 5000

ABSTRACT: Early nutritional events have the potential to affect health outcomes in later life including the development of allergy. Food allergy is usually the first manifestation of allergy. Breast-feeding has been associated with a protective effect against the development of allergy, but the evidence is contradictory and the mechanisms involved are not clear. We hypothesize that milk cytokines, such as transforming growth factor β (TGF-β), play a role in regulating immune responses to dietary antigens. Using a rat pup model of gastrostomy feeding, the immune response profile, at weaning and post-weaning, of allergy-prone Brown Norway rats fed formula supplementation with TGF-β was assessed. We show that feeding formula to allergy-prone rat pups results in increased total IgE immunoglobulin, β-lactoglobulin (BLG) IgG1 antibody, and mucosal mast cell activation, as measured by serum rat mast cell protease II (RMCPII) levels in the gut. Supplementation of formula with physiological levels of TGF-β down-regulated the BLG IgG1 response as well as total IgE and mucosal mast cell activation. Supplementation of formula also resulted in an increase in Th1 cytokines, interleukin (IL)-18, IL-12p40, IL-12p35, and interferon gamma (IFN-γ) and an increase in IL-10. In conclusion, TGF-β supplementation of formula moved the immune response profile of allergy prone (Th2 type) rat pups toward a Th1 profile in the suckling period. Importantly, this immune profile persisted after weaning when TGF-β was no longer present in the diet. **(*Pediatr Res* 59: 650–655, 2006)**

Early nutritional events have the potential to effect health outcomes in later life (1,2) including allergy development. Allergic disease arises due to complex interactions between genetic predisposition and environment including early nutritional events (3). Hereditary factors are important in the development of allergy as infants who go onto develop allergic disease show an early predisposition to Th2 responses to allergens in the first year of life. These responses are short-lived in infants without a predisposition to allergy (4). The mechanism involved in allergy development is at present unclear, although the ability to mount a Th1 immune response is important in preventing persistent Th2 immune responses and development of allergic disease. An imbalance favoring Th2 type immune responses increases the risk of allergic disease.

Food allergy is usually the first manifestation of allergy with susceptible children going on to develop inhalant allergies as well (5). While the incidence of inhalant allergy is increasing, the incidence of food allergy is relatively stable (6). Breast-feeding has been associated with a protective effect against the development of allergy, but the evidence is contradictory and there is debate as to whether breast milk prevents or reduces the incidence of allergic disease (7,8). Van Odijk *et al.* (6) have carried out a multidisciplinary review of the literature on early feeding and impact on later allergy and concluded that breast-feeding protects against the development of atopic disease. This effect was shown to be stronger in infants with a hereditary predisposition to allergy.

Breast milk cytokines have the potential to regulate the immune response to food antigens. Cytokines are present in all mammalian milks and are capable of inhibiting excess inflammation (9–11) and modulating intestinal epithelial proliferation (12). There are a range of candidate cytokines in milk such as TGF-β, the major cytokine present, and IL-10, which may play a role in immunoregulation in the developing infant (13–15). TGF-β and IL-10 are thought to be responsible for maintaining normal homeostasis in the adult gut by creating a cytokine milieu for appropriate antigen processing and promoting oral tolerance development (16). We have previously demonstrated an important role for the maternal milk cytokine TGF-β in regulating the mucosal immune response after formula feeding in nonallergy-prone rat pups (9). High-dose TGF-β has also recently been shown to suppress IgE responses to food antigens in adult mice (17).

Regulation of immune responses during the neonatal period may be beneficial later in life, particularly in individuals with a genetic predisposition to allergy. Neonates with a genetic predisposition to allergy have a preferential Th2 type immune response to antigenic stimuli (18). Neonatal production of

Received October 6, 2005; accepted December 6, 2005.
Correspondence: Irmeli Penttila, PhD, Child Health Research Institute, 72 King William Road, North Adelaide, S.A., Australia, 5006; e-mail: irmeli.penttila@adelaide.edu.au
This work was carried out with support from Dairy Australia.

DOI: 10.1203/01.pdr.0000203149.75465.74

Abbreviations BLG, β-lactoglobulin; **GAPDH,** glyceraldehyde 3-phosphate dehydrogenase; **IFN-γ,** interferon gamma; **MIF,** macrophage migration inhibition factor; **RMCPII,** rat mast cell protease II

IL-4 and IFN-γ remain low in those infants who go on to develop atopy in early childhood (19,20).

The Brown Norway rat has a genetic predisposition to allergy development and has been successfully used as a model to induce allergic IgG and IgE responses to oral antigens (21–24). Given the contradictory evidence of the role of breast-feeding in allergy development, we assessed at weaning (when TGF-β is still present in the diet) and post-weaning (when TGF-β was no longer present in the diet), the immune response profile of allergy-prone Brown Norway rat pups either suckled naturally or fed formula with and without supplementation with the maternal milk cytokine TGF-β.

METHODS

Animals. Pregnant inbred Brown Norway rats were obtained from the Animal Resource Centre (Perth, Western Australia) and the Women's and Children's Hospital breeding colony. The study was carried out with the approval of the Women's and Children's Hospital Animal Ethics Committee, Adelaide, Australia.

