REVIEW ARTICLE

Development of natural tolerance and induced desensitization in cow's milk allergy

Emma M. Savilahti & Erkki Savilahti

The Hospital for Children and Adolescents, University of Helsinki, Helsinki, Finland

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Correspondence
Erkki Savilahti, Biomedicum 1, POB 700, 00029 HUS Helsinki, Finland.
Tel.: +358947171939
Fax: +358947175299
E-mail: erkki.savilahti@helsinki.fi

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Abstract

Cow's milk allergy (CMA) affects 2–3% of infants. It resolves in the great majority spontaneously during childhood. CMA encompasses a spectrum of clinical and immunologic characteristics. Non-IgE-mediated allergy typically resolves earlier than IgE-mediated allergy. The most documented prognostic characteristic is that intense-specific IgE response predicts persistence of CMA. Low serum levels of cow's milk (CM)-specific IgG4 are also associated with persistent CMA. Natural development of tolerance involves an immunologic shift where Th2 responses diminish, and Th1 as well as T regulatory cell responses strengthen. Accordingly, specific IgE levels decrease and specific IgG4, possibly also IgA, levels increase in serum. Specific oral immunotherapy (OIT) with CM induces desensitization in most cases where spontaneous recovery has not yet occurred. Data on long-term tolerance induction are still scarce. According to current research data, the immunologic changes induced by OIT resemble those seen during natural development of tolerance.

Cow's milk (CM) is often the first environmental antigen infants are orally exposed to. It elicits an immunologic response which in the majority leads to tolerance (1). In 2–3% of children, however, the antigen exposure results in cow's milk allergy (CMA) (2–5). CMA can be defined as an abnormal immunologic reaction to CM proteins, which is associated with an adverse clinical reaction. The only treatment option for patients has long been elimination diet until tolerance develops, and reactive treatment of allergic symptoms with antihistamines, locally or systemically administered corticosteroids and/or injectable epinephrine in case of CM ingestion (6). Several novel approaches to treat food allergies are at different stages of pre-clinical and clinical research (6). Data on specific immunotherapies in particular are accumulating, and these antigen-specific therapies may soon be applied in the clinical practice (7, 8). This review first gives an overview on the mechanisms of oral tolerance. It then describes the variation in clinical and immunologic characteristics of CMA, and how these are related to the development of tolerance. Next, we discuss the evidence on the role of specific antibodies in spontaneous development of tolerance. We proceed to the role of T cells, especially T regulatory cells (Tregs). We then briefly review clinical results from oral specific immunotherapies in CMA. Finally, we review current knowledge on the immunologic processes behind desensitization induced by oral immunotherapy (OIT). This reviews focuses on clinical studies and especially on the immunologic mechanisms underlying the development of tolerance.

Oral tolerance and cow's milk allergy

Oral tolerance results from the ability of the intestinal immune system to respond adequately to the multitude of external stimuli it encounters. A physiologically functioning intestinal immune system eliminates pathogens, tolerates harmless environmental antigens (such as food proteins and peptides), and maintains commensal bacterial flora. These tasks are optimally achieved with minimal tissue inflammation.

Food allergies are most commonly seen in infancy (5). Several factors may predispose infants to impaired oral tolerance. Gut barrier is not yet fully developed in infancy (9). The secretion of gastric acid and proteolytic enzymes may in infancy be suboptimal, which results in exposure to more allergenic antigens. IgA deficiency may increase the risk of food allergies; clinical data to date are, however, inconclusive (9, 10). The immune system during pregnancy and in infancy is Th2-deviated. If genetic and/or environmental factors impede the development of Th1/Th2 balance, it may lead to allergy.

A major predisposing factor for CMA is atopic heredity (4). Early exposure to small amounts of CM appears to increase the risk of IgE-mediated CMA, while non-IgE-mediated CMA is associated with early exposure to large amounts of CM (2, 11). Intestinal microbial flora may influence the development of tolerance or allergy (12). In a double-blind, placebo-controlled study, supplementation of peptide formula with Lactobacillus rhamnosus GG (LGG) in the treatment of infants with CMA accelerated the development of tolerance (13). One possible mechanism is that probiotic treatment increases IFN-ϒ expression, which is deficient in CMA (14).

