The Role of Immune Tolerance in Allergy Prevention

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Introduction

Immune tolerance is an essential mechanism which maintains a state of unresponsiveness to autoantigens and food while generating protective immunity against pathogens. This phenomenon was discovered by the fact that exposure to an antigen before the development of an immune response specifically abrogates the capacity to respond to that antigen in later life [1, 2]. Tolerance-inducing strategies have been demonstrated in animal models of autoimmunity, allergy and transplant graft rejection and therefore have opened the way for testing such approaches in human diseases. Immune tolerance can be established by respiratory or oral exposure to the allergen.

Processes that regulate peripheral tolerance involve clonal anergy, clonal deletion or active suppression by regulatory cells (fig. 1). Inhibition of T-cell costimulatory molecules at the cell surface has been reported to play an important role in T-cell tolerance [3–5]. The T cells could be anergized in experimental models that bypass costimulatory signals [6–8]. The interaction between B7 and CD28 may determine whether a T-cell response develops. For example, blocking antibodies to B7–2 inhibit the development of specific IgE and allergic symptoms in mice [9].

Regulatory T Cells

Over the last years, a great deal of interest has focused on regulatory T (Treg) cells that appear to control the development of autoimmune disease, transplant rejection and play a central role in controlling the expression of asthma and allergy.
Animal experiments have shown that Treg cells can suppress both Th1 and Th2 responses in vivo. In vitro engineered allergen-specific Treg cell lines protected mice from developing allergen-induced Th2 responses [10]. In humans it has been speculated that Treg cells secreting IL-10 are involved in the suppression of allergic Th2 responses. Several clinical studies supported this hypothesis [11–13] and others not [14].

Different types of Treg have been described which suppress immune responses via cell-to-cell interactions and/or the production of suppressor cytokines.

Tr1 cells were described [15] following in vitro activation of naïve CD4+ T cells in the presence of IL-10 which gave rise to CD4+ T cells with low proliferative capacity that produced high levels of IL-10, low levels of IL-2 and no IL-4. Such antigen-specific T cells suppressed the proliferation of CD4+ T cells in response to antigen.

Th3 cells were induced by oral feeding of low doses of antigen in a T-cell receptor-transgenic experimental encephalitis model [16, 17]. The CD4+ T cells isolated from mesenteric lymph nodes in such orally tolerized animals

**Fig. 1.** Induction of tolerance through either lack of costimulation (leading to anergy or deletion) or by the suppressive action of T-regulatory cells.
produced high levels of transforming growth factor (TGF)-β, and variable amounts of IL-4 and IL-10 upon activation with the antigen. TGF-β and IL-10 are critical as treatment with neutralizing antibodies abrogated the disease-protective effects of these cells.

CD4+CD25+ Treg cells were described by Sakaguchi et al. [18] as a subfraction of CD4+ T cells which play a critical role in the prevention of autoimmunity, allograft rejection and maintenance of self-tolerance. Elimination of CD4+CD25+ T cells leads to spontaneous development of various autoimmune diseases, such as gastritis, or thyroiditis in genetically susceptible hosts. These regulatory cells suppress immune responses through direct cell–cell contact in a process that is dependent on signalling via CTLA-4, as well as secretion of TGF-β. Several hypotheses exist on the origin of these regulatory cells. It was proposed that thymic differentiation accounts for CD4+CD25+ T cells that are specific for self-peptides and are devoted to the control of autoimmune responses, whereas peripheral differentiation may be required for environmental antigen-specific T cells for which an undesired immune response results in pathology [19].

Other regulatory cells have been reported like CD8+CD25+, which may play a role in oral tolerance [20, 21], or γδT cells and also regulatory dendritic cells.

Oral Tolerance

Oral administration of protein antigens induces immunologic hyporesponsiveness (tolerance) to these antigens. Induction of oral tolerance has been well documented with a number of antigens in several animal models. It was first reported by Wells and Osborne [22] with guinea pigs. They noted that anaphylactic reactions to ovalbumin (OVA) could be inhibited by prior oral administration of OVA to these animals.