Cannulation and maintenance procedure. The pup-in-cups model was carried out essentially as described previously (9). Briefly, on d 4 of life, a water-lubricated length of silastic tubing (0.51 mm) was introduced through the mouth of each pup to the stomach. The pups were then anesthetized using Forthane® (Isoflurathane, Abbott, Australia) and an intragastric cannula implanted. Following surgery, each pup was placed in a polystyrene cup held in a water bath at 42°C and the cannula was connected to a polyethylene milk line attached to a syringe containing formula (Rat Milk Replacer, Wombaroo, Adelaide, Australia). The formula maintained at 4°C and delivered by a multisyringe infusion pump. We have previously established that the changes in intestinal parameters in artificially reared rat pups are directly attributed to the formula and not to the surgical procedure itself, as maternal milk fed via cannula results in no physiologic or immunologic changes in the intestine when compared with naturally suckled pups (9,25).

Experimental design. Rat pups from several different litters were randomly assigned to one of four feeding groups: A) naturally suckled (control); B) naturally suckled and orally challenged by daily gavage with BLG, 11 mg/d, which is equivalent to the total daily amount present in the rat formula; C) formula; or D) formula supplemented with 100 ng/mL TGF-β_2 (R&D Systems, Minneapolis, MN), a dose equivalent to that detected in rat milk at mid-lactation. Endpoint days for the experiments were d 18 (weaning age) or d 28 (post-weaning). All pups were maintained on the cannulae until d 18. Weaned animals remaining in the experiment until d 28 were fed standard rat chow and water *ad libitum* and received no additional TGF-β. Animals in the weaned groups received additional oral gavages with BLG on d 26 and 27.

No bioactive TGF-β was detected in the formula as assessed by inhibition of proliferation of the CCL-64 Mv1Lu epithelial cell line (10). We have previously shown that the immune response profiles of rat pups fed maternal milk via cannula were not different from naturally suckling animals in terms of the immune response to formula components (9). A maternal milk via cannula group was not included in this experiment due to the difficulty in obtaining sufficient milk to feed the pups until d 18. Pups that did not receive at least 90% of their required formula intake or showed any obvious sign of infection were excluded from the trial. Weight gain did not significantly differ between groups over the course of the experiment (data not shown).

Tissue collection. Animals were anesthetized and blood collected by cardiac puncture on d 18 or 28 after birth. Following euthanasia, a section of ileum was removed, fixed in 4% buffered formaldehyde (pH 7.0) for 24 h, and embedded in paraffin for cell counts. Spleens were collected and snap-frozen in liquid nitrogen for cytokine mRNA analysis. Blood samples were centrifuged and serum stored at −80°C until analysis.

Eosinophil and mast cell staining Sections 4 μm in size were cut from paraffin-embedded ileum and placed on gelatin-coated slides. Sections were deparaffinized, rehydrated, and stained for mast cells (identified by stained red granules using Leder chloroacetate esterase stain) and eosinophils (identified by red/orange granules using Giemsa stain). Image Pro Plus software (Media Cybernetics, Silver Spring, MD) was used to capture images for analysis.

Spleen cytokines. The cytokine RNA profile was determined in spleen tissue using the Riboquant multiprobe RNase protection assay system (BD Pharmingen, San Diego, CA) according to the manufacturer's instructions. RNA was extracted using RNAzol B (Tel-Test, Friendswood, TX). *In vitro* transcription with ^{32}P-labeled UTP (Amersham Pharmacia Biotech, Buckinghamshire, UK) was performed using the relevant cytokine probe sets (RcK1 or RcK2), and 30 μg of splenic RNA. Dried gels were exposed to phosphorimaging screens and protected fragments visualized using a phosphorimager (Typhoon, Molecular Dynamics, Australia). Protected fragments were quantified and normalized with respect to the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

RMCP II. Serum RMCPII was determined by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (Moredun Scientific Ltd., Midlothian, Scotland).

BLG IgG1, IgG2b, and IgE antibodies. ELISAs forBLG specific IgG1, IgG2b, and IgE were established using matched antibody pairs (primary and secondary antibodies) and standards obtained from Pharmingen and Zymed Laboratories (San Francisco, CA) (rat myeloma IgE standard). Briefly, 96-well plates (Greiner, Frickenhausen Germany) were incubated with 5 μg/mL BLG (Sigma Chemical Co.) in phosphate-buffered saline for 1 h at 37°C. The wells were washed five times with wash buffer (PBS/0.05% Tween20) and then blocked for 1 h at room temperature with either goat serum (Sigma Chemical Co.) for the IgG1 and IgG2b assays or PBS/1% Polypep® protein digest (Sigma Chemical Co.) for the IgE assay. After incubation, the plates were washed and the appropriate secondary biotinylated antibody was added (concentration: 2 μg/mL). IgG1, IgG2b, and IgE were detected using biotinylated mouse anti-rat IgG1 (clone: RG11/39.4), biotinylated mouse anti-rat IgG2b (clone: RG7/11.1), or biotinylated mouse anti-rat IgE (clone: B41-3), respectively. Plates were incubated at room temperature and then washed. Following the final wash, a solution of ABC reagents (DAKO Pty Ltd., Glostrup, Denmark) in PBS and 0.1% Tween20 was added and incubated for 45 min at room temperature. Plates were washed six times, and TMB substrate was added to the wells for 10 min and the reaction stopped using 50 μL of 1 mol/L H_2SO_4 and read at an absorbance of 450 nm.