Immunologic subtypes of cow's milk allergy

Immunologically, the key classification of CMA is in either IgE-mediated or non-IgE-mediated form (4, 6). The IgE-mediated form is usually defined as the patient having high levels of CM-specific IgE antibodies in serum or having a positive skin prick test (SPT) to CM. Cutoff for CM-specific IgE levels is commonly defined as 0.7 kU/l or for higher sensitivity (and lower specificity), 0.35 kU/l (4). CM-specific SPT is considered positive whether the wheal diameter is 3 mm or more greater than the negative control (4). The reaction to CM in IgE-mediated CMA is characteristically immediate: the symptoms arise within a few minutes to hours. They result from the release of proinflammatory mediators from mast cells and other effector cells upon cross-linking of surface-bound IgE antibodies by antigen. In young infants with immediate symptoms, the IgE response may not yet be increased at the onset of clinical CMA (11). Later, measurements of CM-specific IgE response may therefore classify patients more accurately (11).

In non-IgE-mediated CMA, the reaction is delayed and plausibly T cell mediated. Symptoms usually appear only several hours or days after allergen exposure.

Some patients show both immediate and delayed reactions and meet the criteria for IgE-mediated disease. They are variably classified as having IgE-mediated CMA or a combined form of the disease.

Allergens and diagnosis

The number of potentially allergenic proteins in CM is close to 20 (4), and patients are typically sensitized to several CM proteins. Sensitization to bovine serum albumin (BSA) is associated with allergic symptoms to both CM and beef (15). In cases where CMA affects an infant who is exclusively breastfed, the eliciting CM exposure may arise through breast milk or cutaneous exposure, and CMA may be sustained by cross-reactivity to proteins in human breast milk (16).

The gold-standard for CMA diagnosis is an oral, preferably placebo-controlled and double-blind CM challenge after a successful elimination diet. A history of CM-related symptoms combined with high levels of CM-specific IgE or positive CM-specific SPT make CMA plausible, but do not suffice for a reliable diagnosis. Non-IgE-mediated CMA is more difficult to diagnose than IgE-mediated CMA because symptoms are typically delayed and by definition tests measuring IgE response to CM are negative (6).

Natural history and prognosis

Cow's milk allergy typically emerges during the first year of life (4). The majority of children with CMA recover spontaneously. The pace of clinical recovery has varied in different studies. In most studies, the majority of children have become tolerant to CM by school age (Table 1). Tolerance can, however, develop even later (17). Some studies suggest that the pace of clinical recovery is slower than before, in conjunction with the increase in the prevalence of allergy (5, 18). However, published studies are not fully comparable especially because of different recruitment strategies: 3 (3, 11, 19) are population-based studies, while others (17, 20, 21) are based on patients referred to specialist clinics.

Asthma and allergic rhinitis (17, 22), urticaria (11), anaphylaxis, and severe symptoms overall (4) are associated with persisting CMA. The smaller the CM dose that elicits symptoms in oral challenge, the greater the likelihood for CMA persistence (19, 22).Patients who tolerate heated CM appear to recover earlier than those who do not, and incorporation of heated CM to the diet may accelerate the development of tolerance (23). Terracciano et al. (24) have reported, in contrast, that total avoidance of CM-derived products promotes the resolution of CMA. Polysensitization to other food allergens as well as inhalant allergens is associated with worse CMA prognosis (22). The role of CM-specific IgE in prognostics is discussed later in this review under the topic ''Immunoglobulin class E''.