Immune regulation by the induction of oral tolerance to food antigens is thought to prevent food allergy [23]. It has been shown that induction of oral tolerance is dependent on the age of the host [24], the dose of antigen administered, the nature of the antigen and microbial environment. This last point has been the subject of a number of hypotheses and experimental work in the last years. The interrelationship between microbes and the induction of allergy or oral tolerance involves the commensal bacteria that colonize the gastrointestinal tract. Microbial stimulation seems to provide counter-regulatory signals necessary to overcome the inherent Th2 bias of the mucosa-associated lymphoid tissue to prevent allergic disease [25]. It was reported that administration of a food allergen with a mucosal adjuvant induces allergen-specific IgE and anaphylactic symptoms in strains of mice lacking a functional receptor for bacterial lipopolysaccharide (TLR-4) but not in major histocompatibility complex-matched controls.
The importance of age has been highlighted in several studies. In neonatal rodents, oral exposure to antigen was reported to induce tolerance or priming depending on the age at the first antigen administration [26]. Starting from 7 days of age, oral administration of antigen leads to induction of oral tolerance. In a rat model we showed that oral antigen administration by the age of 14 days leads to the strongest downregulation of specific IgE responses on subsequent challenge with the antigen.

The underlying immunologic mechanisms involved in oral tolerance induction have not been fully elucidated, but recent studies suggest that antigen-presenting cells like intestinal epithelial cells and dendritic cells as well as the above-cited regulatory cells play a central role. Dendritic cells residing within the lamina propria and Peyer's patches express IL-10 and IL-4, which favor the generation of tolerance. It has been suggested that T cells primed in the local mucosal environment lead to tolerance induction. Studies in mice suggest that tolerance can be induced by one of several mechanisms: low-dose tolerance (repeated administration of protein antigens at low doses) which results from the activation of Treg cells that secrete inhibitory cytokines (e.g. TGF-β or IL-10), or high-dose tolerance (administration of a single high dose of protein antigen), which results from either clonal anergy or clonal deletion [27]. Experiments in mice have also recently shown that CD4+CD25+ T cells may play an important role in oral tolerance induction [28–31].

The phenomenon of oral tolerance has been well characterized in selected mouse strains but there are significant strain differences in terms of the ability to induce oral tolerance [32]. Oral tolerance to food antigens was also shown in several rat models [33, 34]. In humans, experimental oral tolerance induction was also attempted [35] by feeding volunteers keyhole limpet hemocyanin. It was observed that tolerance was induced at the T-cell compartment (reduction of T-cell proliferation and delayed-type hypersensitivity responses) but not at the humoral level. Recently published work [27], also using human volunteers fed keyhole limpet hemocyanin, showed that oral administration does not result in tolerance in Crohn's disease or ulcerative colitis patients on the contrary to normal controls. This may reflect an in vivo functional defect in mucosal suppression of immune responses in these patients.

**Dietary Intervention for Induction of Oral Tolerance**

**Importance of Antigen Structure**

The majority of tolerogens are soluble proteins. Larger particulate antigens, aggregated or heat-treated soluble proteins, lose their capacity to induce oral tolerance. Although oral tolerance to dietary proteins has been extensively investigated with intact antigens, few studies with antigen
fragments or digests have been done. We have shown [34] that moderately hydrolyzed whey proteins are able to induce oral tolerance to intact whey proteins, whereas extensively hydrolyzed proteins are unable to achieve this (fig. 2). This was confirmed in a recent publication [36]. Up to now the mechanisms of oral tolerance induction with hydrolysates are not very clear, but medium-sized peptides appear to play a central role. So-called ‘tolerogenic peptides’ have been described in the literature to occur in the serum after feeding OVA to animals. A number of clinical studies have shown that for primary prevention of atopy in infants with a positive family history, partially hydrolyzed infant formulas are found useful to avoid cow’s milk allergy and atopic symptoms [37, 38]. Long-term prevention has been observed in these studies, which may be due to induction of long-lasting oral tolerance with such formulas.

