Cow's milk allergy

Avoidance versus tolerance:

new concepts for allergy management

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2011

ISBN/EAN: 978-90-39355442

Cover design: Harold Kuiper, Terralemon, fresh design for interaction, motion & print, Amsterdam

Thesis lay-out: Bastiaan Schouten

Printed by: GVO | Ponsen & Looijen B.V., Ede, the Netherlands

The printing of this thesis was financially supported by: Danone Research - Centre for Specialised Nutrition, Danone Baby Nutrition, Nutricia Advanced Medical Nutrition, Nutricia Nederland and the J.E. Jurriaanse stichting.
The printing was further supported by: Special Diet Services, Plexx B.V., Quadratech Diagnostics; Costar, Greiner and BD Biosciences.

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Koemelkallergie

Eliminatie versus tolerantie:

nieuwe concepten voor allergiemanagement

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht
op gezag van de rector magnificus, prof.dr. G.J. van der Zwaan,
ingevolge het besluit van het college voor promoties,
in het openbaar te verdedigen op
woensdag 18 mei 2011 des middags te 2.30 uur

door

Elisabeth Catharina Adriana Maria Lobato-van Esch

geboren op 16 mei 1964 te Loon op Zand

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In memory of my brother Marno

In herinnering aan mijn broer Marno

Contents

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General Introduction

Food allergy affects about 4-5% of young children with a prevalence of 2-3% for cow's milk and hen's egg (1), the first and most common types of allergies during early infancy. Food derived allergens, aeroallergens, drugs and insect venoms are the major allergens encountered that are responsible for induction of type1-hypersensitivity reactions after initial sensitization. Type I allergic reactions to food proteins leading to food allergy are characterized by T helper 2 (Th2) polarization of the immune response resulting in the production of allergen-specific IgE (sensitization phase). Binding of IgE to the high affinity receptor FcεR1 on mast cells followed by subsequent cross-linking of the receptors by the allergen provokes mast cell degranulation (effector/challenge phase; figure 1). The release of mediators such as histamine, leukotriens and cytokines results in clinical symptoms involving the skin, gastrointestinal tract, airways and sometimes anaphylaxis within a few minutes to one hour after ingestion of the food. Approximately 80% of cow's milk and 70% of hen's egg allergies are outgrown at young adulthood (2, 3). However, IgE mediated

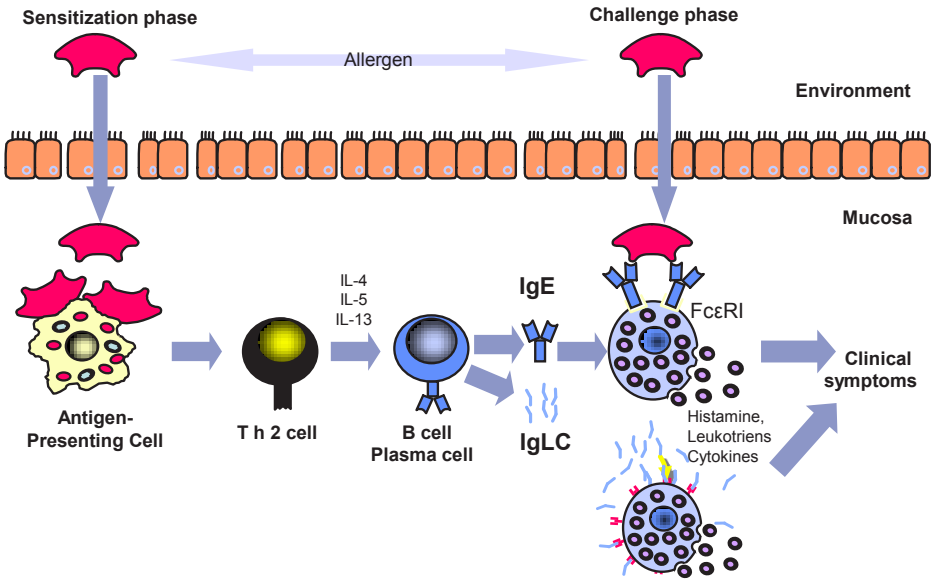

Figure 1. *Schematic overview of the sensitization and the effector/challenge phase in the immunological response to food allergens. During sensitization, allergens cross the mucosal barrier and are sampled by antigen presenting cells and presented via MHC-II to naïve T-cells that develop into Th2-cells. Cytokines produced by Th2 cells induce isotype switching in B-cells, resulting in the production of IgE antibodies that bind to the FcεRI receptor on mast cells. Upon a second encounter with the same allergen cell-bound IgE is cross-linked leading to degranulation and the release of mediators (i.e. histamine) that cause the clinical symptoms. Recent studies showed that immunoglobulin free light chain (IgLC) can induce acute type hypersensitivity responses similar to IgE (8).*

cow's milk allergy might predispose for the development of other allergies and even asthma later in life (4-7) (see review chapter 2 of this thesis).
About 60% of food allergic patients suffer from IgE-mediated allergic responses. However, it should be realized that in allergic diseases like food allergy, atopic dermatitis, rhinitis and asthma, a considerable number of patients exhibit clinical features without detectable local or systemic IgE. A novel potential mechanism for the elicitation of immediate hypersensitivity-like reactions via immunoglobulin free light chain (IgLC) has been described in mice (8). Human studies confirm the significant role for IgLC, in non-IgE mediated rhinitis patients both lambda and kappa IgLC concentrations were enhanced in nasal secretions (9). More studies are needed to provide further insight on the role of IgLC in the development of allergic responses (figure 1).

Whilst it is established that there is a strong genetic factor contributing to food hypersensitivity it is hypothesized that factors responsible for the increasing number of children suffering from allergic reactions may be found in changing environmental conditions, westernized life style, diet, air pollution, and the increased consumption of additives and preservatives in food (6, 10) . In young infants the mucosal barrier and immune system are still immature making them more susceptible to develop undesired immune reactivity to food proteins (10-12). Therefore early intake of solid food is considered a risk factor to develop food allergy. However, there is a delicate balance between avoidance of protein to prevent early sensitization and the need for exposure to develop oral tolerance.

Hypoallergenic infant formulas

Avoidance versus tolerance

Hypoallergenic (HA) infant formulas play an important role in the prevention of allergies. Infants born from parents suffering from an atopic disease or who have siblings affected with an atopic disease, have a higher risk of becoming allergic to dietary antigens. For this group, besides the preferred breast feeding, hypoallergenic formulas are commonly used as a good alternative for standard infant milk formulas. For children suffering from persistent or severe cow's milk allergic symptoms amino acid based infant formulas are preferred. HA formulas are processed by enzymatic treatment, heat treatment and/or ultra filtration of cow's milk proteins. These hydrolysed formulas are generally categorized as partial and extensive hydrolysates based on the degree of hydrolysis and consequently the length of the remaining peptides which determine the degree of allergenicity (figure 2). The main strategy for prevention of cow's milk allergic symptoms is the avoidance of the offended food. There is general scientific and clinical agreement that triggering of the allergic reaction in cow's milk allergic infants can be avoided by extensive HA or amino acid

based formulas which lack the capacity to cross-link allergen specific IgE. For IgE cross-linking it has been concluded from several studies that the distance between two FcεRI molecules is within 8-24 nm, corresponding to approximately 30-100 amino acids (13). Peptides below this size are still able to stimulate the allergen-specific T cells, but unable to cross-link IgE on mast cells. Clinical studies have proven that high risk infants fed HA formulas have a reduced risk of developing cow's milk allergy compared to those fed cow's milk formulas (14-16). Whether partial or extensive HA infant formulas are the best strategy for avoiding initial sensitization in genetically predisposed children is still a matter of debate (17). Specific immune modulation is the most promising strategy to prevent initial sensitization to food antigens and aims to improve the induction of oral tolerance to cow's milk proteins. In addition re-establishment of oral tolerance in patients with diagnosed food allergy by immunotherapy via different routes is studied; subcutaneous, sublingual or the oral route (see review chapter 2 in this thesis).

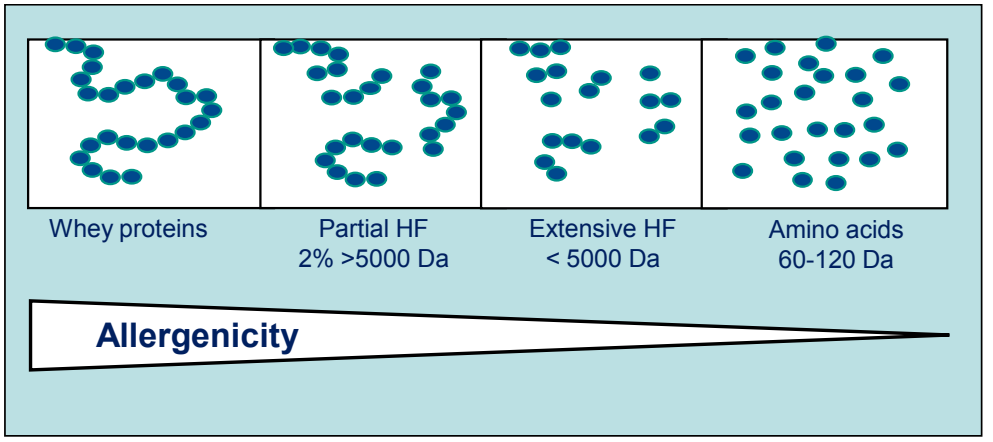

Figure 2. *Reduced allergenicity of hydrolysed milk protein or amino acid based formula in relation to protein size. Cow's milk proteins are hydrolysed with a specific mixture of endopeptidases and exopeptidases resulting in partially hypoallergenic (HA) formulas (partial HF) and extensive HA formulas (extensive HF). HA infant formulas can be based on whey and casein proteins or a combination of both. Extensive HA formulas and amino acid based formulas are commonly used in clinical practice to prevent the allergic reaction in established cow's milk allergic infants. In genetically predisposed children partial and extensive HA infant formulas have been shown to prevent early sensitization to cow's milk proteins.*

Non-digestible oligosaccharides

Generic modulation of the immune system by dietary interventions has been shown to have high potential in preventing allergic sensitization (18-24). Prevention strategies using neutral and acidic oligosaccharides, which are abundantly present in human milk, can selectively support the growth of health promoting commensal bacteria in the gut and showed potential protective properties on the development of allergic disease. Infant milk formulas supplemented with non-digestible oligosaccharides containing neutral short-chain galacto oligosaccharides (scGOS) and long-chain fructo oligosaccharides (lcFOS) in a ratio of 9:1 and scGOS/lcFOS combined with acidic oligosaccharides (AOS) in a ratio of 9:1:1 have been designed to structural and functional mimic some of the health and immune promoting properties of human milk oligosaccharides. In clinical studies performed with a HA formula containing the scGOS/lcFOS (9:1) mixture (Immunofortis®) a reduction in the incidence of atopic dermatitis and allergic manifestations in association with a beneficial immunoglobulin profile was observed in high risk children during the first 6 months of life (25-27). The effects persisted for the next few years which might indicate immune programming (26). The scGOS/lcFOS/AOS (9:1:1) mixture of non-digestible oligosaccharides reduced the occurrence of early atopic dermatitis among low-atopy-risk infants (28). Mechanistic studies in animals using these non-digestible oligosaccharides showed the involvement of regulatory T-cells and a immune deviation towards an Th1 type response in allergic asthma and cow's milk allergy (29-31) . Dietary intervention with these oligosaccharides may be a promising new approach to support the immune system in the establishment of oral tolerance to food proteins in young children.

Safety

It is clear that for safety reasons the potential sensitizing capacity of hydrolysed formulas needs to be assessed before they can be used in young children. According to the European guidelines on HA formulas and follow-on formulas, it is required that objective and scientific data are available as proof of the hypoallergenicity of HA formulas. Although the outcome of several *in vitro* assays are indicative of the residual allergenicity of HA formulas, for safety reasons hypoallergenicity needs to be assessed by showing that the HA formulas are not able to sensitize animals to the protein source they are derived from (Commission Directive 96/4/EC of 16th February 1996 amending Directive 91/321/EEC on infant formulas and follow-on formulas. Official Journal of the European Communities No L 49: 12-16). Guinea pigs sensitized by the oral route have been used as a common model for the identification of the residual sensitizing capacity of new hypoallergenic formulas due to their innate responsiveness. However, a main disadvantage of this guinea pig model is the generation of IgG_{1a} subclass anaphylactic antibody responses instead of

IgE antibody responses which is the main physiological antibody response in allergic humans. This discrepancy between IgG_{1a} and IgE makes this model questionable with regard to the potential extrapolation to the human situation.

Oral tolerance

Oral tolerance provides a unique defense mechanism to maintain the intestinal immune homeostasis. It enables the mucosal immune system to generate a protective inflammatory response against potential pathogens while remaining quiescent when harmless food proteins enter the intestine (32). The usual response to harmless gut antigens is the generation and maintenance of local immunosuppressive conditions and systemic immune hyporesponsiveness, known as oral tolerance. The gut-associated lymphoid tissue (GALT) in the intestine is constituted of a wide variety of innate and adaptive immune cells like dendritic cells (DC), macrophages, intraepithelial lymphocytes, effector T-cells, regulatory T-cells and B-cells which all determine the response to food antigens. These cells are grouped in structures in the inductive sites of the Peyer's patches, mesenteric lymph nodes and lymphoid follicles or are scattered throughout the lamina propria, the effector compartment (33). Intact food derived epitopes that escaped gastrointestinal degradation may be taken up in the intestine through M-cells overlying Peyer's patches or may be absorbed by DC directly in the lamina propria which may sample the antigen by extending their dendrites through the mucosa reaching to the gut lumen as well (34, 35). Disruption of the epithelial tight junctions allows direct access of antigens to the DC in the lamina propria which most likely plays a role in the induction of hypersensitivity responses to food antigens (11). The site of mucosal antigen uptake by DC and consequent presentation to T-cells remains elusive. Recently, it was shown that DC trafficking to the mesenteric lymph nodes is a prerequisite for tolerance induction to harmless food antigens, although regulatory responses induced in the Peyer's patches also contribute to oral tolerance (36-38) (see review chapter 2 in this thesis).

Mucosal dendritic cells

In the absence of pathogens, mucosal DC either ignore the antigen or induce regulatory responses. More than ten years ago it was demonstrated by Viney *et al* that in mice treated with the ligand Flt3L, a growth factor that induces DC proliferation *in vivo*, DC expansion at mucosal sites is correlated with enhanced tolerance to food antigens indicating a key role for mucosal DC in oral tolerance induction (39). Ever since, several subclasses of DC with regulatory properties have been identified in the intestine (see review chapter 2 in this thesis). The conventional myeloid $CD11c^+CD11b^+$ DC and the $CD11c^+B220^+$ plasmacytoid DC have unique regulatory functions and may contribute to oral tolerance induction

(40, 41) most likely by inducing the differentiation of naïve T-cells into regulatory T-cells (38, 41-43). In contrast, $CD8\alpha^+$ DC and $CCR6^+$ DC mainly activate Th1 responses (44), although a population of $CD8\alpha^+B220^+$ DC have been identified in the Peyer's patches with regulatory properties (45). A major subset of DC present in the mesenteric lymph nodes and lamina propria are expressing the integrin alpha chain, $CD103^+$. Recent studies showed that both in mice and human $CD103^+$ DC promote the differentiation of de novo generated $Foxp3^+$ regulatory T-cells and the induction of gut homing receptors by mechanisms involving TGF_β and retinoic acid, a metabolite from dietary derived vitamin A (46-49). DC are primarily non-dividing populations that display a rapid turnover *in vivo*, indicating that this compartment is continuously replenished by blood borne precursors. Epithelial cells are known for their capacity to produce immune modulating mediators like retinoic acid, TGF_β and thymic stromal lymphopoietin (TSLP) which might contribute to a prolonged conditioning of mucosal DC (50). A recent study by ILiev *et al* demonstrated retinoic acid dependent expression of CD103 on DC after interaction of monocyte derived DC with supernatants from human intestinal epithelial cells (51). This indicates cross-talk between intestinal DC and epithelial cells which might contribute to the generation of regulatory T-cells and tolerance induction.

Regulatory T-cells

Regulatory T-cells are expanded in Peyer's patches and mesenteric lymph nodes as soon as 48 hours after feeding of the antigen indicating that the GALT possesses an unique capacity to induce regulatory T-cells in the gut (52). Many different markers, including induced CD25, Foxp3, TNFR (GITR), CTLA-4 and CD45RBlow have been used to describe regulatory T-cells. However, the two markers most commonly used to define regulatory T-cells are CD25 and the forkhead transcription factor (Foxp3). Two major subsets of $CD4^+$ regulatory T-cells, the naturally arising or de novo generated $CD25^+Foxp3^+$ regulatory T-cells and $CD4^+CD25^+$ regulatory T-cells which are characterized by their ability to produce certain cytokines (Th3 and Tr1 regulatory T-cells) are described that contribute to the induction of oral tolerance (32, 53-55). The de novo generation of mucosal $Foxp3^+$ regulatory T-cells from $CD4^+CD25^+$Foxp3- T-cells after oral administrated antigen indicates that inducible $Foxp3^+$ regulatory T-cells might be of importance in re-directing the Th2 immune responses in food allergy (46-49, 54, 55). TGF_β-producing Th3-cells are detected in mice after antigen feeding and this subtype of regulatory T-cells has been implicated as a key mediator for oral tolerance (56). Th3 regulatory T-cells might thereby contribute to the TGF_β dependent induction of Foxp3 regulatory T-cells (57, 58). Tr1 regulatory T-cells represent a truly distinct lineage of regulatory T-cells which are dependent on IL-10 for their differentiation and function and are characterized by the constitutive absence of Foxp3. Also non-conventional regulatory mucosal

T-cells, like $CD8^+$ suppressor T-cells (59, 60) , NKT-cells (61) or gamma delta T-cells (62) might contribute to tolerance induction.

Mouse model for cow's milk allergy

Animal models for cow's milk allergy provide an interesting tool to perform mechanistic research and to investigate safety and efficacy of new therapeutic and preventive approaches for cow's milk allergy. Cow's milk contains two main protein classes, which are the caseins (30g/L) and whey proteins (5g/L). Animal models for cow's milk allergy using oral sensitization are mimicking the human situation as children are most likely sensitized to cow's milk via the oral route. Different mouse strains have been used to study cow's milk allergy showing that cow's milk allergy can be induced in C3H/HeJ (63), C3H/HeOuJ (64), and BALB/c mice (65) upon sensitization with whole cow's milk or β-lactoglobulin which is one of the major whey allergens. In these models cholera toxin is used as a mucosal adjuvant which may affect epithelial integrity and elicits both systemic and mucosal immune responses skewing towards Th2 responsiveness at the level of DC (66). Currently only a few animal models for cow's milk allergy using systemic rather than oral sensitization, have been described to gain insight into the mechanisms of oral tolerance induction (67-69). Oral tolerance to cow's milk proteins has been studied in these models aiming to prevent both systemic as well as mucosal responses (65, 70, 71). To our knowledge, no studies have been described so far on the capacity and potential mechanisms of whey hydrolysates to induce oral tolerance to the native whey protein and the possible contribution of non-digestible oligosaccharides on tolerance induction in animal models using the oral route for both tolerance induction and sensitization.

Scope and outline of this thesis

In the current thesis an adapted mouse model for orally induced cow's milk allergy was used to gain more insight into the mechanisms of sensitization and/or tolerance induction by orally administered whey protein and/or whey hydrolysates. This in order to create more insight into the efficacy and safety of whey hydrolysates.

The major aims of this thesis are:

- To develop tools to determine the sensitizing and residual allergenicity of whey hydrolysates *in vivo* using the mouse model for cow's milk allergy. The acute allergic skin response, serum immunoglobulins, body temperature, anaphylactic shock scores and mucosal mast cell degranulation (measured as mMCP-1) were measured after allergen challenge. In addition, the capacity of whey hydrolysates to cross-link human IgE antibodies on RBL-huFcεRI cells *in vitro* was determined.
- To validate the mouse model for cow's milk allergy in a multicenter ring trial study,

to position this model as a new *in vivo* test for efficacy and safety testing of new hypoallergenic infant formulas.

- To investigate the capacity of partially hydrolysed whey proteins to induce oral tolerance to whey protein in the mouse model for cow's milk allergy. It was hypothesized and investigated whether a unique specific oligosaccharide mixture might contribute to the tolerizing capacity of whey hydrolysates.
- To investigate the contribution of regulatory T-cells on the ongoing cow's milk allergic response in mice by using an *in vivo* regulatory T-cell depleting antibody.
- To address the contribution of IgLC in the induction of hypersensitivity-like responses in mice and patients suffering from atopic dermatitis and cow's milk allergy.
- To investigate whether IgLC contribute to the tolerizing capacity of whey proteins in a mouse model for cow's milk allergy using oral sensitization.

Chapter 1, the general introduction, describes the scientific rationale and aims of the thesis and is further substantiated by **chapter 2** which provides a review on prevention strategies for food allergy.

In **chapter 3** a mouse model for cow's milk allergy is described using oral exposure to whey and/or casein proteins combined with cholera toxin as a mucosal adjuvant. Interestingly, differences were observed in the underlying mechanisms responsible for either whey or casein sensitization.

In **chapter 4** and **chapter 5** the sensitizing capacity and residual allergenicity of partially hydrolysed whey proteins was assessed using the mouse model of cow's milk allergy. In **chapter 4** the acute allergic skin response is presented as a new major read-out to address the putative sensitizing capacity and the residual allergenicity of whey hydrolysates in a mouse model for orally induced cow's milk allergy. In **chapter 5**, the body temperature, anaphylactic shock reactions and mucosal mast cell degranulation (measured as mMCP-1) are included as major read-outs of clinical reactions induced in the cow's milk allergic mice. In addition, the capacity to cross-link human IgE antibodies on RBL-huFcεRI cells *in vitro* was determined. The combination of assays is proposed as a new strategy for the screening of new hypoallergenic formulas aiming to prevent sensitization in atopic children and to avoid clinical symptoms in infants suffering from cow's milk allergy respectively.

In **chapter 6** the first results of a multicenter ring trial study are described. The aim is to validate and to position the mouse model as a pre-clinical tool to test the efficacy and safety of new hypoallergenic infant formulas. In **chapter 7** the determination of allergenic and immunological properties of whey proteins, hydrolysed for different periods of time, is described. By differentiation between IgE-mediated responses and T-cell activation we aimed at selecting whey hydrolysates with reduced allergenic properties which retained the capacity to activate T-cells.

Chapter 8 continues with the topic on tolerance induction. In this chapter the tolerizing capacity of partial and extensive whey hydrolysates is described. Data from this study show that partial but not extensive whey hydrolysates possess the putative capacity to induce oral tolerance to whey in mice. This effect is transferable to naïve mice using mesenteric lymph node cells and is associated with enhanced $Foxp3^+$ regulatory T-cell numbers in the mesenteric lymph nodes. In **chapter 9** it is investigated whether non-digestible carbohydrates, mimicking functional and structural properties of breast milk oligosaccharides, might influence the tolerizing capacity of partially hydrolysed whey proteins. This study shows that a specific mixture of these non-digestible oligosaccharides potentiates the capacity of a partial whey hydrolysate to induce oral tolerance in mice. This effect was associated with an increased percentage of tolerogenic $CD11c^+CD103^+$ DC in the mesenteric lymph nodes. **Chapter 10, 11 and 12** describe the involvement of IgLC in the immune response to orally administered whey proteins. **Chapter 10** describes *in vivo* depletion of $CD25^+$ lymphocytes to reveal the contribution of $CD4^+CD25^+$ T-cells in the allergic response to whey in mice. Compromised function of regulatory T-cells might underlie cow's milk allergy or food allergy in general. These data suggest that $CD25^+$ T-cells, regulatory T-cells and effector cells, play a crucial role in balancing cow's milk allergy between IgE and IgLC-dependent effector responses and that both mechanisms are involved in the generation of acute allergic responses to the same allergen. **Chapter 11** describes the contribution of IgLC to the allergic effector response in casein sensitized mice. Measurement of IgLC in human disease indicates a possible role for IgLC in the allergic response in high risk children suffering from atopic dermatitis as well as truly cow's milk allergic children as determined by double blind placebo controlled food challenge. In **chapter 12** it was investigated whether IgLC may be associated with the tolerizing capacity of whey proteins. In this study we show that additional mechanisms like the presence of elevated levels of IgLC prior to sensitization might protect against the development of allergy and is involved in tolerance induction for food proteins. Finally, the results of our findings are summarized and concluded in **chapter 13**.

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2

Prevention strategies for food allergy might intervene with the development of atopic dermatitis and the atopic march

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Book chapter in: Food allergies: symptoms, diagnosis, and treatment, In press
ISBN: 978-1-61728-748-0
Editor: Patricia M. Rodgers

Abstract

02

Food allergy, atopic dermatitis, rhinitis and allergic asthma are common atopic diseases in Western countries and their prevalence has been rising the past thirty years (1-3). Prevention of food allergy induced by the three major food allergens, hen's egg, cow's milk and peanut is considered important to prevent related diseases like atopic dermatitis, allergic rhinitis and allergic asthma later in life. There is a strong relation between food allergy and eczema in young children under the age of 1 year (4-6). The route of sensitization to food allergen is still not completely elucidated and might involve the skin or airways beside sensitization via the gastrointestinal tract. Breastfeeding is still considered the best way of preventing food sensitization. Prebiotic oligosaccharides and cytokines like IL-10 and TGF_{β} which are abundantly present in human milk, might provide an immunological milieu that favors the induction of oral tolerance in the Th2 prone infant. However, if breastfeeding is not possible or sufficient, hydrolysed formulas are the second best option for children with a genetic predisposition to develop allergic diseases. Recent studies provide evidence that the induction of specific oral tolerance is beneficial above complete avoidance of the suspected food in preventing sensitization to food allergens early in life (7-10). For prevention of clinical symptoms in sensitized children, different approaches of specific immune modulation are currently investigated to desensitize or tolerize cow's milk allergic infants; subcutaneous immunotherapy (SIT), sublingual immunotherapy (SLIT) or oral immunotherapy (OIT). In the current chapter primary prevention of allergic disease or treatment strategies to prevent clinical symptoms in sensitized individuals are described. Furthermore, the use of animal models is discussed in relation to food allergy, atopic dermatitis and the predisposition to other allergies later in life (e.g. atopic march).

Food allergy

Food allergy affects about 4-5% of young children with hen's egg, cow's milk (2-3%) and peanut (>1%) being the three most important diagnosed food allergies in children with incidences of emergency hospitalization after life-threatening food-induced anaphylaxis (11). Whilst it is established that there is a strong genetic compound to food hypersensitivity it is hypothesized that factors responsible for the increasing number of children suffering allergic reactions may be found in changing environmental conditions, westernized life style, diet, air pollution, and the increased consumption of additives and preservatives with food. Moreover, food allergy might partly be associated with the immature gut and the Th2 prone young infant. The type of antigen, antigen dose and age at first exposure are all thought to play a crucial role in the subsequent immune response (12) and most likely results from either a failure in establishing oral tolerance or a breakdown in existing tolerance. The route of sensitization to food allergen is still not completely elucidated and might involve the skin or airways beside sensitization via the gastrointestinal tract. Recent findings show that many cases of hen's egg, cow's milk allergy persist later in life and only 11% of hen's egg, and 18% of cow's milk and peanut allergy resolved at the age of 4 years (13). However, in contrast to peanut 80% of cow's milk and hen's egg allergy resolved at 16 years of age (14, 15).

Hypersensitivity reactions were classified into four different types by Coombes and Gell. Aeroallergens, food allergens, drugs and insect venoms are the major allergens encountered that are responsible for induction of type1-hypersensitivity reactions. This response may arise in individuals who have a propensity to exert an allergic response characterized by production of allergen-specific IgE. Binding of IgE to the high affinity receptor FcεR1 on mast cells followed by subsequent cross-linking of the receptors by the allergen provokes degranulation and the release of mediators such as histamine, leukotriens and cytokines. About 60% of food allergic patients have IgE-mediated allergic responses and IgE mediated allergy is an important indicator of susceptibility to develop atopic diseases like atopic dermatitis and allergic asthma later in life (16, 17). IgE mediated mast cell activation results in clinical symptoms involving the skin (urticaria, angiodema and atopic dermatitis), gastrointestinal tract (nausea, vomiting, abdominal pain, constipation and diarrhea), airways (cough, wheeze) and systemic symptoms within a few minutes to one hour after ingestion of the food which can potentially lead to a life threatening anaphylactic shock. It should be realized that in atopic diseases like food allergy, atopic dermatitis, rhinitis and asthma, a considerable number of patients exhibit clinical features without detectable local or systemic IgE. Mast cells have been implicated in the pathogenesis of IgE-mediated and non-allergic hypersensitivity responses and several in vivo studies indicate that they are involved in allergic asthma and gastrointestinal allergy (18-21). Although antigen-specific mast cell activation results from cross-linking

the high-affinity IgE receptor also other mechanisms can be involved in antigen specific activation in the absence of IgE antibodies. A novel potential mechanism for the elicitation of immediate hypersensitivity-like reactions via immunoglobulin free light chain (IgLC) has been described (22). Transfer of antigen-specific IgLC into naive mice sensitizes them to the respective antigen resulting in local mast cell activation upon challenge, leading to edema formation after skin challenge or acute bronchoconstriction after intranasal challenge (21-24). In a recent study, CD25 depletion in mice orally sensitized to whey proteins was found to alter the nature of the allergic response from IgE- to IgLC dependent (25). Human studies confirm a significant role for IgLC in allergic diseases like atopic dermatitis, allergic rhinitis and food allergy. Children with mild atopic dermatitis have higher concentrations of lambda and kappa IgLC in serum (26). Furthermore, in non-IgE mediated rhinitis patients both lambda and kappa IgLC concentrations were found to be enhanced in nasal secretions (27). A recent study showed that IgLC levels were enhanced in children with Double Blind Placebo Controlled Food Challenge (DBPCFC) diagnosed food allergy (Schouten *et al*, in press). Overall these data suggest that IgLC might be a promising new biomarker in the early diagnoses of allergic diseases like food allergy. More studies are needed to define the correlation between IgLC and allergic disease.

Atopic dermatitis

The prevalence of atopic dermatitis is 10-20% in young children (1). There is a strong relation between food allergy and eczema in young children under the age of 1 year (4-6) although there is some debate on the association between food allergy and atopic dermatitis (28). It is estimated that in one third of the children suffering from atopic dermatitis the skin symptoms are triggered by sensitization to food (5, 6). Moreover, food allergens can exaggerate symptoms of atopic dermatitis by promoting Th2 activation in the skin as dendritic cells (DC) in the gastro intestinal tract are able to carry the allergen to the dermis where they may lead to degranulation of mast cells (29).

Impaired barrier function of the skin is considered to associate with atopic dermatitis (30) and genetic predisposition may underlie this. The protein filaggrin is part of the inner layer of the skin forming the cornified envelope, a specialized structure playing an essential role in the skin barrier (31). Mutations of filaggrin genes are associated with skin diseases like, ichtyosis vulgaris and atopic dermatitis (32, 33). The expression of the protein filaggrin was reduced in the skin of atopic dermatitis patients which was linked to mutations in chromosome 1q20 located genes including filaggrin encoding for the structural proteins of the epidermis (34-36). It is clear that genetic (filaggrin nul/nul mutations and environmental factors like breastfeeding, allergen exposure or infections) may underlie the development

of atopic dermatitis in early life and is often the first manifestation of atopy in a triad of events including rhinitis and allergic asthma (16) called the atopic march.

Atopic march

The atopic march is the natural history of atopic manifestations, characterized by a progression of clinical signs of atopic disease. In general, clinical signs of atopic dermatitis predate the development of asthma and allergic rhinitis, suggesting that atopic dermatitis is the "entry point" for subsequent allergic disease. Early sensitization to food might also be considered as one of the entry points. Sensitization to hen's egg or cow's milk (determined with skin prick test) in genetically predisposed children was predictive of adult asthma (17, 37). It is generally accepted that atopic dermatitis when affecting children before two years of age increases the risk of developing allergic asthma later in life (1). This can be explained by the observations that allergens (e.g. food and aero-allergens) taken up by the damaged skin results in new sensitizations which might underlie the development of food allergy and atopic dermatitis leading to allergic rhinitis and allergic asthma later in life. Epicutaneous sensitization to food and aero-allergens has been supported by clinical and animal studies (38, 39). Removal of the stratum corneum in mice followed by skin exposure to antigen led to eczema like symptoms and consequent airway hyper-responsiveness after allergen challenge (39). Moreover, topical creams containing peanut, applied to inflamed skin have been related to allergic responses in human (40). Skin sensitization after removal of the upper layer of the skin is predominantly regulated by Langerhans cells causes a systemic immune response without the use of an adjuvant. Upon allergen binding to FcεR1 receptors on Langerhans cells migrate to the local lymph nodes were they can induce Th2 polarization. Subsequently, Th2 cells migrate through the circulatory system to various sites including nasal, lung and intestinal mucosa. Inhalation or oral exposure of allergens results in presentation of local DC to interact with these Th2 cells driving the generation of IgE. These interactions may underlie the allergic response resulting in clinical symptoms in lungs or the gastrointestinal tract.

The atopic march hypothesis is mainly based on cross-sectional studies (41-43). Only a few prospective studies showed the relation between atopic dermatitis early in life and the progression into asthma later in life (44, 45). However, concomitant wheezing reflecting early sensitization is an important predictive factor in this relation. In the German Multicenter Atopy Study (MAS; 1314 newborns of whom 499 are high risk infants) early wheezing was found to be the decisive factor in the association between atopic dermatitis and childhood asthma in infants (46). In contrast , a recent Australian study showed that atopic dermatitis early in life is associated with childhood asthma in boys even when early wheezing and early sensitization has been taken into account (45). Hence, besides the clear relation

between atopic dermatitis and allergic disease later in life, early sensitization to inhalant or food allergens in high risk children may be an important factor in pursuing the atopic march (47, 48). It is fair to state that sensitization to food and inhalant-allergens either via the skin, respiratory system or the gastro-intestinal tract may contribute to clinical symptoms of food allergy, atopic dermatitis, allergic asthma and rhinitis.

Prevention of food allergy

Although the causal relationship between food allergy, atopic dermatitis, allergic rhinitis and asthma is still under discussion, prevention of the first and most common allergies early in life might be important to delay or even stop the development of atopic diseases later in life. The two major strategies to prevent sensitization to food allergens are avoidance of the allergic protein (e.g. epitope exclusion) or improving establishment of oral tolerance.

Breastfeeding

Breastfeeding is considered to be the golden standard and the best way of preventing sensitization and might help the establishment of oral tolerance in the Th2 prone young infants. It provides a unique combination of lipids, proteins, carbohydrates, vitamins and minerals. Furthermore there are numerous bioactive compounds with immunological properties such as soluble IgA, antioxidants, oligosaccharides, probiotics, Toll-like receptor ligands, cytokines, hormones, fatty acids and many more. Each compound can individually, additionally or synergistically act on the immune system of the neonate. The breast milk content changes over time to ensure optimal passive and active protection and growth for the child (49-51).

TGF_{β} and IL-10 present in human milk might play a role in diminishing the risk of allergic disease in infancy (52) as these cytokines are considered regulatory cytokines involved in tolerance induction. In addition, prebiotic oligosaccharides which are abundantly present in human milk may have many immunological properties and might facilitate tolerance induction (49, 53). However, breastfeeding cannot totally eliminate the risk of sensitization because of passage of some proteins through the gut barrier and as a consequence their release in human milk (54-56). Prebiotic fibers manufactured to resemble some of these human oligosaccharides are defined as “non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacterial species already resident in the colon, and thus attempt to improve host health” (57). These manufactured prebiotic oligosaccharides might improve infant formulas by generic immune modulation. They possess the capacity to induce a beneficial immunoglobulin profile in infants at risk for allergy (58) and diminish the incidence of atopic dermatitis in high risk children (59). In addition, symptoms of food allergy

(60) and allergic asthma (61) are reduced in mice fed these specific oligosaccharides. Dietary intervention with these oligosaccharides may be a promising new approach by supporting the establishment of oral tolerance induction in young children. However, breastfeeding is still considered the golden standard and hydrolysed formulas are the second best option for children with a genetic predisposition to develop allergic diseases if breastfeeding is not possible or sufficient.

Avoidance versus Oral Tolerance

The complete avoidance of even very low amounts of known allergens is the most followed strategy in high risk children and avoidance of food and house dust mite allergens has shown promising results in preventing allergic asthma (62). Early introduction of solid food was considered a risk factor in the development of food allergies and was partly based on experiments in rodents (12, 63) leading to the recommendation that atopic mothers may wish to avoid dietary antigens such as hen's egg, fish and nuts during pregnancy and lactation. However, recent results indicate that avoidance of dietary compounds failed to prevent food allergy (9, 10, 64) indicating that early introduction of solid food might play a significant role in the prevention of sensitization to food allergens. This is supported by the fact that the prevalence of peanut allergy is significantly lower in countries were peanut is consumed at an early age (8, 65). Furthermore, fish consumption of newborns during first year of life was found to reduce the risk of allergy (rhinitis, eczema) and sensitization for food and airborne allergens by the age of four years (66). Moreover, allergens provided via the oral route are considered important inducers in the maturation of the immune system (67, 68), indicating that early oral exposure to food might be necessary to induce oral tolerance and might be beneficial above avoidance strategies (7).