Total IgE. ELISA for total serum IgE was established as described above with the following modifications. Plates were coated with 2 μg/mL mouse anti-rat IgE (clone: B41-1, Zymed Laboratories) for 1 h at 37°C. After washing wells were blocked with PBS + 1% Polypep® protein digest for 1 h. Test samples or standard (rat myeloma IgE (clone: IR 162) (Zymed Laboratories) (100 μL, in duplicate) were added to washed wells and incubated for 1 h at room temperature. After washing, biotinylated mouse anti-rat IgE (clone: B41-3, concentration: 2 μg/mL) was added to each well for 1 h at room temperature. Following the final wash, a solution of ABC reagents (DAKO Pty Ltd., Glostrup, Denmark) in PBS and 0.1% Tween20 was added and incubated for 45 min at room temperature. Plates were washed six times, and TMB substrate was added to the wells for 10 min and the reaction stopped using 50 μL of 1 mol/L H_2SO_4 and read at an absorbance of 450 nm.

Sample size and statistics. To determine the minimum number of animals required for each group, a power calculation was carried based on past data. Data were analyzed by one-way analysis of variance with a Newman-Keuls multiple comparison test (GraphPad Prism software).

RESULTS

BLG IgG1, IgG2, and IgE antibodies and serum RMCPII levels. Serum was collected from rat pups at d 18 and 28 to determine BLG IgG1, IgG2b, and IgE antibodies, total IgE antibody, and serum RMCPII levels. BLG-specific IgG2b and IgE were found to be below the levels of detection by the ELISA. BLG-specific IgG1 titers were significantly higher in formula-fed pups at d 18 and 28 compared with both naturally suckled and naturally suckled BLG-challenged rat pups (Fig. 1*A*). In contrast, serum from rat pups fed formula supplemented with TGF-β had BLG-specific IgG1 titers comparable with the naturally suckled groups (Fig. 1*A*). BLG-specific IgG2b and IgE were below the level of detection of the ELISA assay.

Total serum IgE was significantly increased in formula-fed pups when compared with rat pups naturally suckled, BLG-challenged, and supplemented with TGF-β at d 18 and 28 (Fig. 1*B*). Provision of TGF-β in the formula prevented the increase in total IgE.

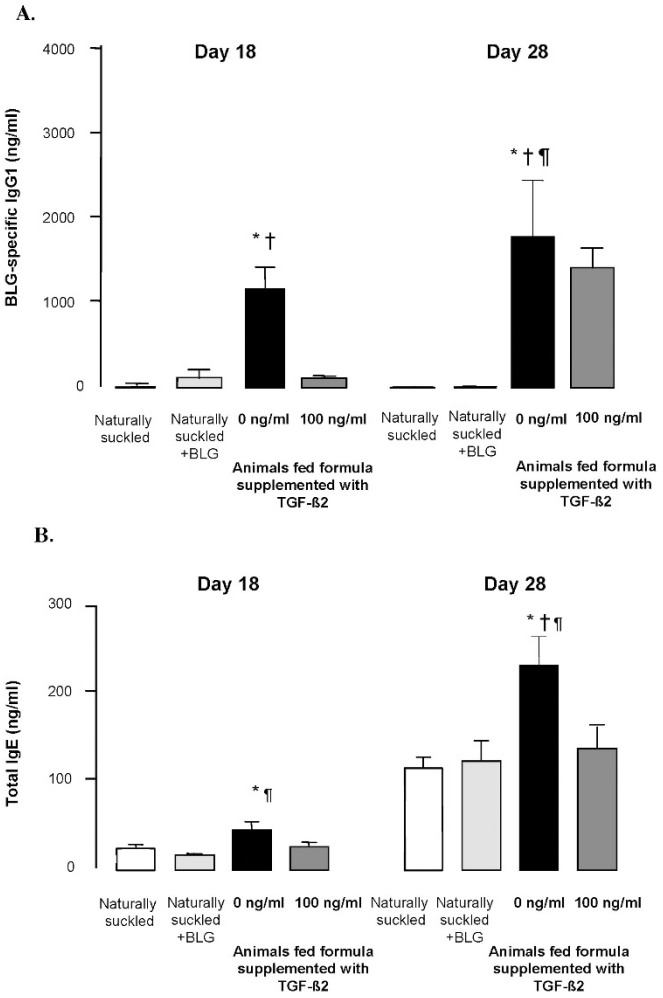

Figure 1. BLG IgG1 and total IgE antibody responses. (*A*) BLG IgG1 antibody levels over a time course in naturally suckled [$n = 8$ (□)], naturally suckled orally challenged with BLG [$n = 8$ (▨)], formula fed [$n = 8$ (■)], and formula supplemented with TGF-β–fed [$n = 8$ (▩) rat pups]. (*B*) IgE antibody over a time course in naturally suckled; naturally suckled, orally challenged with BLG; formula-fed; and formula supplemented with TGF-β–fed rat pups. Naturally suckled [$n = 8$ (□)]; naturally suckled, orally challenged with BLG [$n = 8$ (▨)]; formula fed [$n = 8$ (■)]; and formula supplemented with TGF-β–fed [$n = 8$ (▩)] rat pups. Significance indicated ($p < 0.005$) by the following symbols: *TGF-β *vs* formula fed, †naturally suckled *vs* formula fed, ‡TGF-β *vs* naturally suckled + BLG; ¶[naturally suckled + BLG *vs* formula fed; §TFG-β *vs* naturally suckled; **naturally suckled + BLG *vs* naturally suckled.