Th1/Th2 balance in cow's milk allergy and tolerance

Studies on immune responses to CM proteins in patients with CMA have reported Th2 domination, Th1 repression and/or diminished TGF-β and IL-10 production. A study by Beyer et al. (25) compared cytokine production of gut-residing lymphocytes in patients with CM-associated gastrointestinal immunologic disorders and healthy individuals. Lymphocytes from patients produced more Th2 cytokines, and less IL-10 and TGF-β in response to CM stimulation *in vitro* (25). In another study on lymphocytes of duodenal mucosa, investigators observed lower levels of TGF-β production, but no Th2-skewing, in children with food allergy compared with healthy control subjects (26). Studies on CM protein–specific T-cell clones have shown that T cells from patients with CMA produce significantly higher levels of Th2 cytokines and lower levels of Th1 cytokines compared with healthy individuals (27, 28). Similarly, peripheral blood mononuclear cells (PBMCs) from patients with IgE-mediated CMA reportedly secreted more Th2 cytokines and less Th1 cytokine IFN-ϒ than healthy individuals in response to *in vitro* stimulation with CM proteins (29). A shift toward Th1 responses was seen in individuals who had become tolerant to CM after CMA (29). Development of tolerance appears to also involve a decrease in activated basophils (30).

Regulatory T cells in cow's milk allergy and tolerance

The balance between Th2 and Treg cells essentially determines whether the immune system tolerates an innocuous

Table 1 Summary of recovery from cow's milk allergy (CMA) in six independent studies (3, 11, 17, 19–21)

Age (yrs)	% of patients who recovered from CMA	Patient population (N)	IgE-mediated or non-IgE-mediated or both	References
1	56	39	Both	Host and Halken (3)
2	77			
3	87			
2	51	117	Both	Saarinen et al. (11)
5	74	86	IgE	
5	100	32	Non-IgE	
8–9	85	86	IgE	
2	41	54	IgE	Elizur et al. (19)
4	57			
2	44	162	Both	Vanto et al. (20)
3	69			
4	77			
4	68	66	Both	Garcia-Ara et al. (21)
4	19	807	IgE	Skripak et al. (17)
10	52			

Host et al. (3), Saarinen et al. (11), and Elizur et al. (19) are population based. Skripak et al. (17), Vanto et al. (20) and Garcia-Ara et al. (21) are based on patients referred to specialist clinics.

antigen or develops allergy (31, 32). A genetic defect in Treg function, that is, a variant of human immunodysregulation polyendocrinopathy enteropathy x-linked syndrome (IPEX) caused by a FoxP3 mutation, leads to severe food allergies (33). Moreover, weaker responses of effector T cells to suppressive Tregs may predispose infants to egg allergy (34).

Higher numbers of circulating $CD4^+CD25^+$ T cells were reported in children who had outgrown CMA and reintroduced CM in their diet compared with children who had active CMA (35). *In vitro* depletion of CD4CD25 cells from these newly CM tolerant children increased the CM antigen-specific proliferation of PBMCs (35). Shreffler et al. (36) compared circulating Tregs in four groups: (1) children who reacted to all forms of CM, (2) those who tolerated heated CM but reacted to unheated CM, (3) children who had fully recovered from CMA, and (4) children who had never had CM-related reactions. They found that children who tolerated only heated CM (group 2) had the highest percentage of proliferating CM-specific putative Treg cells, whereas children who had outgrown CMA (group 3) had intermediate percentages. Children who reacted to heated CM (group 1) and those with no history of CMA had lower percentages of these cells (group 4) (36). These cells were identified as $FoxP3^+CD25^{hi}CD27^+$, $CTLA4^+$, $CD45RO^+CD127^-$ (36). The study also provided functional evidence on the suppressive capacity of CD25(hi) cells extracted from patient samples (36). The results suggest that proliferation of allergen-specific Tregs in blood is seen during the development of tolerance, but not so much during the maintenance of tolerance. We investigated *in vitro* β-lactoglobulin stimulated PBMCs from children who either had active CMA or had become tolerant several years earlier, and in children with no atopy (37). Our study thus did not involve patients who had very recently developed tolerance like the previous studies by Karlsson et al. and Shreffler et al. We saw no difference in frequencies of $CD4^+$CD25(high) $FoxP3^+$CD127(low) cells, but the FoxP3 intensity in $CD4^+$CD25(high)CD127(low) cells was higher in children with CMA compared with non-atopic children (37). FoxP3 expression on RNA level, also, was highest in cells extracted from children with active CMA (37). We furthermore found that a combination of Treg- and Th2-related RNA expression markers could discern children with active CMA from those who tolerated CM (37). Our study thus suggests that the balance of Th2 and Treg responses is crucial in distinguishing allergy and tolerance. We also speculated that circulating PBMCs may not accurately reflect the immunologic status at the tissue site, that is, the gut.