Oral tolerance provides a unique defense mechanism to maintain the intestinal immune homeostasis. It enables the mucosal immune system to generate a protective inflammatory response against potential pathogens while remaining quiescent when harmless proteins such as food allergens enter the intestine (69).
At the intestinal mucosa a single layer of epithelium separates the inner milieu from the external environment. Intact food derived epitopes that escaped gastrointestinal degradation may be taken up in the intestine by regulatory DC in Peyers patches or in the villous lamina propria and presented to naïve T-cells in the Peyer's patches or mesenteric lymph nodes resulting in a protective immune response (70, 71). Several subclasses of DC with regulatory properties are present in the intestine and might therefore be important in oral tolerance induction to harmless food antigens and play a key role in preventing oral sensitization to dietary antigens by the induction of regulatory T-cells (72, 73). The conventional myeloid $CD11c^{+}CD11b^{+}$ DC

and the $CD11c^+B220^+$ plasmacytoid dendritic cells (pDC) from the Peyer's patches have unique regulatory functions and may contribute to oral tolerance induction (73, 74). They possess the capacity to suppress T-cell responses (75-77) most likely by inducing the differentiation of naive T-cells into regulatory T-cells (74, 78, 79). $CD103^+$ DC that are predominantly present in the lamina propria take up antigen and migrate to the mesenteric lymph nodes (70, 71, 80-82) where they drive the induction of regulatory T-cells (83). The site of mucosal antigen uptake by DC and consequent presentation to T-cells remains elusive. Uptake of antigens in M-cells in Peyer's patches has been shown to be involved in oral tolerance induction as well (84-88) although mesenteric lymph nodes seem to be the major site for T-cell recognition (79, 89). Also the liver, as an intestinal site for antigen presentation, is considered to play a role in the induction of regulatory T-cells and subsequent tolerance induction (90, 91).

In general, when oral administrated harmless food is taken up by intestinal mucosal DC regulatory T-cells are generated that support oral tolerance induction. These regulatory T-cells encompass natural or inducible $CD4^+CD25^+Foxp3^+$, TGF_β producing Th3 and IL-10-producing Tr1 regulatory T-cells (69, 92, 93). It is believed that the induction of regulatory T-cells, acting via cell-cell contact or suppressive cytokines (e.g. IL-10, TGF_β), are crucial in inducing or maintaining oral tolerance (94-97) although other mechanisms like anergy or clonal deletion of effector Th2-cells (high-dose tolerance) may have overlapping functionality.

Hydrolysed Formulas

Hydrolysed formulas are the second best option for children with a genetic predisposition to develop allergic diseases if breastfeeding is not possible or sufficient. Hypoallergenic formulas are commonly used to manage cow's milk allergy and are generally categorized into partial and extensively hydrolysed hypoallergenic formulas. Cow's milk subjected to enzymatic hydrolysis, heat treatment and/or ultra filtration reduces the molecular weight and consequently the allergenicity of the proteins. Although there is general scientific and clinical agreement that triggering of the allergic reaction in cow's milk allergic infants can be avoided with extensive hydrolysed formulas or amino acid based formulas (e.g. epitope exclusion) debate on the most effective strategy to avoid initial sensitization remains intense (98).

Infants fed hydrolysed formulas have a reduced risk of developing cow's milk allergy compared to those fed cow's milk formulas (99). 10 Years of clinical studies show that both partial and extensive hydrolysed formulas are effective in preventing cow's milk allergy in high risk children (100-104). Clinical studies addressing the effectiveness of hydrolysed formulas in preventing cow's milk allergy were mainly designed as avoidance strategies to prevent sensitization and are limited in answering whether the beneficial effects are due to avoidance of the allergic epitopes or a result of

oral tolerance induction. Partial rather than extensive hydrolysed formulas possess the capacity to induce oral tolerance to the offended protein (88, 105-107). In a recent study using a mouse model for cow's milk allergy we showed, that feeding a partial but not an extensive whey hydrolysate prior to oral whey sensitization reduced the clinical symptoms upon challenge with whey in these animals. These effects coincided with enhanced numbers of intestinal regulatory T-cells and the protective effect was transferable to naïve recipient mice using the mesenteric lymph nodes of the donor mice (van Esch *et al*., submitted). It is clear that for safety reasons the potential sensitizing capacity of these hydrolysed formulas need to be assessed before they can be used in young children. Immunological *in vitro* assays are important tools for the efficacy and safety testing of hydrolysed formulas in prevention of clinical symptoms in already sensitized infants. *In vivo* animal models for cow's milk allergy have the additional advantage that they are useful to predict the efficacy and safety of hydrolysed formulas in humans in terms of primary prevention of cow's milk allergy or potential residual sensitizing properties of the hydrolysed formulas. A combination of both *in vitro* as well *in vivo* models should be considered for optimal safety and efficacy testing in terms of avoidance, tolerance induction or potential sensitizing capacity of hydrolysed formulas (108) (van Esch *et al.*, Toxicology Letters).

Prevention of food allergic clinical symptoms (treatment)

In children already sensitized to the most common allergens present in cow's milk, hen's egg and peanut the standard care is mainly based on strict avoidance strategies and pharmacotherapy for symptom relieve. For basic foods such as cow's milk, hen's egg and peanut this might become more and more difficult because of the widespread use in processed food (109). Therefore, specific immune therapies aiming at restoring tolerance to harmless food proteins have been under intensive investigation the past decade. Different approaches of specific immune modulation are currently investigated which might lead to a specific long lasting immune suppression. The re-establishment of tolerance in sensitized children to the first and most common allergies in early life might even delay or stop the atopic march. Allergen immunotherapy redirects allergen-specific T-cell responses from a Th2 to a Th1 profile along with tolerance induction mediated by regulatory T-cells. There is a strong rationale for improving the efficacy of allergen-specific immunotherapy by reducing the incidence and severity of IgE mediated adverse reactions. Approaches to address this problem include the use of modified allergens, novel adjuvants and alternative routes of administration. It is known that the allergenicity of cooked or baked product is reduced although it remains important to evaluate the potential (residual) antigenicity/allergenicity of new or modified protein products in animal models (110). Allergen specific immune therapies include subcutaneous

immunotherapy, sublingual immunotherapy and oral immunotherapy. These methods generally involve administering small yet increasing doses of antigen aimed to reduce clinical symptoms occurring during the natural exposure to the allergen itself. Treatment is performed in a controlled setting and in the case of sublingual and oral immunotherapy followed by regular home dosing of a maximum tolerated amount of antigen (111). Whether these different strategies for specific immune therapy redirect the immune response or induce a transient effect is still under discussion.

Subcutaneous Immunotherapy (SCIT)

The most effective curative treatment for IgE-mediated allergy is SCIT and has been proven effective in respiratory and bee venom allergy. The history of SCIT began almost one hundred years ago by the observation by Leonard Noon that the subcutaneous route of administration was effective in reducing clinical symptoms of hay-fever. Immune modulation by SCIT include the modulation of T and B cell responses and is accompanied by a significant decrease of allergen specific IgE and increase in allergen specific IgG antibodies, mainly IgG_4. Animal studies showed that these effects where dependent on IL-10 and may act via indoldiamine 2,3-deoxygenase (IDO) dependent metabolites (112-114). However, SCIT is not yet available for the management of food allergy because of the high risk of inducing anaphylaxis by accidental intake of the offending allergen (e.g. peanut) (115, 116). Several specific strategies to reduce the allergenicity of therapeutic preparations, while maintaining their therapeutic benefit, are being developed. Several studies have examined the differential effects of reducing allergen size most particularly by producing peptides. Allergenic peptides are still able to stimulate the allergen-specific T cells, but due to reduced size peptides are unable to crosslink IgE on mast cells thereby avoiding the inducting of clinical reactions (117). This so called peptide immunotherapy has been investigated and seem effective in bee venom allergy (118) and allergic asthma (119, 120). Recently it has been shown that peptide immunotherapy reduced the allergic response in a mouse model of egg allergy (121). From this point of view, also cow's milk hydrolysates might be an option for SCIT in cow's milk allergic infants. However, less invasive therapies might be preferred for the management of cow's milk allergy and egg allergy in the young infant.

Sublingual Immunotherapy (SLIT)

The sublingual route for specific immunotherapy has been routinely used for more than ten years in Europe (122, 123). The first randomized double blind placebo controlled clinical trial was published in 1986. Because of the less invasive character SLIT is considered a safe and well tolerated alternative for SCIT in young children (124, 125) although there is a minor risk of inducing anaphylaxis. Since the distribution of

antigens administered is limited the adverse affects elicited by SLIT are very low (126). Animal models mimicking SLIT for the treatment of allergic diseases are currently being used and might give important insight in the mechanisms underlying SLIT. Three different subtypes of oral DC have been identified in mice (127). $CD11b^{+}CD11c^{-}$, $CD11b^{+}CD11c^{+}$ mucosal DC, $B220^{+}$ pDC and a minor population of $CD207^{+}$ Langerhals cells. However, Langerhals cells represent the predominating DC population within the human oral mucosa and represent a more immature DC subpopulation which might underlie their tolerogenic properties in SLIT. To optimize treatment protocols of SLIT animal studies are used to study (128), improved efficacy using adjuvants aimed to facilitate the generation of regulatory T-cells or Th1 type responses (129) (130) and the use of muco-adhesive substances. The clinical efficacy of SLIT has been proven in the treatment of allergic rhinitis (pollen) and allergic asthma (house dust mite) in children (131-133). The most important concern to be elucidated in SLIT is the optimal dose of allergen used because effectiveness has been shown over a very large range of doses. In a recent randomized, placebo controlled study Pajno *et al*. reported that SLIT may provide an additional therapeutic tool for the treatment of house dust mite sensitized children with atopic dermatitis (122). The allergic form of atopic dermatitis affects the majority of the patients and occurs in the context of an IgE sensitization towards food or environmental allergens. Therefore, SLIT might be a potential strategy for preventing symptoms in food allergy. So far, at least one clinical study described the efficacy of SLIT for hazelnut allergy (134).

Oral Immunotherapy (OIT)

Oral immunotherapy aiming at the tolerizing/desensitizing capacity of orally applied allergens in diagnosed food allergic patients started with several clinical trials in the 1980s and showed promising results (135) although in some cases serious gastrointestinal adverse events were reported. In the last decade the interest in non-invasive routes of specific immunotherapy increased again. Currently this approach aiming at the tolerizing/desensitizing capacity of orally applied allergens in diagnosed food allergic patients undergoes the most intensive research. Meglio *et al* showed that from 21 cow's milk allergic children above 5 years of age 71% tolerated 200 ml of cow's milk after 6 months and were still tolerant in a 4-years follow-up. However, the weakness of this study was that no control group was included to study the natural course of cow's milk allergy (136, 137). The safety and efficacy of cow's milk oral immunotherapy was confirmed in children with severe cow's milk allergy (138). After 1 year the threshold dose was increased in 54% of the children and 36% of the children became tolerant whereas in the control group all children were still cow's milk allergic. Side effects occurred but were controllable with medication. Also for hen's egg and peanut allergy the efficacy of oral immunotherapy was studied and confirmed (139-142) but more research is needed to optimize treatment protocols

(e.g. rush versus semi-rush) and to study the underlying mechanism. It is still not known whether persistent immune modulation or a transient desensitization is induced by oral immunotherapy (143). However, even if desensitization rather than true tolerance is induced, clinical tolerance due to regular intake of the offended food has the advantage of increasing the threshold dose for allergic reactions which can improve the quality of life substantially for the allergic patient. However, it can not be excluded that diseases (e.g. infections) affect the increased threshold dose. Oral immunotherapy with peanut is currently studied in patients with atopic dermatitis. In the Learning Early About Peanut Allergy study 480 children aged 4 to 10 months affected with eczema and egg allergy (as a predictor for peanut allergy) are assigned to either avoid consumption of peanuts or to be fed peanut snacks three times a week. After five years the development of peanut allergy will be compared. This study can give important insight in the capacity of oral immunotherapy to diminish new sensitizations and ultimately to pause the atopic march.

Summary

Prevention of food allergy induced by the three major food allergens, hen's egg, cow's milk and peanut is considered important and might prevent related diseases like atopic dermatitis, allergic rhinitis and allergic asthma later in life. Breastfeeding is still considered the best way of preventing food sensitization. Prebiotic oligosaccharides and cytokines like IL-10, TGF_{β} which are abundantly present in human milk, might provide an immunological milieu that favors the induction of oral tolerance in the Th2 prone infant. The early introduction of food proteins or peptides (e.g. hydrolysed formulas) seems to be the most promising approach to induce oral tolerance and prevent sensitization to harmless food antigens. In addition, food proteins seem to be involved in stimulating maturation of the immature gut. In the allergic child, allergen specific immune therapies are important strategies to induce hypo-responsiveness to the offending food either by restoring tolerance or by transient desensitization. They include subcutaneous immunotherapy, sublingual immunotherapy and oral immunotherapy. Although allergen specific immunotherapy seems very promising and might prevent secondary sensitizations and thereby even diminishing or stopping the atopic march, still much research is needed to further optimize the protocols and to study the underlying mechanisms.

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3

Acute allergic skin reactions and intestinal contractility changes in mice orally sensitized against casein or whey

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International Archives of Allergy and Immunology 2008;147:125-134

Abstract

Background Cow's milk allergy (CMA) is characterized by hypersensitivity against casein or whey, affecting 2.5% of young infants. The pathogenesis of CMA involves IgE as well as non-IgE-mediated reactions and clinical symptoms are found in the skin, lungs and gastrointestinal tract. In this study, local and systemic immunopathology was determined in whey- or casein-allergic mice.

Methods Mice were orally sensitized with casein or whey using cholera toxin as an adjuvant. Serum immunoglobulins and the acute allergic skin reaction (ear swelling 1 h after intradermal allergen challenge) were determined to reveal systemic hypersensitivity. Furthermore, pathophysiological changes were assessed within the intestine.

Results An acute allergic skin reaction was induced in both whey- and casein-sensitized mice. In these mice, whey-specific IgE, IgG_1, IgG_{2a} and casein-specific IgG_1 levels were found to be increased. In addition, the serum mouse mast cell protease-1 (mMCP-1) concentration was enhanced, reflecting mast cell degranulation. Indeed, the number of mMCP-1-positive mast cells within the colon was diminished in both whey- and casein-sensitized mice. Only in casein-sensitized mice isometric contraction of the colon was reduced, reflecting motility alterations.

Conclusion Mice, orally sensitized against casein or whey, revealed an allergen-specific acute allergic skin reaction. In casein-sensitized mice, hypocontractility of the colon reflected pathophysiological changes within the intestine. Allergen induced ear swelling and intestinal contractility changes are novel parameters in animal models of CMA which may add to the search for new therapeutic strategies to relieve symptoms of CMA.

Introduction

Cow's milk allergy (CMA) is one of the leading causes of food allergy in adults (1). In developed countries, approximately 2–3% of infants exhibit CMA. Although most infants outgrow CMA before their fifth year, IgE mediated CMA predisposes the development of other (food) allergies and even asthma (2, 3). Clinical features due to IgE-mediated reactions are expressed as immediate symptoms mostly. Clinical symptoms may involve the skin, respiratory tract and gastrointestinal tract, and can even lead to a systemic anaphylactic reaction (4, 5). However, it should be realized that a particular group of patients, up to 40%, exhibit clinical features of CMA without detectable cow's milk-specific serum IgE (6-8). So far, the only therapeutic approach has been the elimination of cow's milk proteins from the diet. Cow's milk contains 2 main protein classes, caseins (30 g/l) and whey proteins (5 g/l). The caseins consist mainly of αS1-, αS2- and β-casein, whereas whey proteins comprise of β-lactoglobulin, α-lactalbumin, bovine serum albumin, serum immunoglobulins and lactoferrin. Large population studies with cow's milk-allergic infants have shown that the major allergens are β-lactoglobulin, caseins and α-lactalbumin (9, 10). The pathogenesis and development of CMA probably involves a partial dysfunction in immunological tolerance induction during early life combined with enhanced intestinal permeability (5). There is growing evidence that intestinal allergic responses can initiate motility changes through attraction and activation of mast cells and production of TH2 type of cytokines (11). Motility changes of the intestine are a clinical problem in CMA patients, which manifests as either diarrhea or even constipation (12-14).

Animal models of CMA provide a tool to reveal mechanisms involved in CMA and may explore new therapeutic and preventive approaches in those models. However, in most existing food allergy models, the animals are not sensitized via the oral route (but, for example, intraperitoneally), while in reality humans are sensitized orally. Although numerous animal models for food allergy are available using an intraperitoneal sensitization protocol, only a few models use oral sensitization (15-23). Li *et al.* (22) were the first to introduce a model in which mice were sensitized orally against complete cow's milk, while Frossart *et al.* (23) showed oral sensitization to β-lactoglobulin. In these models, mice are sensitized by means of intragastric (i.g.) gavage using cholera toxin (CT) as an adjuvant. The primary read out parameters are IgA and IgE, and in particular cow's milk-specific IgE. Furthermore, Li *et al.* (22) described systemic anaphylaxis upon i.g. challenge with cow's milk. They described vascular leakage, mast cell degranulation and enhanced serum histamine levels in CMA animals. The systemic allergic responses to food allergens that are described in these models resemble features found in the clinic; however, the mechanisms behind the development of CMA are still only partially understood.

To further assess mechanisms underlying CMA and/or test new concepts for prevention and/or treatment for CMA, in the present study new tools to address the pathological changes in casein and whey allergy in mice have been developed. Measurement of the acute allergic skin response, as an equivalent of the human skin prick test, was introduced to reflect a systemic sensitization. Intestinal contractility changes were measured to address local pathophysiological changes after oral challenge with the specific allergens.

Methods

Chemicals

Casein and whey were obtained from DMV International (Veghel, The Netherlands). Cholera toxin was purchased from Quadratech Diagnostics (Epsom, UK) and PBS from Cambrex Bio Science (Verviers, Belgium). Biotin-labeled rat anti-mouse IgE, IgG_1 and IgG_{2a}, and unlabeled rat anti mouse IgE were obtained from BD Biosciences (Alphen aan den Rijn, The Netherlands). All other chemicals were obtained from Sigma-Aldrich-Chemie (Zwijndrecht, The Netherlands).

Oral Sensitization and Challenge of Mice

Three- to five-week-old specific pathogen-free female C3H/HeOuJ mice (4–6 mice per group) were purchased from Charles River Laboratories (Maastricht, The Netherlands), maintained on cow's milk protein-free mouse chow (Special Diets Services, Witham, UK) and housed in the animal facility at the Utrecht University. Animal care and use were performed in accordance with the guidelines of the Dutch Committee of Animal Experiments. Mice were sensitized i.g. with 0.5 ml homogenized casein or whey (40 mg/ml PBS) with CT (20 µg/ml PBS) as an adjuvant, using a blunt needle. Control mice received CT alone or PBS. Mice were boosted weekly for a period of 4–6 weeks, 1 week after the last sensitization mice were challenged i.g. with 100 mg casein or whey in 1 ml PBS (figure 1). Blood samples were collected and centrifuged (15 min at 13,500 rpm). Sera were stored at –70 ° C. Mice were sacrificed by cervical dislocation 30 min after i.g. challenge.

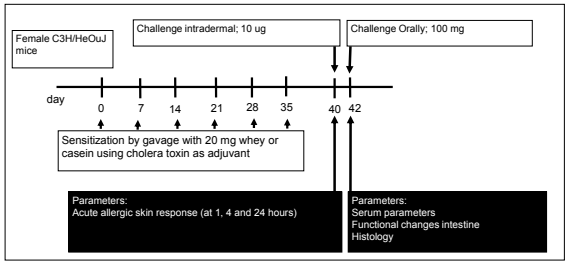

Figure 1. *A schematic overview of the sensitization and challenge protocol and the parameters that are analyzed.*

Allergen-Specific Skin Response

The acute allergen-specific skin response was measured after injection of the specific protein in the ear pinnae intradermally (i.d.). Before i.g. challenge (t = 0), the control, casein- and whey-sensitized mice were injected i.d. in the left ear with 20 µl homogenized casein or whey (0.5 mg/ml in PBS), respectively. In the right ear, 20 µl PBS was injected as a vehicle control. Also, the CT and PBS sham-sensitized mice received a casein or whey ear challenge using PBS injections as control. Ear thickness was measured in duplicate using a digital micrometer (Mitutoyo, Veenendaal, The Netherlands), at t = 0 as well as 1, 4 and 24 h after challenge. The allergen-specific net ear swelling was calculated by subtracting the basal thickness (0 h) from the thickness measured at 1, 4 and 24 h after injection in the left ear. In addition, the control (right ear) swelling measured at the same time points was subtracted. The ear swelling is expressed as delta µm.

Measurement of Serum Immunoglobulins and Mouse Mast Cell Protease-1

Concentrations of total IgE and levels of casein- or whey-specific IgE, IgG_1 and IgG_{2a} were determined in serum of sacrificed mice by means of ELISA. Microlon plates (Greiner, Alphen aan den Rijn, The Netherlands) were coated with 100 µl whey or casein (20 µg/ml) in coating buffer or rat anti-mouse IgE (1 µg/ml; carbonate-bicarbonate buffer, 0.05 M , pH = 9.6) for 18 h at 4°C. Plates were washed and blocked for 1 h with 5% BSA. Serum samples were applied in several dilutions (4–100 times) and incubated for 2 h at room temperature. Plates were washed and incubated with biotin-labeled rat anti-mouse IgE, IgG_1 or IgG_{2a} (1 µg/ml) for 90 min at room temperature and washed. The plates were incubated with streptavidin-horseradish peroxidase for 1 h, washed and developed with o-phenylendiamine. After 5 min, the reaction was stopped with 4 M H_2SO_4 and absorbance was measured at 490 nm on a Benchmark microplate reader (Bio-Rad, Hercules, Calif., USA). Serum concentrations of mouse mast cell protease-1 (mMCP-1) were determined as described previously using a commercially available ELISA kit (Moredun Scientific Ltd., Penicuik, UK) (24).

Immunohistology of the Colon

For determination of histopathological alterations, Swiss rolls of 4 cm colon were prepared (25). The colon was carefully dissected, opened longitudinally over the mesenteric line and luminal contents were removed by gently washing in saline. The colon was placed with the mucosal side down. Colons were rolled from the distal to the proximal end, fixed in ice-cold 10% formaldehyde in PBS (for 24–48 h) and embedded in paraffin (Leica EG1150c; Leica Microsystems, Rijswijk, The Netherlands). Sections of 5 µm were cut using a microtome (Leica RM2165; Leica Microsystems), stretched on water (Tissue Flotation Bath TFB45; Medite, Nunningen, Switzerland) and placed

on poly-L-lysine-coated slides. Deparaffinized Swiss roll sections were stained with mMCP-1 to detect mucosal mast cells (26). In short, after fixation in acetone, the sections were blocked with 10% normal goat serum, followed by incubation with rat anti-mouse mMCP-1 (kindly donated by Dr. H.R.P. Miller, Edinburgh, UK). The primary antibody was detected with a biotinylated polyclonal goat anti-rat antibody (Pharmingen, Aalst, Belgium), followed by incubation with streptavidin-horseradish peroxidase (Sanquin, Utrecht, The Netherlands). Color was developed with AEC chromogen staining kit (Sigma Chemical Co., St. Louis, Mo., USA) and sections were counterstained with hematoxylin. Mast cell counts were performed by counting 1 complete section of every animal.

Isometric Contraction of the Colon

The colon, caudal from the cecum, was dissected free of connective tissue and mesenterium. Colon parts of 1 cm length were mounted in an organ bath, using 2 small clamps, containing 10 ml tyrode buffer (in mM: NaCl 136.89, KCl 2.68, $MgCl_2$ 1.05, $CaCl_2$ 1.77, NaH_2PO_4 0.42, $NaHCO_3$ 11.9 and glucose 5.55) (27-29). The tissue was kept at 32°C, to prevent spontaneous contraction, and continuously gassed with a 5% CO_2 and 95% O_2 gas mixture. One clamp was attached to a fixed point in the organ bath and the other clamp was connected to an isometric transducer (Harvard Apparatus Ltd., Edenbridge, UK) with an analog recorder (BD40; Kipp & Zn., Delft, The Netherlands). Contractions were measured under a constant preload of 1.00 g. The preparations were equilibrated for 1 h in the organ bath before starting a log dose-response curve for carbachol (10^{-8} until 10^{-3} M). After every dosage, the organ bath was flushed twice and the tissue was allowed to recover for 10 min before addition of the next concentration. Also, basal activity and spontaneous contraction frequencies of the tissue were recorded.

Water Percentage in the Stool

Twenty-four hours after oral challenge, feces were collected from every animal. These samples were weighted, dried for several days and weighted again. The difference in weight is the evaporated water. The relative amount of water in the feces (relative to the total weight of the feces) was calculated and statistically evaluated.

Statistics

All data except for the isometric contractions were analyzed using one-way ANOVA and post hoc Dunnett's test. Isometric contraction data were analyzed using repeated measures ANOVA and post hoc Dunnett's test. Statistical analyses were conducted using GraphPad Prism software (version 4.0). For correlations, the Spearman rank order correlation coefficient test was used in SPSS 13.0 for Windows. Data are

represented as means ± SEM.

Results

Acute Allergen-Specific Skin Reaction

In order to study allergic skin responses, mice were challenged i.d. in the ear pinnae with casein or whey 1 week after the last oral sensitization. One hour after dermal challenge, the allergen-specific acute ear swelling response was maximal when compared to PBS and CT sham-sensitized control mice. The delta ear swelling in the casein- and whey-sensitized animals was 71.2 ± 8.4 and 137.9 ± 21.7 µm, respectively, while this was neglectable in PBS and CT sham-treated mice (8.1 ± 9.4 and –4.6 ± 4.7 µm, respectively; fig. 2, $p < 0.01$, $n = 4$ in PBS group, $n = 6$ in all other groups). All sensitized mice reacted positively to the allergen, indicating that there are no non-responders. Four hours after challenge, the ear swelling turned to basal levels in the casein group, while in the whey-sensitized group the swelling remained slightly enhanced up to 24 h after swelling (37.5 ± 12.6 µm in whey-sensitized vs. –3.9 ± 11.4 µm in CT-sensitized animals, $p < 0.05$). In additional experiments (data not shown) it was found that the marginal 24 h response was not followed by a response at later time points. In sham-sensitized animals, no differences were found between casein and whey challenge at any time points. Furthermore, whey challenge in casein-sensitized mice or vice versa did not result in any significant swelling response, indicating the antigen specificity of the skin reaction (data not shown).

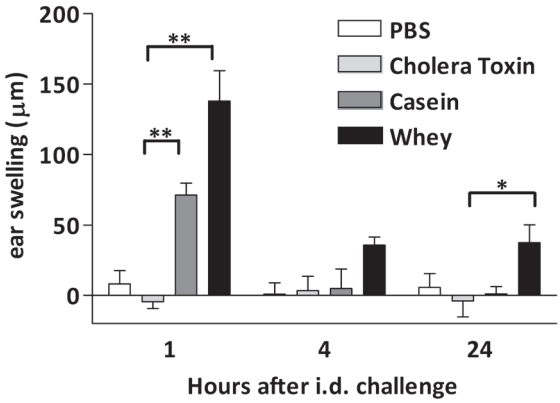

Figure 2. *Induction of an acute ear swelling in casein and whey sensitized mice in comparison with sham-sensitized (CT and PBS) mice. Delta ear swelling (µm) is calculated by subtracting the specific ear swelling induced by the corresponding antigen with vehicle (PBS) induced swelling at 1, 4 and 24 hours. * p<0.05, ** p<0.01, n=4 in PBS group, n=6 in all other groups*

Increased Total IgE and Antigen-Specific Serum IgE, IgG_1 and IgG_{2a} Levels

Half an hour after oral challenge, serum was obtained from both sensitized and non-sensitized animals. Total IgE concentrations were increased in casein- and whey-sensitized mice (400 ± 32 and 364 ± 19 ng/ml, respectively) in comparison with CT and PBS sham-sensitized mice (243 ± 37 and 218 ± 31 ng/ml, respectively; data not shown, $p < 0.01$ or $p < 0.05$, $n = 4$ in PBS group and $n = 6$ in all other groups). Casein-specific IgE as well as casein-specific IgG_{2a} levels were not increased (fig. 3a and c). In contrast, casein-specific IgG_1 levels were enhanced in the casein-sensitized mice (0.537 ± 0.135 ODA490) when compared to CT and PBS sham-sensitized animals (0.087 ± 0.017 and 0.062 ± 0.013, respectively; fig. 3b, $p < 0.01$). In the whey-sensitized animals, the whey-specific IgE (1.662 ± 0.136), IgG_1 (0.988 ± 0.069) and IgG_{2a} (0.587 ± 0.081) levels were augmented when compared to the CT sham-sensitized controls (0.000–0.311 ± 0.032; fig. 3d–f, $p < 0.01$).

Correlation Serum Immunoglobulins and the Acute Allergic Skin Reaction

To investigate the correlation between serum immunoglobulins and the ear swelling response, linear regression analyses were performed using data from 4–5 independent experiments. The serum dilutions used to calculate the correlations were: 10 times for IgE, 100 times for IgG_1 and 50 times for IgG_{2a}. In whey-sensitized mice the acute ear swelling was found to correlate positively with whey-specific IgE (fig. 4c; $p = 0.015$, $r^2 = 0.276$, $n = 21$), whey-specific IgG_1 (fig. 4d; $p = 0.043$, $r^2 = 0.154$, $n = 27$), but not with whey-specific IgG_{2a} (data not shown; $p = 0.314$, $r^2 = 0.038$, $n = 29$). Ear swelling of casein-sensitized mice was not found to correlate with casein-

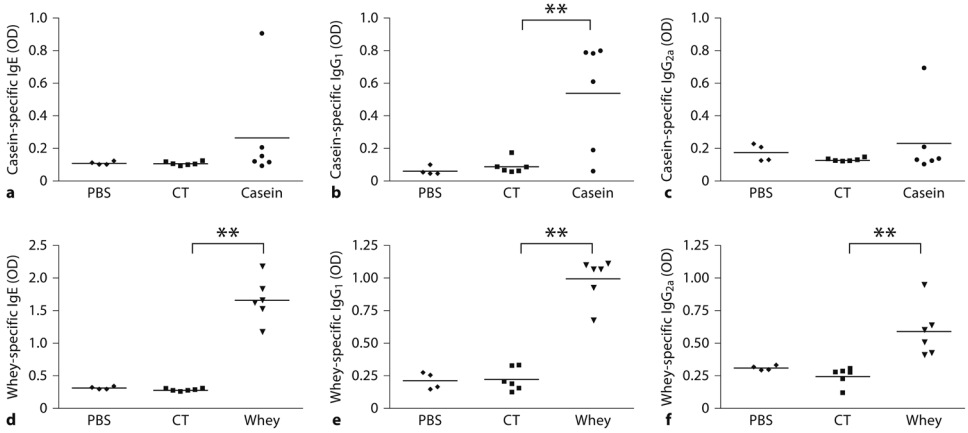

Figure 3. *Enhanced serum immunoglobulins in casein and whey sensitized mice A, B, C In the casein sensitized mice only casein specific IgG_1 levels were enhanced, no specific IgE and IgG_{2a} was measured. D, E, F In the whey sensitized mice specific IgE, IgG_1, and IgG_{2a} serum levels were enhanced. ** p<0.01, n=4 for PBS and n=6 for all other groups*

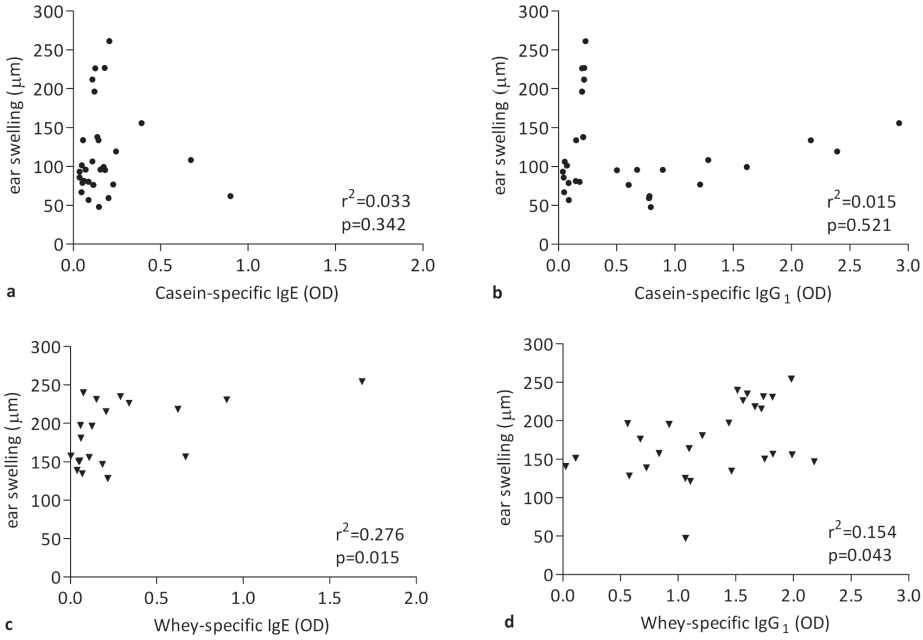

Figure 4. *Regression analyses between ear swelling and immunoglobulins for casein and whey sensitized mice. Correlation between ear swelling and A casein specific IgE (n=29) B casein specific IgG_1 (n=29) C whey specific IgE (n=21) and D whey specific IgG_1 (n=27).*

specific IgE (fig. 4a; p = 0.342, r^2 = 0.033, n = 29), casein-specific IgG_1 (fig. 4b; p = 0.521, r^2 = 0.015, n = 29) and casein-specific IgG_{2a} (data not shown; p = 0.488, r^2 = 0.18, n = 29).

Increased mMCP-1 Levels in Serum

mMCP-1 is a protease specific for mouse mucosal mast cells and will appear in the bloodstream after mast cell degranulation. To assess mast cell degranulation, mMCP-1 levels were determined in the serum 30 min after oral challenge. mMCP-1 serum concentrations of casein-and whey-sensitized mice were enhanced when compared to CT and PBS controls (3.890 ± 0.769 and 6.472 ± 2.341 vs. 0.357 ± 0.139 and 0.878 ± 0.338 ng/ml, respectively; fig. 5; n = 4–6, p < 0.01).

Decreased Number of mMCP-1-Positive Mast Cells in the Colon

After oral challenge, mice were sacrificed and the colon was obtained for histological examination. mMCP-1-positive cells were counted after immunohistochemical staining. A decreased number of mucosal mast cells was counted in the colon of casein- (7.33 ± 5.13; p < 0.05) and whey-sensitized animals (6.33 ± 5.32; p < 0.01) when compared to non-sensitized control mice (20.67 ± 10.27; fig. 6; n = 6).

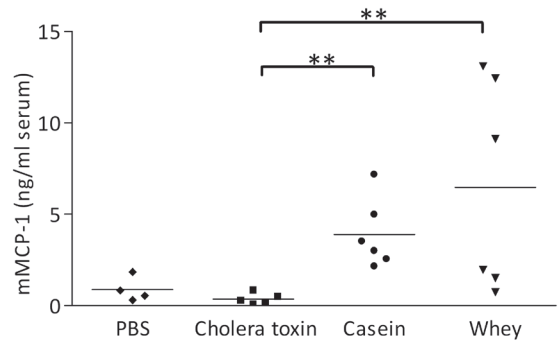

Figure 5. *Serum mMCP-1 concentrations are increased in casein and whey sensitized mice in comparison to sham-sensitized (CT and PBS) mice. ** p<0.01, n=4 for PBS, n=5 for CT and n=6 for all other groups*

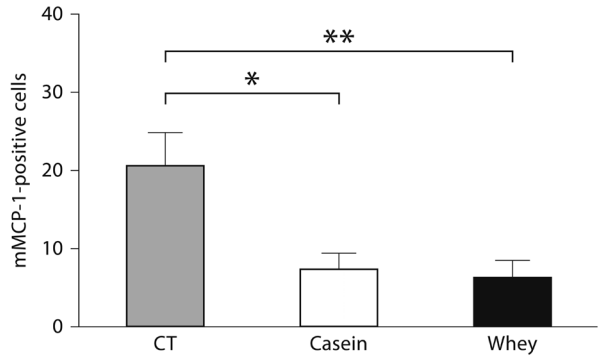

Figure 6. *Decreased mast cell counts in colon of casein and whey sensitized mice. Immunohistochemistry staining of mMCP-1 in 5 µm sections. The mean number of mMCP-1 positive cells was declined in casein and whey mice when compared to CT mice. * p<0.05, ** p<0.01, n=6 for all groups*

Altered Isometric Contraction of the Colon

Isometric contraction of the colon was determined by assessing contractility upon exposure to muscarinic receptor agonist carbachol (noncumulative dose response, 10^{-8} – 10^{-3} M). Reduced contractility for all carbachol concentrations was found for the colon of casein-sensitized mice when compared to sham-treated mice (fig. 7; $p < 0.01$, basal contractility did not differ between groups). This hyporesponsiveness of the colon cannot be characterized by a right movement of the EC_{50} concentration, but only by a reduction in maximal contraction force (E_{max}) (4,975 ± 1,046, 2,242 ± 296 and 5,933 ± 1,071 mg in control, casein and whey groups). The hyporesponsiveness

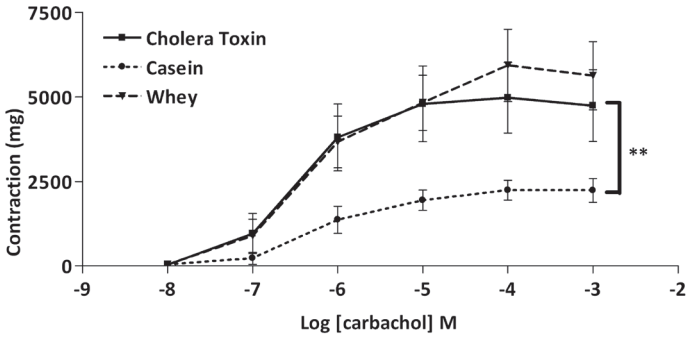

Figure 7. *Contractility changes in the colon of casein sensitized mice after carbachol stimulation. Contractility of the colon of casein mice was decreased when compared to CT and whey treated mice. ** p<0.01, n=6 in all groups*

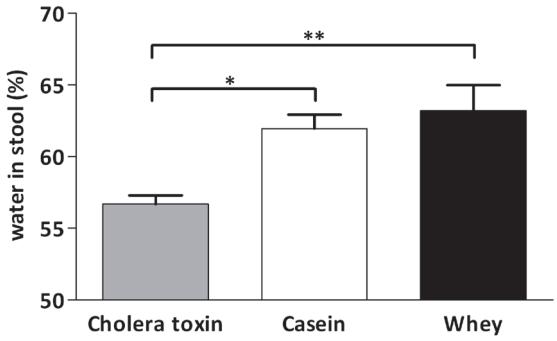

Figure 8. *Water percentage water in the stool of casein and whey sensitized mice compared to CT controls. The stool water content was found to be increased in casein and whey mice, 24 hours after oral challenge. * p<0.05, ** p<0.01, n=6 in all groups*

of the colon in the casein-sensitized mice was found to be consistently present in all experiments, while none of these effects were seen in the whey-sensitized animals.