**Intervention during Pregnancy**

Animal models have shown that active induction of tolerance to dietary antigens before birth, via nutrition of the pregnant mother, is an effective
means of primary prevention in the offspring. Several authors [39–41] demonstrated tolerance induction to soya proteins, bovine serum albumin or cow’s milk proteins in the offspring of rabbits and guinea pigs respectively fed these different dietary proteins. We have shown in recent experiments done in a rat model of IgE suppression that oral tolerance to cow’s milk proteins can be transferred from the mother to the offspring and that this phenomenon can also be achieved with cow’s milk protein hydrolysates [42]. In these experiments it was interesting to observe that the downregulation of the IgE response was antigen-specific and that cow’s milk protein peptides were as efficient as intact cow’s milk proteins for inducing oral tolerance in the offspring by feeding mothers during pregnancy (fig. 3).

**Fig. 3.** In utero induction of oral tolerance in rats with intact or hydrolyzed whey proteins. Protocol: Throughout pregnancy adult Sprague-Dawley rats were given intact whey proteins (group A), trypsin hydrolyzed whey proteins (group B) or water (group C) in drinking bottles in addition to conventional chow. Four weeks after birth of the offspring, rats were immunized subcutaneously with β-lactoglobulin (βLG), ovalbumin (OVA) and Al(OH)3 as adjuvant. Fourteen days later, rats were sacrificed and serum analyzed for specific IgE. Results: The offspring of mothers fed whey (group A) or a whey hydrolysate (group B) during pregnancy had a strongly suppressed IgE anti-βLG antibody response if compared to controls (group C).

**Intervention during Breastfeeding**

Breast milk contains a number of factors which may promote the development of the infant’s immune system. Low levels of food allergens in breast milk, arising from the mother’s diet, may also play an important role in the induction of oral tolerance of the infant. Further, recent work has shown that the quality of fatty acids ingested by the mother may have effects on the development of immunological tolerance to dietary antigens in the offspring. It was reported that Sprague-Dawley rats fed a diet deficient in essential fatty acids, rather than the one enriched with essential fatty acids, favored the induction of oral tolerance in neonatal rats via their mothers [43]. Similar
further experiments showed that the dietary ratio of n-6 to n-3 fatty acids influences the induction of tolerance to OVA in neonatal rats [44].

To date in humans, it has not been clearly demonstrated if an allergen-reduced diet by lactating mothers has a long-term protective effect on the occurrence of atopic symptoms in infants despite observations that avoidance of milk and eggs during lactation may benefit some breastfed high-risk infants with eczema.

**Importance of Gut Flora**

The effect of the gastrointestinal microflora on the induction and maintenance of oral tolerance to dietary antigens has been studied in several animal models with contrasting results. Oral administration of OVA was able to induce oral tolerance in axenic (germfree) mice, but the maintenance of tolerance was found to be of shorter duration than with conventional mice [45]. On the contrary, in other work [46], the intestinal bacterial flora was shown to be required for the development of an IgE-production system susceptible to oral tolerance induction. It also appears that the contribution of the microflora to oral tolerance depends on the antigen used [47]. It is further well known that bacterial endotoxins (e.g. cholera toxin) may abrogate oral tolerance to an antigen co-administered through the oral route. A recent study shows further that, in mice, infection with *Helicobacter felis* can prevent the development of oral tolerance to OVA. These results indicate that chronic infection with *Helicobacter* inhibits the establishment of oral tolerance by preventing IgE suppression, normally induced after OVA feeding [48].

In humans, it was observed that in comparison with healthy infants, babies who developed allergies were less often colonized with enterococci during the first month of life and with bifidobacteria during the first year of life. It was therefore proposed that early colonization may affect the development of mucosal tolerance, as perinatal administration of probiotics to infants at risk of allergy induced a reduction in eczematous symptoms later on [49].