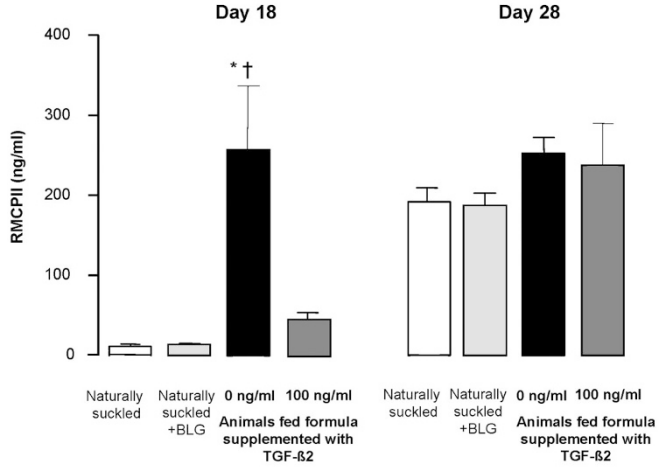

Figure 2. Mucosal mast cell activation as measured by serum RMCPII levels. Serum RMCPII levels over a time course in naturally suckled; naturally suckled, orally challenged with BLG; formula-fed; and formula supplemented with TGF-β–fed rat pups. Naturally suckled [$n = 8$ (□)]; naturally suckled, orally challenged with BLG [$n = 8$ (▨)]; formula fed [$n = 8$ (■)]; and formula supplemented with TGF-β–fed [$n = 8$ (▩) rat pups]. Significance indicated ($p < 0.005$) by the following symbols: *TGF-β *vs* formula fed; †naturally suckled *vs* formula fed, ‡TGF-β *vs* naturally suckled + BLG; ¶[naturally suckled + BLG *vs* formula fed; §TFG-β *vs* naturally suckled; **naturally suckled + BLG *vs* naturally suckled.

Serum RMCPII was significantly increased in formula-fed pups at d 18 compared with all other groups (Fig. 2); however, this increase was not seen at d 28.

Eosinophil and mast cell infiltration in the ileum. The data in Fig. 3*A* and *B* show the number of mast cells and eosinophils infiltrating the lamina propria of rat pups in each of the feeding groups. There were no significant difference in mast cell or eosinophil numbers between the four animal groups at either time point. At d 18, eosinophil numbers in the TGF-β–supplemented formula–fed rat pups were increased, but the number of cells was not significantly different from the naturally suckling groups.

Expression of cytokine mRNA in the spleen. To determine whether formula feeding influenced the infant's cytokine profile, we assessed spleen mRNA expression (Table 1). At d 18, formula feeding resulted in increased IL-4 and IL-1ra cytokine mRNA ratio (when normalized to the housekeeping gene GAPDH) along with a decrease in IL-12p35 and IL-1α mRNA when compared with the naturally suckled group. At d 28, MIF, IL-12p40, IL-18, and IL-1α cytokine mRNA were also decreased. At d 18, the mRNA for the Th1-associated cytokines, IFN-γ, macrophage migration inhibition factor (MIF), IL-18, IL-12p35, IL-1Ra, and IL-12p40 was significantly higher in the TGF-β–supplemented animals when compared with rat pups fed formula alone or those in the naturally suckled groups. IL-4 (a Th2 cytokine) was significantly higher in the TGF-β–supplemented animals, whereas IL-6 was significantly lower. The Th1 cytokines discussed above were also significantly higher at d 28 in the TGF-β–supplemented group. TGF-β supplementation of formula also resulted in an increase in IL-10 mRNA at d 18 and 28.

DISCUSSION

Maternal milk has been associated with a reduction in the risk of allergy development. Human milk contains significant quantities of TGF-β, which has been associated with regulation of atopic disease in infants (13,26). The data in this study provide evidence of a role for maternal milk and TGF-β in directing immune development in infant rat pups with a genetic predisposition to allergy both during the suckling period and also after weaning. TGF-β supplementation of formula moved the immune response profile of these allergy-prone (Th2 type) rat pups toward a Th1 profile during the suckling period. Importantly, this immune response profile persisted when rat pups were rechallenged with the formula component BLG after weaning when TGF-β was no longer present in the diet.