Water Percentage in the Stool

The relative amount of water in the feces was increased in the casein- (fig. 8; 61.97 ± 0.97%, $p < 0.05$, $n = 6$) and whey-sensitized animals (63.22 ± 1.78%, $p < 0.01$, $n = 6$) when compared to the control mice (56.68 ± 0.62%, $n = 6$).

Discussion

One of the most common food allergies in childhood is CMA. CMA is diagnosed when symptomatic patients have enhanced serum levels of cow's milk protein-specific IgE (radioallergosorbent test) and/or a positive skin prick test (SPT). In addition, a double-blind placebo-controlled oral challenge can be performed, which is the most reliable test for food allergy (9, 30, 31). Currently, no immunotherapy is available for CMA patients, hence patients need to avoid cow's milk allergens in the diet and use hydrolyzed milk formulas (9). In the present study, sensitization against the cow's milk proteins whey and casein is investigated in mice that were sensitized orally. Specific immunoglobulin levels were induced and local and systemic symptoms were evaluated by studying effects in the gastrointestinal tract and skin. The investigated parameters closely resemble diagnostic tools that are used in the clinic; they are summarized in table 1.

Besides screening for cow's milk-specific serum IgE, the SPT is used for the diagnoses of CMA in humans (30). In the current study, the allergen-induced ear swelling is introduced as a possible equivalent for the SPT. It is a new tool to determine systemic sensitization for casein and whey in mice. Oral sensitization with casein or whey consistently resulted in a positive acute allergic ear swelling response upon local allergen challenge. Furthermore, it can be concluded that sensitization via the oral route was able to induce systemic sensitization in mice treated either with casein or whey. In other models for skin hypersensitivity, comparative values for ear swelling responses have been found (32).

Although both whey- and casein-sensitized animals showed evidence of systemic allergy according to the ear swelling response, differences were found in serum immunoglobulin responses between the groups. Although total IgE concentrations

Table 1. *Overview of major parameters tested in both models. $^{+}$: increased compared to control (CT) mice; - : decreased compared to control (CT) mice; ND : no difference observed regarding to control (CT) mice.*

	Parameter	Casein	Whey
Sensitization	IgE	ND	$^{+}$
	IgG_1	$^{+}$	$^{+}$
	ear swelling	$^{+}$	$^{+}$
Mast cell degranulation	mMCP-1	$^{+}$	$^{+}$
Physiology	water percentage	$^{+}$	$^{+}$
	motility	-	ND

and specific IgG_1 levels were enhanced in both the casein- and whey-sensitized mice, specific IgE and IgG_{2a} were found to be increased only in the whey-sensitized mice. In the clinic, 40% of CMA patients have a negative radioallergosorbent test for cow's milk proteins; however, they reveal to be positive in the SPT (6-8). In a recent study, the SPT was found to correlate better with the double-blind food provocation test than serum immunoglobulins, although larger studies are necessary to confirm these findings (33). Typically, in the casein-sensitized mice, no specific IgE was detected, while the mice indeed showed systemic allergy as seen in the acute allergen-induced ear swelling response. In contrast, the whey-sensitized mice showed the classical characteristics of type I IgE-mediated allergy, scoring positive for allergen-specific IgE and acute ear swelling, underlining the validity of this tool (34-36). In whey-sensitized mice, the validity of the acute skin reaction test as a tool to determine systemic sensitization was supported by the finding that challenge-induced ear swelling correlated with whey-specific IgE and IgG_1. The acute allergic ear swelling in casein-sensitized mice was not associated with enhanced specific IgE levels, which may imply that there is no role for IgE in this model under the current protocol. In CMA patients, casein-specific IgE can be detected in the serum, but levels vary. It is known that patients with persistent CMA over the age of 9 years had elevated levels of casein-specific IgE compared to younger children with CMA. Therefore, low levels of casein-specific IgE early in life could indicate a non-persistent form of CMA (31). Instead of IgE, the acute ear swelling in mice might have been triggered by IgG_1, which is generally known to play a crucial role in mast cell activation in rodents. It has been shown that immunoglobulins can significantly interfere, positively and negatively, with mast cell responses (reviewed by Bruhns *et al.* (37)). In addition, it is known from the literature (38) that even in FcεRI-deficient mice, anaphylactic reactions are possible, indicating that IgE is not always a prerequisite for anaphylaxis. Although in casein-sensitized mice no significant correlation was found between ear swelling and levels of IgG_1, the scatter plot indeed shows a subgroup of mice in which the ear swelling is positively associated with the level of IgG_1. Hence, although casein-allergic mice lack specific IgE, enhanced IgG_1 levels in these mice may at least partly reflect allergic sensitization. Apart from IgE and IgG_1, Redegeld *et al.* (39) have found that mast cell degranulation can occur with immunoglobulin light chain. Immunoglobulin light chain is produced in excess during the formation of immunoglobulins. Hence, casein-specific immunoglobulin light chain may have caused the ear swelling in casein mice with an acute allergic skin response that could not be explained by the presence of IgE nor IgG_1. However, this remains speculative and further research is necessary.

Whey-sensitized mice show a slight but significant ear swelling at 24 h. Together with the higher allergen-specific IgG_{2a} levels in these animals, this might reflect a late-

phase response after whey sensitization. Frossard *et al.* (40) also found enhanced serum levels of IgE/IgG_1 and IgG_{2a}, characteristic for a mouse TH2 and TH1 type of immune response, respectively, against β-lactoglobulin in a C3H/HeOuJ mice model for β-lactoglobulin. These levels of IgG_{2a} are in accordance with enhanced levels of IgG_{2a} that have been found in a similar mouse model for peanut allergy (22, 23, 41).

Clinical features of CMA are generally known to be elicited by mucosal mast cells (4, 22, 42). Those mast cells are present in the intestinal mucosa and additionally drawn to the site of allergen challenge. In casein- and whey-sensitized mice, mast cell numbers of the colon were found to be declined in comparison to sham-sensitized mice. Mucosal mast cells were stained with mMCP-1, a β-chymase present in the mast cell granules, which end up in the bloodstream after degranulation (43). In both whey- and casein-sensitized mice, serum mMCP-1 concentrations were increased, 30 min after i.g. challenge, reflecting mast cell degranulation. Upon oral allergen exposure, mucosal mast cells may have degranulated, resulting in enhanced mMCP-1 serum levels. After mast cell degranulation, the mast cell itself is not visible anymore with mMCP-1 staining, which might explain the drop in mast cell numbers in the colon of casein- and whey-sensitized mice (44).

One of the most prominent symptoms of food allergy is discomfort in the gastrointestinal tract which can be abdominal pain, diarrhea or sometimes constipation (4, 5). The collected stool samples of casein- and whey-sensitized animals contained a higher water percentage than the controls, which is suggestive of diarrhea. Besides diarrhea, dysmotility of the intestine is a problem in CMA patients (12, 13). The casein-sensitized mice showed contractility changes in organ bath studies, while whey-, CT and PBS-sensitized mice did not. Isometric contractions of colon segments of casein-sensitized animals were hyporeactive in comparison to CT controls. It is known that allergy has adverse effects on the gut health, causing dysmotility (45, 46). In this study, smooth muscle contractility differences between control and casein-sensitized animals may reflect changes in motility, which is supported by findings of Kobayashi *et al.* (47) in a model for diarrhea. Motility measurements are often performed when studying inflammatory bowel diseases, in which similar intestinal symptoms can be found. In these studies, local intestinal inflammation is indicated as causative factor for motility changes reflecting alterations in smooth muscle contractions (48). Hence, local intestinal inflammation may have induced hypomotility occurring in the casein mice. However, histological evaluation did not reveal any obvious signs of inflammatory cell infiltrates after oral challenge. Possibly during sensitization, casein might have caused local inflammation, hereby reducing sensitivity of cholinergic neurons for

carbachol stimulations, known to alter colonic motility (49). In addition, local mast cell degranulation may have induced motility changes (11). Mule *et al.* (29, 50) have shown that activation of protease-activated receptors (PAR) can cause colon smooth muscle relaxation as well as contraction. PAR agonists such as mast cell-derived tryptase may have caused PAR activation in both casein- and whey-sensitized mice. It remains speculative why hypomotility of the colon was only observed in casein-sensitized mice; however, local levels of tryptase and PAR expression may differ between casein- and whey-sensitized mice and will be the focus of future investigation.

Additionally, casein and whey proteins differ with regard to physical, physiological and dietary properties and therefore may cause differential effects within the intestine. For example, it is known that there is a difference in digestive speed of casein and whey (51). Casein protein is a slowly digested dietary protein, while whey protein is a fast one (52). In addition, both proteins have different effects on satiety and gastrointestinal hormone response (53). Hence, these proteins differentially influence the intestinal physiology. Furthermore, it is known that casein by itself can cause DNA damage in the colon, which is associated with a thinner mucus barrier. In the same study, whey did not cause these effects (54). Two studies have shown hypomotility of the intestine after ingestion of casein, which might relate to the presence of casomorphins that were found to reduce motility (55, 56). Hence, casein may also have intrinsic properties that can cause hypomotility. At this stage, the exact mechanism behind the hypocontractility found in the casein-sensitized mice is not known and will be the topic of future studies.

Taken together, altered motility suggests subtle local changes in intestinal discomfort and provides a new tool to measure local intestinal alterations as a consequence of allergic sensitization in mice.

Both casein and whey sensitization consistently resulted in an acute allergic skin reaction after allergen challenge, which was associated with specific IgE and IgG_1 serum levels in whey-sensitized mice and specific IgG_1 serum levels in casein-sensitized mice. Decreased numbers of mMCP-1-positive mucosal mast cells within the colon and enhanced mMCP-1 levels in the serum suggest a pathophysiological role for mucosal mast cells in this model. In addition, the stool water content of allergic mice was enhanced, reflecting occurrence of diarrhea 24 h after oral allergen challenge. In the casein-sensitized mice, reduced intestinal smooth muscle contraction was observed. This suggests dysmotility of the colon shortly after oral exposure to the allergen. Indeed, constipation and/or diarrhea are among the symptoms in patients affected with CMA. Overall, the tools described in this study

might open new avenues to unravel underlying mechanisms of whey and casein allergy in mice.

Acknowledgement

We would like to thank Dr. Anneke Rijnierse for assistance with mMCP-1 immunohistochemistry and helpful discussions.

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4

Acute allergic skin response as a new tool to evaluate the allergenicity of whey hydrolysates in a mouse model of orally induced cow's milk allergy

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Pediatric Allergy and Immunology 2010;21:e780-e786

Abstract

Hypoallergenic milk formulas are used for cow's milk allergic infants and may be a good option for infants at risk. Clinical studies have shown that the protein source or the hydrolysis methodology used may influence the effectiveness in infants stressing the importance of adequate pre-clinical testing of hypoallergenic formulas in an *in vivo* model of orally induced cow's milk allergy. This study was undertaken to introduce a new read-out system to measure the residual allergenicity of whey hydrolysates on both the sensitization and challenge phase of orally induced cow's milk allergy in mice. Mice were sensitized orally to whey or a partial whey hydrolysate (pWH) to measure the residual sensitizing capacity. To predict the residual allergenicity of hydrolysates, whey allergic mice were challenged in the ear with pWH, extensive whey hydrolysate or an amino acid-based formula. An acute allergic skin response (ear swelling at 1 h), whey-specific serum antibodies, and local MCP-1 concentrations were measured. In contrast to whey, oral sensitization with pWH did not result in the induction of whey-specific antibodies, although a minor residual skin response to whey was observed after challenge. Skin exposure to whey hydrolysates showed a hydrolysation dependent reduction of the acute allergic skin response in whey allergic mice. In contrast to whey, skin exposure to pWH did not enhance tissue MCP-1 levels. The acute allergic skin response in mice orally sensitized to cow's milk proteins reveals a new pre-clinical tool which might provide information about the residual sensitizing capacity of hydrolysates supporting the discussion on the use of hypoallergenic formulas in high risk children. This mouse model might be a relevant model for the screening of new hypoallergenic formulas aimed to prevent or treat cow's milk allergy.

Introduction

Beside hen's egg allergy, cow's milk allergy is considered to be the first and most common type of allergy during early infancy (1). In particular in infants with a genetically elevated risk of allergy the incidence of cow's milk allergy rises sharply. Although most infants outgrow cow's milk allergy before their fifth year, immunoglobulin (Ig)E mediated allergy is an important indicator of susceptibility for the development of atopic diseases like atopic dermatitis and allergic asthma later in life (2, 3). Therefore, attempts to prevent cow's milk allergy deserve high priority either by avoidance of the allergenic protein or by inducing oral tolerance (4). Breastfeeding is considered to be the golden standard and the best way of preventing sensitization and cow's milk hypoallergenic formulas are considered a good alternative for infants at high risk or infants diagnosed for cow's milk allergy. Cow's milk hydrolysates are generally categorized into partial and extensive hypoallergenic formulas based on the degree of hydrolysis and consequently the length of the remaining peptides. The allergenicity is considered to reduce in accordance with declining peptide size. For infants suffering from cow's milk allergy extensive hypoallergenic formulas or amino acid based formulas constitute a good option (5, 6). Although there is general scientific and clinical agreement how triggering of the allergic reaction in cow's milk allergic infants can be avoided debate on the most effective strategy to avoid initial sensitization remains intense (7). The effectiveness of partial- and or extensive hypoallergenic formulas in preventing cow's milk allergy in high risk children has been studied in several clinical trials over the past ten years but results remain inconclusive (8-11). The protein source and hydrolysis methodology used varies significantly which might influence the effectiveness of hypoallergenic formulas (12). For safety and efficacy reasons the allergenicity of these hypoallergenic formulas need to be assessed which can not solely be based on the peptide size distribution or immunological *in vitro* assays. *In vivo* animal models for cow's milk allergy might be useful to predict the efficacy of hypoallergenic formulas in humans in terms of prevention of cow's milk allergy or avoidance of clinical symptoms in already sensitized infants. Although some animal models address the sensitizing capacity and the residual allergenicity of hypoallergenic formulas they predominantly use parental rather than oral sensitization or in the case of guinea pigs the effects are IgG_1 rather than IgE mediated (13, 14).

In this current study an acute allergic skin response is presented as a new major read-out system to address the putative sensitizing capacity and the residual allergenicity of whey hydrolysates in a mouse model of orally induced cow's milk allergy, which mimics the human route of sensitization.

Materials and methods

Cow's milk proteins and hydrolysates

Whey and β-lactoglobulin were obtained from DMV international, Veghel, the Netherlands. The amino acid based formula from Danone Research Centre for Specialised Nutrition, the Netherlands. A partial whey hydrolysate (pWH) was manufactured at Danone Research Centre for Specialised Nutrition by enzymatic hydrolysis under specified conditions. The enzymatic process was stopped by fast cooling. The pWH was further characterized by analysis of the peptide size (85% < 1 kD, 8% < 2 kD, 4% < 5 kD, 1% < 10 kD, 0.6% < 20 kD and 1.4% >20kD) by means of high pressure liquid chromatography. Subsequently, filtering of the pWH using a filter with a cut-off of 3 kD resulted in an extensive whey hydrolysate. These experimental whey hydrolysates were used in the animal studies as mentioned below.

Mice

Three- to 4-week-old specific pathogen free female C3H/HeOuJ mice were purchased from Charles River Laboratories (Maastricht, the Netherlands), maintained on a cow's milk protein free standard mouse chow (AIN-93G soya, Special Diets Services, Witham, Essex, UK) and housed in the animal facility at the Utrecht University. Animal care and use were performed in accordance with the guidelines of the Dutch Committee of Animal Experiments.

Sensitizing capacity of pWH

Mice were sensitized orally, using a blunt needle, on days 0, 7, 14, 21 and 28 to 20 mg whey homogenized in PBS (0.5 ml, Cambrex Bio Science, Verviers, Belgium) mixed with 10 µg cholera toxin (Quadratech Diagnostics, Epsom, UK) as an adjuvant (n=6 per group). Sham-sensitized mice received cholera toxin only.

To study the residual sensitizing capacity of whey hydrolysates a third group of mice was sensitized using 20 mg pWH (in 0.5 ml PBS) with 10 µg cholera toxin as depicted in Figure 1(A). One week after the last sensitization the acute allergic skin responses to whey and pWH were measured, blood samples were collected and spun down for 15 minutes at 13500 rpm. Sera were stored at -70 ^{o} C for determination of whey-specific antibodies.

Residual allergenicity of whey hydrolysates

To study the allergenicity of whey hydrolysates on their residual capacity to induce clinical symptoms mice were sensitized to whey using cholera toxin as adjuvant to induce cow's milk allergy as depicted in figure 1(B). One week after the last sensitization the acute allergic skin responses to whey, β-lactoglobulin, pWH, extensive whey hydrolysate or an amino acid based formula were measured (n=6 per group). Ears of whey and pWH skin challenged mice were collected after 5 hours

for the determination of tissue cytokines and were stored at -70 ° C until further analysis.

Whey-specific serum immunoglobulins

To determine sensitization to whey and pWH, whey-specific IgE, IgG_1 and IgG_{2a} levels were measured in serum by means of ELISA. Microlon plates (Greiner, Alphen aan de Rijn, the Netherlands) were coated with 20 µg whey in coating buffer (Sigma) in

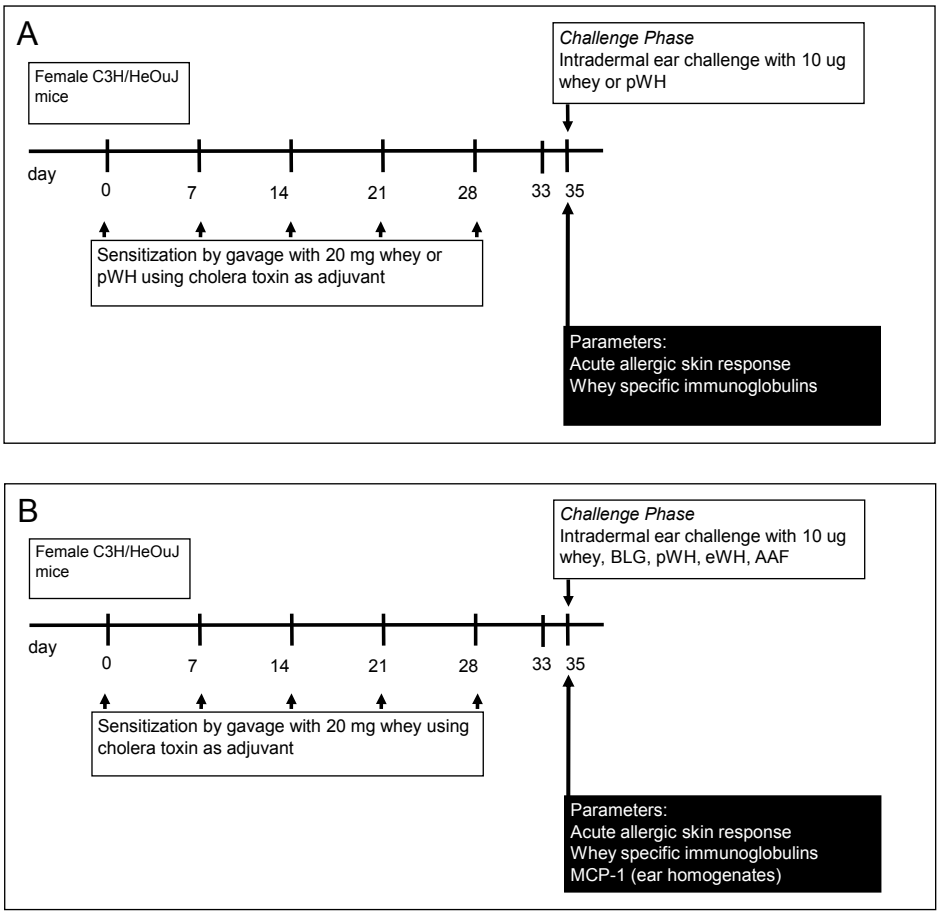

Figure 1. *Sensitizing capacity (a) and residual allergenicity (b) of whey hydrolysates were investigated in a mouse model of orally induced cow's milk allergy. Mice were sensitized orally to whey or partial whey hydrolysate (pWH) at days 0, 7, 14, 21 and 28 using cholera toxin as an adjuvant. One week after the last sensitization mice were challenged in the ear with 10 µg whey, pWH, extensive whey hydrolysate (eWH), β-lactoglobulin (BLG) or an amino acid-based formula (AAF).*

demi for 18 hours at 4°C. Plates were washed and blocked for 1 hour with buffer containing 50 mM Tris, 2 mM EDTA en 137 mM NaCl /0.05% Tween and 5% BSA. Serum samples were incubated for 2 hours at room temperature. Plates were washed and incubated with 1 µg biotin labeled rat anti-mouse IgE, IgG_1 or IgG_{2a} (Pharmingen, Alphen a/d Rijn, the Netherlands) for 1 hour at room temperature. After washing the plates were incubated with streptavidin-horse radish peroxidase (Sanquin, Amsterdam, the Netherlands) for 1 hour, washed and developed with o-phenylendiamine (Sigma). The reaction was stopped after 10 minutes with 4M H_2SO_4 and absorbance was measured at 490 nm on a Benchmark microplate reader (Biorad, Veenendaal, the Netherlands). For measuring whey-specific IgE and IgG_{2a} in whey sensitized mice a serum dilution factor of 10 and for whey-specific IgG_1 a dilution factor of 50 was used. The whey-specific antibodies were expressed as optical density (OD).

Acute allergic skin response

The acute allergic skin response (ear swelling at 1 hour) was determined in anaesthetized mice after intra dermal injection of 10 µg pWH, extensive whey hydrolysate, β-lactoglobulin or an amino acid based formula in the ear pinnae and compared to the ear swelling after whey skin challenge (10 µg). Based on earlier experience ear swelling can be measured as early as 20 minutes with an optimum at one hour. As a negative control sham-sensitized mice were skin challenged with whey. Ear thickness was measured in duplicate using a digital micrometer (Mitutoyo, Veenendaal, the Netherlands). The allergen specific net ear swelling was calculated by correcting the allergen-induced increase in ear thickness with the non-specific ear swelling due to local injection in the sham-sensitized group.

Tissue cytokines

To study the capacity of a pWH to induce an inflammatory response after allergen challenge whey sensitized mice where challenged in the ear with whey or pWH. Ears were collected 5 hours after challenge as described previously (15). In short, whole ears were collected and snap frozen in liquid nitrogen and taken up in buffer. Homogenates were spun down before analysis of cytokine content. MCP-1 levels were measured using a cytometric bead array for mouse inflammatory cytokines (Becton Dickinson, Alphen a.d. Rijn, the Netherlands) according to the manufacturer's protocol. Beads were analyzed at a FACS Calibur flow cytometer (Becton Dickinson, Erebodegem, Belgium).

Statistics

All data were analyzed using one way ANOVA and post hoc Dunnett's test. A probability value of $p<0.05$ was considered significant. Statistical analyses were

conducted using GraphPad Prism software. Data are represented as mean ± SEM (n=6 per group).

Results

Sensitizing capacity of pWH

To study the residual sensitizing capacity of whey hydrolysates, mice were sensitized orally to whey as a positive control or pWH, using cholera toxin as adjuvant. Subsequently, the mice were skin challenged with whey or pWH (Fig.1A). In whey sensitized mice, whey-specific IgE, IgG_1 and IgG_{2a} levels were enhanced (Fig. 2A-C). In contrast, sensitization with pWH did not generate any detectable whey-specific IgE or IgG_{2a} antibodies. Whey-specific-IgG_1 was slightly enhanced in two out of six mice compared to sham-sensitized mice however this did not reach significance (Fig. 2A-C). Interestingly, the acute skin response after whey skin challenge in the ear was not completely prevented in pWH sensitized mice suggesting the presence of some intact sensitizing whey epitopes in pWH. This residual ear swelling response was not induced upon skin challenge with pWH in these mice (Fig. 3).

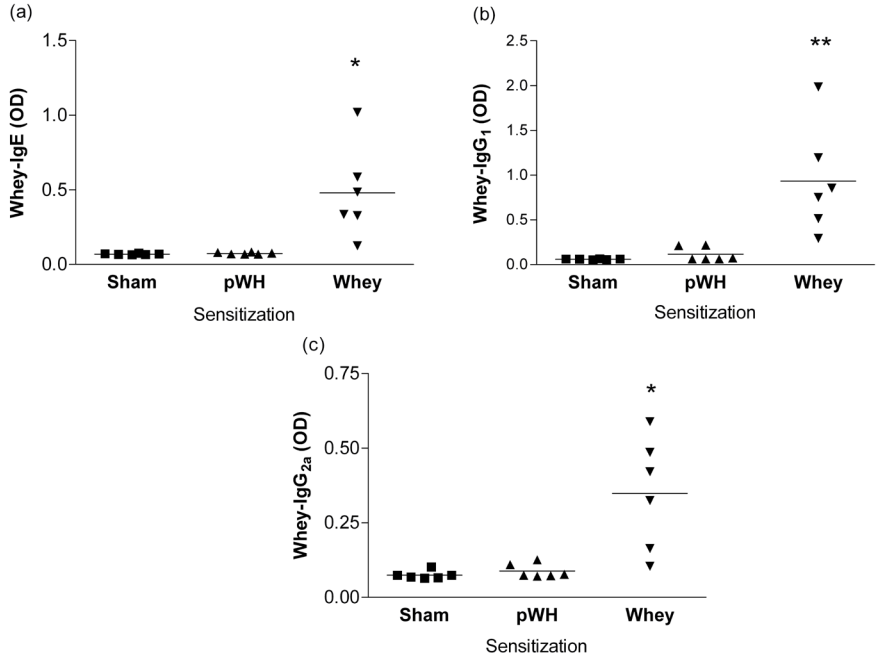

Figure 2. *No generation of whey-specific IgE, IgG_1 and IgG_{2a} antibodies in serum of partial whey hydrolysate-sensitized mice in contrast to whey sensitization. Sham-sensitized mice received cholera toxin only. Whey-specific IgE (a), IgG_1 (b) and IgG_{2a} (c) were measured on day 35 in serum. Data are expressed as means ± SEM of six mice per group. *p < 0.05, **p < 0.01 compared to sham-sensitized mice.*

Residual allergenicity of whey hydrolysates

To study the residual potency of whey hydrolysates to trigger an allergic response whey, pWH, β-lactoglobulin or an extensive whey hydrolysate were injected in the ears of mice orally sensitized to whey (Fig. 1B). An amino acid based formula was used as a negative control and induced no acute allergic skin response when compared to sham-sensitized mice (data not shown). The acute allergic skin response was reduced in a hydrolysation grade dependent fashion (Fig. 4). In addition, the acute allergic skin response to β-lactoglobulin was determined in whey sensitized mice. The response observed after β-lactoglobulin skin challenge (110.8 µm ± 6.7 µm) was similar to the response after whey challenge (135.9 µm ± 6.5 µm) showing that β-lactoglobulin is probably the dominant whey protein inducing allergic skin responses in whey allergic mice. To study the cellular response upon mast cell degranulation, MCP-1 levels were measured in ear homogenates. MCP-1 levels were increased significantly in whey sensitized mice after whey skin challenge, while pWH skin challenge did not result in enhanced MCP-1 levels (Fig. 5).

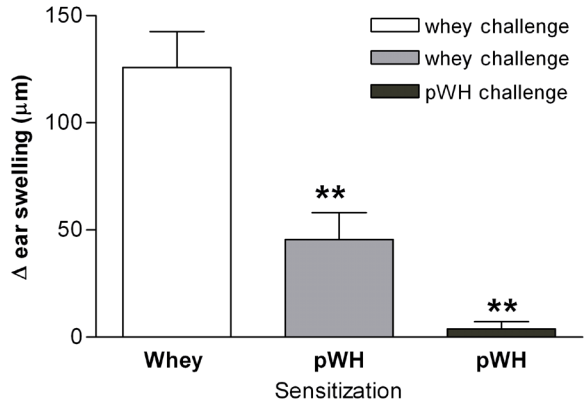

Figure 3. *Reduced acute allergic skin response after skin challenge with whey in mice sensitized to pWH when compared to whey sensitized mice. The acute allergic skin response was not induced upon pWH skin challenge in these mice. Data are expressed as means ± SEM of six mice per group. **p < 0.01 compared to whey-sensitized/challenged mice.*

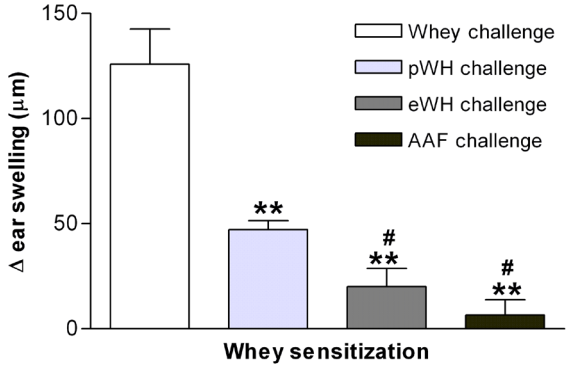

Figure 4. *Hydrolysation grade dependent reduction of the acute allergic skin response after skin challenge with pWH, extensive whey hydrolysate (eWH) or an amino acid-based formula (AAF) compared to skin challenge with whey in whey allergic mice. Data are expressed as means ± SEM of six mice per group. p < 0.05 compared to sham-sensitized mice, ##p < 0.01 compared to sham-sensitized mice, **p < 0.01 compared to whey challenged mice.*

Figure 5. *Enhanced MCP-1 concentrations in ear homogenates obtained 5 h after skin challenge with whey, but not after pWH skin challenge in whey-sensitized mice. Data are expressed as means ± SEM of six mice per group. **p < 0.01 compared to sham-sensitized mice.*

Discussion

Several clinical studies have been performed to address the sensitizing capacity of partial- and extensive hypoallergenic formulas. Differences in peptide size, variations in protein sources and hydrolysation methods of hypoallergenic formulas can modify the preventive effect of these formulas (12) stressing the importance of adequate pre-clinical testing of hypoallergenic formulas in an *in vivo* model for orally induced cow's milk allergy.

In the current study a mouse model of orally induced cow's milk allergy was used in order to assess the sensitizing capacity and the residual allergenicity of hypoallergenic formulas. Previous studies by our group have confirmed that whey-specific IgE and IgG_1 serum levels correlate with an allergen specific acute skin response after ear challenge with whey (16). In contrast to mice orally sensitized to whey, pWH sensitization did not induce any detectable whey-specific IgE antibodies. Interestingly, in these mice the acute allergic skin response to whey was not completely lost suggesting the presence of some intact sensitizing epitopes in the pWH. Although no whey-specific IgE and IgG_1 were detected in serum, these antibodies may have been present on the mast cell surface and have caused the residual acute ear swelling in pWH sensitized mice. These observations indicate that the acute skin response might be a sensitive method to determine the sensitizing capacity of hypoallergenic formulas in addition to whey-specific antibodies as compared to measurement of whey-specific antibodies solely. In contrast to whey skin challenge, no residual skin response was observed after pWH challenge suggesting that despite the presence of some sensitizing epitopes seem to be present in pWH, the capacity of pWH to cross-link low surface density of IgE or IgG_1 bound to mast cells required to induce an allergic response is probably lost. Allergen specific antibodies and skin prick test have been proven useful to diagnose cow's milk allergy in humans (17-19). A positive skin prick test was invariable associated with an adverse reaction on formal open challenge (20) and in a recent study the skin prick test was found to correlate better with the double blind food provocation test than serum antibodies (21). Although the acute skin response in mice is not fully identical to the human skin prick test, it is considered to be a scientific parameter for sensitization as well as an allergic effector response. The current finding that an acute allergic skin response is a sensitive read-out for cow's milk allergy in mice is in agreement with human studies which suggest that in IgE-mediated cow's milk allergy the skin prick test is more sensitive than the measurement of specific antibodies as a screening tool for cow's milk allergy (22, 23) .

Until now several *in vivo* models have been used to study the residual allergenicity of hypoallergenic formulas. Guinea pigs sensitized by the oral route have been used as the recommended model of reference for testing safety of new hypoallergenic

formulas due to their predisposed responsiveness (13). However, the generated antibody response is of the IgG_{1a} subtype which is different from humans. Both rat and mouse models for cow's milk allergy have been used to test allergenicity of cow's milk hydrolysates (14, 24). In these studies, instead of oral sensitization systemic sensitization was used which might induce a different immunological response because of differences between the mucosal and the systemic part of the immune system (25). The model presented in this study includes oral sensitization with serum IgE and an acute allergic skin response as a major read-out reflecting the human situation.

Apart from testing the putative sensitizing capacity of whey hydrolysates, the acute allergic skin response was used to provide additional information on the residual allergenicity of hypoallergenic formulas. The whey protein fraction consists in majority of β-lactoglobulin, α-lactalbumin and bovine serum albumin. Similar to whey, β-lactoglobulin induced an acute allergic ear swelling, showing that, like in human, β-lactoglobulin is the dominant whey protein in cow's milk. In contrast to whey, skin exposure to pWH was found to trigger a much less pronounced allergic skin response and an amino acid based formula was unable to induce any ear swelling in whey sensitized mice. Furthermore, an extensive hydrolysate provoked a less pronounced reaction than pWH, suggesting hydrolysation grade dependency. In parallel with a diminished allergic skin response, skin exposure to pWH was not found to enhance tissue MCP-1 concentrations whereas whey did. MCP-1 is a chemo attractant for monocytes and activated T cells, NK cells and basophils. Besides its regulation of leucocyte recruitment, MCP-1 also promotes Th2 type inflammatory responses which might point to an intermediate or late phase response (26). Although skin challenge with pWH was still able to evoke a limited acute allergic skin response in whey allergic mice, the capacity to induce an allergic late phase response may be lacking.

In the present study the acute allergic skin response is presented as a new read-out system to evaluate the sensitizing capacity and the residual allergenicity of whey or β-lactoglobulin based cow's milk hydrolysates in a mouse model of cow's milk allergy. Similar to the human situation, the oral sensitization route was used. This pre-clinical tool might support the discussion around the use of hypoallergenic formulas to prevent sensitization in high risk children and might be a relevant model for the screening of new hypoallergenic formulas aimed to avoid allergic symptoms in cow's milk allergic infants.

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5

In vivo and *in vitro* evaluation of the residual allergenicity of partially hydrolysed infant formulas

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Toxicology Letters 2011; Mar 25;201(3):264-9

Abstract

Hypoallergenic infant formulas are commonly used for genetically predisposed children and infants diagnosed with cow's milk allergy. This study describes both *in vitro* and *in vivo* approaches to assess residual allergenicity of partially hydrolysed infant formulas. Electrophoretic patterns indicated that β-lactoglobulin and other whey proteins were largely degraded. For safety reasons, according to the European commission-guidelines, it is required that the sensitizing capacity of hypoallergenic formulas is tested in an animal model. In contrast to whey sensitization, no elevated levels of whey-specific IgE, anaphylactic reactions or drop in body temperature were observed in sensitized mice exposed to whey hydrolysates. This indicates that the whey hydrolysates lost their putative sensitizing capacity in a mouse model using oral sensitization, which is highly relevant in relation to the human situation. In combination with the lost capacity of hydrolysed infant formulas to cross-link human IgE antibodies on RBL-huFcεRI *in vitro*, both the sensitization and the challenge phase of the allergic response were studied. This combination of assays is proposed as a strategy for the screening of new hypoallergenic formulas aimed at preventing sensitization in atopic children and avoiding clinical symptoms in infants suffering from cow's milk allergy.

1. Introduction

Food allergens, aeroallergens, medications and insect venoms are the most common allergens encountered that are responsible for inducing Type I or IgE-mediated hypersensitivity reactions (1). Type I allergic responses to cow's milk proteins leading to cow's milk allergy are characterized by a T helper 2 (Th2) response resulting in the production of allergen-specific IgE (sensitization phase). Binding of IgE to the high affinity receptor FcεRI on mast cells or basophils followed by cross-linking of the IgE-antibodies by the food allergen elicits degranulation and the release of mediator (effector/challenge phase). Clinical symptoms may occur in the skin, gastrointestinal tract or airways. Cow's milk allergy is considered to be the first and most common type of allergy during early infancy.

While breastfeeding is considered to be the golden standard for infant nutrition, hypoallergenic formulas are a good alternative for infants at high risk or infants diagnosed with cow's milk allergy. Hypoallergenic formulas are processed by enzymatic treatment, heat treatment and/or ultra filtration of cow's milk proteins. These hydrolysed formulas are generally categorized as partial and extensive hydrolysates based on the degree of hydrolysis and can be characterized by assessing the molecular weight distribution of the residual proteins. However, residual allergenicity cannot be ascertained solely based on peptide size distribution. Several clinical studies have been performed to address the sensitizing capacity of partial and extensive hypoallergenic formulas in high risk children (2-5). Differences in peptide size, variations in protein sources and hydrolysation methods of hypoallergenic formulas can modify the preventive effect of these formulas (6) stressing the importance of adequate pre-clinical testing of hypoallergenic formulas.