**References**

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Discussion

Dr. Kaminogawa: My first question is, why does microflora increase the induction of oral tolerance? My second question is, is it useful to increase the induction of oral tolerance with probiotics or not? Thirdly, I don't think that the regulatory T cell is so important in the induction of oral tolerance; I have some evidence, but please give me your opinion.
Dr. Fritsché: To the first question, I think that the flora is really important but the proof for this is not complete because there are contradictory results from animal models. It was shown that, in germ-free animals, the flora does not appear to be important for oral tolerance induction, only for its maintenance [1]. On the contrary, other work has shown that the intestinal bacterial flora is essential for the induction of oral tolerance [2]. So the question is open here, but I believe strongly that flora might be important for at least the maintenance of oral tolerance. The other question was on the effect of probiotics?

Dr. Kaminogawa: Yes, are probiotics important for the induction of oral tolerance or not? What do you think?

Dr. Fritsché: The experiments by different groups on probiotics have shown that one might have allergy prevention by giving the mothers different probiotic strains either during late pregnancy or early infancy. This study was done by Isolauri’s group, and clearly there is some downregulation of eczematic symptoms in the groups receiving probiotics. So it appears important. Is this downregulation due to the induction of oral tolerance? We don’t know. It may be another phenomenon, it may be an immune deviation directly without going through anergy, deletion or T-regulatory cells. This has not been clarified to date. Please remind me of your third question.

Dr. Kaminogawa: You said that regulatory T cells are most important in the induction of oral tolerance, but I don’t think so. You mentioned publications on the importance of the CD4+CD25+ T cells, but we cannot repeat these experiments.

Dr. Fritsché: Published work exists in animal models [3] where the authors depleted the CD4+CD25+ cells and they could not induce anymore tolerance. In the human situation as well, there is upregulation of CD4+CD25+ cells in infants being orally tolerized to cow’s milk proteins by the age of 2–3 years [4]. So there is indirect proof that this population of cells is very important.

Dr. M. Hoekstra: I think the last study you referred to was recently published by a group from Norway in the Journal of Experimental Medicine [4]. They showed that the children who outgrew their cow’s milk allergy had an increased number of regulatory T cells.

Dr. Heymans: In the past decades clinical evidence has shown that partial hydrolysates have a comparable preventive effect for food allergy to that of breast milk. Now we are using cow’s milk as a whole protein with no physiological background. I think the only reason we do it is because we have a lot of cows, especially in this country, even more than inhabitants, and they produce enormous amounts of milk. So why shouldn’t we use a partial hydrolysate in a normal formula? It has beneficial effects as we have shown; it has the same nutritional effects [5]. So why we shouldn’t do it?

Dr. Fritsché: My personal view is that we should use it. There is no disadvantage using partially hydrolyzed formulas for so-called normal infants because one can never be sure that these infants really are normal, because the only history markers are the parents or siblings who are atopic or not. By restricting the administration of partially hydrolyzed formulas to at-risk groups of infants, one may end up missing a lot of indications because it has been shown that normal infants, representing only 15% at risk of atopy, represent the majority of cases who later on contract atopies and allergies.

Dr. Heymans: So let’s ask Dr. Hernell his opinion about this because he is involved in these regulations.

Dr. Hernell: One first question is, what is a partial hydrolysate?

Dr. Fritsché: Unfortunately I don’t have the slides to show you exactly what it is. It is a cow’s milk whey-based formula, 100% whey proteins.

Dr. Hernell: I know what it is prepared from, but how can you define a partial hydrolysate in a useful way?
Dr. Fritsché: We have different definitions. In Europe one of the definitions is that its antigen-active capacity should be reduced by a factor of 100, at least according to the European Commission directives. So you have on one hand this allergenicity reduction, and on the other hand we have proven that it should also induce oral tolerance to cow’s milk proteins. I think there are two parameters here which very strongly narrow the room of activity of such formulas. This is based on in vitro and animal studies, but in the human situation they should really prevent cow’s milk allergy. It is clear, you have to do human studies to show their efficacy.

Dr. Hernell: There are some long-term clinical studies on allergy prevention, but I don’t think they are very convincing with respect to partial hydrolysates. Again I don’t think you can really use an in vitro definition based on molecular size or a reduction in the number of epitopes to classify a partial hydrolysate. It is really difficult to know what is meant when you talk about the partial hydrolysate. My second question refers to your statement that with an extensive hydrolysate oral tolerance doesn’t develop. What happens after intact food protein has been introduced? I think that is what is important, rather than what is found during the experimental period.