Data on gut-residing Tregs in food allergy are rather scarce. Westerholm-Ormio et al. (38) studied duodenal biopsies from children with food allergy, or Crohn's disease, or no pathology. $FoxP3^+$ cells were more frequent in patients with food allergy compared with healthy subjects or with patients with Crohn's disease (38). Furthermore, untreated food allergy was associated with higher numbers of $FoxP3^+$ cells compared with patients on an elimination diet (38). The lower ratio of FoxP3 mRNA expression to the number of $FoxP3^+$ cells in patients with untreated food allergy suggested a lower activity of these cells in comparison with healthy subjects (38). Studies on gut-residing lymphocytes showed lower secretion of IL-10 and TGF-β in patients with food allergy compared with

healthy individuals (25, 26), which may result in lower numbers of Tregs in the gut mucosa.

Specific antibodies in the natural development of tolerance

Physiological antibody response to cow's milk

Lymphocyte priming to CM antigens appears to begin prenatally (39). Transplacental transfer conveys IgG class antibodies to CM from the mother to the fetus. Immune responses to food antigens are most intensive during first months of life and decline later in infancy. The physiological response to CM exposure is dominated by IgG class antibodies (40), IgG1 subclass in particular (1). Specific IgG levels increase within weeks after introduction of CM formula and peak 3–4 months later (1, 40). Specific IgA and IgM to CM are absent at birth, but even minimal exposure during breastfeeding induces antibody production (41). Also specific IgE antibodies are part of a physiological response, although in lower quantities than other immunoglobulins (42).

Immunoglobulin class E

When CM oral challenge is positive and a CM-specific IgE response exceeds a specified level, CMA is categorized as IgE-mediated, as discussed earlier regarding immunologic subtypes of CMA. A strong CM-specific IgE response, measured as SPT wheal size and/or serum CM IgE level, predicts the persistence of CMA (11, 17, 20, 22, 43). Periodic measurement of CM-specific IgE assists in assessing the speed of recovery. Increasing CM-specific IgE levels predict persistence of allergy, whereas decreasing levels point to faster recovery (17, 21, 43, 44). Non-IgE-mediated CMA resolves usually faster than IgE-mediated CMA (11). Several studies have reported that profiles of IgE binding to CM epitopes differ between persistent and transient forms of CMA (45–48). Persistent CMA tends to manifest in wider variety of sequential IgE epitopes (45–48). IgE binding to certain sequential epitopes, especially in caseins, (45, 46, 49) as well as stronger avidity of IgE binding to CM epitopes may also predict persistence of CMA (47). CM-specific IgE levels decline as tolerance develops, but may remain at levels usually considered pathologically long after tolerance has been achieved (43).

Immunoglobulin class G

A specific IgG4 response to an antigen may be physiological, the result of continuous exposure to the antigen (50). Early exposure to CM appears to elicit pronounced specific IgG production (51, 52). Upregulation of allergen-specific IgG4 production appears furthermore to be related to the development of tolerance. Most evidence comes from studies on allergen-specific immunotherapy showing that specific IgG4 levels increase and functionally block specific IgE binding as allergic symptoms subside (53). Increase in specific IgG4 levels during allergen immunotherapy has been further associated with increased Treg numbers and IL-10 secretion (53).