Residual allergenicity of hydrolysed formulas can be determined *in vitro* by measuring the binding of the hydrolysed proteins to serum antibodies of sensitized animals or humans using an *in vitro* cell based degranulation assay reflecting the effector phase of the allergic response. According to the EC-directive on infant formulas and follow-on formulas, it is required that objective and scientifically verified data are available as proof of the claimed hypoallergenicity. For safety reasons, the hypoallergenicity of hydrolysed infant formulas needs to be assessed by showing that the hypoallergenic formulas are not able to sensitize animals to the protein source they are derived from (Commission Directive 96/4/EC of 16th February 1996 amending Directive 91/321/EEC on infant formulae and follow-on formulae. Official Journal of the European Communities No L 49: 12-16). Guinea pigs sensitized by the oral route have been used as a common model for the identification of the residual sensitizing capacity of new hypoallergenic formulas due to their innate responsiveness. A main disadvantage of the guinea pig model is the generation of IgG_{1a} subclass anaphylactic antibody responses instead of IgE antibody responses, which are the main physiological antibody responses in allergic humans. This makes

the suitability of the guinea pig model questionable with regard to the potential extrapolation to the human situation, although this model was frequently used due to the absence of other suitable oral sensitization models. Recently, however, a predominantly IgE-mediated mouse model of orally induced cow's milk allergy was introduced (Schouten *et al.*, 2008). Using this model, the residual allergenicity of partially and extensive hydrolysed whey proteins was determined (7), indicating that this model can potentially be used to study the sensitizing capacity of hydrolysed whey formulas.

In the present study, a strategy for comprehensive and detailed analyses of the potential allergenicity of whey-based hydrolysed infant formulas is described. By assessing the sensitizing capacity in an *in vivo* mouse model using oral sensitization in combination with the capacity to cross-link human IgE antibodies on RBL-huFcεRI cells *in vitro*, both the sensitization and challenge phase of the allergic response can be studied.

2. Material and methods

2.1 Materials

Whey protein concentrate 80 (WPC80; indicated as whey) was obtained from DMV International (Veghel, The Netherlands) and hydrolysed with an established mixture of endopeptidases and exopeptidases (confidential enzyme composition used by Danone) resulting in partially hydrolysed whey proteins (hWhey). This is an experimental hydrolysate solely produced for these experiments and not for usage in an end product. The partially whey based hydrolysed formula CS pre Aptamil HA (Hydrolysed formula) (Nutricia, Zoetermeer, The Netherlands) and an amino acid based formula (AA based formula) were also included, the latter serving as a negative control.

2.2 Sodiumdodecylsulfate-polyacrylamide gelelectrophoresis (SDS-PAGE) and Western blotting

The pattern of the residual proteins in the hWhey and the Hydrolysed formula was analyzed with SDS-PAGE. A Bio-Rad (Hercules, CA, U.S.A) Mini-PROTEAN II system was used with a 15% Tris–HCl gel. Molecular weight markers were used as a reference. The proteins were diluted in a 1:3 ratio with reducing sample buffer (6.05 g Tris, 8.0 g SDS, 3.2 g dithiothreitol, 20 mg bromophenol blue in 60 ml H_2O and 40 ml glycerol 87%, pH 6.8), 40 µg of protein was loaded on the gel. De proteins were either stained in the gel using silver staining according to Wray *et al.* (8) or blotted to a PVDF membrane (Roche Diagnostics, Basel, Switzerland). The membranes were incubated with monoclonal antibodies against α-lactalbumin or β-lactoglobulin (1:30,000; Bethyl Laboratories Montgomery, TX, USA). The binding of antibody was visualized by using Lumi-light Plus Western blotting substrate (Roche Diagnostics)

and chemiluminescence signal was measured with the Chemidoc XRS (BioRad).

2.3 Measurement of residual whey proteins in hWhey and the Hydrolysed formula
For the determination of residual whey proteins in the hWhey and the Hydrolysed formula, a sandwich-type ELISA was performed according to the manufacturers' protocol (Vitrochemie, Nijmegen, The Netherlands).

2.4 Degranulation of rat basophil leukemia cells expressing human FcεRI (RBL-huFcεRI)
To investigate the cross-linking capacity of the Hydrolysed formula, the release of β-hexosaminidase was measured using the RBL-huFcεRI cell line. Degranulation of RBL-huFcεRI cells sensitized with a pooled serum of cow's milk allergic (CMA) patients was performed as described previously (9). In short, confluent growing RBL-huFcεRI cells were sensitized with huIgE or with a human serum pool of 10 CMA patients. The human serum sensitized cells were incubated with Whey, Hydrolysed formula and AA based formula for 1 hour at 37°C. For this assay a valuable unique serum pool obtained from truly cow's milk allergic patients as assessed by double blind placebo controlled food challenge was used and because of ethical reasons we decided to use these sera solely for testing of real end product hydrolysates and not experimental hydrolysates and therefore decided not to include the hWhey group in the RBL-assay. The cross-linking capacity of the hWhey was therefore not determined in this assay. The β-hexosaminidase activity released into the medium was expressed as percentage of the maximum release observed with purified huIgE and α-huIgE.

2.5 Oral sensitization of mice with whey and whey hydrolysates
Three-week-old specific pathogen-free female C3H/HeOuJ mice were purchased from Charles River Laboratories (Saint Germain sur l'Arbresle, France), and maintained on semi-purified cow's milk protein-free mouse chow (Research Diet Services, Wijk bij Duurstede, The Netherlands). Animal care and use were performed in accordance with the guidelines of the Dutch Committee of Animal Experiments. To investigate the residual sensitizing capacity of hWhey and the Hydrolysed formula, mice were orally sensitized with a blunt needle on day 0, 7, 14, 21, 28 and 35 with 20 mg whey, hWhey or an adjusted amount of hydrolysed formula homogenized in PBS (0.5 ml, Cambrex Bio Science, Verviers, Belgium) mixed with 10 µg cholera toxin (Quadratech Diagnostics, Epsom, UK) as an adjuvant. (The amount of hydrolysed whey was equal for the hWhey and the Hydrolysed formula). Non-sensitized mice received cholera toxin in PBS only. One week after the last oral sensitization, anaphylactic reactions, drop in body temperature and acute allergic skin response were determined after an intradermal whey challenge (10 µg whey/ 10 µl PBS per ear). After 24 hours,

mice were challenged orally with 50 mg whey and 30 minutes later blood samples were collected. Blood samples were centrifuged for 15 minutes at 13,500 rpm and sera were stored at -20°C (Figure 1).

2.6 Measurement of whey-specific serum immunoglobulins

Concentrations of whey-specific IgE and IgG_1 were determined in serum by means of ELISA as described previously (10). In short, Microlon plates (Greiner Bio-one, Monroe, USA) were coated with whey for 18 hours at 4°C. Plates were washed after each incubation step. Serum samples were applied and incubated for 2 hours at room temperature (RT), followed by incubation with biotin-labeled rat anti-mouse IgE or IgG_1 for 90 minutes at RT. The plates were incubated with streptavidin-horseradish peroxidase for 1 hour at RT and developed with o-phenylendiamine (Sigma-Aldrich). The reaction was stopped with 4 M H_2SO_4 and absorbance was measured at 490 nm on a Benchmark microplate reader (Bio-Rad, Hercules, CA, USA).

2.7 Anaphylactic shock score and body temperature

To evaluate the residual sensitization capacity of hWhey and Hydrolysed formula, the anaphylactic reactions and body temperature were determined as a clinical related symptom after intradermal whey challenge. To establish the severity of the shock, a validated anaphylactic scoring table (Table 1) was used, as adapted from Li *et al.* (11). To measure changes in body temperature, all mice were given an implantable electronic ID transponder (Bio Medic Data Systems, Delaware, USA) on

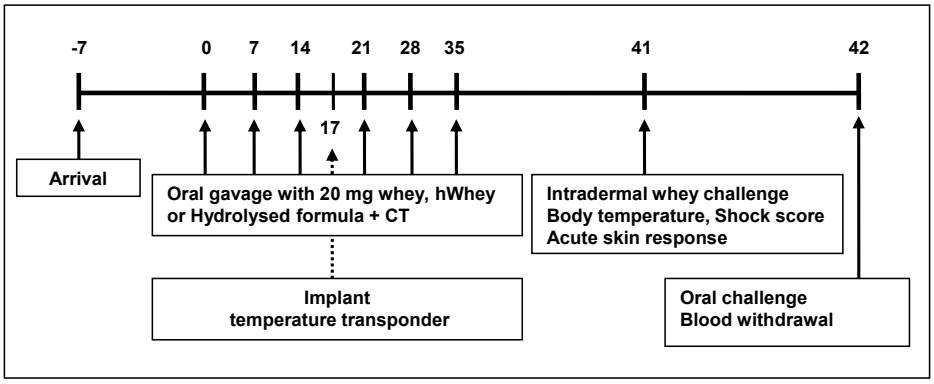

Figure 1. *Mice were sensitized weekly by oral gavage with whey, hydrolysed whey (hWhey) or Hydrolysed formula in PBS using cholera toxin (CT) as an adjuvant. Non-sensitized mice received CT in PBS only. One week after the last sensitization the body temperature, anaphylactic shock score and an acute allergic skin response were determined after intradermal whey challenge in the ear pinnae.*

Table 1: *Anaphylactic symptom scoring table*

Score	Symptoms
0	No symptoms
1	Scratching nose and mouth
2	Swelling around the eyes and mouth; pillar erecti; reduced activity; higher breatihing rate
3	Shortnes of breath; blue rash around the mouth and tail; higher breathing rate
4	No activity after stimulation, shivering and muscle contractions
5	Death by shock

day 16 to measure the individual body temperature.

2.8 Acute allergic skin response

An acute allergic skin response in whey sensitized mice was determined 1 hour after intradermal challenge with 10 µg whey in the ear pinnae. As a negative control, non-sensitized mice were challenged in the ear pinnae with whey. Ear thickness was measured in duplicate using a digital micrometer (Mitutoyo, Veenendaal, The Netherlands). The allergen-specific net ear swelling was calculated by correcting the allergen-induced ear thickness with the basal ear thickness. The delta ear swelling is expressed as µm.

2.9 Statistical analysis

Statistical analysis of the whey-specific immunoglobulins and β-hexosaminidase release of RBL-huFcεRI cells and body temperature were performed using a 1-way ANOVA and post hoc Bonferroni's multiple comparison test. The anaphylaxis scores were statistically analyzed using the Kruskal-Wallis test followed by the Dunn's multiple comparison test. All statistical analyses were conducted using GraphPad Prism software (version 4.03). P-values are depicted as * for $p<0.05$, ** for $p<0.01$, and *** for $p<0.001$.

Animal studies were performed with n=7 mice per group based on power analyses.

3. Results

3.1. SDS-PAGE and Western blotting

Electrophoretic patterns indicated that β-lactoglobulin (β-Lg), a major allergen in whey, was completely degraded after hydrolysis in both hWhey and Hydrolysed formula. A small residual band of α-lactalbumin (α-La) was seen in hWhey, but not in the Hydrolysed formula. In the Hydrolysed formula, a large band of low molecular

weight peptides was found. In the AA based formula, no protein bands were detected (Figure 2). Positive staining on the Western blot after incubation with anti-α-La and anti-β-Lg antibodies of the bands corresponding to α-La and β-Lg were detected in whey, but not in the hWhey, Hydrolysed formula or in the AA based formula (data not shown).

3.2. Residual whey proteins in hWhey and Hydrolysed formula

The whey powder contained 159.2 ± 14.7 µg/mg whey proteins, after hydrolysis only 1.8 ± 0.3 µg/mg and 0.9 ± 0.1 µg/mg of residual whey protein was detected in the hWhey and Hydrolysed formula respectively (Figure 3). This is a reduction of 97% (hWhey) and 98.4% (Hydrolysed formula) in whey proteins when compared to whey. The whey protein content of the AA based formula was below the detection limit of the assay.

3.3 Degranulation assay with RBL-huFcεRI cells

The capacity of the Hydrolysed formula to elicit degranulation of RBL-huFcεRI cells was investigated (Figure 4). The degranulation of the RBL-huFcεRI cells which were sensitized with purified huIgE and cross-linked with α-huIgE was considered as the maximum release (100%). Incubation of the serum sensitized RBL-huFcεRI cells

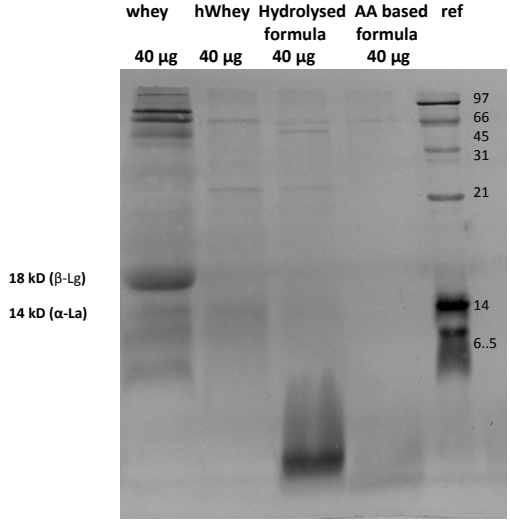

Figure 2. *SDS-PAGE patterns of whey, hWhey, Hydrolysed formula and AA based formula. The reference (ref) is a standard broad-range marker: phosphorylase b (97 kDa), serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21 kDa), lysozyme (14 kDa) and aprotinin (6.5 kDa). The band at 66 kDa in all lanes reflects the non-specific coloring from the sample buffer.* β- *lactoglobulin (*β*-LG) and α-lactalbumin (α-LG).*

with whey resulted in a release of 88.7 ± 3.8%. The Hydrolysed formula showed no enhanced degranulation (46.7 ± 6.2%) as compared to the CMA pool in the absence of anti-IgE or protein (35.6 ± 2.7%). The AA based formula has been tested in a previous assay and did not induce degranulation (data not shown).

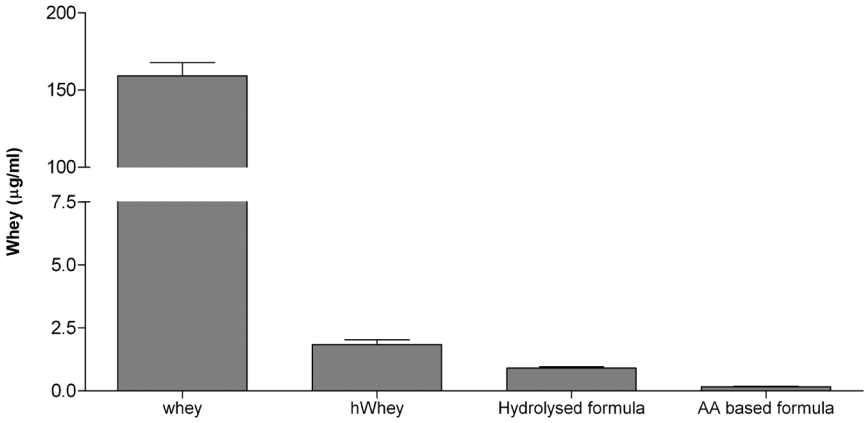

Figure 3. *The residual concentrations of whey were determined in whey, hWhey, Hydrolysed formula and AA based formula by an ELISA assay (Data are expressed as mean ± SD.)*

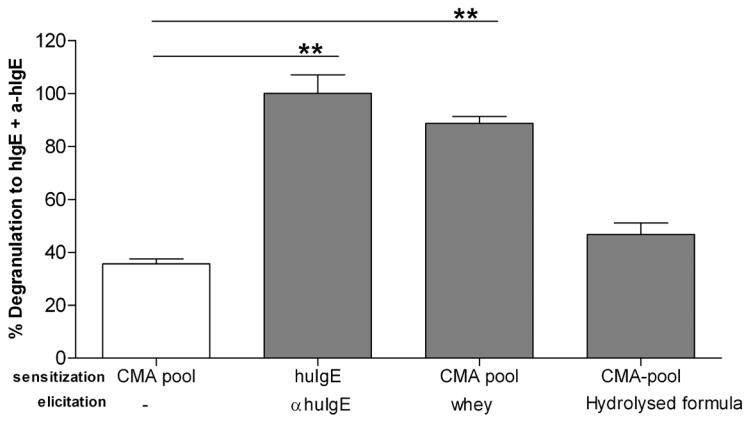

Figure 4. *β-Hexosaminidase release by RBL-huFcεRI cells sensitized with serum from cow's milk allergic (CMA) patients. Degranulation was determined after incubation of the sensitized RBL-huFcεRI cells with whey or Hydrolysed formula. The maximum release (100%) was induced with huIgE and α-huIgE. (Data are expressed as mean ± SD; *p<0.05, **p<0.01 compared to the control.)*

3.4 Whey-specific IgE and IgG_1

The whey-specific IgE and IgG_1 levels were strongly elevated in the whey-sensitized group compared to the non-sensitized mice, whereas sensitization with the hWhey and the Hydrolysed formula did not induce a whey-specific IgE response (Figure 5A) and strongly reduced whey-specific IgG_1 levels (Figure 5B). After the oral sensitization period no further increase in whey-specific IgE and IgG_1 levels were observed due to the intra dermal whey challenge (data not shown).

3.5 Anaphylactic shock score

To study whether challenge with the whole whey protein induced clinical symptoms in the whey, hWhey and the Hydrolysed formula sensitized mice, anaphylactic shock reactions were measured after intradermal whey challenge. Figure 6 shows the anaphylactic reactions one hour after allergen challenge. Six out of the seven

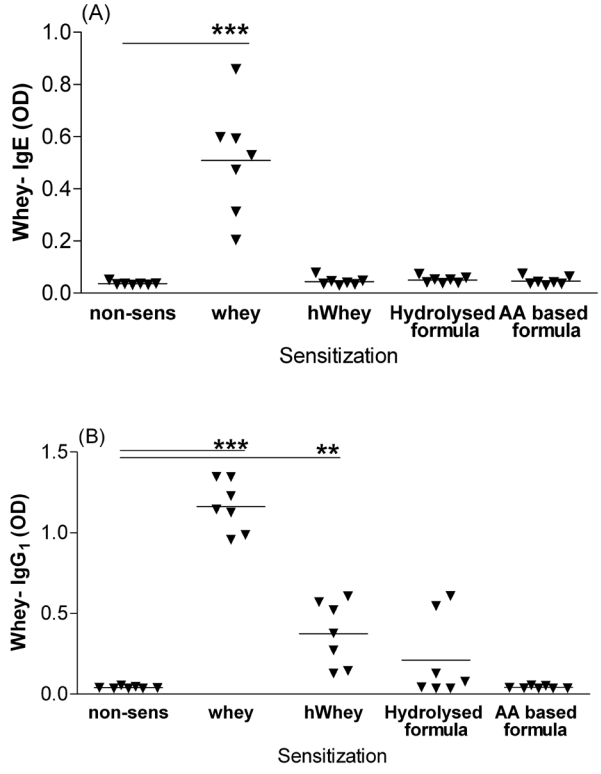

Figure 5. *Levels of whey-specific IgE (A) and IgG_1 (B) were determined in sera from whey, hWhey, Hydrolysed formula or AA based formula sensitized mice collected on day 42. (Data are expressed as individual values; **p<0.01, ***p<0.001 compared to non-sensitized mice.)*

mice sensitized with whey showed mild to severe anaphylactic shock symptoms. However, no shock symptoms were observed in the hWhey, the Hydrolysed formula or the AA based formula sensitized group, indicating that no allergic response to whey was induced in mice exposed to the whey hydrolysates.

3.6 Body temperature

When animals experience an anaphylactic shock upon allergen challenge, a drop in the body temperature can be observed. The starting body temperature of all animals was approximately 36°C before whey challenge. A rapid decline to 32°C was observed in the whey-sensitized mice. In agreement with the anaphylactic shock score, no drop in body temperature after the intradermal whey challenge was induced in mice sensitized with the hWhey or the Hydrolysed formula (Figure 7).

3.7 Acute allergic skin response

Previous studies have confirmed that the acute allergic skin response correlates with whey-specific IgE levels (12). As expected because of the absence of whey-specific IgE, exposure of mice to the hWhey or the Hydrolysed formula did not induce an acute allergic skin response after intradermal whey challenge. However, although whey-sensitization induced whey-specific IgE antibodies, the whey-sensitized control group failed to show an acute allergic skin response, probably because of the superseding anaphylactic shock in these mice (data not shown).

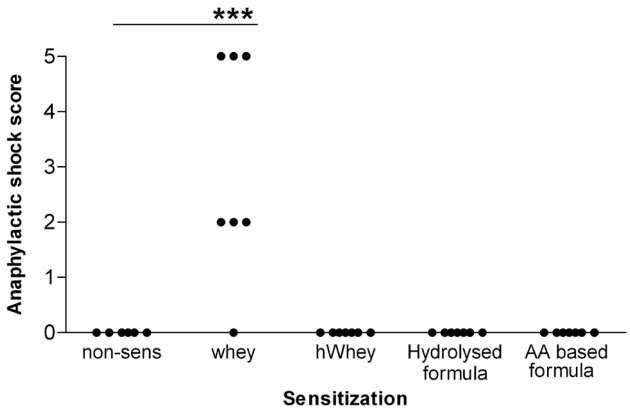

Figure 6. *Anaphylactic symptoms were determined after intradermal whey challenge in mice sensitized to whey, hWhey, Hydrolysed formula or AA based formula. (Data are expressed as mean ± SD ; ***p<0.001 compared to non-sensitized mice.)*

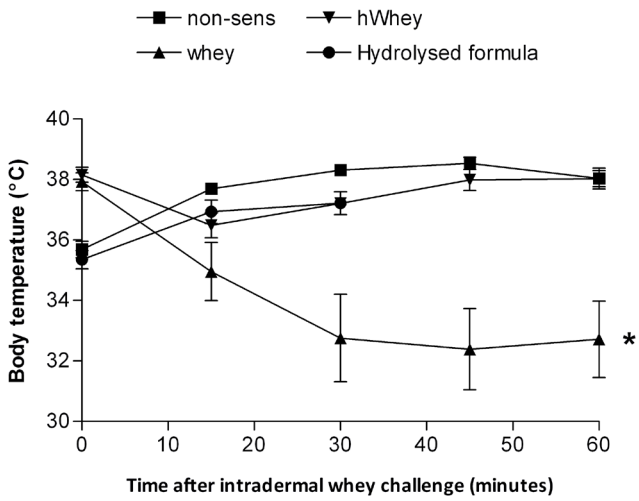

Figure 7. *The body temperature was measured at different time-points after intradermal whey challenge in mice sensitized to whey, hWhey or Hydrolysed formula. (Data are expressed as individual values; *p<0.05 compared to non-sensitized mice.)*

4. Discussion

Hydrolysed infant formulas are commonly used as an alternative for standard infant milk formulas in genetically predisposed infants or infants diagnosed with cow's milk allergy. The hypoallergenicity of new infant and follow-on formulas must be assessed to confirm that the formulas are not able to sensitize animals as described by the European guidelines. In our opinion, a combination of several techniques, covering both the sensitization phase and the elicitation or effector phase of the allergic response, is necessary to obtain a comprehensive and detailed picture of the hypoallergenicity of hydrolysed formulas.

Whey, which represents 20% of the total protein content of cow's milk, contains primarily β-Lg, α-Lg and bovine serum albumin. Of these proteins, β-Lg is considered the major allergenic component of the cow's milk derived whey fraction. Complete degradation of β-Lg was observed and no binding of antibodies against β-Lg and α-Lg was observed in the whey hydrolysates giving a first indication of the reduced antigenicity of the hydrolysates. Moreover, the Hydrolysed formula lost the capacity to cause degranulation of the RBL cell-line transfected with the human IgE receptor. This degranulation assay, which may serve as a model for the effector phase of the allergic response (as a reflection for clinical effects in allergic children), is considered a highly relevant *in vitro* model for the human situation because of predictive value of recognition of residual proteins by the human immune system (13, 14).

Besides the obtained *in vitro* data, it is required to determine the residual sensitizing capacity of hypoallergenic infant formulas in respect to safety. Recently, we

positioned a mouse model of cow's milk allergy using oral sensitization to assess both the sensitizing capacity of whey hydrolysates and the residual potency of whey hydrolysates to induce an allergic effector response upon challenge in whey allergic mice using an acute allergic skin response as the major read-out (7). In the current study, this model was slightly adapted to assess the residual sensitizing capacity of hWhey and the Hydrolysed formula with anaphylactic shock score and body temperature as major read-out parameters for clinical symptoms.

Oral sensitization to whey resulted in the induction of whey-specific IgE and IgG_1 antibodies and after challenge acute clinical symptoms were observed, as measured by severe anaphylactic reactions and a rapid drop in body temperature. Oral sensitization to hWhey and the Hydrolysed formula did not result in the induction of allergen-specific IgE responses. As expected by the absence of whey-specific IgE antibodies, no anaphylactic shock reactions and no drop in body temperature were observed after whey challenge in mice sensitized with hWhey or the Hydrolysed formula. Exposure to hWhey or Hydrolysed formula did, however, show low levels of whey-specific IgG_1 compared to whey-sensitized mice. Thus, although an immune response was observed to the whey hydrolysates, no allergic IgE response and clinical symptoms occurred in this model. In allergic mice experiencing a severe anaphylactic reaction and a rapid drop in body temperature no local acute allergic skin response could be determined anymore. It can be hypothesized that after a whey challenge, due to the large systemic anaphylactic response, local vasodilatation is not detectable anymore.

For safety reasons, the hypoallergenicity of hydrolysed infant formulas needs to be assessed by showing that the hypoallergenic formulas are not able to sensitize animals. Although some attempts were made to use the oral sensitization route, in many animal studies no oral sensitization was used (15, 16). Although a rat model using oral sensitization with whey has been described which generates both specific IgG and IgE antibodies of similar specificity to those produced by humans (17), hypoallergenicity testing was not performed in this model so far. Guinea pigs sensitized via the oral route are the most commonly used model of reference for testing the safety of new hypoallergenic formulas due to their predisposed responsiveness (18). However, a main disadvantage of this guinea pig model is the generation of IgG_{1a} subclass anaphylactic antibody responses instead of IgE antibody responses which is the main physiological antibody response in allergic humans. This discrepancy between IgG_{1a} and IgE makes this model questionable with regard to the potential extrapolation to the human situation. In the described whey based mouse model for cow's milk allergy, which also uses the oral route of sensitization, allergen-specific IgE could be induced and, upon challenge, clinical symptoms were measured to establish the residual allergenicity of whey hydrolysates.

In summary, a mouse model of cow's milk allergy using oral sensitization with relevant

clinical read-out parameters was used to determine the residual sensitizing capacity of hydrolysed whey proteins. In combination with *in vitro* assays determining the amount of intact protein by ELISA and the residual capacity of hWhey and Hydrolysed formula to cross-link human IgE antibodies and subsequent degranulation of the huFcεRI transfected RBL cell-line, a comprehensive and detailed assessment of the hypoallergenicity of hydrolysed formulas is provided. This combination of assays is proposed as a strategy for the screening of new hypoallergenic formulas aimed at preventing sensitization in atopic children and avoiding clinical symptoms in infants suffering from cow's milk allergy.

5. Acknowledgements

The authors would like to thank L. Buelens, B. Schouten and A. Nauta for their excellent assistance.

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Interlaboratory evaluation of a mouse model for cow's milk allergy to assess the allergenicity of hydrolysed cow's milk based infant formulas

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Abstract

Background This study is part of a multi-phase project which aims to validate a new mouse model for cow's milk allergy to assess the potential allergenicity of hydrolysed cow's milk-based infant formulas. The EC-directive 1991/321/EEC on infant formulas requires that objective and scientifically verified data should be available to the claim hypoallergenicity. For safety reasons, hypoallergenic formulas should not be able to sensitize animals. Despite guinea pig assays are frequently used for this purpose, no validated animal models are currently available to assess the potential residual sensitizing capacity.

Objective In this first phase of a multicenter ring trial the transferability of a recently developed mouse model for cow's milk allergy was evaluated in 4 independent research facilities.

Methods C3H/HeOuJ mice were sensitized by oral administration of whey (2 and 20 mg) and cholera toxin at weekly intervals for 5 weeks. One week after the last sensitization, the acute allergic skin response (ear swelling at 1 hour) and anaphylactic symptoms were determined upon intradermal whey injection into the ear pinnae. Subsequently, mice were challenged orally with 50 mg whey and blood samples were taken after 30 minutes. Serum was analyzed for whey-specific antibodies and mMCP-1. All protocols, test substances, and procedures were standardized and animals were from the same age and obtained from the same breeder.

Results All participating research facilities were able to sensitize mice to whey as shown by elevated levels of whey-specific IgE/IgG_1/IgG_{2a} and serum mMCP-1 as a reflection of mast cell degranulation. An acute allergic skin response after intradermal whey challenge compared to non-sensitized mice was observed in 3 out of 4 research facilities. Anaphylactic symptoms were present in all 4 research facilities although minor responses were measured at one of the facilities.

Conclusion In this ring trial a mouse model for cow's milk allergy was simultaneously introduced at 4 independent research facilities in the Netherlands. The first results indicated that it is possible to transfer this model with low interlaboratory variation.

Introduction

Food allergens belong to the most common allergens encountered responsible for inducing Type I or IgE-mediated hypersensitivity reactions (1). Type I allergic responses to cow's milk proteins leading to cow's milk allergy are characterized by a Th2 response resulting in the production of allergen-specific IgE.

Hypoallergenic cow's milk (HA) formulas are processed by enzymatic treatment, heat treatment and/or ultra filtration of cow's milk proteins to reduce their allergenicity and HA formulas are commonly used as an alternative for standard infant milk formulas in genetically predisposed infants or infants diagnosed with cow's milk allergy. According to the European guidelines on HA formulas and follow-on formulas, it is required that objective and scientifically verified data are available as proof of the hypoallergenicity of HA formulas. Although the outcome of several *in vitro* assays is indicative of the residual allergenicity of HA formulas (2) for safety reasons hypoallergenicity needs to be assessed by showing that the HA formulas are not able to sensitize animals to the protein source they are derived from (3).

In vivo animal models for cow's milk allergy are therefore considered indispensable to predict the safety of HA formulas in humans in terms of preventing sensitization to cow's milk proteins. However, no validated animal models are currently available to assess the potential residual sensitizing capacity of HA formulas. Guinea pigs sensitized by the oral route have been used as a common model for the identification of the residual sensitizing capacity of new HA formulas due to their innate responsiveness to proteins. A main disadvantage of the frequently used guinea pig model is the generation of IgG_{1a} subclass anaphylactic antibodies instead of IgE antibodies. This makes the suitability of the guinea pig model questionable with regard to the potential extrapolation to the human situation. Both rat and mouse models for cow's milk allergy have been used also to test allergenicity of cow's milk hydrolysates (4, 5). However, in these studies systemic sensitization was used which might induce a different immunologic response because of differences between the mucosal and the systemic part of the immune system, thereby making extrapolation to the human situation even more difficult (6).

Recently, a novel IgE-mediated mouse model for orally induced cow's milk allergy was introduced to further assess mechanisms underlying cow's milk allergy and/or test new concepts for prevention and/or treatment of cow's milk allergy (7). In this model a new parameter, the acute allergic skin response, as an equivalent of the human skin prick test, was introduced to determine the residual allergenicity of partially and extensive hydrolysed whey proteins (8). Recently, this model was used to assess the sensitizing capacity of hydrolysed whey proteins and showed clear capacity to assess safety of hydrolysed formulas (9).

This study is part of a multi-phase project which aims to validate the novel mouse model for cow's milk allergy to assess the potential allergenicity of hydrolysed

cow's milk-based infant formulas. In the first phase of this project, described in the current manuscript, a multicenter transferability study was performed to validate the model in 4 independent research facilities within the Netherlands.

Material and methods

Participating Institutes

This multicenter transferability study was performed at the following 4 independent research facilities in the Netherlands; Utrecht Institute for Pharmaceutical Sciences, Faculty of Science, Utrecht University, Utrecht, Danone Research-Centre for Specialised Nutrition, Wageningen, TNO, Quality & Safety, Zeist and the Institute for Risk Assessment Sciences, Utrecht University, Utrecht. All institutes are located in the Netherlands

Materials

Whey protein concentrate 80 (WPC80; indicated as whey) was obtained from DMV International (Veghel, the Netherlands). C3H/HeOuJ mice were purchased from Charles River Laboratories (Saint Germain sur l'Arbresle, France) and semi-purified cow's milk protein-free mouse chow was obtained from Research Diet Services, Wijk bij Duurstede, the Netherlands. All animals were from the same batch and supplier and delivered at each facility simultaneously. The whey proteins used to sensitize the animals were of the same batch and provided to all participants. Cholera toxin was purchased from Quadratech Diagnostics, Epsom, UK. Biotin labeled rat anti-mouse IgE, IgG_1 and IgG_{2a} were obtained from Pharmingen, Alphen a/d Rijn, the Netherlands. Streptavidin-horse radish peroxidase was obtained from Sanquin, Amsterdam, the Netherlands. PBS was purchased from Cambrex Bio Science, Verviers, Belgium. All other chemicals were obtained from Sigma-Aldrich, Zwijndrecht, the Netherlands. All protocols and procedures used at the different facilities were standardized.

Oral sensitization of mice with whey and whey hydrolysates

Three to four week-old specific pathogen-free female C3H/HeOuJ mice, raised and bred on a milk-free diet for at least two generations, were purchased from Charles River Laboratories (Saint Germain sur l'Arbresle, France), and maintained on semi-purified cow's milk protein-free mouse chow. Animal care and use were performed in accordance with the guidelines of the Dutch Committee of Animal Experiments. Mice were orally sensitized using a blunt needle with 2 and 20 mg whey homogenized in 0.5 mL PBS mixed with 10 µg cholera toxin as an adjuvant on day 0, 7, 14, 21 and 28. Non-sensitized mice received cholera toxin in PBS. One week after the last oral sensitization, the acute allergic skin response (ear swelling at 1 hour) and anaphylactic reactions were determined after intradermal whey

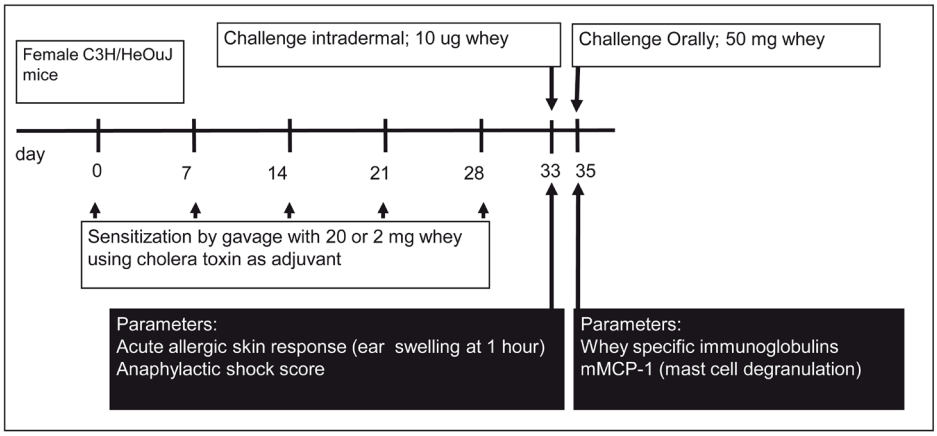

Figure 1. *A schematic overview of the sensitization and challenge protocol and the parameters that are analyzed.*

challenge (10 µg whey/20 µL PBS per ear) as described before (10). After 24 hours, mice were challenged orally with 50 mg whey in 0.5 mL PBS and 30 minutes later blood samples were collected. Blood samples were centrifuged for 15 minutes at 13,500 rpm and sera were stored at -20°C until analysis of whey-specific antibodies and mMCP-1 (Figure 1).

Measurement of serum specific antibodies and mouse Mast Cell Protease I

Whey-specific IgE and IgG_1 and IgG_{2a} levels were measured in serum by means of ELISA. Microlon plates (Greiner, Alphen aan de Rijn, the Netherlands) were coated with 20 µg of whey in coating buffer for 18 hours at 4°C. Plates were washed and blocked for 1 hour with buffer containing 50 mM Tris, 2 mM EDTA en 137 mM NaCl /0.05% Tween and 0.5% BSA. Serum samples were incubated for 2 hours at room temperature. Plates were washed and incubated with 1 µg biotin labeled rat anti-mouse IgE, IgG_1 or IgG_{2a} for one hour at room temperature. After washing the plates were incubated with streptavidin-horse radish peroxidase for one hour, washed and developed with o-phenylendiamine. The reaction was stopped after 10 minutes with 4M H2SO4 and absorbance was measured at 490 nm on a Benchmark microplate reader (Biorad, Veenendaal, the Netherlands). Results were expressed as arbitrary units (AU). Serum concentrations of mouse mast cell protease-1 (mMCP-1) were determined according to the manufacturer's protocol using a commercially available ELISA kit (Moredun Scientific Ltd., Midlothian, UK).

Acute allergic skin response

An acute allergen-specific skin response in whey sensitized mice was determined 1

Table 1: *Anaphylactic symptom scoring table*

Score	Symptoms
0	No symptoms
1	Scratching nose and mouth
2	Swelling around the eyes and mouth; pillar erecti; reduced activity; higher breatihing rate
3	Shortnes of breath; blue rash around the mouth and tail; higher breathing rate
4	No activity after stimulation, shivering and muscle contractions
5	Death by shock

hour after intradermal challenge with 10 µg whey in the ear pinnae. As a negative control, non-sensitized mice were challenged in the ear pinnae with whey. Ear thickness was measured in duplicate using a digital micrometer (Mitutoyo, Veenendaal, the Netherlands). The allergen-specific net ear swelling was calculated by correcting the allergen-induced ear thickness with the basal ear thickness. The delta ear swelling is expressed as µm.