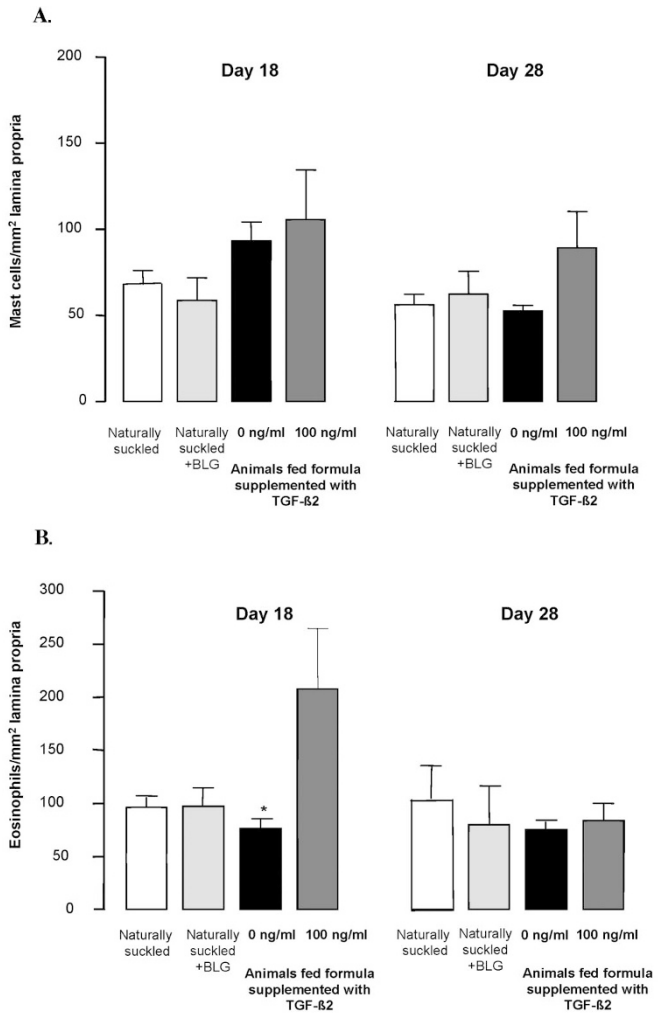

Figure 3. Mast cell and eosinophil numbers in the ileum of naturally suckled and formula-fed rat pups. (*A*) Mast cell staining in the lamina propria of the small intestine of rat pups naturally suckled [$n = 8$ (□)]; naturally suckled, orally challenged with BLG [$n = 8$ (▨)]; formula-fed [$n = 8$ (■)]; and formula supplemented with TGF-β–fed [$n = 8$ (▩)] rat pups. (*B*) Eosinophil staining in the lamina propria of the small intestine of rat pups naturally suckled [$n = 8$ (□)]; naturally suckled, orally challenged with BLG [$n = 8$ (▨)]; formula fed [$n = 8$ (■)]; and formula supplemented with TGF-β–fed [$n = 8$ (▩)] rat pups. Significance indicated ($p < 0.005$) by the following symbols: *TGF-β *vs* formula fed; †naturally suckled *vs* formula red, ‡TGF-β *vs* naturally suckled + BLG; ¶naturally suckled + BLG *vs* formula fed; §TFG-β *vs* naturally suckled; **naturally suckled + BLG *vs* naturally suckled.

In the rat, there is a dichotomy of T-cell function (*i.e.* Th1- and Th2-like immune response development) even though it is not as clear-cut as it is for mice. IgG1 is associated with a Th2 response as is IgE. IgG2a and IgG2b are associated with a Th1-like response (27,28). In Brown Norway rat pups fed formula, we found an increase in BLG IgG1 but not IgG2a, IgG2b, or IgE at d 18 and 28 (Fig. 1*A*). This is in accord with other studies carried out in adult Brown Norway rats fed *ad libitum* with a sensitizing antigen, where specific IgG1 but not IgE were detected (22). Even though specific IgE was not detected, these rats showed a marked delayed-type hypersensitivity (DTH) responsiveness to the sensitizing oral antigen ovalbumin when challenged.

Approximately half of infants and adults with cow's milk allergy do not have circulating milk-specific IgE antibodies (29,30). IgE has a high affinity for the Fcε receptors on mast cells and basophils. IgE preferentially binds to these cells, limiting the amount of free circulating IgE antibodies (31). In the formula-fed rat pups, this could also account for the lack of detectable BLG-specific IgE antibodies in circulation as well as the increased levels of mast cell activation after formula feeding.

Cell infiltrate (mast cells and eosinophils) into the gut are associated with allergy as are mast cell degranulation and release of histamine and RMCPII (32). RMCPII is an enzyme that uses type IV collagen, a component of the basolateral membrane of gut epithelial cells. RMCPII is thought to contribute to the disruption of the epithelial basement membrane, potentially leading to increased permeability and antigen exposure and immune sensitization in the periphery (33). Although we showed no increase in mast cells numbers (or eosinophils), the actual activation state of the mast cells was increased (as measured by RMCPII concentration in serum) after formula feeding at d 18. Importantly, the immune response profile after formula feeding [*i.e.* total IgE, BLG-specific IgG1, and mucosal mast cell activation (RMCPII)] was down-regulated by TGF-β supplementation of the formula in both in preweaning and weaned rat pups that were no longer receiving TGF-β.