Natural development or maintenance of tolerance to food antigens without therapeutic intervention also involves specific IgG4. Ruiter et al. (54) showed that non-atopic individuals had higher levels of CM-specific IgG4 levels than subjects with CMA. We investigated CM-specific antibodies in a population drawn from a prospective, birth cohort-based study on the emergence of CMA that followed children with CMA from birth to the age of 8–9 yr (43). We observed that serum CM-specific IgG4 levels increased in children who recovered from CMA, and once tolerance had been achieved, the levels were higher compared with levels in children with active CMA (43). In a subpopulation from the same study, we furthermore showed that IgG4 binding to CM epitopes increased over time in patients who recovered from CMA by the age of 3 yr compared with children who had active CMA at the age of 8–9 yr (48). Wang et al. (47) have also reported IgG4 binding to a wider spectrum of CM epitopes in CMA that had subsided compared with active allergy. Kim et al. compared children who tolerated heated, but not unheated CM with children who reacted to all kinds of CM. They observed that casein IgG4 levels increased in children who tolerated heated CM, and many of them also developed tolerance to unheated CM (23).Observations in hen's egg allergy are similar to those in CMA. An increase in ovalbumin-specific IgG4 and decrease in specific IgE coincided with clinical improvement of symptoms in a population of patients with hen's egg allergy (55). In children with milk and/or egg allergy, those who had low levels of IgG4 to ovalbumin and/or β-lactoglobulin required a prolonged elimination diet (56).

The balance of IgE and IgG4 antibodies thus appears to be important in the development of tolerance. It plausibly reflects the underlying cytokine milieu and balance between T effector cells and Tregs. IL-4 induces class switching in B cells to both IgE and IgG4, whereas IL-10 promotes IgG4 and inhibits IgE production (57, 58).

Immunoglobulin class A

The large majority of IgA antibodies are secreted to mucosal surfaces, largely in the gut, in secretory form. SIgA plays an important role in oral tolerance. Indeed, high intestinal IgA in infancy may reduce the risk of IgE-mediated allergies (59). Low expression of sIgA in jejunal mucosa is reportedly associated with food allergy and high serum IgE levels (60). Low levels of sIgA in colostrum also appear to increase the risk for CMA, measured as total or CM-specific antibodies (61, 62).

Serum IgA levels in infancy are low compared with sIgA or with serum IgM and IgG. High allergen-specific IgA levels in saliva and serum have been associated with the emergence of allergy (63, 64). Allergen-specific IgA in serum may, however, also contribute to the development of tolerance. Successful aeroallergen immunotherapy appears to induce production of specific IgA in serum (65, 66). The production of IgA is induced rather by innate immunity signals than T helper cells (58). It is associated with local TGF-β expression, and IgA antibodies induce IL-10 production from monocytes (66). We have shown in a prospective clinical study that CM-

specific IgA levels in serum at the time of diagnosis were lower in children whose CMA persisted beyond the age of 8 yr compared with those who recovered earlier (43). Measurement of CM-specific IgA in serum could thus assist in the prognostics of CMA. We furthermore observed a positive correlation between CM-specific IgA in serum and saliva in children who had recovered from CMA, but not in children with persisting CMA (43). A study on children with allergic rhinitis also found a positive correlation with specific IgA levels in serum and saliva, and high specific IgA levels were associated with allergen tolerance (67). The data on the association of specific IgA and allergy are thus to date controversial.

Clinical data on specific oral immunotherapies of CMA

Studies on immunotherapies for food allergies have recently been reviewed (7, 8). Specific OIT to CM has successfully desensitized the majority of patients in a number of studies (Table 2) (68–74). Maintenance of desensitization relies on regular consumption of CM. Optimal result of OIT would be permanent tolerance, which would permit even infrequent CM digestion without allergic symptoms. Evidence for longer term tolerance is, however, scarce to date. A major obstacle for applying OIT in the clinic is the relatively high frequency and unpredictability of adverse reactions.

Immunologic changes in OIT induced desensitization

Several reviews on the mechanisms of specific immunotherapy have been recently published especially to honor 100 years of immunotherapy in 2011 [e.g., Akdis and Akdis, 2011 (53)]. We focus here on specific OIT for food allergens, especially for CM.

Desensitization to CM through OIT has been associated with a decrease in CM-specific IgE levels in some studies (68, 74–76), whereas others have reported no change (71, 72, 77). CM-specific IgG4 levels increases in successful OIT (71, 72, 76). Table 2 summarizes these results. The changes in specific antibody responses in OIT are in line with observations on natural development of tolerance in CMA.