Anaphylactic shock score

The anaphylactic reactions were determined as a clinical related symptom after intradermal whey challenge. To establish the severity of the shock, a validated anaphylactic scoring table (Table 1) was used, as adapted from Li *et al.* (11).

Statistical analysis

Data were statistically analyzed using the Kruskal-Wallis test followed by the Dunn's multiple comparison test. The acute allergic skin response was analyzed using a 1-way ANOVA and post hoc Dunnett's multiple comparison test. All statistical analyses were conducted using GraphPad Prism software (version 4.03). P-values are depicted as * for $p<0.05$, ** for $p<0.01$, and *** for $p<0.001$.

Results

Whey-specific antibodies

All participating research laboratories were able to sensitize mice to whey as shown by elevated levels of whey-specific IgE, IgG_1 and IgG_{2a}. However, the optimal sensitization dose differed per facility. Similarly IgG_1 and IgG_{2a} varied between laboratories.

Significantly elevated levels of whey-specific IgE (figure 2) were observed in mice sensitized with 2 mg whey in research facility 2 (119.2 ± 87.3 AU; $p<0.05$), research facility 3 (793.1 ± 429.2 AU; $p<0.05$) and research facility 4 (337.2 ± 149.1 AU;

p<0.001) compared to non-sensitized mice (0.93 ± 0.35, 0, 14.9 ± 10.9 and 0 AU for research facility 1, 2, 3 and 4, respectively). In mice sensitized with 20 mg whey, significantly elevated levels of whey-specific IgE were observed in research facility 1 (768.6 ± 335.2 AU; p<0.01) and 4 (29.8 ± 12.2 AU; p<0.05).

Significantly elevated levels of whey-specific IgG_1 (figure 3) were measured in mice sensitized with 2 mg whey in research facility 1 (95,590 ± 38,960 AU; p<0.01), research facility 2 (17,310 ± 8650 AU; p<0.05), research facility 3 (109,200 ± 31,820 AU; p<0.001) and research facility 4 (137,800 ± 24,910 AU; p<0.001) compared to non-sensitized mice (317.1 ± 269.0, 0, 17.4 ± 10.8 and 0 AU for research facility 1, 2, 3 and 4 respectively). In mice sensitized with 20 mg whey, significantly elevated levels of whey-specific IgG_1 were observed in research facility 1 (138,300 ± 40,710 AU; p<0.01), research facility 3 (137,800 ± 49,890 AU; p<0.01) and research facility 4 (81,720 ± 22,410 AU; p<0.01).
Significantly elevated levels of whey-specific IgG_{2a} (figure 4) were measured in mice

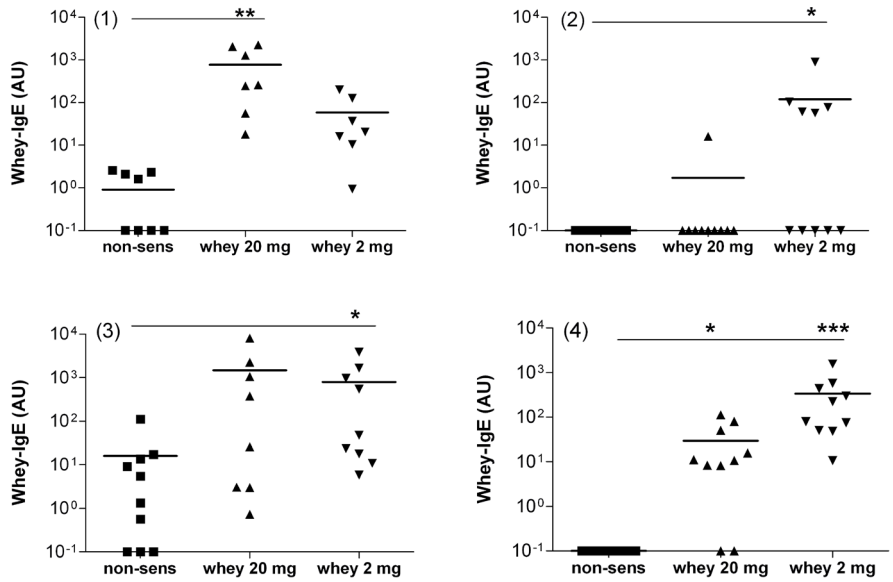

Figure 2. *Whey-specific IgE levels in serum of whey sensitized mice*
*In mice sensitized with 20 mg whey, specific IgE levels were significantly enhanced in research facilities 1 and 4 (Figure 1; 1 and 4). In mice sensitized with 2 mg whey, specific IgE levels were significantly enhanced in research facilities 2,3 and 4 (Figure 1; 2, 3 and 4). Data are expressed as individual values; *p<0.05, **p<0.01 ***p<0.001 compared to non-sensitized mice.*

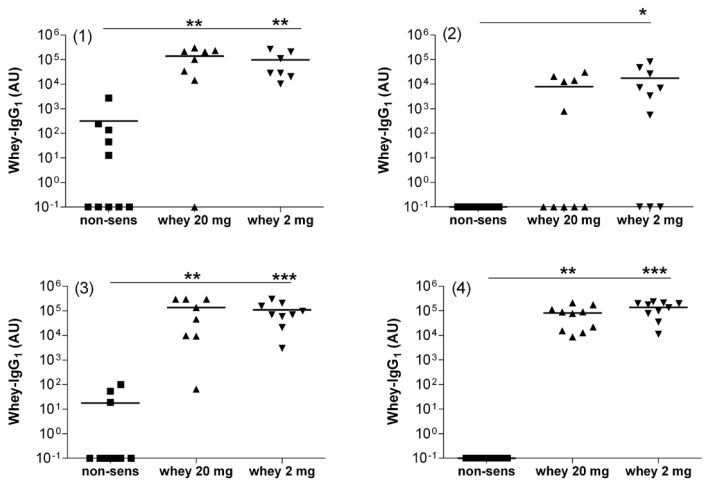

Figure 3. *Whey-specific IgG_1 levels in serum of whey sensitized mice*
*In mice sensitized with 20 mg whey, specific IgG_1 levels were significantly enhanced in research facilities 1, 3 and 4 (Figure 2; 1, 3 and 4). In mice sensitized with 2 mg whey, specific IgE levels were significantly enhanced in all 4 research laboratories (Figure 2; 1-4). Data are expressed as individual values; *p<0.05, **p<0.01, ***p<0.001 compared to non-sensitized mice.*

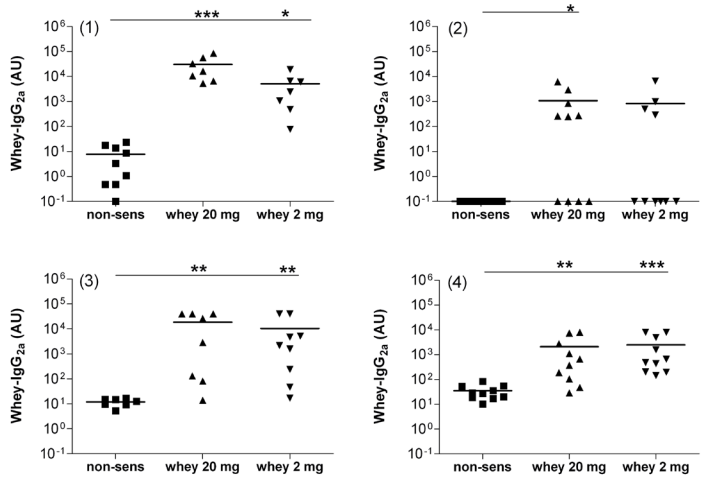

Figure 4. *Whey-specific IgG_{2a} levels in serum of whey sensitized mice*
*In mice sensitized with 20 and 2 mg whey, specific IgG_{2a} levels were significantly enhanced in all 4 research laboratories, except for sensitization with 2 mgwhey in facility 2. Data are expressed as individual values; *p<0.05, **p<0.01, ***p<0.001 compared to non-sensitized mice.*

sensitized with 2 mg whey in research facility 1 (5085 ± 2512 AU; $p<0.05$), research facility 3 (10420 ± 5624 AU; $p<0.01$) and research facility 4 (2463 ± 1029 AU; $p<0.001$) compared to non-sensitized mice (7.6 ± 2.9, 0, 11.8 ± 1.5 and 35.0 ± 7.2 AU for research facility 1, 2, 3 and 4, respectively). In mice sensitized with 20 mg whey, elevated levels of whey-specific IgG_{2a} were observed in research facility 1 (26,360 ± 10,450 AU; $p<0.001$), research facility 2 (1068 ± 630.2 AU; $p<0.05$), research facility 3 (18,660 ± 6955 AU; $p<0.01$) and research facility4 (2078 ± 980.7 AU; $p<0.01$).

Acute allergic skin response

Previous studies have confirmed that the acute allergic skin response correlates with whey-specific IgE and IgG_1 levels (7). As expected from the presence of whey-specific IgE an acute allergic skin response after intradermal whey challenge was observed in whey-sensitized mice in only two laboratories. In research facility 1, despite high levels of whey-specific IgE in whey (20 mg) sensitized mice, this group failed to show an acute allergic skin response, probably because of the superseding anaphylactic shock in these mice (figure 5).

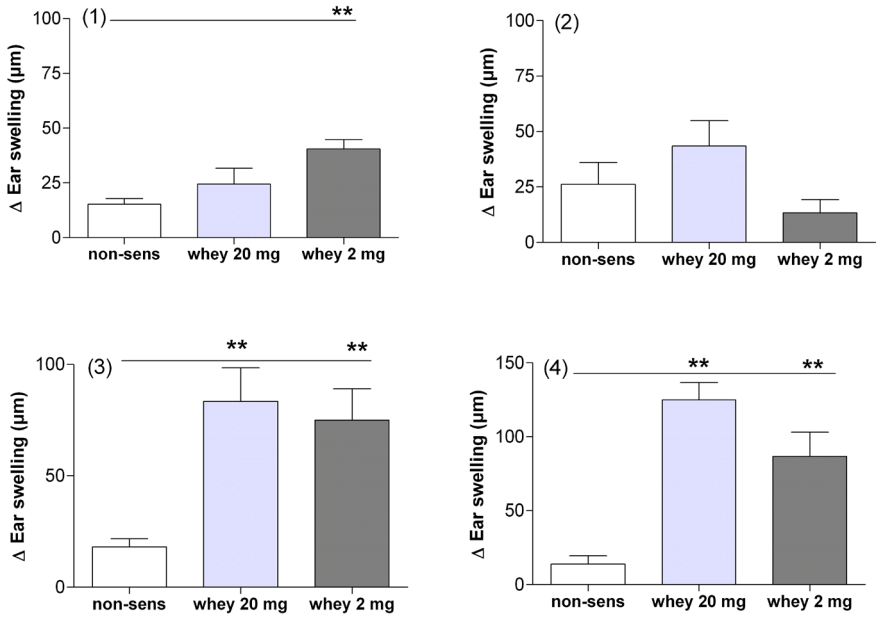

Figure 5. *Acute allergic skin response after whey challenge*

*In mice sensitized with 20 mg whey, a significant acute ear swelling response after intradermal ear challenge was observed in research facility 3 and 4 (Figure 5; 3 and 4). In mice sensitized with 2 mg whey, a significant acute ear swelling response to whey was observed in research facilities 1, 3 and 4 (Figure 5; 1, 3 and 4). Data are expressed mean values ± SEM; **p<0.01 compared to non-sensitized mice.*

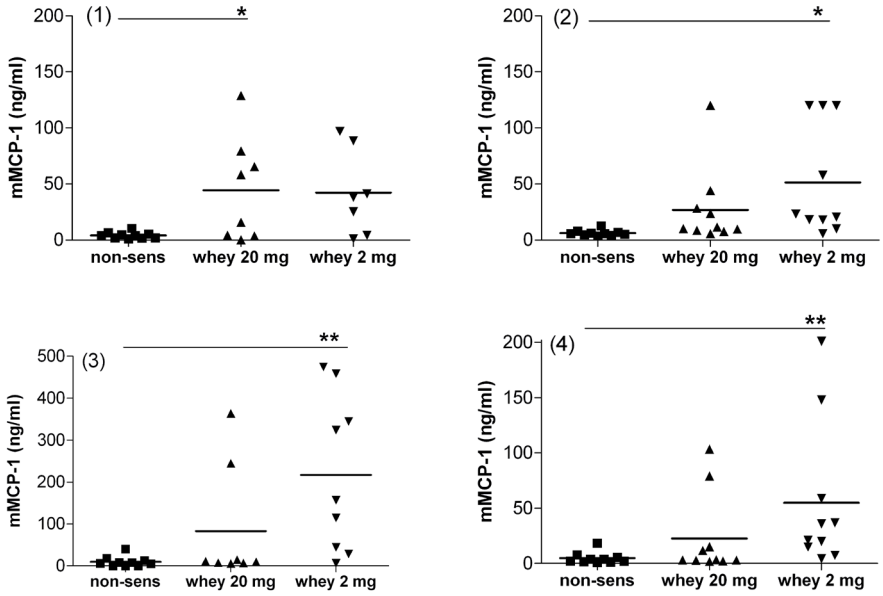

Figure 6. *Increased mMCP-1 concentrations in whey sensitized mice*
*In mice sensitized with 20 mg whey, serum mMCP-1 concentrations after oral challenge were enhanced in research facility 1 (Figure 7; 1). In mice sensitized with 2 mg whey, enhanced mMCP-1 concentrations were observed in research facilities 2, 3, 4 (Figure 7; 2, 3, 4). Data are expressed as individual values; *p<0.05, **p<0.01 compared to non-sensitized mice.*

Although 5 out of 10 mice of research facility 2 showed elevated levels of whey-specific IgE in whey (20 mg) sensitized mice no acute allergic skin response was observed in these mice.

mMCP-1 serum oncentrations
To investigate whether exposure to whey induced mucosal mast cell degranulation in whey sensitized mice, mMCP-1 serum concentrations were measured 30 minutes after oral whey challenge as a reflection of mast cell degranulation (figure 6). All research facilities showed elevated concentrations of mMCP-1 indicating that mucosal mast cells were triggered. In 3 out of 4 laboratories significantly increased mMCP-1 concentrations were observed in mice sensitized with 2 mg whey only. The 4th research facility showed increased mMCP-1 concentrations in mice sensitized to 20 mg whey. Interestingly, the elevated levels of whey-specific IgE induced by either 2 or 20 mg whey corresponded with the mMCP-1 serum concentrations indicating that in all research facilities the allergen induced mast cell response was associated with induced whey-specific IgE levels. Mice sensitized with 2 mg whey showed elevated mMCP-1 serum concentrations in research facility 2 (51.4 ± 15.6 ng/ml;

p<0.05), research facility 3 (216.9 ± 62.0 ng/ml; p<0.01) and l research facility 4 (55.0 ± 21.0 ng/ml; p<0.01) compared to non-sensitized mice (4.1 ± 0.9, 6.3 ± 0.8, 10.0 ± 3.8 or 4.8 ± 1.7 ng/ml for research facility 1, 2, 3 and 4 respectively). In mice sensitized with 20 mg whey, significantly elevated mMCP-1 serum concentrations were observed in research facility 1 (44.4 ± 16.4 ng/ml; p<0.05).

Anaphylactic shock score

To study clinical symptoms in whey sensitized mice, anaphylactic shock reactions were measured after intradermal whey challenge (figure 7). In research facility 1 all mice showed moderate to severe anaphylactic shock reactions which was fatal for one mice sensitized with 2 mg whey. Research facility 2 observed in 1 out of 10 mice anaphylactic symptoms in whey sensitized mice. The results on anaphylactic shock reactions in research facilities 3 and 4 were comparable, 6 to 7 out of 10 mice showed an anaphylactic response in mice sensitized with 20 mg whey. In mice sensitized with 2 mg whey4 to 5 out of 10 mice showed anaphylactic shock reactions.

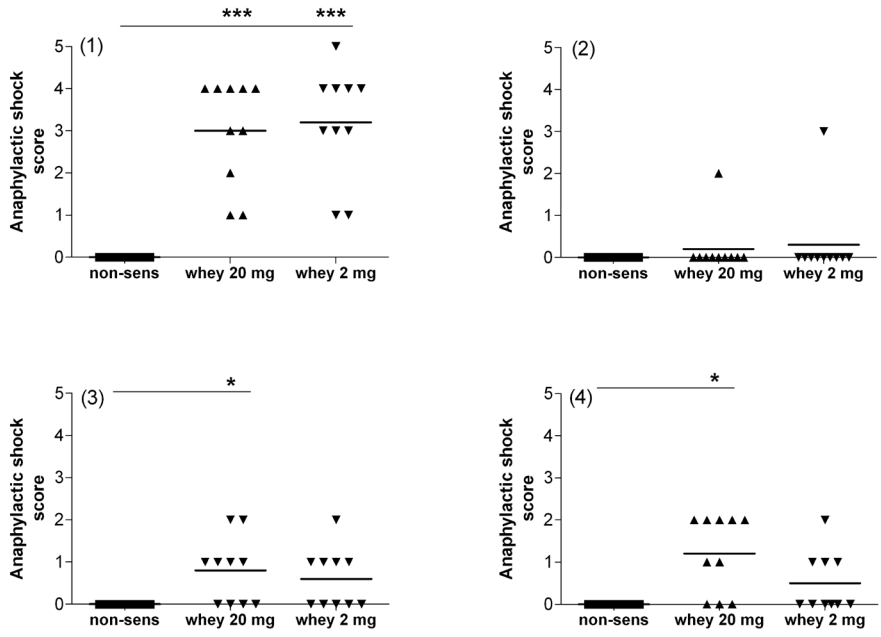

Figure 7. *Anaphylactic shock symptoms after whey challenge*

*In mice sensitized with 20 mg whey, anaphylactic shock symptoms after intradermal ear challenge were observed in research facilities 1, 3 and 4 (Figure 7; 1, 3 and 4). In mice sensitized with 2 mg whey, significant anaphylactic shock scores were only observed in research facilities 1 (Figure 7; 1). Data are expressed as individual values; *p<0.05, ***p<0.001 compared to non-sensitized mice.*

Discussion

The present study is part of a multi-phase project which aims to validate a mouse model for cow's milk allergy to assess the hypoallergenicity of new infant and follow-on formulas according the European Commission-directive 1991/321/EEC on infant formulas. No validated animal models are currently available to assess the sensitizing capacity of HA infant formulas, although guinea pig assays are frequently used for this purpose.

Recently, it was demonstrated that in mice orally sensitized to whey, allergic responses, such as the acute allergic skin response, correlated with whey-specific IgE and IgG_1 serum levels (7). Indeed, enhanced levels of whey-specific IgE, IgG_1 and IgG_{2a} were observed in all research facilities although the optimal sensitization dose was not identical for all research facilities. All animals used were from the same age and supplier, delivered at each facility simultaneously and whey proteins used to sensitize the animals were of the same batch. mMCP-1 was measured to indicate mast cell degranulation after cross-linking of cell bound IgE in response to oral whey challenge. Interestingly, although the optimum sensitization dose used to induce whey-specific IgE differed between the research facilities in all this occasions it was associated with enhanced mMCP-1 serum concentrations upon oral whey challenge as a reflection of an allergic effector response at each research facility.

In research facilities 3 and 4, an acute allergic skin response was observed with the induction of anaphylactic shock reactions after intradermal ear challenge. No acute allergic skin response or anaphylactic shock reactions after whey challenge were measured in research facility 2, which was most likely due to the fact that the majority of whey sensitized mice did not induce whey-specific IgE. Remarkably, only a minor acute allergic skin response was observed in these animals in research facility 1. In a previous study it was shown that in allergic mice experiencing a severe anaphylactic reaction and a rapid drop in body temperature no local acute allergic skin response could be determined anymore (2). Based on the latter observations it was concluded that the anaphylactic shock reactions interfere with the induction of an acute allergic skin response. In the next phase of this multicenter ring trial, body temperature will be included as an additional parameter, next to the acute allergic skin response.

To conclude, despite minor differences in optimal sensitization dose, results indicate that animals were sensitized at all different institutes with elevated specific antibodies and mMCP-1 levels. Anaphylactic shock reactions, as a clinical readout, were observed in three research facilities. Results indicate that severe anaphylactic reactions interfere with the establishment of a local acute allergic skin response. The combined measurement of specific antibodies, mMCP-1 and anaphylactic

shock reactions gives a comprehensive and detailed picture of the allergic response in whey-sensitized mice.

In this ring trial a mouse model for cow's milk allergy was simultaneously introduced at 4 independent research facilities in the Netherlands. The presented results indicate that it is possible to transfer this model to 4 independent research facilities with only minor differences. These results are promising and in the next phase of the validation process whey hydrolysates will be tested and body temperature as an additional parameter will be included. This future ring trial will indicate whether the proposed mouse model using oral sensitization will be suitable as a new model for hypoallergenicity testing of HA infant formulas.

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7

Enzymatic treatment of whey proteins in cow's milk results in differential inhibition of IgE-mediated mast cell activation compared to T cell activation

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Submitted for publication

Abstract

Background Cow's milk (CM) hydrolysates are frequently used as milk substitutes for children with CM allergy. In hydrolysates, allergenic epitopes within cow's milk proteins are diminished by enzymatic treatment. The aim of this study was to examine the allergenic and immunogenic properties of whey proteins during hydrolysis.

Methods During hydrolysis, samples were obtained at 0, 10, 15, 30, 45, 60, 75 and 90 minutes and degradation was checked by HPLC and SDS-PAGE. Allergenic potential was analyzed by IgE crosslinking capacity of human FcεRIα-transduced rat basophilic leukaemia cells sensitized with serum of CM allergic patients. Whey-sensitized C3H/HeOuJ mice were ear-challenged intracutaneously with the hydrolysates. Immunogenicity was tested using whey-specific human T cell clones and T cell lines at the level of proliferation and release of IL-4, IL-10, IL-13 and IFN-γ.

Results After 15 minutes hydrolysis the majority of the proteins were degraded. Hydrolysis for 15 minutes resulted in 92% inhibition of mast cell degranulation and in 82% reduction of ear swelling in the mouse model. In contrast, the T cell stimulatory capacity was affected less by hydrolysis, the reduction of human T cell proliferation was only 9%. This was further reduced to 57% and 74% after 30 and 45 minutes hydrolysis, respectively. Cytokine production followed the same pattern.

Conclusion These *in vitro* and *in vivo* models demonstrate that a relatively low grade of enzymatic digestion of CM allergen can reduce the allergenicity to a much greater degree than the immunogenicity at the level of T cell reactivity.

Introduction

Cow's milk allergy (CMA) is the most common allergy in early childhood, with an estimated prevalence of 3% in the paediatric population (1). Food allergy is defined as an adverse immunological (hypersensitivity) response to food and can be divided into IgE-mediated and non-IgE-mediated reactions (2). Disorders with acute onset of symptoms after ingestion are usually IgE-mediated and affect one or more target organs: the skin (urticaria, angio-oedema), respiratory tract (rhinitis, asthma), gastrointestinal tract (pain, vomiting, diarrhoea), and cardiovascular system (anaphylactic shock) (3). In IgE-mediated reactions, allergen-specific IgE is bound to the high affinity IgE receptor (FcεRI) on the plasma membrane of basophils and/or mast cells. Upon re-exposure, the allergen will crosslink IgE and trigger the release of inflammatory mediators such as histamine, prostaglandins and leukotrienes. The optimal conditions for this release depend on the concentration of allergen-specific IgE antibody on the membrane, the concentration of allergen and the affinity of the IgE for the allergen (4, 5).

The only curative treatment for IgE-mediated allergy is specific immunotherapy (SIT). The immunological mechanisms by which SIT is effective include the modulation of T and B cell responses and is accompanied by a significant decrease of allergen specific IgE and increase in allergen specific IgG antibodies, mainly IgG_4 (6). However, SIT is not available for CMA. Several strategies to reduce the allergenicity of therapeutic preparations, while maintaining their therapeutic benefit, are being developed. Peptide immunotherapy (PIT) is one such approach. Short synthetic peptides, comprising T cell epitopes of major allergens, show a major potential advantage by abolishing the capacity to crosslink allergen-specific IgE and activate mast cells and basophils (7, 8). PIT studies in cat allergic and insect venom allergic subjects show promising results that support the potential of this approach (7, 9).

Whey proteins represent nearly 20% of the total bovine milk proteins. The known major allergenic proteins of whey are β-lactoglobulin (β-Lg) and α-lactalbumin (α-La). Several peptides with considerable biological roles have been identified in enzymatic hydrolysates of whey, of which some seem to have immunomodulatory potential and might therefore be beneficial for humans (10-13). Hydrolysates, also called hypoallergenic (HA) formulas, are intended for use by infants with diagnosed CMA. HA formulas are processed by enzymatic hydrolysis of protein sources such as bovine whey and/or casein, followed by further processing such as heat treatment and/or ultrafiltration. The products have been classified according to the degree of protein hydrolysis as 'extensively' or 'partially' hydrolyzed products. Product properties may be characterized by biochemical techniques, and reduction of allergenicity may be assessed *in vitro* with various immunological methods, and *in vivo* with skin prick test (SPT), patch test or challenge tests (14). It is known that processing of milk leads to changes in clinical allergenicity. For example, heat

treatment reduces the allergenicity of β-Lg by inducing conformational changes and by increasing its susceptibility to enzymatic digestion, both of which disrupted B cell epitopes (15).
In the current study we have examined the allergenic and immunological properties of whey proteins during hydrolysis for different periods of time. By careful differentiation between IgE-mediated responses and T cell activation we aimed at selecting hydrolysates with retained immunogenicity, but reduced allergenic properties.

Materials & Methods

Hydrolysis of whey

The bovine whey used in this study was a mixture of Deminal 90 (Friesland Foods Domo, Zwolle, The Netherlands) and whey protein concentrate (WPC) 60 (Milei, Leutkirch, Germany). 1277 g Deminal 90 and 551 g WPC 60 were dissolved in 8.12 L of deionized water and heat-treated for 2 minutes at 72°C. Whey was enzymatically treated with an established mixture of microbial endopeptidases and exopeptidases (enzyme composition confidential by Danone) at 58°C and adjusted to pH 7.75 with lye (12.3 g $Ca(OH)_2$, 9.6 g $Mg(OH)_2$ and 9.3 g KOH in 360 ml H_2O). During hydrolysis, protein samples were obtained at 0, 10, 15, 30, 45, 60, 75 and 90 minutes. At each time point, hydrolysis was stopped by inactivation of the enzymes through heating to 100°C.

High-performance liquid chromatography (HPLC) analysis of the hydrolysates

Analytical HPLC (Akta Explorer, Pharmacia, Uppsala, Sweden) was performed to analyze the chemical fingerprint of the whey hydrolysates (time points 0, 10, 15 and 30 minutes) according to the "WPImacrospher" method. A Macrospher RP300 (C4) 5 µ 250x4.6mm column (Alltech, Lexington, USA) was used with two solvent systems: 0.15% (v/v) trifluoroacetic acid (TFA; Sigma-Aldrich, St. Louis, USA) in demiwater (A) and 0.13% (v/v) TFA in acetonitril (B). The elution gradient profile was set as follows: 0-1 min 17.5% B, 1-3 min 30% B, 3-10 min linear to 65% B and 10-12 min 17.5% B, while flow rate was set at 1.0 ml/min and the detector wavelength was set to 220 and 280 nm. α-La and β-Lg were identified according to their retention time and comparison to pure α-La (Sigma-Aldrich) and β-Lg (Sigma-Aldrich).

Sodiumdodecylsulfate-polyacrylamide gelelectrophoresis (SDS-PAGE) and colloidal gold staining

The pattern of the residual proteins of the whey hydrolysates (time points 0, 10, 15 and 30 minutes) was analyzed with SDS-PAGE. A Bio-Rad (Hercules, CA, U.S.A) Mini-PROTEAN II system was used with a 15% Tris–HCl gel. Molecular weight markers were used as a reference. The proteins were diluted in a 1:3 ratio with reducing

sample buffer (6.05 g Tris, 8.0 g SDS, 3.2 g dithiothreitol, 20 mg bromophenol blue in 60 ml H_2O and 40 ml glycerol 87%, pH 6.8) and 10 µg of protein was loaded on the gel. De proteins were blotted to a PVDF membrane (Roche Diagnostics, Basel, Switzerland) and the total protein pattern was visualized with a colloidal gold staining (AuroDyeTM Forte kit, GE Healthcare, Uppsala, Sweden).

Degranulation of huFcεRI-RBL cells (RBL-hEIa-2B12 cells)

The cell-line RBL-hEIa-2B12, which was transfected with the α-chain of human Fcε receptor type 1 (hFcεR1) complex was a kind gift of Dr. Teshima, Japan (16). Degranulation of RBL-hEIa-2B12 cells with pooled serum of diagnosed CMA patients was performed as described previously (17). In short, confluent growing RBL-hEIa-2B12 cells ($1x10^5$/well) in 96 wells flat bottom culture plate were sensitized with 5 µg/ml human purified IgE (Chemicon/Millipore, Billerica, USA) or with 1:50 diluted serum of a pool of 10 CMA patients (β-Lg specific IgE 74.6 kUA/l measured by ImmunoCAP (Phadia,Uppsala, Sweden) for 16 hours at 37°C. CMA was confirmed in these patients by double-blind, placebo-controlled food challenge (DBPCFC) (18). The cells sensitized with purified IgE were stimulated with 5 µg/ml anti-human IgE antibody (BD Pharmingen, Franklin Lakes, USA) in Tyrode's buffer with 0.1% HSA (Sigma-Aldrich), pH 7.4) for 1 hour. The release induced by α-IgE on purified IgE-sensitized cells served as maximum amount of release. The sensitized cells with CMA pooled serum were stimulated with anti-human IgE (5 µg/ml), whey or the whey hydrolysates sampled at different time points; 0, 10, 15, 30, 45, 60, 75 and 90 minutes (1 µg/ml Tyrode's buffer) for 1 hour. β-Hexosaminidase activity was determined by a fluorescence assay using 4-methylumbelliferyl-N-acetyl-α-D-glucosamine (Fluka/Sigma-Aldrich) as a substrate. The β-hexosaminidase activity released into the medium was expressed as a percentage of maximum amount of release with anti-IgE.

Oral sensitization and challenge of mice with whey

Three- to five-week-old specific pathogen free female C3H/HeOuJ mice (n=4 in non-sensitised group and n=6 in sensitised groups) were purchased from Charles River Laboratories (Wilmington, USA), maintained on cow's milk protein free mouse chow (Special Diets Services, Witham, Essex, UK) and housed in the animal facility at the University of Utrecht. Animal care and use were performed in accordance with the guidelines of the Dutch Committee of Animal Experiments. As previously described (19), mice were sensitized intragastrically (i.g.) at day 0, 7, 14, 21 and 28 with 0.5 ml homogenized whey (40 mg/ml PBS) (DMV international, Veghel, The Netherlands) with cholera toxin (CT, 20 µg/ml PBS) (Quadratech Diagnostics, Epsom, Surrey, UK) as an adjuvant, using a blunt needle. Control mice received CT only. Mice were boosted weekly for a period of 5 weeks.

Allergen specific skin response

The acute allergic skin response (ear swelling at 1 hour) to evaluate the allergenicity of whey hydrolysates (20) was determined in anaesthetized mice after intradermal injection of 10 µg/20 ul PBS (0,5 mg/ml) whey hydrolysates (time points 0, 10 and 15 minutes of hydrolysis) and compared to the ear swelling after whey skin challenge (10 µg/20 ul PBS). As a negative control, sham-sensitized mice were skin challenged with whey. The allergen-specific net ear swelling was calculated by correcting the allergen-induced increase in ear thickness with the non-specific ear swelling due to local injection in the non-sensitized mice. The delta ear swelling is expressed as µm.

Human T cell cultures

Epstein Barr virus-transformed human B lymphocytes (EBV-B cells) were cultured in RPMI-1640 (Gibco/Life technologies) supplemented with 10% FBS (Gibco). Established T cell clones (TCCs) and T cell lines (TCLs) (21) were maintained in Iscove's Modified Dulbecco's Medium (IMDM) (Gibco) supplemented with 2% pooled human AB serum, 5% Yssel's medium (22) and 50 U/ml interleukin-2 (IL-2) and IL-4 (a kind gift from Novartis Research Institute, Vienna, Austria). TCCs and TCLs were specific for β-Lg. As a control, a casein-specific TCC was included. For the experiments performed to test the antigen-specificity and cytokine production, IL-2 and IL-4 were omitted from the medium. All media were supplemented with penicillin (100 IU/ml), streptomycin (100 mg/ml) and glutamine (1 mM) (Gibco).

T cell stimulation and cytokine production

T cell stimulation was performed in triplicate in 96-well U-bottom plates (Greiner Bio-one, Monroe, USA). Each well contained $4x10^4$ T cells and $4x10^4$ irradiated (55 Gy) autologous EBV-B cells, as antigen-presenting cells, pre-incubated overnight with 50 µg/ml total cow's milk proteins, casein, β-Lg or whey hydrolysates. EBV-B cells, incubated without antigen, were used as negative controls. After 24 hr, proliferation was measured by [3H]-Thymidine (TdR) incorporation (1 µCi/well (Amersham/ GE Healthcare, Buckinghamshire, UK), which was added for an additional 18 hr. Proliferative responses are measured as the mean [3H]-TdR incorporation (counts per minute) of triplicate wells ± SEM. Prior to addition of [3H]-TdR, supernatant was collected for the measurement of cytokine production from the triplicate wells and stored at -20°C. Cytokine production was measured by ELISA according to the manufacturer recommendations (Sanquin, Amsterdam, The Netherlands). The detection limit was 0.6 pg/ml for IL-4, 1.2 pg/ml for IL-10, 2 pg/ml for IFN-ɣ and 0.5 pg/ml for IL-13.

Analysis

The analysis of the mouse results (n=4 in non-sensitised group and n=6 in sensitised groups) and T cell stimulation were analyzed using one way ANOVA and post hoc Dunnett's test. Data are represented as mean ± SEM. Statistical analysis of the *in vitro* mast cells degranulation was done by Student's T tests. Statistical analyses were conducted using GraphPad Prism software version 4.03. Values of $p<0.05$ were considered significant.

Results

Proteolytic pattern of the hydrolysates analyzed by HPLC and SDS-PAGE

Hydrolysis of bovine whey with a mixture of microbial endopeptidases and exopeptidases was carried out for 0, 5, 10, 15, 30, 45, 60, 75 and 90 minutes. Figure 1 shows the proteolytic pattern analyzed by HPLC (A) and SDS-PAGE (B) corresponding to the whey hydrolysates obtained after 0, 10, 15 and 30 minutes of hydrolysis. After hydrolysis for 10 minutes, the protein pattern of α-La (14 kD) and β-Lg (18 kD) was still comparable to the unprocessed whey. After hydrolysis for 15 minutes, the majority of α-La and β-Lg was already partially degraded and after hydrolysis for 30 minutes, both proteins were largely degraded according to both methods.

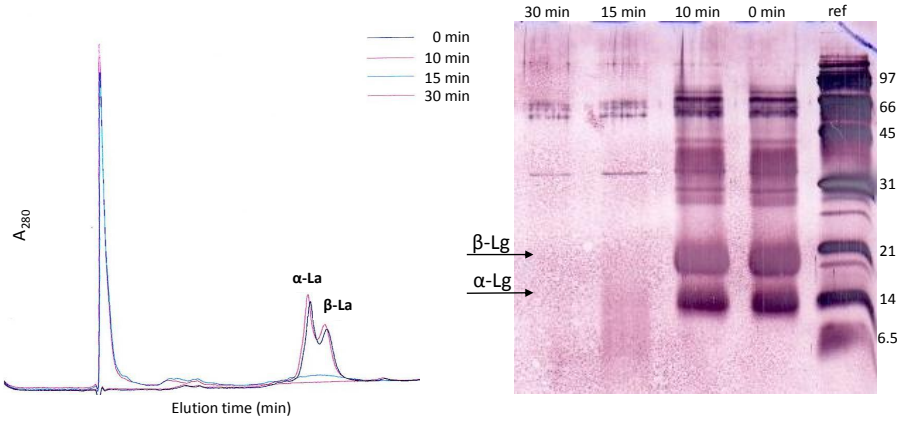

Figure 1. *High-performance liquid chromatography (HPLC) chromatograms (A) and sodiumdodecylsulfate-polyacrylamide gelelectrophoresis (SDS-PAGE) protein pattern (B) of whey hydrolysis. Samples were withdrawn at 0, 10, 15 and 30 minutes of hydrolysis. The HPLC peaks corresponding to α-Lactalbumin (La) and β-Lactoglobulin (Lg) were determined by comparison to pure α-La and β-Lg. The SDS-PAGE reference (ref) is a standard broad-range marker: phosphorylase b (97 kD), serum albumin (66 kD), ovalbumin (45 kD), carbonic anhydrase (31 kD), trypsin inhibitor (21 kD), lysozyme (14 kD) and aprotinin (6.5 kD).*

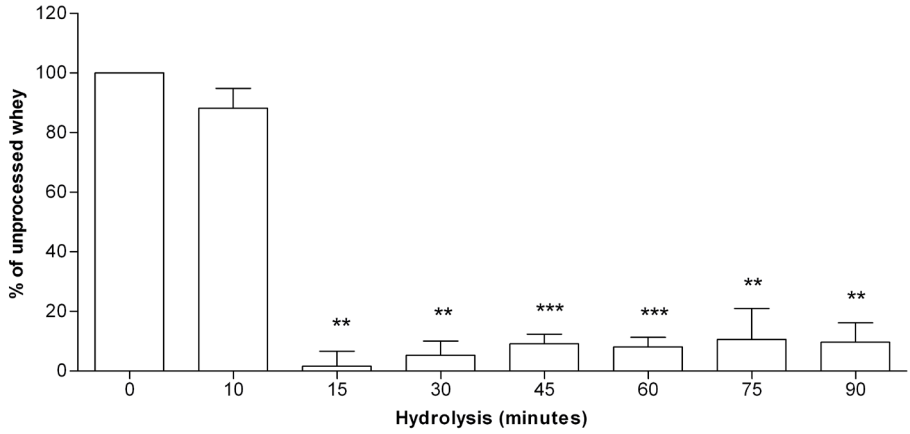

Figure 2. *Effect of hydrolyzed whey proteins on IgE-mediated degranulation of HuFcεRI-α transfected RBL cells. Degranulation was determined by measuring the amount of β-hexosaminidase released in the cell-free supernatant. β-hexosaminidase release of unprocessed whey (0 minutes) was set to 100% and samples were expressed as a percentage of unprocessed whey release. Bars represent average release (n=3) ± SEM. ** p<0.01, *** p<0.001.*

IgE crosslinking and mast cell activation potential of the hydrolysates

The HuFcεRI-α transfected RBL cells were incubated with a pool serum from CMA individuals and subsequently incubated with the whey hydrolysates. Mast cell degranulation was reduced by 12% when whey was added that was hydrolyzed for 10 minutes, whereas 15 minutes of hydrolysis resulted in 98% inhibition (figure 2). No further inhibition was observed at later time points.