We have previously shown that formula feeding in Hooded Wistar (nonallergy-prone) rats results in an increase in Th1 cytokines and an increase in eosinophil and mast cell infiltrate in the intestine (9). In the allergy-prone Brown Norway rat, IL-4 mRNA was increased at d 18 and Th1 cytokine mRNA (IL-12 and IL-18) were decreased at both d 18 and 28 in formula-fed rat pups compared with the naturally suckled group (Table 1). In contrast, rat pups fed formula supplemented with TGF-β had an increase in splenic (systemic) Th1-like cytokines including IL-18, IL-12p40, IL-12 p35, and IFN-γ. This suggests that supplementation of formula with TGF-β has the potential to down-regulate the humoral and mast cell response to formula antigens and importantly supplementation resulted in a redirection of the immune response profile away from a Th2-like response even after weaning. Importantly, TGF-β supplementation resulted in an increase in IL-10 at d 18 and 28. T regulator cells, which produce IL-10, are associated with improvement of cow's milk allergy in affected infants (34,35).

The mechanism by which tolerance and nonreactivity to food antigens in nonallergic individuals is at present unclear. Several studies have suggested that the development of a Th1-type immune response to food antigens underlies the mechanism of a nonallergic state and also for the resolution of food allergy (36). However, the production of IL-10 and TGF-β during the induction phase of the immune response may also play a critical role (37,38). The finding that in formula-fed, allergy-prone rat pups, TGF-β supplementation of formula results in an increase in IL-10 and Th1-type cytokines and an associated decrease in Th2-associated IgG1 as well as mast cell activation provides evidence for TGF-β being involved in promoting appropriate immune priming to

Table 1. *Cytokine mRNA in the spleen of naturally suckled and formula ± TGF-β–fed rat pups*

	D 18				D 28			
Cytokine	Naturally suckled	Naturally suckled + BLG	Formula fed	Formula + TGF-β fed	Naturally suckled	Naturally suckled + BLG	Formula fed	Formula + TGF-β fed
TNF-α	41.4 ± 9.9	36.9 ± 13.9	57.7 ± 9.9	8.9 ± 1.6*§	40.4 ± 4.8	50.1 ± 12.6	64.9 ± 17.7	32.8 ± 10.8
IFN-γ	16.4 ± 3.0	11.4 ± 1.4	8.6 ± 1.6	139.6 ± 42.8*§‡	38.1 ± 7.1**†	19.5 ± 4.1	20.7 ± 1.6	54.4 ± 6.2*§‡
IL-1β	182.7 ± 56.6†¶	87.7 ± 7.8	84.5 ± 11.0	84.5 ± 11.0	141.0 ± 26.8†¶	68.7 ± 18.3	58.1 ± 5.7	583.5 ± 64.9*§‡
MIF	599.9 ± 115.5	493.2 ± 63.9	418.1 ± 83.4	2755.2 ± 655.3*§‡	468.3 ± 48.6†	383.8 ± 47.4	211.5 ± 29.3	755.2 ± 49.8*§‡
IL-18	107.4 ± 37.4	135.1 ± 14.5	110.5 ± 20.5	431.5 ± 152.7*§‡	115.7 ± 12.6†¶	63.0 ± 26.2	42.5 ± 13.3	379.6 ± 63.5*§‡
IL-12p40	8.7 ± 2.2	7.0 ± 0.9	8.1 ± 0.8	82.6 ± 35.2*§‡	24.2 ± 4.8	15.5 ± 5.2	8.9 ± 2.0	79.6 ± 17.7*§‡
IL-1Ra	39.8 ± 22.2†	60.2 ± 7.4	85.7 ± 15.1	449.7 ± 204.1*§‡	62.2 ± 11.2†¶	28.9 ± 7.8	25.8 ± 6.7	325.3 ± 55.7*§‡
IL-12p35	11.8 ± 2.5	4.2 ± 1.4	2.3 ± 0.6	33.9 ± 13.9*§‡	1.0 ± 0.4	0.8 ± 0.3	0.6 ± 0.2	29.2 ± 6.7*§‡
IL-10	8.1 ± 2.5	3.4 ± 0.9	4.5 ± 1.2	42.9 ± 18.6*§‡	10.1 ± 2.7	6.6 ± 1.6	5.1 ± 1.4	32.8 ± 10.3*§‡
IL-5	5.2 ± 2.2	4.9 ± 1.2	6.3 ± 2.3	4.1 ± 1.1	3.0 ± 0.3	2.2 ± 0.8	3.0 ± 0.5	16.1 ± 2.8*§‡
IL-4	2.6 ± 0.8†¶	2.2 ± 0.7	5.5 ± 0.9*,¶	8.9 ± 1.6*§‡	6.9 ± 0.7	6.2 ± 1.4	6.8 ± 1.2	28.6 ± 4.9*§‡
IL-6	10.2 ± 2.3†¶	14.7 ± 3.2	12.0 ± 3.0	2.1 ± 0.7*§‡	3.5 ± 0.7	2.6 ± 1.2	3.6 ± 0.9	8.2 ± 1.2*§‡
IL-2	3.6 ± 1.4	2.4 ± 1.0	2.0 ± 0.4	3.2 ± 0.6	1.9 ± 0.7	1.5 ± 0.3	1.9 ± 0.4	20.7 ± 2.9*§‡
IL-1α	6.5 ± 2.7	2.5 ± 0.6	1.8 ± 0.3	1.8 ± 0.3	62.2 ± 11.2	28.9 ± 7.8	25.8 ± 6.7	32.5 ± 55.7