Bedoret et al. (76) recently reported that during rapid high-dose oral desensitization with CM combined with omalizumab (IgE monoclonal antibodies) $CD4^+$T-cell response to CM initially declined rapidly, but recovered during the immunotherapy with a shift from IL-4 to IFN-ϒ production.

Data on the immunologic mechanisms in specific OIT to other food allergens are plausibly relevant to CM OIT, too. In a double-blind placebo-controlled trial on peanut OIT, the secretion of IL-5 and IL-13 by PBMCs decreased and the expression of FoxP3 increased from baseline in the active group, while no change occurred in the placebo group (78). Jones et al. (79) have reported several immunologic changes during peanut OIT. They observed that specific IgE decreased, whereas IgG4 increased, and that factors in serum inhibited the binding of IgE to peanut proteins (79). Activation of basophils declined, and the secretion of IL-10, IL-5, IFN-ϒ, and TNF-α from PBMCs increased after peanut protein stimulation (79). Furthermore, the number of $FoxP3^+$ cells first increased and then returned to baseline (79).Genes related to apoptotic pathways showed downregulation in T-cell micro-

Table 2 Clinical and immunologic results from studies on cow's milk (CM) oral immunotherapy. Studies with <10 patients are not included

Study	Study setting	Treatment control N	Desensitization achieved; partial desensitization treatment control N (%)	CM-specific IgE IgG4 (if defined) levels during desensitization
Meglio et al. (69)	Open	21	15 (71); ND	↔
	No control group	0		
Morisset et al. (80)	Open	27	24 (89); ND	↓
	Control:CM elimination	30	18 (60)	
Skripak et al. (71)	Double-blind	12	12 (100); 0	↔
	Placebo-controlled	7	0 (0)	↑
Longo et al. (73)	Open	30	9 (36); 16 (54)	↓
	Control:CM elimination	30	0	
Pajno et al. (72)	Single-blind	15	10 (67); 1 (6)	↔
	Control:soy-milk	15	0 (0)	↑
Martorell et al. (68)	Open	30	27 (90); ND	↓
	Control:CM elimination 2-yr-olds	30	7 (23)	
Alvaro et al. (74)	Open	66		↓
	No control group 22 non-anaphylactic (NA) 44 anaphylactic (A)	22, 44	NA: 16 (73); 6 (27) A: 35 (80); 7 (16) No control	

ND, not determined.

arrays (79). Peanut OIT thus appears to induce changes in specific antibody balance, inactivation of basophils, activation of T regs and secretion of tolerogenic cytokines, and changes in T cell survival.

Conclusions

The majority of infants develop oral tolerance to food antigens. Genetic background, prenatal priming, transmission of maternal antibodies, breast milk composition, gut barrier function, and intestinal flora are some of the central factors that affect the risk of developing CMA. Optimal timing and dosage of CM exposure to avoid sensitization is presently unknown.CMA usually appears shortly, within weeks, of introduction of CM in infancy and resolves by school age. Intense-specific IgE response to CM predicts prolonged persistence of CMA. Development of tolerance in CMA appears to involve a shift from Th2 dominant response toward Th1 type responses. Increase in numbers and/or activity of Tregs furthermore suppresses Th2 responses. Tregs express IL-10 and TGF-β, which among other functions induce in B cells the production of IgG4 and IgA. Specific IgG4 and possibly also IgA antibodies promote tolerance to allergens. Specific IgE levels gradually decrease as tolerance develops. Similar changes in specific antibody levels occur during CM OIT that successfully induces desensitization.

We still do not fully understand what happens when CMA spontaneously subsides. One of the key questions is what triggers the development of tolerance. CMA is a model for natural shift from allergy to tolerance, and research in the field thus has potential implications in understanding and treating other allergies, too. Research on CM OIT is active and shows promising clinical results. It is bound to also provide data on the mechanisms of desensitization, or tolerance at best. Some of the further topics for future research are the roles of innate immunity and dendritic cells in the natural and induced development of tolerance in CMA.

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