Dr. Fritsché: This is exactly the reason why I recommend partial hydrolysates, at least something is being done to the immune system. With extensively hydrolyzed formulas nothing at all is being done; the immune system cannot be modulated with extensive hydrolyzed formulas. So when intact formulas are reintroduced later on there is a great chance that the immune system has not evolved very much, which may result in allergic sensitization to intact proteins rather than tolerization.

Dr. Hernell: I think that is an interesting hypothesis but has it actually been proven?

Dr. Fritsché: No, it has not been proven. We are presently doing a comparative study in infants between extensively and partially hydrolyzed formulas on an exclusive basis, without breastfeeding. This should give us some clues for indicating one or the other formula.

Dr. Hernell: What are the outcome variables in that study?

Dr. Fritsché: The usual ones: SCORAD, double-blind placebo-controlled challenge.

Dr. M. Hoekstra: But also parameters of the immune system?

Dr. Fritsché: Yes, absolutely, everything we can do.

Dr. M. Hoekstra: When you refer to the immune system, do you mean IgE or other immune parameters?

Dr. Fritsché: We are doing IgE, IgG1, IgG4, and we are doing cytokine of lymphocyte stimulation profiles.

Dr. M. Hoekstra: And looking at regulatory T cells as well?

Dr. Fritsché: No, not in this study. It is pretty difficult to get enough blood from babies at this young age.

Dr. Aggett: I share Dr. Hernell’s reaction to the need to know what the partially hydrolyzed formula actually is. In terms of your studies, the thing that it is lacking, and is going to be lacking for many subsequent studies, is actually knowing what should be given to the babies. It would be very important to have some feeling to being able to characterize the proteins, not just grossly but also knowing what epitopes are present because what it would be fascinating to know what is inducing oral tolerance. Is it an intact antigen or is it a partial epitope? It is an epitope that is actually released so it has a greater opportunity to interact with whatever sensitizers are present. Do you have any feeling about these aspects?

Dr. Fritsché: We did a lot of work trying to identify the epitopes linked to, for example, tryptic peptides of β-lactoglobulin. We have not succeeded in this task because it is perhaps a multi-epitope here, but we have purified some candidates which could be associated with middle molecular weight peptides. We know that
intact traces of \( \beta \)-lactoglobulin of cow's milk proteins are not present in partially hydrolyzed formulas and, if you remember a picture I showed you, if you try to induce oral tolerance in animal models with intact proteins and partially hydrolyzed formulas, you have the same success of downregulation of the IgE response at all different doses. It means that if there were some contamination of intact proteins in partially hydrolyzed formulas then the low dose would not induce anymore tolerance compared to intact formulations, but there must be protein peptides associated with inducing tolerance. But as I said we are not at the end of this process yet.

**Dr. Siafakas:** It is a common practice to give corticosteroids to premature babies for lung maturity. I wonder if there are any animal or human studies with regard to the prenatal administration of corticosteroids regarding the impact that this on oral immunity postnatally?

**Dr. Fritsché:** I am sorry I am not aware of such studies.

**Dr. Exl-Preysch:** I would like to come back to the question of eHF and pHF in allergy prevention. The GINI study could perhaps give us some hints concerning the induction of oral tolerance [5, 6]. First of all, the comparison of two eHF with each other gave the result that one (eHF-casein) was effective and the other (eHF-whey) was not effective at all. Therefore the still existing dogma that 'eHF is better than pHF' has to fall, because pHF was as good as the effective eHF-casein after 3 years and the other eHF not at all.

In addition, the sensitization dates after 1 year showed clearly that the pHF had the lowest levels of sensitization against any allergens they looked into (cow's milk protein, egg allergens, 5 food allergens, 5 aero-allergens). Those results indicated for the first time what we have been finding in animal models for years: pHF induces oral tolerance at a higher level than eHF! That is what we are searching for in allergy prevention!