Values expressed as mean SEM ($\times 10^3$) units normalized to GAPDH. N = 8 for all groups. Significance indicated ($p < 0.005$) by the following symbols: * TGF-β vs formula fed; † naturally suckled vs formula fed; ‡ TGF-β vs naturally suckled + BLG; ¶ naturally suckled + BLG vs formula fed; § TGF-β vs naturally suckled; ** naturally suckled + BLG vs naturally suckled.

food antigens. The fact that down-regulation of the allergic response is maintained even after weaning has occurred has important implications and also leads to future studies assessing the role of IL-10 with and without TGF-β.

Acknowledgments. The author thanks Prof. Robert Gibson and Dr. Joanna Hawkes for their helpful comments and also Bruno Sergi and Helen Bougesis for their excellent technical assistance. This work was carried out with funding support from Dairy Australia

REFERENCES

1. Singhal A, Cole TJ, Lucas A 2001 Early nutrition in preterm infants and later blood pressure: two cohorts after randomised trials. Lancet 357:413–419
2. Moore VM, Davies MJ 2005 Diet during pregnancy, neonatal outcomes and later health. Reprod Fertil Dev 17:341–348
3. Muraro A, Dreborg S, Halken S, Host A, Niggemann B, Aalberse R, Arshad SH, Berg Av A, Carlsen K, Duschen K, Eigenmann P, Hill D, Jones C, Mellon M, Oldeus G, Oranje A, Pascual C, Prescott S, Sampson H, Svartengren M, Vandenplas Y, Wahn U, Warner JA, Warner JO, Wickman M, Zeiger RS 2004 Dietary prevention of allergic diseases in infants and small children. Part I: immunologic background and criteria for hypoallergenicity. Pediatr Allergy Immunol 15:103–111
4. Prescott SL, Macaubas C, Smallacombe T, Holt BJ, Sly PD, Holt PG 1999 Development of allergen-specific T-cell memory in atopic and normal children. Lancet 353:196–200
5. Kjellman NI, Nilsson L 1998 From food allergy and atopic dermatitis to respiratory allergy. Pediatr Allergy Immunol. 9:13–17
6. Van Odijk J, Kull I, Borres MP, Brandtzaeg P, Edberg U, Hanson LA, Host A, Kuitunen M, Olsen SF, Skerfving S, Sundell J, Wille S 2003 Breastfeeding and allergic disease: a multidisciplinary review of the literature (1966-2001) on the mode of early feeding in infancy and its impact on later atopic manifestations. Allergy 58:833–843
7. Wright AL, Stern DA, Halonen M 2001 The association of allergic sensitization in mother and child in breast-fed and formula-fed infants. Adv Exp Med Biol. 501:249–255
8. Sears MR, Taylor DR, Poulton R 2004 Response to "Breastfeeding, asthma, and atopic disease: an epidemiological review of the literature" by Oddy and Peat. J Hum Lact 20:148–150; author reply 150–152
9. Penttila IA, Flesch IE, McCue AL, Powell BC, Zhou FH, Read LC, Zola H 2003 Maternal milk regulation of cell infiltration and interleukin 18 in the intestine of suckling rat pups. Gut 52:1579–1586
10. Zhang M, Zola H, Read L, Penttila I 2001 Identification of soluble transforming growth factor-beta receptor III (sTbetaIII) in rat milk. Immunol Cell Biol 79:291–297
11. Pittard 3rd, WB Bill K 1979 Immunoregulation by breast milk cells. Cell Immunol 42:437–441
12. Wagner CL, Forsythe DW 2000 Effect of human milk and recombinant EGF, TGFalpha, and IGF-1 on small intestinal cell proliferation. Adv Exp Med Biol 478:373–374
13. Hawkes JS, Bryan DL, James MJ, Gibson RA 1999 Cytokines (IL-1beta, IL-6, TNF-alpha, TGF-beta1, and TGF-beta2) and prostaglandin E2 in human milk during the first three months postpartum. Pediatr Res 46:194–199
14. Saito S, Yoshida M, Ichijo M, Ishizaka S, Tsujii T 1993 Transforming growth factor-beta (TGF-beta) in human milk. Clin Exp Immunol 94:220–224
15. Garofalo R, Chheda S, Mei F, Palkowetz KH, Rudloff HE, Schmalstieg FC, Rassin DK, Goldman AS 1995 Interleukin-10 in human milk. Pediatr Res 37:444–449
16. Weiner HL 2001 The mucosal milieu creates tolerogenic dendritic cells and T(R)1 and T(H)3 regulatory cells. Nat Immunol 2:671–672
17. Okamoto A, Kawamura T, Kanbe K, Kanamaru Y, Ogawa H, Okumura K, Nakao A 2005 Suppression of serum IgE response and systemic anaphylaxis in a food allergy model by orally administered high-dose TGF-{beta}. Int Immunol 17:705–712
18. Lin H, Mosmann TR, Guilbert L, Tuntipopipat S, Wegmann TG 1993 Synthesis of T helper 2-type cytokines at the maternal-fetal interface. J Immunol 151:4562–4573
19. Kondo N, Kobayashi Y, Shinoda S, Takenaka R, Teramoto T, Kaneko H, Fukao T, Matsui E, Kasahara K, Yokoyama Y 1998 Reduced interferon gamma production by antigen-stimulated cord blood mononuclear cells is a risk factor of allergic disorders—6-year follow-up study. Clin Exp Allergy 28:1340–1344
20. Williams TJ, Jones CA, Miles EA, Warner JO, Warner JA 2000 Fetal and neonatal IL-13 production during pregnancy and at birth and subsequent development of atopic symptoms. J Allergy Clin Immunol 105:951–959
21. Penninks AH, Knippels LM 2001 Determination of protein allergenicity: studies in rats. Toxicol Lett 120:171–180
22. Knippels LM, Penninks AH, Spanhaak S, Houben GF 1998 Oral sensitization to food proteins: a Brown Norway rat model. Clin Exp Allergy 28:368–375
23. Knippels LM, Penninks AH 2002 Assessment of protein allergenicity: studies in Brown Norway rats. Ann N Y Acad Sci 964:151–161
24. Knippels LM, Penninks AH 2003 Assessment of the allergic potential of food protein extracts and proteins on oral application using the Brown Norway rat model. Environ Health Perspect 111:233–238
25. Dvorak B, McWilliam DL, Williams CS, Dominguez JA, Machen NW, McCuskey RS, Philipps AF 2000 Artificial formula induces precocious maturation of the small intestine of artificially reared suckling rats. J Pediatr Gastroenterol Nutr 31:162–169
26. Kalliomaki M, Ouwehand A, Arvilommi H, Kero P, Isolauri E 1999 Transforming growth factor-beta in breast milk: a potential regulator of atopic disease at an early age. J Allergy Clin Immunol 104:1251–1257
27. Klein SL, Bird BH, Glass GE 2001 Sex differences in immune responses and viral shedding following Seoul virus infection in Norway rats. Am J Trop Med Hyg 65:57–63
28. Bowman LM, Holt PG 2001 Selective enhancement of systemic Th1 immunity in immunologically immature rats with an orally administered bacterial extract. Infect Immun 69:3719–3727
29. Hill DJ, Hosking CS, Reyes-Benito LV 2001 Reducing the need for food allergen challenges in young children: a comparison of *in vitro* with *in vivo* tests. Clin Exp Allergy 31:1031–1035
30. Host A, Jacobsen HP, Halken S, Holmenlund D 1995 The natural history of cow's milk protein allergy/intolerance. Eur J Clin Nutr 49:S13–S18
31. Samsoe-Jensen T, Hauge-Kristensen K 1960 *In-vitro* fixation of skin-sensitizing antibodies to skin cells and mesenchymal tissue. Acta Allergol 15:202–207