Acute allergen specific skin reaction

In order to study allergic skin responses, whey sensitized mice were challenged with complete whey or whey hydrolysates i.d. in the ear pinnae one week after the last oral sensitization. Acute mouse ear oedema was markedly reduced when whey was applied that was hydrolyzed for 10 minutes (62%), and even further reduced (82%) when whey was used that had been hydrolyzed for 15 minutes (figure 3).

T cell stimulation and cytokine production

The different hydrolysates were tested on their ability to activate β-Lg-specific T cell clones (TCCs) and T cell lines (TCLs). Whey that had been hydrolyzed for 10 and 15 minutes demonstrated only a slight reduction of T cell activation when compared to unprocessed whey (resp. 7% and 9%). After 30 minutes of hydrolysis a pronounced

inhibition of T cell activation of 63% was seen, followed by an inhibition of 82% and 88% for the 45 and 60 minutes hydrolysates. No further inhibition was observed at later time points (figure 4). No T cell stimulation was found when casein-specific T cell clones were stimulated with the hydrolyzed whey material.

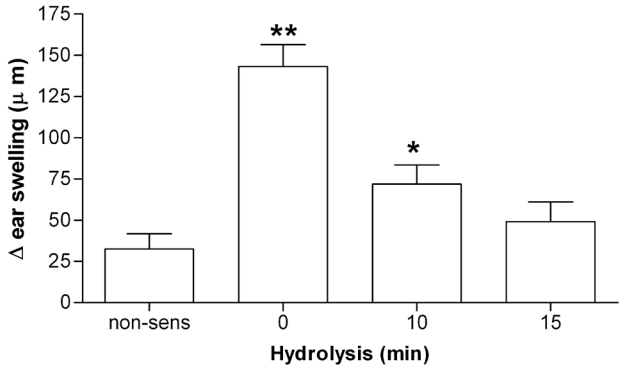

Figure 3. *Allergic skin responses in mice challenged i.d. in the ear pinnae, one week after the last oral whey sensitization, with whey hydrolysates sampled at time points 0, 10 and 15 minutes of hydrolysis. Bars represent average delta ear swelling (n=4 in non-sensitised group and n=6 in sensitised groups) ± SEM. * p<0.05, ** p<0.01.*

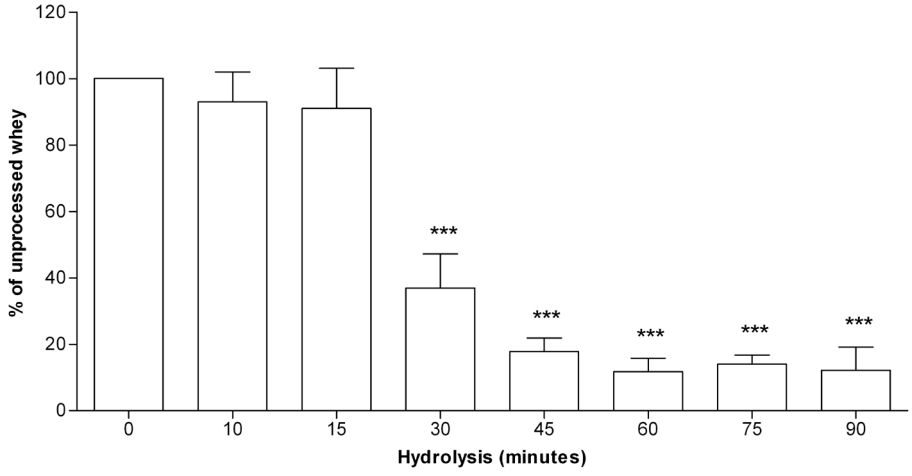

Figure 4. *Percentage of proliferation of β-Lg specific T cell clones (TCCs) and T cell lines (TCLs), incubated with whey hydrolysates. Proliferation of unprocessed whey (0 minutes) was set to 100% and the samples were expressed as a percentage of unprocessed whey proliferation. Bars represent average results from proliferation of 4 different TCCs/TCLs tested in duplicate ± SEM. *** p<0.001.*

The whey-induced production of IL-4, IL-10, IL-13 and IFN-γ cytokine production by activated T cells was measured in the supernatants of the TCCs and TCLs. The cytokine release by the T cells upon activation with the whey hydrolysate samples was inhibited to the same extent as T cell proliferation (figure 5).

Discussion

The current study shows that hydrolysis of whey proteins has a time-dependent effect on their capacity to cause IgE-mediated mast cell activation and T cell activation, which coincides with the decrease in their size. Although this study focused on the manipulation of whey in cow's milk, we anticipate that our approach might also work for other proteins, including cow's milk caseins.

The cow's milk hydrolysates that are currently available display strongly reduced immunogenicity and improved digestibility. The extensive- and amino-acid based hypoallergenic formulas have no apparent immunogenic properties and are well tolerated by cow's milk allergic infants (14). In the current study we aimed at

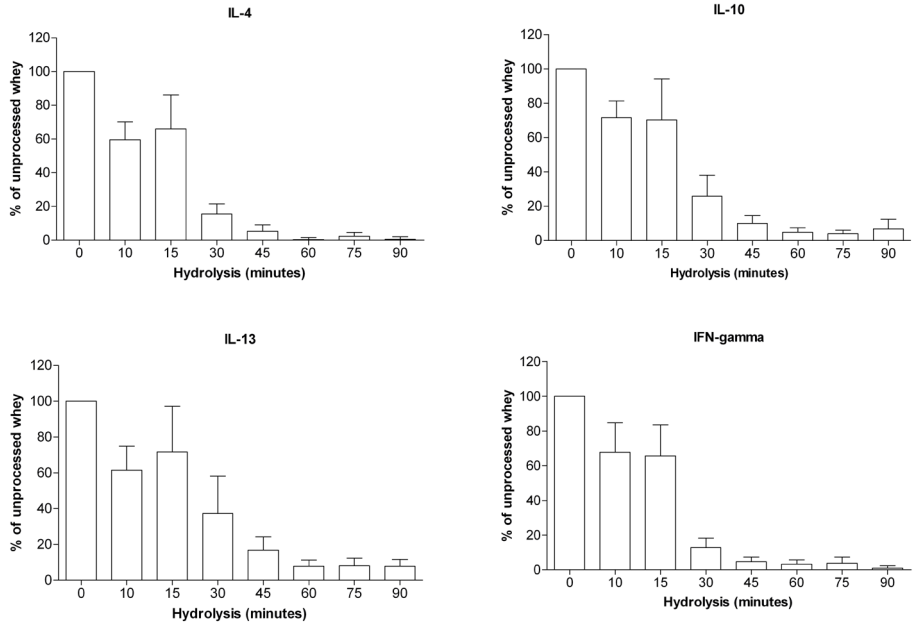

Figure 5. *Effect of different hydrolysates on the release of interleukin-4 (IL-4), IL-10, IL-13 and IFN-γ in supernatants of TCCs/TCLs proliferation cultures. Cytokine production of unprocessed whey (0 minutes) was set to 100% and samples were expressed as a percentage of unprocessed whey cytokine production. Bars represent average results from the cytokine production of 4 different TCCs/TCLs ± SEM. * p<0.05, ** p<0.01, *** p<0.001.*

improving our insight in the changes induced by hydrolysis of whey proteins. By analysis of the time-dependent effects of the process of hydrolysis on different allergenicity and immunogenicity parameters we demonstrated differential effects. The most important finding was that the effects of the hydrolysis on the *in vitro* and *in vivo* IgE-allergen interactions were observed at time points distinct from the effect on T cell activation. After 15 minutes of hydrolysis the IgE-binding and crosslinking capacity was diminished without affecting the T-cell activation. IgE-crosslinking was assessed using an *in vitro* RBL-assay and was confirmed using an *in vivo* mouse model of cow's milk allergy. In contrast, cytokine production and proliferation of human T-cell clones was only reduced after stimulation with whey hydrolysates that were hydrolyzed for at least 30 minutes.

In allergen immunotherapy, several studies have examined the differential effects of reducing allergen size, most particularly by producing peptides. For IgE crosslinking it has been concluded from several studies that the distance between 2 FcεRI molecules is within 8-24 nm, corresponding to approximately 30-100 amino acids (4). Peptides below this size are still able to stimulate the allergen-specific T cells, but unable to crosslink IgE on mast cells. Peptide immunotherapy for cat allergy has been investigated in great detail by Larché and coworkers who found that pro-inflammatory cytokines were reduced while the immunosuppressive cytokine IL-10 was increased. In addition, there was reduction in skin, lung and nasal sensitivity (7, 8). Also for the bee venom allergen PLA2 immunotherapy with 3 different peptides resulted in the clinical benefit in 3 out of 5 treated patients that now could tolerate a live bee sting challenge without reaction (23). However, the potential pitfall of the peptide-based immunotherapy is that there is wide variation in the types of MHC-II expression between individuals, leading to the definition of relatively large mixes of peptides to cover all relevant MHC-II phenotypes of patients. It is likely, although not proven, that by the partial hydrolysis of the whey proteins a large majority of the MHC-II binding structures in the whey proteins are still intact. In contrast to the study by Larché, we could not find a differential effect on the T cell cytokines by the hydrolysates (Figure 5). This is most likely due to the more fixed cytokine profile in the T cell clones and lines compared to the primary T cells used in the peptide immunotherapy studies (7).

In addition to the application of hydrolysed cow's milk protein in the treatment of established cow's milk allergy, there is also the possibility to use hydrolysates in prevention of cow's milk allergy. Until now different results have been obtained with different ranges of hydrolysis of the cow's milk proteins in rodent models (20, 24-26) and clinical studies (27-30). In prevention strategies there is not really a need to check for IgE binding, because the specific IgE antibodies have not yet been produced. However, better insight in the activation of T cells might be of importance to induce tolerance also in this preventive approach. Also in this situation there is

need in better defining the immunologic activation profiles of these hydrolysates. One could hypothesize that for induction of immunological tolerance some immunogenic activity within the hydrolysate is required. Therefore, our study was aimed at the more detailed analysis of the hydrolysis by *in vitro* and *in vivo* methods. Although the current approach seems promising, this should be evaluated in more detail in *in vitro* studies as well as in cow's milk allergic patients to confirm that the partial hydrolysate has the preferred tolerogenic activity.

Acknowledgements
The authors would like to thank Gerrit Witte for his excellent technical assistance.

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Oral tolerance induction by partially hydrolysed whey protein in mice is associated with enhanced numbers of Foxp3^{+} regulatory T-cells in the mesenteric lymph nodes

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Submitted for publication

Abstract

Hypoallergenic formulae are considered a good option for infants at risk for cow's milk allergy. The aim of this animal study was to investigate whether whey hydrolysates (WH) have the capacity to induce oral tolerance to whey. Whey, partial or extensive WH was given via gavages to naïve mice prior to oral whey sensitization using cholera toxin as an adjuvant. The acute allergic skin response, mouse mast cell protease-1 (mMCP-1), whey-specific IgE, IgG_1 and effector Th2-cells, Th1-cells and $Foxp3^+$ regulatory T-cells were determined in the mesenteric lymph nodes (MLN). MLN cells from tolerized mice were adoptively transferred to naïve recipient mice prior to whey sensitization. In contrast to the extensive WH, pre-treatment of naïve mice with whey or partial WH reduced the acute allergic skin response and mast cell degranulation after whey challenge. However, only treatment with whey prevented the generation of serum specific IgE/IgG_1. In partial WH tolerized mice the number of $Foxp3^+$ regulatory T-cells in the MLN was increased compared to whey sensitized mice. Both whey and partial WH treatment showed a tendency towards a decreased number of effector Th2-cells. Transfer of MLN cells from tolerized mice protected recipient mice from developing an acute allergic skin response. These results show that partial WH with limited sensitizing properties reduced the effector response upon whey challenge. This effect is transferable using MLN cells and was associated with enhanced $Foxp3^+$ regulatory T-cell numbers in the MLN. Partial WH retained the capacity to induce active immune suppression in mice which may be relevant for allergy prevention.

Introduction

Prevention of food allergy deserves high priority either by avoidance of the allergen (e.g. epitope exclusion) or the induction of oral tolerance. Oral tolerance enables the mucosal immune system to remain quiescent when harmless proteins such as food allergens enter the intestine while a protective inflammatory response is generated against potential pathogens. Oral tolerance induction most likely results from the deletion of antigen-specific Th2-cells as well as active suppression by regulatory T-cells that encompass natural, or the novo generated, $CD4^+CD25^+Foxp3^+$ and TGF_β- and IL-10-producing regulatory T cells (1-4). Human data showed that regulatory T-cells were associated with tolerance induction (5, 6). Moreover, children born with dysfunctional Foxp3, a key gene for development of $CD25^+$ regulatory T-cells, develop severe dermatitis with high levels of IgE and occasionally eosinophilia which suggest a suppressive role for $Foxp3^+$ regulatory T-cells in allergic immune reactions in humans. Hypoallergenic formulae are commonly used to manage cow's milk allergy and are generally categorized into partial and extensive hypoallergenic formulae based on the degree of hydrolysis and consequently the length of the remaining peptides. Several clinical trials show the effectiveness of partial whey hypoallergenic formulae in reducing clinical symptoms of allergy in high risk children (7-11). However, these studies are limited in answering whether the beneficial effects are due to avoidance of the allergic epitope or a result of oral tolerance induction. Animal models using oral sensitization represent a good tool to investigate oral tolerance to food proteins (12-15). However, the tolerizing capacity of whey hydrolysates and its underlying mechanism has not been investigated in animal models using oral sensitization so far. In the current study the tolerizing capacity of a partial WH and extensive WH was assessed in mice using a mouse model for orally induced cow's milk allergy. Moreover, the number of $Foxp3^+$ regulatory T-cells was determined and the contribution of the MLN cells in the transfer of tolerance was investigated. This study implies that partial and not extensive WH possess the putative capacity to induce clinical tolerance to whey in mice and that $Foxp3^+$ regulatory T-cells from the MLN may contribute to this effect.

Material and Methods

Cow's milk proteins and hydrolysates

Whey was obtained from DMV International, Veghel, the Netherlands. A partial whey hydrolysate (WH) was manufactured at Danone Research Centre for Specialised Nutrition by enzymatic hydrolysis under specified conditions. The enzymatic process was stopped by fast cooling. The partial WH was further characterized by analysis of the peptide size (85% < 1 kD, 8% < 2 kD, 4% < 5 kD, 1% < 10 kD, 0.6% < 20 kD and 1.4% >20kD) by means of high pressure liquid chromatography. Subsequently, filtering of the partial WH using a filter with a cut-off of 5 kD resulted in an extensive

WH. These experimental whey hydrolysates were used in all animal studies as mentioned below.

Reagents and antibodies

Cholera toxin is purchased from Quadratech Diagnostics, Epsom, UK. Biotin labeled rat anti-mouse IgE, IgG_1 and perCp-conjugated anti-CD4, FITC-conjugated-CD4 (L3T4), PE-conjugated CD25 and isotype control mAb were from Pharmingen, Alphen a/d Rijn, the Netherlands. APC-conjugated Foxp3, PE-conjugated CD69, FITC-conjugated T1St2, FITC-conjugated CD69, PE-conjugated CXCR3 and isotype controls were obtained from eBioscience, San Diego, CA, USA. All other chemicals were obtained from Sigma-Aldrich, Zwijndrecht, the Netherlands.

Mice

Three- to 4-week-old pathogen free female C3H/HeOuJ mice were purchased from Charles River Laboratories (Maastricht, the Netherlands), maintained on cow's milk protein free standard mouse chow (AIN-93G soja, Special Diets Services, Wijk bij Duurstede, the Netherlands) and housed in the animal facility at the Utrecht University. Animal care and use were performed in accordance with the guidelines of the Dutch Committee of Animal Experiments.

Oral tolerance induction, oral sensitization and challenge of mice

Prior to whey sensitization mice were treated orally (daily; day -7 until day -2) with 50 mg of partial or extensive WH or whey (positive control for oral tolerance induction) or PBS (sham), using a blunt needle. Subsequently, mice were sensitized orally on day 0, 7, 14, 21 and 28 with 20 mg of whey per mouse homogenized in PBS (0.5 mL, Cambrex Bio Science, Verviers, Belgium) mixed with 10 µg cholera toxin as an adjuvant. Non-sensitized mice received cholera toxin in PBS only. One week after the last sensitization the acute allergic skin response (ear swelling at 1 hour) after intra dermal whey challenge was measured. Mice were challenged orally with 0.5 mL of whey (200 mg/mL PBS) and 18 hours later blood samples were collected,

Table 1 *Treatment groups*

Group	Pre-treatment	Sensitization	Challenge
Non-sens	PBS	Cholera toxin in PBS	Whey
CNTRL	PBS	Whey-cholera toxin	Whey
Whey	Whey	whey-cholera toxin	Whey
Partial WH	Partial WH	Whey-cholera toxin	Whey
Extensive WH	Extensive WH	Whey-cholera toxin	Whey

centrifuged for 15 minutes at 20000 g and stored at –70°C (see table 1 and flow chart in figure 5).

Acute allergic skin response

An acute allergen specific ear swelling in whey sensitized mice was determined at 1 hour after intra dermal challenge with 10 µg total whey protein in the ear pinnae. As a negative control non-sensitized mice were challenged in the ear with similar whey protein. Ear thickness was measured in duplicate using a digital micrometer (Mitutoyo, Veenendaal, the Netherlands). The allergen-specific net ear swelling was calculated by correcting the allergen-induced increase in ear thickness with the non-specific ear swelling due to local injection in the non-sensitized mice. The ear swelling is expressed as delta µm.

Measurement of serum specific antibodies and mMCP-1

Whey-specific IgE and IgG_1 levels were measured in serum by means of ELISA. Microlon plates (Greiner, Alphen aan de Rijn, the Netherlands) were coated with 20 µg of whey in coating buffer (Sigma) for 18 hours at 4°C. Plates were washed and blocked for 1 hour with buffer containing 50 mM Tris, 2 mM EDTA en 137 mM NaCl /0.05% Tween and 0.5% BSA. Serum samples were incubated for 2 hours at room temperature. Plates were washed and incubated with 1 µg biotin labeled rat anti-mouse IgE or IgG_1 (Pharmingen, Alphen a/d Rijn, the Netherlands) for one hour at room temperature. After washing the plates were incubated with streptavidin-horse radish peroxidase (Sanquin, Amsterdam, the Netherlands) for one hour, washed and developed with o-phenylendiamine (Sigma). The reaction was stopped after 10 minutes with 4M H_2SO_4 and absorbance was measured at 490 nm on a Benchmark microplate reader (Biorad, Veenendaal, the Netherlands) and results were expressed as arbitrary units (AU). Serum concentrations of mouse mast cell protease-1 (mMCP-1) were determined according to the manufacturer's protocol using a commercially available ELISA kit (Moredun Scientific Ltd., Midlothian, UK).

Flow cytometer analysis of regulatory T-cells and effector Th1- and Th2-cells

MLN were removed and cut into small pieces and incubated with 0.2% collagenase IV and 2kU/ml DNAse 1 (Roche Diagnostics, Almere, The Netherlands) for 30 minutes at 37 ^{0}C, resuspended and incubated for another 30 minutes. The enzymatic reaction was stopped by adding 50 µl FCS. Single-cell suspensions (5 x 10^5) of MLN were blocked with PBS containing 5% FCS, 1% BSA and subsequently incubated for 20 minutes with either mAb against CD4 and CD25 for regulatory T-cells, mAb against the activation market CD69 and T1ST2 for the detection of effector Th2-cells, mAb against CD69 and CXCR3 for the detection of effector Th1-cells or isotype controls (Pharmingen, Alphen a/d Rijn, the Netherlands). For detection of Foxp3

(FJK-16), cells were permeabilized and incubated with anti-Foxp3 according to the manufacturer's protocol (eBioscience, San Diego, CA, USA). Stained cells were analyzed using a BD FACSCalibur flow cytometer.

Adoptive transfer experiments

Oral treatment with partial WH or total non-hydrolysed whey protected the mice from developing an allergic effector response. Regulatory cells generated in the MLN may be responsible for the transfer from local (gut associated) towards systemic tolerance. To test whether tolerance could be transferred using MLN cells an adoptive transfer experiment was performed. MLN were obtained from donor mice that were whey, partial WH or sham treated prior to sensitization. Single cell MLN suspensions were prepared using cell strainers and three different groups (sham, whey and partial WH) were pooled (n=12 per group). Single cell suspensions were injected intra veneously (100 µL, $4x10^6$ cells per mouse) in six different groups of recipient mice (n=6 per group). The recipient mice were non-sensitized or whey-sensitized as described above without prior pre-exposure to whey or partial WH. The acute allergic skin reaction was measured to determine allergic sensitization (see figure 5).

Statistics

All data were analyzed using one way ANOVA and post hoc Dunnett's test. Whey-specific antibodies were analysed using Kruskall-Wallis as variances differed between groups. A probability value $p<0.05$ was considered significant. Statistical analyses were conducted using GraphPad Prism software. Data are represented as mean ± SEM of 12 mice per group. Power calculations have been performed to determine samples size. Data, except for activated T-cell numbers n=6 were shown as mean ± SEM from n=2 independent experiments.

Results

Reduced whey-specific antibodies in mice orally exposed to whey prior to whey sensitization

In whey-sensitized mice (CNTR) whey-specific IgE and IgG_1 levels were augmented in serum when compared to non-sensitized mice (figure 1, $p<0.05$). No inhibitory effect on IgE or IgG_1 levels was observed when mice were pre-treated with the partial WH before sensitization indicating that sensitization to whey was not completely prevented, the extensive WH showed similar results (figure 1). As expected, pre-treatment of mice with whey prior to whey sensitization prevented the induction of whey-specific IgE ($p<0.05$) and IgG_1 ($p<0.01$).

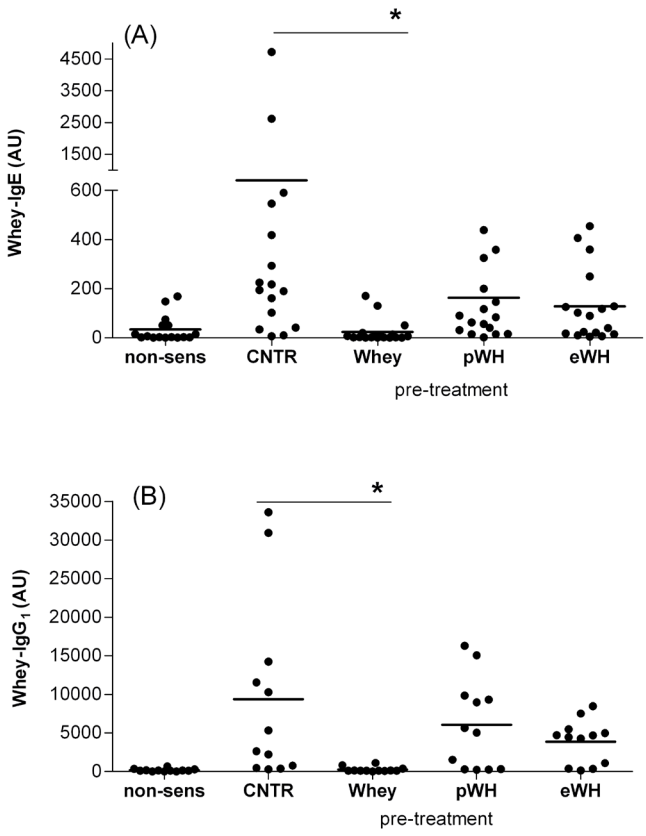

Figure 1. *Reduced whey-specific serum IgE (A) and IgG_1 (B) concentrations in whey pre-treated sensitized mice compared to control (CNTR) sensitized mice ($p<0.050$). No effect of the partial (p)WH or extensive (e)WH on serum IgE/IgG_1 concentrations was observed. Mice were treated with PBS (CNTR) or 50 mg whey, pWH or eWH prior to whey sensitization using cholera toxin as an adjuvant. Non-sensitized mice received cholera toxin only. Data were shown as mean ± SEM from n=2 independent experiments.*

Reduced acute skin response in whey and partial WH treated mice

In order to study whether oral administration of whey, partial or extensive WH prior to sensitization induced systemic tolerance at the effector level, the acute allergen specific skin response was measured in mice after intra dermal whey challenge. In whey sensitized control mice, ear challenge with whey induced a significant ear swelling at 1 hour compared to non-sensitized mice (figure 2; 120.9 ± 12.4 µm vs 25.4 ± 6.8 µm; $p<0.01$). Interestingly, administration of the partial WH prior to sensitization reduced the acute allergic skin response (83.6 ± 5.6µm; $p<0.05$) as well although the induction of whey-specific IgE was not significantly reduced.

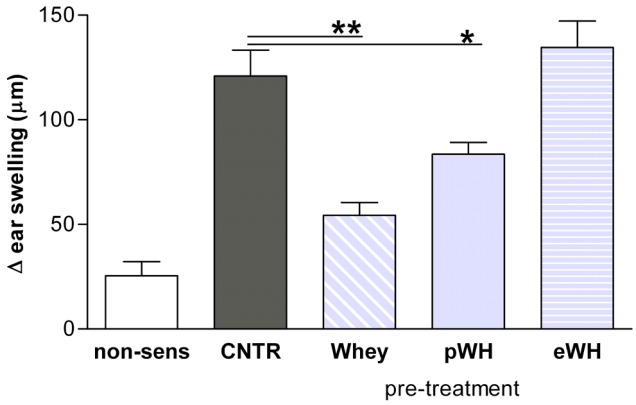

Figure 2. *Reduced acute allergic skin responses in whey (p<0.01) or partial (p)WH (p<0.050) pre-treated sensitized mice compared to control (CNTR) sensitized mice. No effect of the extensive (e)WH on the acute allergic skin response was observed. Mice were treated with PBS (CNTR) or 50 mg whey, pWH or eWH prior to whey sensitization using cholera toxin as an adjuvant. Non-sensitized mice received cholera toxin only. Data were shown as mean ± SEM from n=2 independent experiments.*

Administration of an extensive WH prior to sensitization did not affect the acute ear swelling response (134.6 ± 12.55 μm) compared to control whey sensitized mice (figure 2). As expected by the absence of detectable serum levels of whey-specific IgE or IgG_1, administration of whey prior to oral sensitization showed a significant reduction in the acute ear swelling compared to whey sensitized control mice (54.3 ± 6.1 μm; p<0.01).

Reduced serum mMCP-1 after oral challenge in whey and partial WH treated mice

To assess whether mucosal mast cell degranulation upon oral challenge was affected, mMCP-1 serum concentrations were determined. Administration of whey or partial WH prior to sensitization significantly reduced mMCP-1 concentrations (24.7 ± 5.6 ng/mL and 35.1 ± 6.2 ng/mL respectively) compared to whey sensitized control mice (69.8 ± 13.4 ng/mL) (p<0.05, figure 3). Pre-treatment of mice with the extensive WH did not affect the serum mMCP-1 concentration (67.6 ± 16.3 ng/mL) compared to control treated whey sensitized mice.

Number of regulatory T-cell, effector Th2- and Th1-cells in the MLN

To investigate whether active Th2-cell suppression might underlie the observed protective effect of whey and the partial WH, the number of $Foxp3^+$ regulatory T-cells and effector Th2-cells were determined in the MLN. Oral administration of the partial WH prior to sensitization increased the relative numbers of $CD4^+CD25^+Foxp3^+$

regulatory T-cells compared to whey sensitized control mice (figure 4A; 4.2 ± 1.1 vs 3.1 ± 0.5; p<0.05). The numbers of regulatory T-cells was not increased in whey pre-treated mice. However, a tendency towards increased numbers of effector Th1-cells compared to whey sensitized control mice was observed in the latter group indicating that these cells might contribute to the observed protective effects of whey (Fig 4B; p=0.10). Both whey and pWH treatment prior to whey sensitization showed a trend in reducing the number of activated Th2-cell after oral whey challenge (Fig 4C; p=0.092 for both). No effect on T-cells numbers was observed in extensive WH pre-treated mice (figure 4).

Adoptive transfer of MLN

MLN single cell suspensions from whey or partial whey tolerized (donor) mice were injected into recipient mice prior to PBS (non-sensitized) or whey-sensitization (scheme figure 5). As expected an acute allergic skin response was measured in recipient mice upon whey challenge when mice received MLN cells from control donor mice (figure 5, P<0.01). Recipient mice transferred with MLN cells of donor mice tolerized with whey or partial WH prior to whey sensitization developed a reduced acute allergic skin response compared to recipients transferred with control MLN cells (figure 5, P<0.01). These results indicate that regulatory mechanisms generated in the MLN contribute to the protective effects of whey and partial WH.

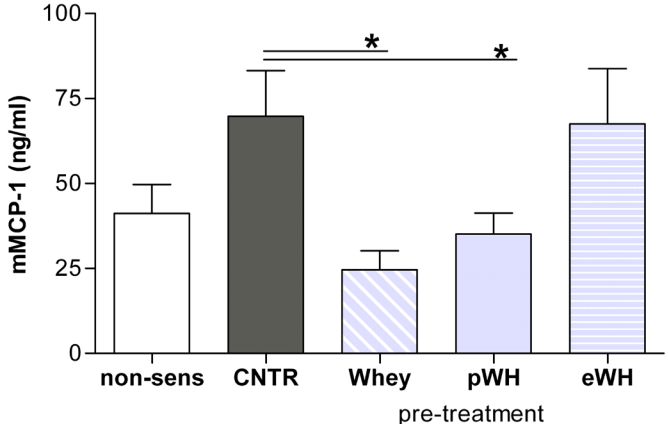

Figure 3. *Reduced serum mMCP-1 serum concentration were observed in whey or partial (p) WH pre-treated sensitized mice compared to control (CNTR) sensitized mice (p<0.050). No effect of extensive WH (eWH) on mMCP-1 was observed. Mice were treated with PBS (CNTR) or 50 mg whey, pWH or eWH prior to whey sensitization using cholera toxin as an adjuvant. Non-sensitized mice received cholera toxin only. Data were shown as mean ± SEM from n=2 independent experiments.*

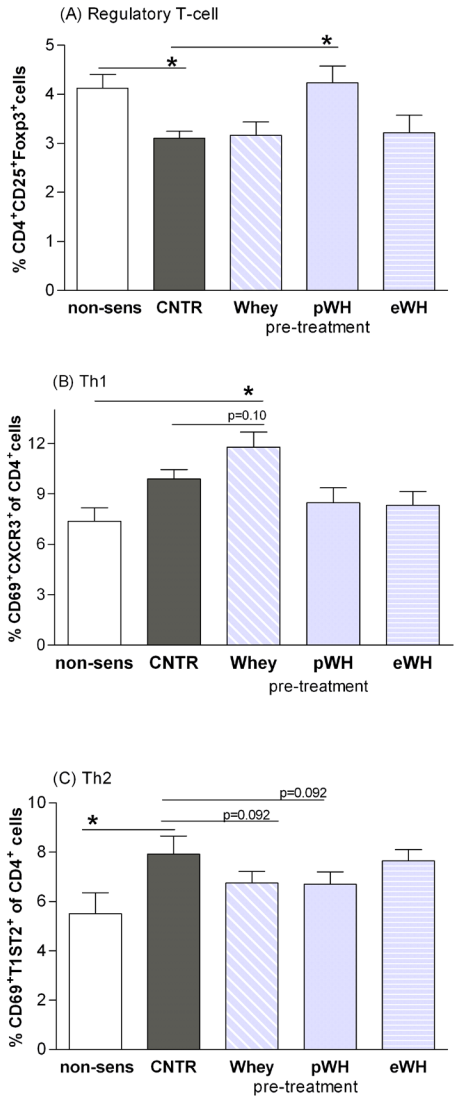

Figure 4. *Increased numbers of Foxp3⁺ regulatory T-cell in MLN were observed in partial (p)WH pre-treated sensitized mice compared to control (CNTR) sensitized mice (A; P<0.050). Data were shown from n=2 independent experiments. Whey pre-treatment tended to increase the number of activated Th1-cells compared to whey sensitized mice while this was significant compared to sham sensitized mice (B ; p=0.10, p<0.05). Administration of whey and pWH prior to sensitization tended to reduce the number of recently activated Th2 cells (C; p=0.092). No effect was observed after pre-treatment with an extensive (e)WH. Mice were treated with PBS (CNTR) or 50 mg whey, pWH, eWH prior to whey sensitization using cholera toxin as an adjuvant.*

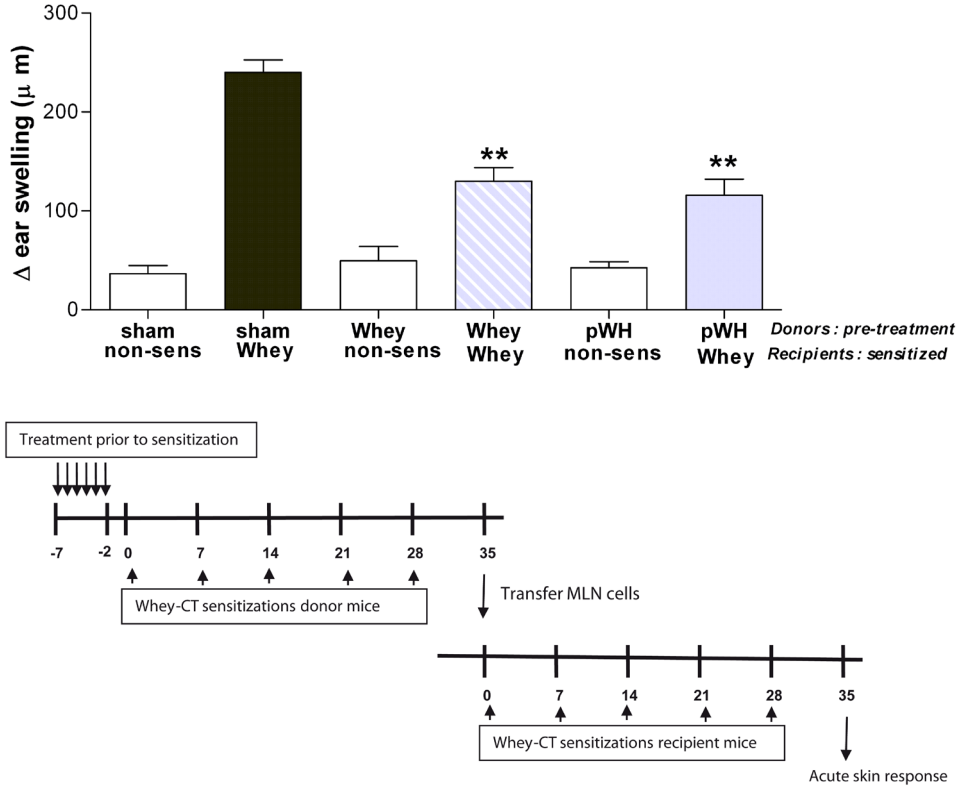

Figure 5. *Adoptive transfer of MLN cells of both whey or partial (p)WH pre-treated sensitized donor mice significantly reduced the acute allergic skin response in whey sensitized recipient mice when compared to recipient mice receiving sham-treated MLN cells (P<0.01). Donor mice were pre-treated with PBS (sham), whey or pWH prior to whey sensitization using cholera toxin as an adjuvant. MLN cells from donor mice were injected in recipient mice and subsequently sensitized to whey using cholera toxin as an adjuvant. Non-sensitized mice received cholera toxin only.*

Discussion

Establishment of oral tolerance to harmless food proteins might be of importance to prevent the development of food allergies and other allergies later in life. In the current study it was demonstrated that partial and not extensive WH retained the putative capacity to prevent allergic symptoms when given orally prior to sensitization, in a mouse model of orally induced cow's milk allergy. The protective effects coincided with elevated numbers of regulatory T-cells in the intestine and could be adoptively transferred using MLN cells.

In a recent study of our group, a partial WH was found to have limited sensitizing

capacities in a mouse model of cow's milk allergy using oral sensitization (16, 17). In the current study it was investigated whether this partial WH still was able to protect against sensitization to whey. As expected, whey treatment (as a positive control) prior to sensitization prevented the induction of whey specific-IgE and IgG_1 and as a result the allergic effector response upon whey challenge. Our results show that treatment of naïve mice with the partial WH prior to oral whey sensitization, like the non-hydrolysed whey, reduced the acute allergic skin response and mast cell degranulation upon intra dermal or oral whey challenge respectively. Surprisingly, partial WH treatment of naïve mice prior to oral sensitization decreased the acute allergic skin response and mast cell degranulation without affecting whey-specific IgE/IgG_1 serum levels. This implies that feeding a partial WH did not prevent sensitization to whey but protects against the development of an allergic effector response (e.g. clinical symptoms). One of the possible explanations of a reduced effector response in the presence of whey-specific IgE/IgG_1 may be that IgE receptor signaling of mast cells is diminished by active suppression involving regulatory T-cells which can be mediated via cell-cell contact or mediator release (18, 19). No protective effect of the extensive WH was observed.