I would also like to draw your attention to the fact that years ago we conducted a study in Switzerland on a regular newborn population that was either fed allergen-reduced (breastfeeding and/or HE pHF) formula or regularly fed (breastfeeding and/or regular infant formula) [7–9]. Even after 2 years the population fed the allergen-reduced formula had only half of the skin symptoms than the regularly fed infant population. Again, a reason why we should feed all non- or partially breastfed infants an allergy-reduced pHF formula!

**Dr. Hernell:** You mentioned that there is a critical window during which it is easier to induce oral tolerance which occurs around day 14 if I remember correctly, I believe that has also been shown in mice, but how much is really known about if there is a critical window in humans?

**Dr. Fritsché:** In humans I think there are no data showing this.

**Dr. M. Hoekstra:** It is speculated that it would be the first 18 or 24 months of life. Compared to a rat life of 2 years, the equivalent of 14 days in a rat’s life would be 18 months in humans. But I think there is no more evidence than that.

**Dr. Bueno:** I want to come back to the question related to the role of T cells, particularly in oral tolerance, and you mentioned the change in the Th3 profile. What is your opinion about the fact that some specific cytokines like TGF-\( \beta \) could be interesting surrogate markers for oral tolerance?

**Dr. Fritsché:** Absolutely, the cytokines secreted by Th1 and Th3 are very important, TGF-\( \beta \) and IL-10. I think there are a number of animal studies which have shown the importance of IL-10 at least in modulating the immune system for downregulation, that is clear, they are mandatory. About the CD4+CD25+ cells, people actually think that it is a cell-to-cell contact but nobody knows if they do not also secrete cytokines which may also be very important.

**Dr. Sinaasappel:** In your scheme I missed the possible role of dendritic cells in the intestine. I was wondering how you imagine that the antigens are exposed to the body?
Dr. Fritsché: I think dendritic cells are in the center of the actual research activities in this domain because they have the capacity to leave the mucosa and fish the antigens through the mucosa layer. They have been categorized into different classes of dendritic cells, those inducing Th1 and Th2, so activity is strongly focused on this class of cells.

Dr. M. Hoekstra: In your rat experiments comparing partial and extensive hydrolysates, did you look at the intestinal inflammatory response in these two groups?

Dr. Fritsché: Yes, we looked at the triggering effect on intestinal mast cells because here you have sensitized mast cells lining the mucosa which are or are not covered by IgE.

Dr. M. Hoekstra: But you didn’t look at antigen-presenting cells or T-cell infiltrate?

Dr. Fritsché: Not in these old experiments.

Dr. M. Hoekstra: We would really like to know something about the mechanism. Why it does work in partial but not in extensive hydrolysates?

Dr. Schmitz: I would like to ask two questions. One is related to what you said about the partial hydrolysate having a kind of tolerance-inducing ability. How would you reconcile this with the fact that it has been well shown in clinical practice that sometimes partial hydrolysates have raised acute allergic reactions when refeeding the child with normal cow’s milk? The second question relates to what you said about quantity, nature and time of feeding of antigens. What makes the difference between casein, for example, or β-lactoglobulin to which allergy will decrease after 18 months or 2 years usually, and ovalbumin to which allergy will last for years and years? Have we a clue to the reason why in one case it tends to disappear and in the other case it does not?

Dr. Fritsché: I think no animal model for the moment has answered this question but it is observed in the human situation that sensitization to eggs is very early and it lasts longer; for peanuts it is very strong and it never goes away, so I don’t think I can answer your question. Now on the refeeding of hydrolyzed formulas, do you mean that these were infants who were prevented with hydrolyzed formulas only or mixed with breastfeeding?

Dr. Schmitz: If I remember correctly, it was in children fed only with partially hydrolyzed formula.

Dr. Fritsché: But perhaps the formulas were used not in normal or at-risk infants but in already sensitized infants; because we always advise not to give partially hydrolyzed formulas to already sensitized infants.

Dr. Heymans: There are publications on children with proven cow’s milk allergy [11–13]. It is stated that it can’t be used it as treatment, it can only be used in prevention.

References

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