32. Ju HR, Matsuura I, Yamada K, Sugano M, Imaizumi K 1995 Systemic release of mucosal mast-cell protease in primed Brown Norway rats after feeding with beta-lactoglobulin. Biosci Biotechnol Biochem 59:771–775
33. Turner MW, Boulton P, Strobel S 1995 Experimental intestinal hypersensitivity. Effect of four anti-allergic drugs on protein uptake, permeability to sugars and mucosal mast-cell activation. Clin Exp Allergy 25:448–455
34. Tiemessen MM, Van Ieperen-Van Dijk AG, Bruijnzeel-Koomen CA, Garssen J, Knol EF, Van Hoffen E 2004 Cow's milk-specific T-cell reactivity of children with and without persistent cow's milk allergy: key role for IL-10. J Allergy Clin Immunol 113:932–939
35. Karlsson MR, Rugtveit J, Brandtzaeg P 2004 Allergen-responsive CD4+CD25+ regulatory T cells in children who have outgrown cow's milk allergy. J Exp Med 199:1679–1688
36. Schade RP, Van Ieperen-Van Dijk AG, Van Reijsen FC, Versluis C, Kimpen JL, Knol EF, Bruijnzeel-Koomen Van Hoffen E 2000 Differences in antigen-specific T-cell responses between infants with atopic dermatitis with and without cow's milk allergy: relevance of TH2 cytokines. J Allergy Clin Immunol 106:1155–1162
37. Jutel M, Akdis M, Budak F, Aebischer-Casaulta C, Wrzyszcz M, Blaser K, Akdis CA 2003 IL-10 and TGF-beta cooperate in the regulatory T cell response to mucosal allergens in normal immunity and specific immunotherapy. Eur J Immunol 33:1205–1214
38. Zemann B, Schwaerzler C, Griot-Wenk M, Nefzger M, Mayer P, Schneider H, de Weck A, Carballido JM, Liehl E 2003 Oral administration of specific antigens to allergy-prone infant dogs induces IL-10 and TGF-beta expression and prevents allergy in adult life. J Allergy Clin Immunol 111:1069–1075