Oral tolerance induction by partial whey hydrolysates in mice was widely studied in the past more than ten years ago using systemic sensitization rather than oral sensitization. In contrast to the current study, these animal studies showed that partial hydrolysed formulae prevented the generation of whey-specific IgE antibodies for β-lactoglobulin in mice (20-22). Children suffering from cow's milk allergy are most likely sensitized via the oral route; therefore animal models using oral sensitization instead of systemic sensitization might give a more accurate reflection of the sensitization route in children. In mouse models using oral sensitization with cholera toxin as an adjuvant it was shown that orally administered food proteins prior to oral sensitization prevented the generation of serum-specific antibodies (12-15) and decreased local Th2-cell responses to beta-lactoglobulin (12, 15). However, the tolerizing capacity of whey hydrolysates has not been investigated in a mouse model using oral sensitization so far. The tolerogenic features of the partial WH were associated with enhanced local numbers of $CD25^+Foxp3^+$ regulatory T-cell in the MLN and showed a strong tendency towards decrease in percentage of activated Th2-cells in the MLN of whey sensitized mice. The number of $CD25^+Foxp3^+$ regulatory T-cells in the MLN was decreased in whey sensitized mice compared to non-sensitized mice indicating that regulatory T-cells contribute to the development of the allergic response. Hence, feeding the partial WH prior to sensitization restores the number of regulatory T-cells to the level in non-sensitized mice. Studies in mice and humans have demonstrated that a novel transcription-repressor protein, Foxp3, is exclusively expressed by naturally occurring and locally induced $CD4^+CD25^+$ regulatory T-cells (5). Support for a role for regulatory T-cells in mediating oral tolerance has been

obtained from feeding experiments in mice (23-25) and human studies (5). Foxp3+ regulatory T-cells have been shown to convert local generated tolerance to systemic tolerance (26). The MLN connects the local and the systemic immune system creating one of the possibilities to convert the gut induced regulatory responses to systemic tolerance. To confirm whether the inhibitory effects of the partial WH on the acute allergic effector response (e.g. skin response and mMCP-1 levels) could be converted at the level of MLN, an adoptive transfer using MLN derived cells was conducted. Indeed, adoptive transfer of MLN cells of tolerized donor mice reduced the acute allergic skin response of whey-sensitized recipient mice. Hence, MLN cells from partial WH as well as whey tolerized mice could transfer tolerance to recipient mice. The protective effect of MLN cells of the partial WH tolerized donor mice may relate to the increased number of Foxp3+ regulatory T-cells found in the MLN of these mice. In whey tolerized mice, regulatory T-cell numbers remained low when naïve mice were fed whey prior to sensitization. However, a strong tendency towards a decrease in the relative number of effector Th2-cells was observed. In addition, number of Th1-cells was enhanced compared to non-sensitized mice. This implies that other mechanisms might contribute to the tolerogenic effect of the whole protein whey. A concomitant Th1 response may be involved in redirecting the Th2-type allergic response, contributing to the protective effects of whey feeding prior to sensitization. Further investigation is needed to examine whether effector Th1-cells or regulatory T-cells, other than Foxp3+ regulatory T-cells may contribute to the observed protective effects of whey tolerization.

In this study it was shown that partial WH and not extensive WH treatment of naïve mice prior to whey sensitization reduced the induction of clinical symptoms to whey. The tolerizing capacity of partial WH coincided with elevated numbers Foxp3+regulatory T-cells in the MLN and the protective effects could be transferred using MLN cells. These results indicate that Foxp3+ regulatory T-cells might contribute to a reduced effector response (e.g. clinical tolerance) induced by partial WH in mice. This might be of relevance in the prevention of cow's milk allergy in children.

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A specific mixture of non-digestible oligosaccharides enhances the tolerizing capacity of a partial whey hydrolysate in a mouse model for cow's milk allergy

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Submitted for publication

Abstract

Introduction Hypoallergenic (HA) infant formulas are considered a good alternative for infants at high risk for developing allergy if breastfeeding is not possible. Dietary intervention studies with HA infant formulas combined with non-digestible oligosaccharides, mimicking structural and functional properties of oligosaccharides present in human milk, have been shown to reduce allergic symptoms in these children. However, the mechanisms by which these non-digestible oligosaccharides exert their effect are yet to be explored. Objective: In a mouse model for cow's milk allergy, the contribution of a specific oligosaccharides mixture on the tolerizing capacity of a partial whey hydrolysate (WH) in relation to effects on intestinal Foxp3$^+$ regulatory T-cells and tolerogenic CD103$^+$ dendritic cells (DC) was investigated.

Methods Mice were sensitized orally once a week for five weeks with whey using cholera toxin as adjuvant. Prior to sensitization mice were pre-treated orally with 50 mg partial WH or PBS (as control), or fed a specific non-digestible oligosaccharide mixture diet containing short chain-Galacto-, long chain-Fructo- and pectin derived Acidic-oligosaccharides in a ratio of 9:1:1 (GFA-mixture) with or without the partial WH by gavage dosing for six consecutive days. One week after the last sensitization, mice were challenged orally and intradermally and the acute allergic skin response, mucosal mast cell mediator mMCP-1 and whey-specific antibodies were measured. The presence of Foxp3$^+$ regulatory T-cells and CD103$^+$ DC was determined in mesenteric lymph nodes.

Results Oral pre-treatment of mice with the partial WH induced tolerance as reflected by a reduced acute allergic skin response and a suppressed mMCP-1 release without affecting whey-specific IgE levels. This effect was associated with increased CD103$^+$ DC and Foxp3$^+$ regulatory T-cell numbers. Interestingly, a combination of the partial WH and a specific mixture of non-digestible oligosaccharides completely abolished the acute allergic skin response and mMCP-1 release. In addition, whey-specific IgE levels were decreased when compared to pre-treatment with the GFA-mixture in absence of partial WH gavage and a further increase in intestinal CD103$^+$ DC numbers was observed.

Conclusion A specific mixture of non-digestible oligosaccharides enhanced the capacity of a partial WH to induce oral tolerance. This effect was associated with increased numbers of CD103$^+$ DC in the mesenteric lymph nodes, known to contribute to tolerance induction. This suggests an important mechanistic role of these cells in the observed tolerance inducing capacity of non-digestible oligosaccharides combined with partial WH.

Introduction

Cow's milk allergy is besides hen's egg allergy, the first and most common type of allergy during early infancy and breastfeeding is considered to be important for the prevention of allergic disease. It provides a unique combination of lipids, proteins, carbohydrates, vitamins and minerals. Furthermore there are numerous bioactive compounds present in human milk with immunological properties such as soluble IgA, oligosaccharides, beneficial bacteria, Toll-like receptor ligands, cytokines, fatty acids and many more (1). Each compound can individually, additionally or synergistically act on the immune system of the neonate.

One of the potential protective mechanisms of breastfeeding may be the prebiotic activity of neutral and acidic oligosaccharides which are abundantly present in human milk (2, 3). To mimic some of the health and immune promoting properties of human oligosaccharides, a specific mixture of non-digestible oligosaccharides containing short-chain galacto oligosaccharides (scGOS) and long-chain fructo oligosaccharides (lcFOS) in a ratio of 9:1 was designed (scGOS/lcFOS; Immunofortis®). It was demonstrated that an intervention diet stimulated the growth of Bifidobacteria and Lactobacilli similar to counts found in breastfed infants (2, 4-6). Clinical studies confirmed the positive effects of the scGOS/lcFOS mixture observed as a reduction in the incidence of atopic dermatitis and allergic manifestations in association with a beneficial immunoglobulin profile in high risk children (7-9). Based on the above described findings, non-digestible oligosaccharides are considered a good additive for infant formulas.

The mechanisms by which these non-digestible oligosaccharides exert their effect are yet to be explored. Animal studies showed that a specific mixture of non-digestible oligosaccharides, containing short chain-Galacto-, long chain-Fructo- and pectin derived Acidic-oligosaccharides in a ratio of 9:1:1 (GFA-mixture), was effective in decreasing allergic symptoms in mouse models of allergic asthma (10) and cow's milk allergy through the induction of Th1 and/or regulatory T-cell mediated immune modulation (11, 12). Moreover, in a murine influenza vaccination model it was shown that the increased delayed type hypersensitivity response, indicative for an improved response against influenza, induced by the GFA-mixture was most effective when the GFA-mixture was introduced before onset of the disease (13). Based on these data it can be hypothesized that non-digestible oligosaccharides in human milk may fulfill a supportive role in the induction of immune responses in general and therefore might play an important role in oral tolerance induction to harmless food allergens like cow's milk proteins as well, hence the prevention of food allergy. Dietary exposure to the GFA-mixture may therefore be able to enhance the capacity of a partial whey hydrolysate (WH) to induce oral tolerance in a mouse model for cow's milk allergy.

Mucosal surfaces contain specialized dendritic cells (DC) capable of sensing external stimuli entering the gut. These DC enables the mucosal immune system to generate a protective inflammatory response against potential pathogens or other danger signals while remaining quiescent when harmless proteins such as food allergens enter the intestine. $Foxp3^+$ regulatory T-cells have been associated with their capability to actively suppress unwanted immune activation and are functionally involved in the generation of oral tolerance (14). A unique property of mucosal DC is their capability to induce $Foxp3^+$ regulatory T-cells in response to orally administered antigens (14, 15). $CD103^+$ mucosal DC reside in the lamina propria and mesenteric lymph nodes (MLN) and are involved in the local generation of regulatory T-cells, including $Foxp3^+$ regulatory T-cells (16-19), but their contribution to oral tolerance induction to cow's milk proteins has yet to be explored. Recently, oral tolerance was shown to originate in the intestinal lamina propria, and to depend on antigen transport by DC from the lamina propria to the MLN (20). It is important that once established, the effects of oral tolerance are not limited to the intestine but become systemic. The MLN provide the bridge between the mucosal and the systemic immune system and therefore systemic oral tolerance to food antigens may be generated via the induction of regulatory T-cells in the MLN by antigen presenting migratory DC arriving from the intestinal mucosa.

In the current study it was hypothesized that a specific GFA-mixture could contribute to improve the tolerizing capacity of a partial WH. Using a mouse model for cow's milk allergy, the tolerizing capacity of the partial WH, combined with the GFA-mixture, was investigated in relation to effects on $CD103^+$ DC and $CD4^+CD25^+Foxp3^+$ regulatory T-cells in the MLN known to play an important role in oral tolerance induction.

Methods

Cow's milk proteins and partial WH

Whey was obtained from DMV International, Veghel, the Netherlands and hydrolysed with an established mixture of endopeptidases and exopeptidases (confidential enzyme composition used by Danone) resulting in partially hydrolysed whey proteins. The enzymatic process was stopped by fast cooling. The partial WH was characterized by analysis of the peptide size (85% < 1 kD, 8% < 2 kD, 4% < 5 kD, 1% < 10 kD, 0.6% < 20 kD and 1.4% >20kD) by means of high pressure liquid chromatography. This experimental whey hydrolysate was used in the animal studies as mentioned below.

Reagents and antibodies

Cholera toxin was purchased from Quadratech Diagnostics, Epsom, UK. Biotin

labeled rat anti-mouse IgE, IgG_1, FITC-conjugated anti-CD4 (L3T4), PE-conjugated anti-CD25, APC-conjugated anti-CD103, PerCP-Cy5.5-conjugated anti-CD11c and isotype controls were obtained from Pharmingen, Alphen a/d Rijn, the Netherlands. APC-conjugated Foxp3 was obtained from eBioscience, San Diego, CA, USA. PBS was obtained from Cambrex Bio Science (Verviers, Belgium), streptavidin-horse radish peroxidase was obtained from Sanquin, Amsterdam, the Netherlands. Collagenase IV and DNAse 1 were obtained from Roche Diagnostics, Almere, the Netherlands. All other chemicals were obtained from Sigma-Aldrich, Zwijndrecht, the Netherlands.

Diets

Semi-purified cow's milk protein free AIN-93G-based diets were composed and mixed with non-digestible oligosaccharides by Research Diet Services (Wijk bij Duurstede, the Netherlands). The specific oligosaccharide mixture contained a 2 w/w% (9:1:1) mixture of short-chain galacto-oligosaccharides (scGOS, obtained by enzymatic elongation of lactose with galactose by β-galactosidase), long-chain fructo-oligosaccharides (lcFOS, derived from chicory inulin) and acidic oligosaccharides (pAOS, produced from pectin) and is indicated as GFA-mixture throughout the manuscript. scGOS (Vivinal GOS, Borculo Domo, Zwolle, the Netherlands), and lcFOS (Raftiline HP, Orafti, Wijchen, the Netherlands) consists of approximately 50% GOS and FOS, 19% is maltodextrin (glucidex 2, Roquette, France), 16% lactose, 14% glucose and 1% galactose. The pAOS powder (Sudzucker AG, Mannheim, Germany) consists of approximately 75% galacturonic acid oligomers, 10% monomers and 15% of moisture and ash. All oligosaccharides were exchanged for the same amount of total carbohydrates resulting in a comparable carbohydrate composition in the diets. The diets were stored at -20˚C prior to use.

Animals

Three- to 4-week-old pathogen free female C3H/HeOuJ mice were purchased from Charles River Laboratories (Maastricht, the Netherlands), maintained on cow's milk protein free standard mouse chow (AIN-93G soja, Research Diets Services, Wijk bij Duurstede, the Netherlands). Animal care and use were performed in accordance with the guidelines of the Dutch Committee of Animal Experiments.

Oral tolerance induction, oral sensitization and challenge of mice

Prior to whey sensitization mice were fed a control diet and treated orally (daily; day -7 until day -2) with PBS as a control or 50 mg partial WH using a blunt needle. Another group of mice were fed the GFA-mixture for 6 days with or without oral administration of the partial WH (50 mg, once a day). Subsequently, mice were sensitized orally by gavage dosing, on day 0, 7, 14, 21 and 28 with 20 mg whey per animal homogenized in PBS (0.5 ml) mixed with 10 µg cholera toxin as an adjuvant,

as previously described (21, 22). Non-sensitized mice received cholera toxin in PBS only. At day 33, five days after the last sensitization, the acute allergic skin response (ear swelling at 1 hour) after intradermal whey challenge was measured. Mice were subsequently challenged orally with 0.5 ml of whey (100 mg/ml PBS) and 18 hours later blood samples were collected, centrifuged for 15 minutes at 20,000 g and stored at –70°C until analyses. A schematic representation of the used tolerance induction, oral sensitization and challenge protocol is provided in figure 1.

Acute allergic skin response

An acute allergen-specific skin response was determined in whey sensitized mice, 1 hour after intradermal challenge with 10 µg whey in the ear pinnae. As a negative control non-sensitized mice were challenged in the ear with whey. Ear thickness was measured in duplicate using a digital micrometer (Mitutoyo, Veenendaal, the Netherlands). The allergen-specific net ear swelling was calculated by correcting the allergen-induced increase in ear thickness with the non-specific ear swelling due to local injection in the non-sensitized mice. The ear swelling is expressed as delta µm.

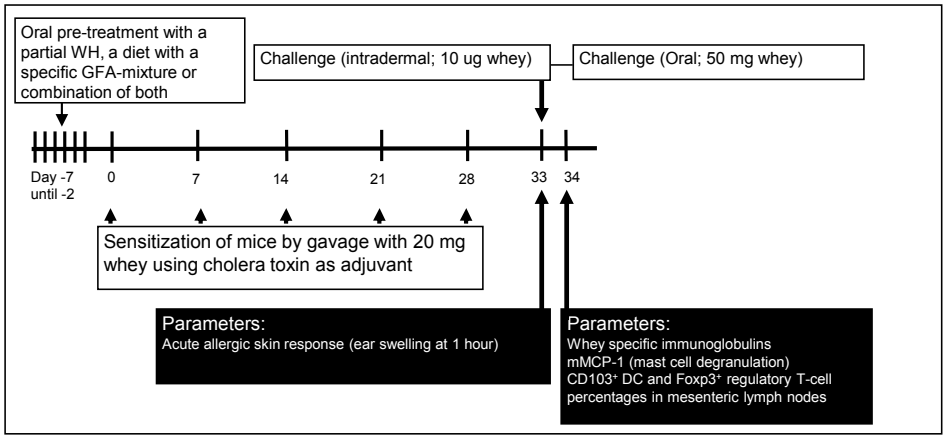

Figure 1. *Mice fed a control diet were sensitized orally by gavage dosing, on day 0, 7, 14, 21 and 28 using cholera toxin as an adjuvant. Prior to whey sensitization mice were treated orally (daily; day -7 until day -2) with PBS or a partial (p)WH while being fed the control diet or a diet containing the GFA-mixture for 6 days with or without oral administration of the partial WH (50mg, once a day). Five days after the last sensitization (day 33), the acute allergic skin response was determined after intradermal whey challenge. At day 33 also an oral challenge was given with 50 mg whey and at day 34 the mice were sacrificed and whey-specific immunoglobulins, serum mMCP-1 concentrations and dendritic cell and regulatory T-cell percentages in mesenteric lymph nodes (MLN) were determined.*

Measurement of serum specific antibodies and mMCP-1

Whey-specific IgE and IgG_1 levels were measured in serum by means of ELISA. Microlon plates (Greiner, Alphen aan de Rijn, the Netherlands) were coated with 20 µg of whey in coating buffer for 18 hours at 4°C. Plates were washed and blocked for 1 hour with buffer containing 50 mM Tris, 2 mM EDTA en 137 mM NaCl /0.05% Tween and 0,5% BSA. Serum samples were incubated for 2 hours at room temperature. Plates were washed and incubated with 1 µg biotin labeled rat anti-mouse IgE or IgG_1 for one hour at room temperature. After washing the plates were incubated with streptavidin-horse radish peroxidase for one hour, washed and developed with o-phenylendiamine. The reaction was stopped after 10 minutes with 4M H_2SO_4 and absorbance was measured at 490 nm on a Benchmark microplate reader (Biorad, Veenendaal, the Netherlands). Results were expressed as arbitrary units (AU). Serum concentrations of mouse mast cell protease-1 (mMCP-1) were determined according to the manufacturer's protocol using a commercially available ELISA kit (Moredun Scientific Ltd., Midlothian, UK).

Flow cytometric analysis of regulatory T-cells and DC

MLN were removed and cut into small pieces and incubated with 0.2% collagenase IV and 2kU/ml DNAse 1 for 30 minutes at 37 ^{0}C, resuspended and incubated for another 30 minutes. The enzymatic reaction was stopped by adding 50 µl FCS. Single-cell suspensions (5 x 10^5) of MLN were blocked with PBS containing 5% FCS, 1% BSA and subsequently incubated for 20 minutes with either mAb against CD4 and CD25 for regulatory T-cells, mAb against CD103 and CD11c for the detection of DC or isotype controls. For detection of Foxp3 (FJK-16), CD4 and CD25 stained cells were permeabilized and incubated with anti-Foxp3 according to the manufacturer's protocol. Stained cells were analyzed using a BD FACS Calibur flow cytometer.

Statistics

The data were analyzed using one way ANOVA and post hoc Dunnett's test for the acute allergic skin response, regulatory T-cells and DC percentages. Kruskal-Wallis was used for whey-specific antibody levels, and mMCP-1 concentrations. A probability value of $p<0.05$ was considered significant. Statistical analyses were conducted using GraphPad Prism software. Data are represented as mean ± SEM of 6 mice per group. Data for whey-specific antibodies, acute allergic skin response and mMCP-1 are a representative from n=2 independent experiments.

Results

Whey-specific immunoglobulins

In whey-sensitized mice (CNTR) whey-specific IgE (fig. 2A: 1321 ± 791 AU; $p<0.05$) and IgG_1 (fig. 2B: 62130 ± 44710 AU; $p<0.01$) levels were increased in serum

when compared to non-sensitized mice. In mice fed the control diet no significant inhibitory effect on IgE or IgG_1 levels was observed when mice were pre-treated with the partial WH prior to sensitization (188 ± 122 AU and 9367 ± 2314 AU; for IgE (fig 2A) and IgG_1 (fig. 2B) respectively). However, when the partial WH was administered orally in mice fed the GFA-mixture a reduction in whey-specific IgE levels was observed (fig 2A: 73 ± 29 AU; $p<0.05$) when compared to sensitized mice pre-fed the GFA-mixture without partial WH by gavage dosing. As observed in a previous study, pre-treatment of mice with whey prior to whey sensitization prevented the induction of whey-specific IgE (data not shown).

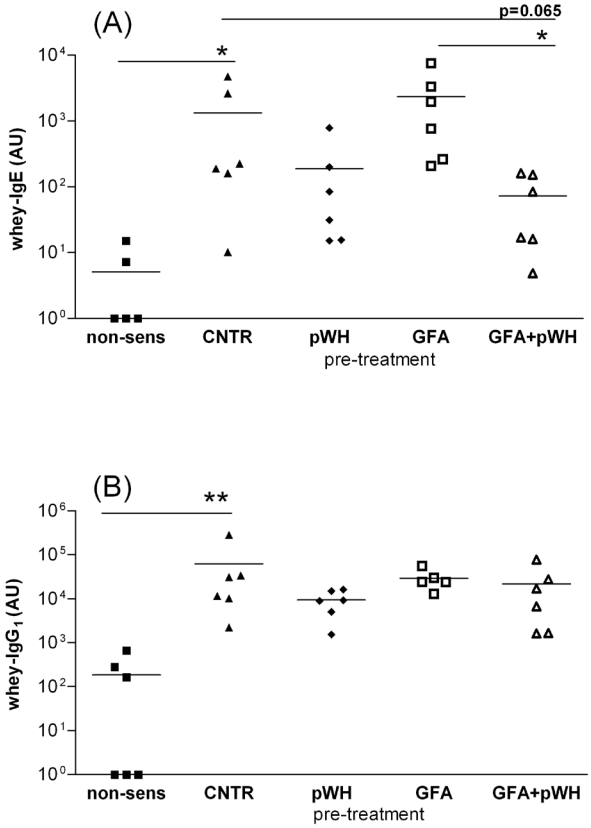

Figure 2. *At day 34, whey-specific IgE (A) and IgG_1 (B) concentrations were measured in serum of non-sensitized and whey sensitized mice pre-treated with PBS (CNTR) the partial (p) WH, a diet containing the GFA mixture (GFA) or a combination of both. Data are expressed as individual values of n=6 animals ± SEM; *p<0.05, **p<0.01. Data are representative for n=2 experiments.*

Acute allergic skin response

In order to study whether the GFA-mixture improved the protective effects of the partial WH, the acute allergic skin response was measured in mice after intradermal whey challenge (fig. 3). In control mice (CNTR), ear challenge with whey induced a significant ear swelling at 1 hour in whey sensitized animals (121.1 ± 19.0 µm; $p<0.01$) compared to non-sensitized mice (36.7 ± 5.7 µm). Administration of the partial WH prior to whey sensitization significantly reduced the acute ear swelling response (67.2 ± 10.7 µm; $p<0.05$) without affecting the induction of whey-specific IgE. Feeding the GFA-mixture for five days did not change the acute allergic skin response in whey sensitized mice. Interestingly, when the partial WH was administered when mice were fed the GFA-mixture the acute allergic skin response to whey was further reduced comparable to the level of non-sensitized mice (32.6 ± 8.1 µm; $p<0.01$ as compared to CNTR). As expected by the absence of whey-specific IgE no acute allergic skin response was observed in whey pre-treated mice (data not shown).

mMCP-1 serum concentrations after oral challenge

To assess whether the GFA-mixture improved mucosal tolerance, mMCP-1 serum concentrations were determined as a reflection of mucosal mast cell degranulation (fig. 4). In the current study administration of the partial WH showed a tendency to reduce the mMCP-1 levels (42.1 ± 16.2 ng/ml) compared to control whey sensitized mice (69.2 ± 14.6 ng/ml). Interestingly, when the partial WH was administered in

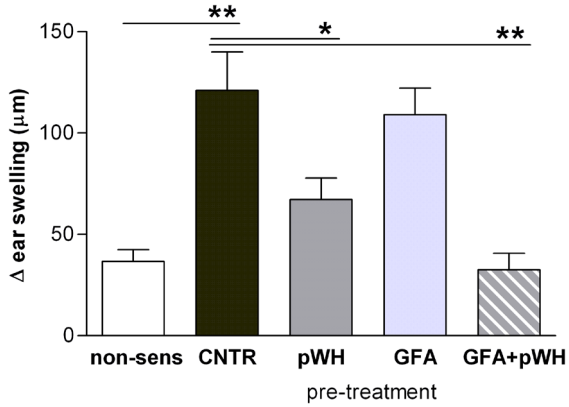

Figure 3. *At day 33, an acute allergic skin response was measured 1 hour after intradermal whey challenge in the ears of non-sensitized and whey sensitized mice (CNTR). Whey sensitized mice were pre-treated with PBS (CNTR) the partial (p)WH, a diet containing the GFA mixture (GFA) or a combination of both. Data are expressed as mean values of n=6 animals ± SEM; *p<0.05, **p<0.01. Data are representative for n=2 experiments.*

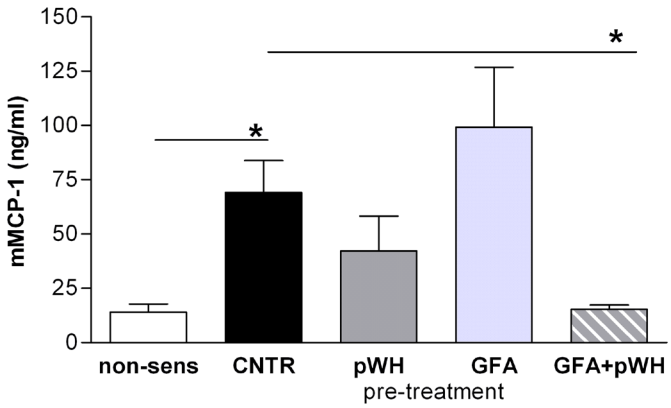

Figure 4. *mMCP-1 serum concentrations were measured 18 hours after oral whey challenge in non-sensitized and whey sensitized mice as a reflection of mast cell degranulation. Whey sensitized mice were pre-treated with PBS (CNTR) the partial (p)WH, a diet containing the GFA mixture (GFA) or a combination of both. Data are expressed as mean values of n=6 animals ± SEM; *p<0.05.*

the presence of the GFA-mixture the serum mMCP-1 concentration was significantly reduced (15.3 ± 2.0 ng/ml; $p<0.05$) compared to PBS treated whey sensitized mice (CNTR). No effect on mMCP-1 release (99.1 ± 27.7 ng/ml) compared to the control diet was observed in mice fed the GFA-mixture.

09

Percentage of $CD11c^+CD103^+$ DC in MLN

To investigate whether the oligosaccharide diet affected the percentage of DC with a regulatory phenotype present in the MLN in association with the protective effects of the partial WH, the percentages of $CD11c^+CD103^+$ DC were determined (fig. 5). No change in regulatory $CD11c^+CD103^+$ DC (2.3 ± 0.2% of total cells) was observed in control whey sensitized mice (CNTR) compared to non-sensitized mice (2.6 ± 0.3% of total cells). Interestingly, increased relative numbers of $CD11c^+CD103^+$ DC were observed in mice fed the GFA-mixture for five days prior to sensitization with whey (3.2 ± 0.2% of total cells; $p<0.05$) and in mice pre-treated with the partial WH prior to whey sensitization (3.1 ± 0.2% of total cells; $p<0.05$). Moreover, when the partial WH was administered orally in the presence of the GFA-mixture a further increase in the percentage of $CD11c^+CD103^+$ DC was observed (3.8 ± 0.2%; $p<0.01$) compared to control treated whey sensitized mice This may imply that $CD11c^+CD103^+$ DC numbers contribute to the improved tolerance to a partial WH in presence of the GFA-mixture.

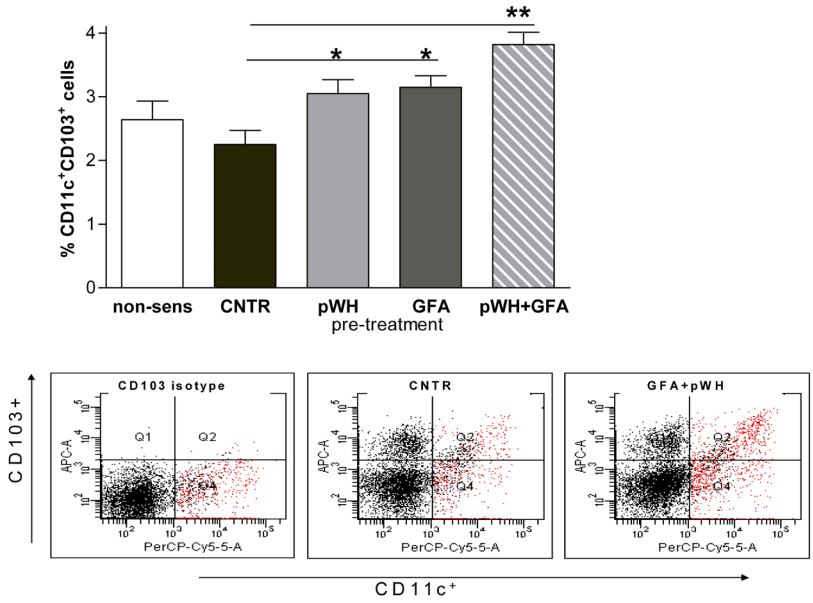

Figure 5. *CD11c⁺CD103⁺ DC percentages were determined in the MLN of non-sensitized and whey sensitized mice pre-treated with PBS (CNTR) the partial (p)WH, a diet containing the GFA mixture (GFA) or a combination of both. A representative dot-plot is shown from MLN cells of whey sensitized control mice (CNTR) and whey sensitized mice pre-treated with GFA⁺ pWH stained with CD11c and CD103 mAb or isotype control. Data are expressed as mean values of n=6 animals ± SEM; *p<0.05, **p<0.01.*

09

Percentage of Foxp3⁺ regulatory T-cells in MLN

MLN cell suspensions were analyzed to investigate whether the percentage of Foxp3⁺ regulatory T-cells was increased in the MLN in association with the protective effects in mice fed the GFA-mixture (fig. 6). Administration of the partial WH prior to whey sensitization increased the relative number of Foxp3⁺ regulatory T-cells in the MLN (5.8 ± 0.5% of total cells) compared to whey sensitized mice fed the control diet (4.1 ± 0.4% of total cells; $p<0.05$). In mice fed the GFA-mixture this did not result in a further increase in the percentage of regulatory T-cells (4.5 ± 0.2% of total cells) although in these mice the allergic effector response was further reduced after oral administration of the partial WH, as determined by the allergen-specific skin response and mMCP-1 in serum.

Discussion

Tolerance towards dietary components known as oral tolerance evokes a body-wide non-responsiveness against the plethora of food antigens. DC trafficking from the lamina propria to the MLN have been shown to be essential for the induction

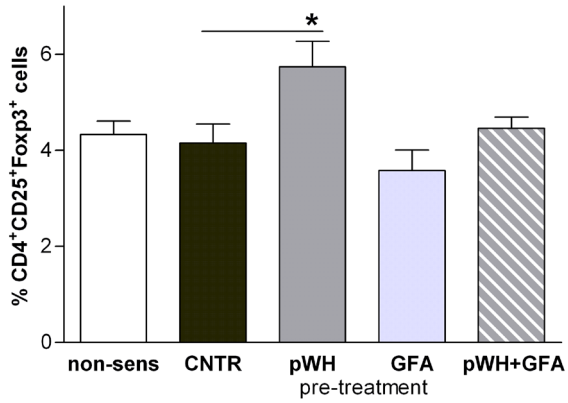

Figure 6. *Foxp3⁺ regulatory T-cell percentages were determined in the MLN of non-sensitized and whey sensitized mice pre-treated with PBS (CNTR) the partial (p)WH, a diet containing the GFA mixture (GFA) or a combination of both. Data are expressed as mean values ± SEM of n=6 animals; *p<0.05.*

of oral tolerance (20, 23, 24). In the current study we showed that non-digestible oligosaccharides, which mimic structural and functional aspects of human oligosaccharides in breast milk, improve the efficacy of oral tolerance induction using a partial WH. This effect was associated with increased numbers of $CD11c^+CD103^+$ DC in the MLN. To our knowledge, no studies have been described so far on the possible contribution of non-digestible oligosaccharides on tolerance induction.

09

While breastfeeding is considered to be the golden standard for infant nutrition, HA formulas are a good alternative for infants at high risk or infants diagnosed with cow's milk allergy. These HA infant formulas are generally categorized as partial and extensive WH based on the degree of hydrolysis. Several studies investigated the putative capacity of partial whey hydrolysates to induce oral tolerance to the native whey protein (25-27). In the current study, it was shown that a specific GFA-mixture fed for six days prior to whey sensitization further improved the partial WH induced reduction of the acute allergic skin response and mast cell degranulation. The acute allergic effector response is induced by binding of allergen-specific IgE-antibodies to the high affinity receptor FcεRI on mast cells or basophils and the subsequent cross-linking of the IgE-antibodies by whey resulting in mast cell degranulation and mediator release which causes the allergic symptoms. Interestingly, oral treatment of naïve mice with the partial WH in the presence of the GFA-mixture, and not the partial WH alone, reduced whey-specific IgE levels if compared to mice fed the GFA-mixture alone. The observed effects on the acute allergic skin response, mast cell degranulation and whey-specific immunoglobulins indicate that the GFA-mixture

fed for six days induced a tolerogenic milieu which contributes to the establishment of oral tolerance. In recent studies we showed that feeding the GFA-mixture to mice at the time of sensitization contributed to a generic immune modulation. When provided before and during sensitization with whey the GFA-mixture protected mice against the development of allergic symptoms via a mechanism that indicate to be dependent on regulatory T-cells (11). So far, the supportive role of non-digestible oligosaccharides on oral tolerance induction to whey or partial WH has not been described.

$CD103^+$ DC are the major subset of DC present in the lamina propria and MLN (17, 28) and both in mice and human $CD103^+$ DC promote the differentiation of $Foxp3^+$ regulatory T-cells by mechanisms involving TGF_β and the dietary component retinoic acid (17-19, 29) and their role in tolerance induction has been suggested recently (30). Interestingly, our study shows that the protective effect of the partial WH coincided with increased percentages of $CD11c^+CD103^+$ DC and $Foxp3^+$ regulatory T-cells in the MLN. In addition, combining the partial WH with the GFA-mixture before sensitization resulted in an improvement of oral tolerance induction using partial WH in association with increased percentages of $CD11c^+CD103^+$ DC. Intestinal epithelial cells are in close proximity to DC in the lamina propria and have been shown to condition mucosal DC specialization. Epithelial cells are known for their capacity to produce retinoic acid and TGF_β which has been implicated to contribute to prolonged conditioning of mucosal $CD103^+$ DC (31, 32). We can only speculate that the prolonged effects induced by the GFA-mixture fed to mice for five days prior to sensitization may involve immune modulating mediators secreted by memory cells of the adaptive immune system in close collaboration with the intestinal epithelium and/or mucosal DC.

Although $CD11c^+CD103^+$ DC percentages were increased, no further increase of $Foxp3^+$ regulatory T-cells was observed in the animals pre-treated with the partial WH and fed the GFA-mixture. Regulatory T-cells can be divided in $Foxp3^+$ regulatory T-cells and regulatory T-cells like the Th3 (TGF_β producing) or Tr1 (IL-10 producing) regulatory T-cells. These regulatory T-cell subtypes might have contributed to the observed effects as induced by the GFA-mixture. However, the relation between $CD11c^+CD103^+$ DC and Th3 or Tr1 regulatory T-cells has not been described in literature. Hence, future studies exploring these mechanisms are needed to determine the mechanisms by which the GFA-mixture increases the percentage of $CD11c^+CD103^+$ DC in the MLN and improved tolerance induced by the partial WH.

The current study demonstrated that a diet containing a GFA-mixture enhanced the efficacy of tolerance induction by a partial WH. This resulted in an abolished acute

allergic skin response to whey, a prevention of mucosal mast cell degranulation and a tendency towards reduced IgE serum levels. We show that this effect coincided with elevated numbers of tolerogenic $CD11c^{+}CD103^{+}$ DC in the MLN. This study indicates that non-digestible oligosaccharides might proof their benefit as an adjuvant to optimize immune modulation and to support oral tolerance induction for specific antigens.

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10

Depletion of $CD4^+CD25^+$ T cells switches the whey-allergic response from immunoglobulin E- to immunoglobulin free light chain-dependent

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Clinical and Experimental Allergy 2010; 40(9): 1414-21

Abstract

Background Symptoms of allergy are largely attributed to an Immunoglobulin (Ig)E-mediated hypersensitivity response. However, also a considerable number of patients exhibit clinical features of allergy without detectable systemic IgE. Previous work showed that immunoglobulin free light chains (IgLC) may act as an alternate mechanism to induce allergic responses. $CD4^+CD25^+$ T-cells are crucial in the initiation and regulation of allergic responses and compromised function might affect the response to allergens.

Objective To examine the contribution of $CD4^+CD25^+$ T-cells and IgLC on the whey allergic response.

Methods Mice were sensitized orally with whey using cholera toxin as an adjuvant. $CD25^+$ T-cells were depleted *in vivo* using a CD25 mAb. The acute allergic skin response to whey and *ex vivo* colon reactivity was measured in presence or absence of F991, a specific inhibitor of IgLC. Serum whey-specific antibodies and IgLC in serum and mesenteric lymph node supernatants were measured. Depletion of $CD4^+CD25^+$ T-cells was confirmed in the spleen.

Results Anti-CD25 treatment strongly reduced whey-specific antibody levels and resulted in a partial depletion of effector T-cells and a major depletion of $Foxp3^+$ regulatory T-cells. Surprisingly, despite the abolished specific IgE response, the acute allergic skin response to whey was not affected. IgLC levels were enhanced in serum and mesenteric lymph node supernatants of CD25-depleted sensitized mice. F991 inhibited the acute skin response and colon hyperreactivity in anti-CD25 treated mice indicating that these responses were mainly IgLC dependent.

Conclusions Depletion of $CD4^+CD25^+$ T-cells resulted in a switch from an IgE- into an IgLC-dependent acute skin response and functional hyperresponsiveness of the colon. Our data suggest that $CD25^+$ T-cells play a crucial role in balancing cow's milk allergy between IgE and IgE-independent responses and both mechanisms might play a role in allergic responses to the same allergen.

Introduction

The incidence of atopic diseases is increasing worldwide. It is hypothesized that factors responsible for this may be found in changing environmental conditions, westernized life style, air pollution, and the increased consumption of additives and preservatives with food. Similar trends have been observed for the prevalence of autoimmune diseases such as type I diabetes and multiple sclerosis. Regulatory and effector T-cells play a crucial role in balancing the immune system and a compromised function of $CD4^+CD25^+$ T-cells may provide an explanation for the increased immunological response to allergens and auto-allergens.

Cow's milk allergy is one of the leading causes of food allergy in children (1, 2). Clinical symptoms affect the skin, respiratory and gastrointestinal tract and are mostly caused by IgE-induced hypersensitivity (3, 4). However, it should be realized that in atopic diseases like food allergy, rhinitis and asthma, a considerable number of patients exhibit clinical features of allergy without detectable local or systemic IgE (5-7). Mast cells have been implicated in the pathogenesis of atopic and non-atopic diseases and several *in vivo* studies indicate that they are involved in allergic asthma and gastrointestinal allergy (8-12). Although antigen-specific mast cell activation results from crosslinking the high-affinity IgE receptor, FcεR1, also other mechanisms can be involved in antigen specific activation in the absence of IgE antibodies. In previous studies, a novel mechanism for the elicitation of immediate hypersensitivity-like reactions via immunoglobulin free light chain (IgLC) has been described in skin and airways (13-16). Moreover, IgLC-elicited hypersensitivity responses can be inhibited by local or systemic application of the IgLC antagonizing peptide F991 (13, 15, 16). Immunoglobulin light chains are assembled with immunoglobulin heavy chains into complete antibodies. However a substantial part of the synthesized immunoglobulin light chains is secreted as a free polypeptide. Elevated levels of IgLC are found in asthma and rhinitis patients suggesting a role for IgLC in human atopic diseases (16-18).

In the present study, the contribution of $CD4^+CD25^+$ T-cells and IgLC on acute allergic symptoms and intestinal responses in cow's milk allergy was assessed using *in vivo* CD25-depletion. The results indicate that after depletion or functional impairment of $CD4^+CD25^+$ T-cells the nature of the acute allergic response to whey changed from IgE- into IgLC-dependent. This suggests that depending on the immunologic milieu an IgLC- or IgE-dependent allergic response to the same allergen can be elicited.

Materials and methods

Reagents and antibodies

Whey was obtained from DMV International, Veghel, the Netherlands. Cholera toxin is purchased from Quadratech Diagnostics, Epsom, UK. Biotin labeled rat anti-mouse IgE, IgG_1, and perCp-conjugated anti-CD4, PE-conjugated antiCD25 and

isotype control mAb were from BD Biosciences, Alphen a/d Rijn, the Netherlands. APC-conjugated Foxp3 and isotype control from eBioscience, San Diego, CA, USA. Rat anti-CD25 mAb (clone PC61) was kindly provided by Dr. Louis Boon, Bioceros BV, Utrecht, the Netherlands. All other chemicals were obtained from Sigma-Aldrich, Zwijndrecht, the Netherlands.

IgLC-binding compound, F991

F991 is a 9-mer peptide and has been demonstrated to specifically inhibit IgLC-induced hypersensitivity responses (13, 15). F991 was synthesized by Fmoc Chemistry (Ansynth, Roosendaal, the Netherlands).

Mice

Three- to 4-week-old pathogen free female C3H/HeOuJ mice were purchased from Charles River Laboratories (Maastricht, the Netherlands), bred and raised on cow's milk protein free standard mouse chow (AIN-93G soja, Special Diets Services, Witham, Essex, UK) and housed in the animal facility at the Utrecht University. Animal care and use were performed in accordance with the guidelines of the Dutch Committee of Animal Experiments.

Oral sensitization and in vivo administration of anti-CD25 mAb

Mice were sensitized orally, using a blunt needle, on day 0, 7, 14, 21 and 28 with 20 mg whey per animal homogenized in PBS (0.5 ml, Cambrex Bio Science, Verviers, Belgium) mixed with 10 µg cholera toxin (Quadratech Diagnostics, Epsom, UK) as an adjuvant. Non-sensitized mice received cholera toxin only. At day 14 just before the third sensitization mice were injected intraperitoneally with 400 µg rat anti-mouse CD25 mAb (PC61). Non-sensitized mice and control mice received a non-specific IgG_1 isotype control antibody (Sigma-Aldrich-Chemie, Zwijndrecht, the Netherlands), according to the same schedule. One week after the last sensitization the ear swelling at 1 hour after intradermal whey challenge and *ex vivo* colon reactivity to carbachol were measured. Blood samples, mesenteric lymph nodes and spleen were collected. Blood samples were spun down for 15 minutes at 13500 rpm. Sera and mesenteric lymph node (MLN) cell supernatants were stored at -70 ^{o}C for determination of whey-specific antibodies and total IgLC.

Flow cytometer analysis of $CD4^{+}CD25^{+}$ T-cells

The efficacy of systemic CD25-depletion was confirmed in spleen using cell-surface monoclonal antibodies directed against CD4 (L3T4), CD25 (PC61) and Foxp3 (FJK-16). Single-cell suspensions prepared from spleen were incubated for 30 minutes with blocking buffer (PBS containing 5% FCS, 1% BSA). Subsequently, 5 x 10^5 cells were incubated for 20 minutes with 100 µl FACS buffer (PBS with 1% FCS, 1% BSA)

containing perCp-conjugated anti-CD4, PE-conjugated anti-CD25 or isotype controls. For detection of Foxp3, cells were subsequently permeabilized and incubated with anti-Foxp3 according to the manufacturer's protocol. Stained cells were analyzed using a BD FACSCalibur flow cytometer.

Measurement of serum immunoglobulins

Whey-specific IgE and IgG_1 levels were measured in serum by means of ELISA. Microlon plates (Greiner, Alphen aan de Rijn, the Netherlands) were coated with 20 µg whey in coating buffer (Sigma) for 18 hours at 4°C. Plates were washed and blocked for 1 hour with buffer containing 50 mM Tris, 2 mM EDTA en 137 mM NaCl /0.05% Tween and 5% BSA. Serum samples were incubated for 2 hours at room temperature. Plates were washed and incubated with 1 µg biotin labeled rat anti-mouse IgE or IgG_1 (Pharmingen, Alphen a/d Rijn, the Netherlands) for one hour at room temperature. After washing the plates were incubated with streptavidin-horse radish peroxidase (Sanquin, Amsterdam, the Netherlands) for one hour, washed and developed with o-phenylendiamine (Sigma). The reaction was stopped after 10 minutes with 4M H_2SO_4 and absorbance was measured at 490 nm on a Benchmark microplate reader (Biorad, Veenendaal, the Netherlands). For measuring whey-specific IgE a serum dilution factor of 4 and for whey-specific IgG_1 a dilution factor of 50 was used for antibody detection. The whey-specific antibodies were expressed as OD.

Acute allergic skin response

An acute allergen specific ear swelling in whey sensitized mice was determined at 1 hour after intradermal challenge with 10 µg whey in the ear pinnae. As a negative control non-sensitized mice were challenged in the ear with whey. Ear thickness was measured in duplicate using a digital micrometer (Mitutoyo, Veenendaal, the Netherlands). The allergen-specific net ear swelling was calculated by correcting the allergen-induced increase in ear thickness with the non-specific ear swelling due to local injection in the non-sensitized mice. The delta ear swelling is expressed as µm. To investigate the contribution of IgLC on the acute allergic skin response, F991 was applied topically in a cetomacrogol cream (1mg F991/g cream)on the inside of the ear pinnae three hours before intra dermal ear challenge as described earlier (15). The cream (1 cm stripe equaling 20 mg of cream) was dispensed using an eye-cream tube and spread out using an inert spatula. Cetomacrogol cream FNA is a hydrophilic cream that is normally used in indifferent treatment of skin conditions and was readily taken up by the mouse skin.

Measurement of IgLC in serum and MLN supernatants

On day 35, MLN's were incubated in RPMI 1640 (1 x 10^6 cells/ml) without any

supplements for 24 hours in the absence of allergen. Supernatant was collected and analyzed for IgLC. Serum samples were precipitated as described (19). In short, 20 µl of serum was depleted from albumin by resuspension in 80 µl ice-cold 10% TCA/acetone followed by incubation for 90 min at -20 °C. Samples were centrifuged for 20 minutes at 15000g at 4 °C. Supernatant was removed and pellet was washed with 1 mL ice-cold acetone and dissolved in Laemmli buffer. A small volume was run on a 12% SDS-PAGE gel followed by electro transfer to PVDF (Bio-Rad). Gels were run under non reducing conditions. Membranes were blocked o/n in PBS-T with 2% non-fat dry milk. IgLC were detected with anti-mouse kappa light chain mAb conjugated to horse radish peroxidase (Southern Biotech, Alabama, USA). Immunoreactive bands were visualized with ECL and X-Ray Film (Pierce, Etten-Leur, the Netherlands). Films were scanned and analyzed on a GS710 Calibrated Imaging Densitometer equipped with Quantity One v. 4.0.3 software (Bio-Rad, Veenendaal, the Netherlands).

Isometric contraction of the colon

The colon, caudal from the caecum, was dissected free of connective tissue and mesenterium. Colon parts of 1 cm length were mounted in an organ bath, using two small clamps, containing 10 mL Tyrode buffer (NaCl, 136.89; KCl, 2.68; $MgCl_2$, 1.05; $CaCl_2$ 1.77; NaH_2PO_4, 0.42; $NaHCO_3$, 11.9 and glucose, 5.55 (mM)). The organ bath was kept at 32° C, to reduce spontaneous contraction of the colon, and was continuously gassed with a 5% CO_2 and 95% O_2 gas mixture. One clamp was attached to a fixed point in the organ bath and the other clamp was connected to an isometric transducer (Harvard Apparatus Ltd., Kent, UK) with an analog recorder (BD 40 Kipp & Zn., Delft, the Netherlands). Contractions were measured under a constant preload of 1.00 gram. The preparations were equilibrated for one hour in the organ bath before starting a log dose-response-curve for carbachol (10^{-8} until 10^{-5} M). After every dosage the organ bath was flushed two times and the tissue was allowed to recover for 10 minutes before addition of the next concentration.

F991 was administered by intraperitoneal injection (50 µg/100 µl) eighteen hours and one hour before removal of the colon to investigate the contribution of IgLC on colon reactivity in anti-CD25 treated mice.

Statistics

All data except for the isometric contractions were analyzed using one way ANOVA and posthoc Dunnett's test. Isometric contraction data were analyzed using Repeated Measures ANOVA and post hoc Dunnett's test. A probability value $p<0.05$ was considered significant. Statistical analyses were conducted using GraphPad Prism software. Data are represented as mean ± SEM of 6 mice per group.

Results

Anti-CD25 treatment abolished the production of whey-specific Immunoglobulins

In whey-sensitized mice whey-specific serum IgE and IgG_1 levels were augmented at day 35 when compared to non-sensitized mice. Surprisingly, administration of anti-CD25 mAb abolished the induction of whey-specific IgE and strongly reduced IgG_1 levels (Figure 1). Previous studies showed no detectable levels of IgE at day 14 of the sensitization protocol indicating that no whey-specific IgE was present when anti-CD25 mAb was administrated. Although not significant different from non-sensitized mice low levels of whey-specifc IgG_1 were present at day 14 (data not shown).

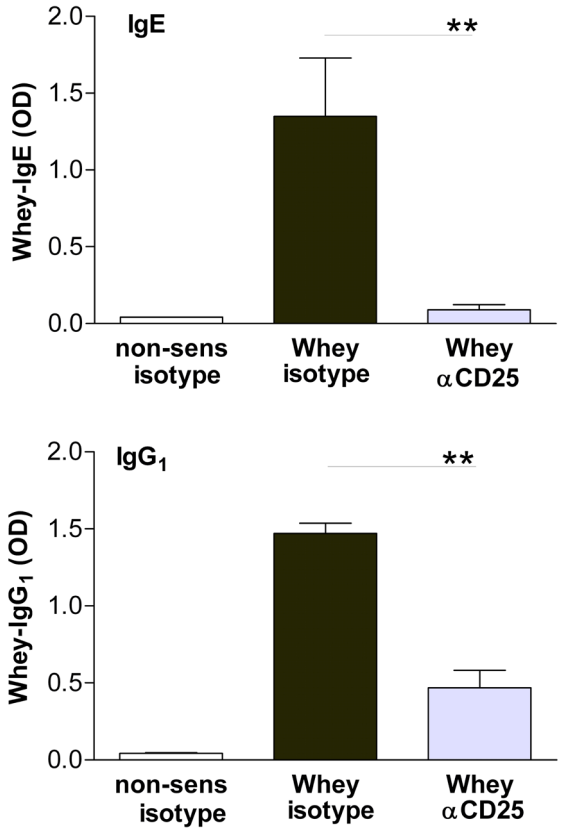

Figure 1. *Whey-specific antibodies were found in mice after sensitization to whey compared to non-sensitized mice. Administration of anti-CD25 mAb (αCD25) significantly reduced whey-specific IgE (4 times dilution) and IgG_1 (50 times dilution) levels compared to isotype controls (**p<0.01, n=6). Whey sensitized mice received 400 µg αCD25 mAb or isotype control mAb. Non-sensitized mice received an isotype control mAb.*

Depletion of $CD4^+CD25^+$ T-cells

To discriminate between $CD25^+$ regulatory T-cells and activated $CD25^+$ effector T-cells Foxp3 was used as an additional marker to identify regulatory T-cells. In the current experiments, $CD4^+CD25^+Foxp3^+$ T-cells were depleted *in vivo* after administration of anti-CD25 mAb 1 hour before the third sensitization (0.25 ± 0.091% of total cells vs 8.73 ± 0.74% of total cells for isotype controls in spleen, $p<0.01$). Anti-CD25 treatment also partially depleted $CD4^+CD25^+$Foxp3- T-cells (2.88 ±0.30% of total cells vs 4.40±0.23% of total cells for isotype controls, $p<0.05$) indicating that PC61 treatment applicated systemically at day 14 during sensitization depleted regulatory T-cells but also partially depleted or affected the generation of $CD4^+CD25^+$ Foxp3- T-cells systemically.

Anti-CD25 treatment did not affect the acute allergic skin response

In the next experiments, it was determined whether the reduced IgE and IgG_1 production in anti-CD25 treated mice also resulted in an impaired allergic skin reaction after challenge with the allergen as well. As expected, intra dermal skin challenge with whey induced a significant ear swelling at 1 hour in sensitized mice compared to non-sensitized mice (142.1±16.2 vs 30.3 ±14.5 µm; Figure 2). Unexpectedly, in the anti-CD25 treated animals, no statisitical significant reduction in the acute ear swelling response was observed (142.1±16.2 vs 120.2 ± 9.7 µm; Figure 2). These results indicated that the reduction in specific IgE and IgG_1 levels did not correlate

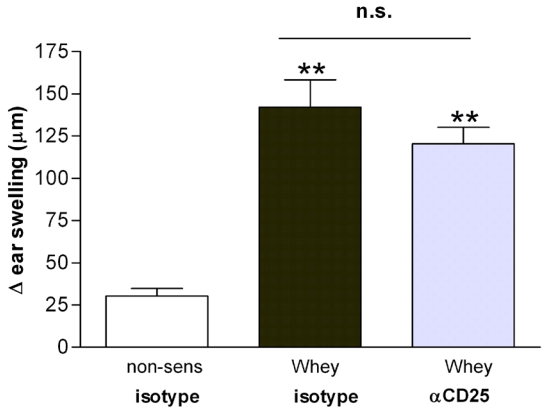

Figure 2. *The acute allergic skin response after local whey challenge was not affected in anti-CD25 mAb (αCD25) treated whey sensitized mice (P> 0.27). Local whey challenge (i.d. in ear) in both anti-CD25 mAb-treated and isotype control-treated whey sensitized animals was enhanced compared to non-sensitized mice (**p<0.01, n=6). Whey sensitized mice received 400 µg αCD25 mAb or isotype control mAb. Non-sensitized mice received an isotype control mAb.*

with the ear swelling response in the CD25-depleted mice and suggested that other mechanisms causing an allergic skin response may be involved.

IgLC levels in serum and mesenteric lymph node supernatants

In previous studies IgLC was shown to mediate antigen-specific mast cell-dependent responses. Therefore, it was examined whether IgLC levels in serum and MLN supernatants from non-sensitized and whey-sensitized mice were detectable. Immunoblot analysis showed that IgLC protein expression was strongly increased in serum and MLN supernatants of whey sensitized mice after anti-CD25 mAb treatment, while no increase in IgLC levels was found in the whey-sensitized control group as compared to the non-sensitized controls (Figure 3).

IgLC dependent acute allergic skin response in anti-CD25 treated whey sensitized mice

To investigate whether the increase in IgLC was responsible for the sensitization of anti-CD25 treated mice, the whey-induced ear swelling response was analyzed in

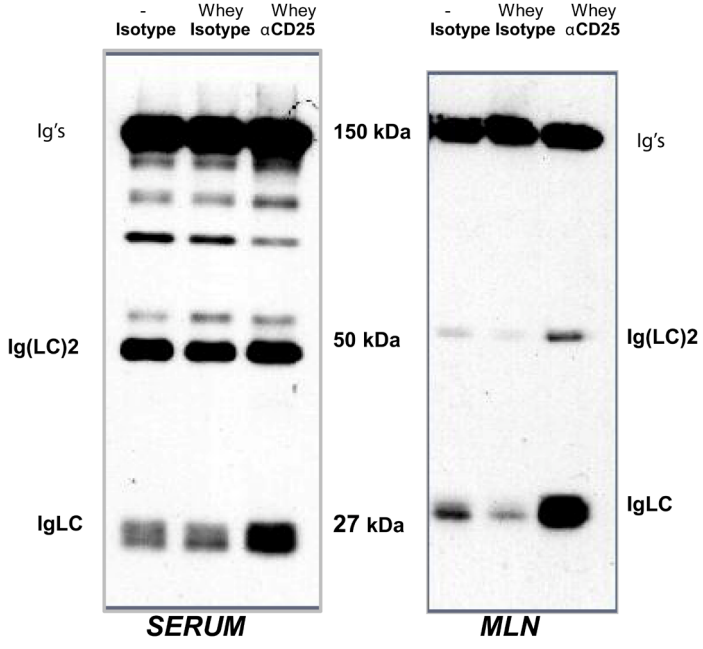

Figure 3 *Anti-CD25 mAb (αCD25) treatment in whey sensitized mice increased the IgLC levels in serum and MLN supernatants. Whey sensitized mice received 400 µg αCD25 mAb or control mAb on day 14 during sensitization. Non-sensitized mice received an isotype control mAb. Representative blots are shown from n=3 independent experiments. Gels were run under non reducing conditions. IgLC = monomer free light chain, Ig(LC)2= dimer IgLC.*

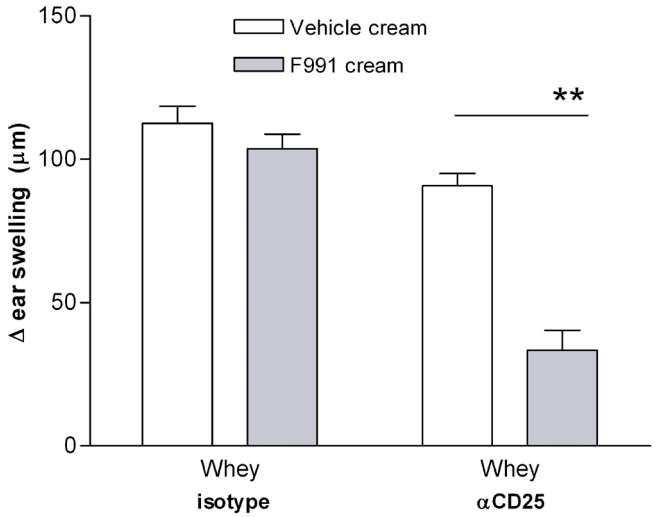

Figure 4 *The acute allergic skin response was measured in the presence or absence of the IgLC inhibitor F991. In mice treated with anti-CD25 mAb (αCD25), F991 inhibited the acute allergic skin reponse to whey when compared to vehicle cream treated mice (**p<0.01, n=6). Whey sensitized mice received 400 µg αCD25 mAb or isotype control mAb.*

absence or presence of the IgLC antagonist F991 (topical application), vehicle cream was applied as a control. F991 is a 9-mer peptide which has been demonstrated to specifically inhibit IgLC-induced hypersensitivity responses. As expected, F991 did not inhibit the ear swelling response induced in whey-sensitized mice treated with the isotype control antibody (112.5 ± 6.0 vs 103.7 ± 5.0 µm; Figure 4), indicating that no IgLC was involved here and this response was mediated by IgE and/or IgG_1. However, the acute ear swelling in anti-CD25 mAb treated whey-sensitized mice was significantly inhibited by F991 (90.8 ± 4.2 vs 33.3 ± 6.9 µm; Figure 4) suggesting that indeed the increased IgLC was responsible for systemic sensitization and the whey-induced allergic skin response in these mice.

IgLC dependent colon hyperreactivity in anti-CD25 treated whey sensitized mice

To investigate whether gastrointestinal symptoms were also affected by CD25 depletion, the *ex vivo* isometric contraction of the colon in response to carbachol was measured. In previous experiments it was shown that no differences in colon reactivity were detected in whey sensitized mice compared to non-sensitized mice (20). CD25 depletion resulted in a significant hyperreactivity of the colon when compared to control allergic mice (Figure 5). To investigate whether also IgLC was involved in the induction of the colon hyperreactivity after CD25 depletion, whey-sensitized mice were treated *in vivo* with the IgLC inhibitor F991. Indeed, the

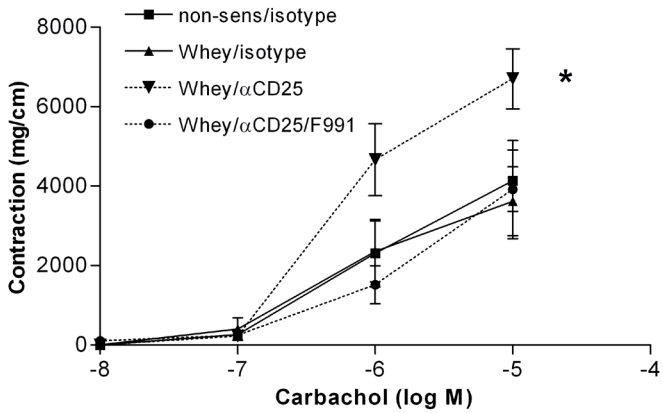

Figure 5. *Hyperreactivity of the colon in sensitized mice was observed after anti-CD25 mAb (αCD25) treatment compared to isotype controls (*p<0.05, n=6). This hyperreactivity was abolished after administration of the IgLC inhibitor F991. Whey sensitized mice received 400 µg αCD25 mAb or isotype control mAb. F991 or PBS was injected i.p. 24h and 1 hour before dissection of the colon.*

carbachol induced hyperreactivity of the colon was abolished after treatment of the anti-CD25 treated mice with F991 demonstrating IgLC dependency of this response (Figure 5).

Discussion

Acute allergic symptoms are generally intimately associated with IgE-induced hypersensitivity; however also IgE-independent mechanisms may be involved (21). Previously, it was shown that in mice orally sensitized to whey, allergic responses correlated with whey-specific IgE and IgG_1 levels in serum (20). The current study describes the involvement of $CD4^+CD25^+$ T-cells in balancing the immune system between IgE and IgE-independent effector responses in allergic disease. Depletion of $CD4^+CD25^+$ T-cells resulted in an abolished specific IgE response. Surprisingly the allergic skin response to whey was not affected and was explained by the presence of IgLC in CD25-depleted mice. This study suggests that $CD25^+$ T cells may determine the nature of allergic responses and could modulate a switch from an IgE- to an IgLC driven response.

In the past years, a novel mechanism for the elicitation of immediate hypersensitivity-like reactions via IgLC has been described. Transfer of antigen-specific IgLC into naive mice sensitizes them to the respective antigen resulting in local mast cell activation upon challenge, leading to edema formation after skin challenge or acute bronchoconstriction after intranasal challenge (13, 16). IgLC-elicited hypersensitivity

responses can be inhibited by local or systemic application of a specific antagonist F991 (13, 15). In the current study, F991 was used to investigate whether the acute allergic skin response (ear swelling upon allergen challenge) in the CD25-depleted mice could be ascribed to IgLC. F991 does not interfere with IgE or IgG-mediated mast cell activation (13). Indeed, the allergic skin reaction was strongly inhibited by F991. An involvement of IgG_1 in the remaining part of the acute skin response cannot be excluded because CD25 depletion did not completely reduce whey-specific IgG_1 levels. In accordance with the indicated immunoglobulin switch, it was observed that the IgLC concentration in serum and production by MLN cells upon *in vivo* anti-CD25 mAb treatment were significantly increased. Recent studies have shown enhanced IgLC serum concentrations in human affected with immune disorders, like rheumatoid arthritis (22, 23) and multiple sclerosis (24, 25). Moreover elevated IgLC concentrations are found in rhinitis and asthmatic patients suggesting a role for IgLC in human allergic disease (16, 17).

It has been shown that anti-CD25 mAb (PC61) selectively depletes natural occurring $CD4^+CD25^+Foxp3^+$ regulatory T-cells in autoimmune disease and more recently in allergic disease (26, 27). Selective depletion of $CD4^+CD25^+Foxp3^+$ regulatory T-cells using the PC61 mAb was found to enhance allergen specific IgE and allergic symptoms in mouse models of peanut allergy, allergic asthma (28) and allergic conjunctivitis (29). In contrast to our study where the CD25 mAb was administered at day 14 only, the CD25 mAb was applied prior to sensitization and on day 14 of the sensitization protocol . In the latter study the mice were also thymectomised to further exclude the prescence of thymus derived regulatory T-cells. However, CD25 is not a unique marker for regulatory T-cells as it is also transiently expressed by allergen specific effector T-cells which drive the allergic response after sensitization. $CD25^+$ regulatory T-cells counteract these effects and *in vivo* both cells are important players in the adaptive immune response. Parallel to the present study, *in vivo* application of anti-CD25 mAb in a mouse model of chronic Toxoplasma gondii infection was found to deplete both regulatory and effector T-cells (30).

Depletion of $CD25^+$ T-cells during sensitization resulted in a combined depletion of $CD4^+Foxp3^+$ regulatory and to a lesser extent $CD4^+$ effector T-cells. Different mechanisms can underlie the induced IgLC dependent acute allergic responses. Regulatory $CD25^+$ T-cells might directly interact with B-cells (31, 32) and loss of these cells may lead to aberrant immunoglobulin responses. On the other hand allergen specific $CD25^+$ effector T-cells are required for B-cell isotype switch and differentiation of the B-cell into IgE/IgG_1 secreting plasma cells. *In vitro* polyclonal stimulation of mouse spleen cells showed that the release of IgLC versus the amount incorporated in complete immunoglobulins depends on the strength of stimulation (33) suggesting that suboptimal function of $CD25^+$ effector T-cells might affect the differentiation of B-cells resulting in the production of IgLC upon sensitization

rather than inducing whey-specific antibodies. Hence, compromised function of regulatory T-cells combined with dysfunctional effector T-cell responses might have been responsible for induction of IgLC rather than IgE/IgG_1.

Apart from investigating the acute allergic skin response, the effects on intestinal motility using *ex vivo* carbachol induced muscle contraction was investigated. These reactions are generally not associated with specific serum IgE (34) and it is known that food allergy may have adverse effects on gut health causing motility disturbances (35, 36). In previous studies it was shown that motility of the colon was not affected in mice sensitized to whey (20). In this study, it is shown that depletion of $CD25^+$ T-cells results in a hyper-responsiveness of the colon. Development of this hyper-responsiveness could be inhibited by *in vivo* treatment with the specific IgLC antagonist F991 showing the involvement of IgLC in this change in motility response. Hence, this suggests that $CD4^+CD25^+$ T-cells may contribute to the mechanism underlying non-IgE mediated motility disorders of the intestine in food allergy. In line with these results, the development of tracheal hyperreactivity in a mouse model for non-atopic asthma was shown to be IgLC dependent (16).

The current study showed that $CD4^+CD25^+$ T-cells may play a role in balancing between IgE dependent and IgE-independent responses in allergic disease. Depletion of $CD25^+$ T-cells changed the nature of the allergic response to cow's milk proteins in mice from a specific-IgE dependent acute allergic response into an IgLC-dependent response. Moreover, depletion of $CD25^+$ T-cells induced an IgLC-dependent colonic hyperresponsiveness. This suggests that depending on the immunologic milieu an IgLC- or IgE-dependent allergic response to the same allergen can be elicited. Further studies are needed to gain insight in the underlying mechanisms and the contribution of regulatory and/or effector T-cells in the balance between IgE and IgE-independent allergic responses and if these two independent mechanisms may contribute to each other's allergic response.

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Contribution of IgE and immunoglobulin free light chain in the allergic reaction to cow's milk proteins

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Journal of Allergy and Clinical Immunology 2010;125:1308-14.

Abstract

Background Cow's milk allergy (CMA) is affecting 2.5% of young infants. In previous murine studies it was observed that allergic sensitization to the major cow's milk allergens, casein and whey, led respectively to immunoglobulin (Ig)E-independent and IgE-dependent clinical responses.

Objectives In this study, the involvement of immunoglobulin free light chains (IgLC) in the hypersensitivity response to cow's milk proteins (CMP) was explored in mice and IgLC serum levels were determined in children affected by CMA or atopic dermatitis (AD).

Methods Mice were orally sham- casein- or whey-sensitized. Acute allergen specific skin responses were determined, serum immunoglobulins and IgLC were measured. IgLC dependency was validated using IgLC blocker F991 in active and passive sensitized mice. IgLC serum concentrations were measured in a cohort of CMA infants and infants with AD.

Results After sensitization, no specific IgE was detectable in serum of casein-sensitized mice, while in whey-sensitized mice specific IgE was enhanced. Instead, IgLC levels were increased in serum from casein-sensitized mice. Furthermore, blocking IgLC strongly diminished the allergic skin responses not only in casein-sensitized mice, but also in mice transferred with splenocytes supernatants of casein-sensitized mice. Both in CMA and AD patients serum IgLC concentrations were significantly enhanced.

Conclusions This study indicates that sensitization with CMP can lead to both IgE-dependent and Ig free light chain-dependent allergic hypersensitivity responses. Also in children affected with CMA or AD serum IgLC concentrations were increased implying the relevance of IgLC measurements in the diagnoses of human allergic disease.

Introduction

Cow's milk allergy (CMA) is a complex disorder, arising early in life and affecting 2-5% of the children of the western world. CMA is also associated with an increased risk to develop asthma later in life (1, 2). The majority of CMA patients are allergic to the major cow's milk proteins (CMP) casein and/or whey (3, 4). Usually reactions to a skin prick test (SPT) are measured and/or titers of cow's milk specific immunoglobulin (Ig)E are analyzed in the serum of patients. However, there are CMA patients which exhibit acute clinical features of CMA without detectable titers of specific IgE (3, 5, 6). Therefore the 'gold standard' for diagnosis is still an orally induced double blind placebo controlled food challenge (DBPCFC) (7-10). The main disadvantages of an DBPCFC is the time consuming character of the test, it's costs and the potential of inducing a severe allergic reaction. Although the incidence of anaphylaxis is low, its potential risk is quite stressful for the patient and parents (7). Therefore the search for another clinical tool to diagnose CMA is ongoing. So far, no (immuno)therapy is available and merely avoidance of cow's milk and the use of hydrolyzed formulae are currently the only effective strategies to prevent symptoms of CMA (10).

Previously two preclinical models for CMA have been introduced in which mice were sensitized orally for whey or casein (11). In these models the acute allergic skin reaction was monitored as a possible equivalent of the SPT. In both models all mice exhibit an enhanced ear swelling upon intra dermal (i.d.) allergen challenge, which reflects systemic sensitization. The whey model resembles a typical type I allergy with high levels of whey-specific IgE and IgG_1. However, despite developing a pronounced acute allergic skin reaction upon local allergen challenge, the response to casein was not associated with detectable levels of casein-specific IgE. Although the casein-sensitized mice did have enhanced specific titers of IgG_1 this was found not to correlate quantitatively with the skin reaction (11). Therefore an alternative explanation for the induction of the acute allergic skin reaction was explored. In previous studies, it was demonstrated that antigen-induced acute allergic responses could be elicited via immunoglobulin free light chains (IgLC) (12-14). For instance, transfer of trinitrophenol-specific IgLC into naïve mice sensitized them to the respective antigen. Local challenge of the passively sensitized animals with the appropriate antigen resulted in the induction of mast cell degranulation, leading to a local inflammation. This IgLC-elicited hypersensitivity response can be inhibited by local or systemic application of a specific antagonist, a 9-mer peptide F991. Acute allergic responses induced by IgG or IgE are not inhibited by F991 (12, 14). Immunoglobulin free light chains (IgLC) are present in serum and their production is augmented under inflammatory conditions including allergic asthma as well as some autoimmune diseases (12, 15, 16). For example, in patients suffering from multiple sclerosis free kappa light chains correlate with disability prognosis (17) and

in rheumatoid arthritis there is a significant correlation between kappa and lambda fLC and the disease activity score (18).

In this study it is demonstrated that circulatory IgLC are increased after oral sensitization with casein. Moreover, acute allergic skin reactions to casein are inhibited by F991, which indicates that IgLC play a pivotal role in the allergic sensitization. Clinical data indicate that IgLC concentrations are increased in children with CMA and in children at risk for allergy with mild atopic dermatitis (AD).

Methods

Chemicals

Casein and whey were obtained from DMV international, Veghel, The Netherlands. Cholera toxin is purchased from Quadratech Diagnostics, Epsom, UK. PBS from Cambrex Bio Science, Verviers, Belgium. Biotin-labeled rat anti-mouse IgE and IgG_1 from BD Biosciences, Alphen aan den Rijn, The Netherlands. The IgLC antagonist F991 (AHWSGHCCL) was synthesized by Fmoc chemistry, Ansynth, Roosendaal, the Netherlands. All other chemicals were obtained from Sigma-Aldrich-Chemie, Zwijndrecht, The Netherlands.

Oral sensitization and challenge of mice

Three- to 5-week-old specific pathogen free female C3H/HeOuJ mice (n = 6 per group) were purchased from Charles River Laboratories (Maastricht, the Netherlands), maintained on cow's milk protein free mouse chow (Special Diets Services, Witham, Essex, UK) and housed in the animal facility at the Utrecht University. Animal care and use were approved by and performed in accordance with the guidelines of the Animal Ethics Committee of the Utrecht University. Mice were sensitized i.g. with 0.5 mL homogenized casein or whey (40 mg/mL PBS) with cholera toxin (CT, 20 µg/mL PBS) as an adjuvant, using a blunt needle. Control mice received CT alone or PBS. Mice were boosted weekly for a period of 5 weeks. One week after last sensitization an i.d. ear challenge was performed. After 24 hours blood samples were collected and centrifuged (15 min at 16000 x g). Sera were stored at – 70°C. Mice were sacrificed by cervical dislocation. Cells of spleen and mesenteric lymph nodes of sensitized mice were collected and cultured in serum free RPMI (1 x 10^6 cells/mL) for 24 hours (14). Supernatants were collected in order to measure Ig and/or IgLC levels.

Measurement of specific serum immunoglobulins

Levels of casein- or whey-specific IgE and IgG_1 were determined in serum by means of ELISA. Microlon plates (Greiner, Alphen aan den Rijn, The Netherlands) were coated with 100 µL whey or casein (20 µg/mL) in coating buffer (carbonate-

bicarbonate buffer, 0.05 M, pH=9.6) for 18 hours at 4oC. Plates were washed and blocked for 1 hour with 5% BSA. Serum samples were applied in several dilutions and incubated for 2 hours at room temperature. Plates were washed and incubated with biotin-labeled rat anti-mouse IgE or IgG_1 (1 µg/mL) for one and a half hour at room temperature and washed. The plates were incubated with streptavidin-horseradish peroxidase (HRP) for one hour, washed and developed with o-phenylendiamine. After 5 min the reaction was stopped with 4M H_2SO_4 and absorbance was measured at 490 nm on a Benchmark microplate reader (Biorad, California, USA). Results were expressed as arbitrary units (AU), with pooled sera from casein and whey alum-i.p. immunized mice used as a positive reference serum to make the titration curve, as an internal standard.

Immunoblotting IgLC

Prior to use, serum samples were precipitated to deplete high amounts of albumin using trichloroacetic acid/acetone as previously described (19). Samples were fractionated by SDS-PAGE under non-reducing conditions, electroblotted to PVDF membrane (Bio-Rad Laboratories, Veenendaal, The Netherlands) overnight and probed with anti-mouse kappa HRP Ab (Southern biotech Birmingham, Alabama USA). Immunoreactive bands were visualized using enhanced chemiluminescence. Optical density (OD) of the immunoreactive bands was quantified with a calibrated densitometer (BioRad, Veenendaal, the Netherlands).

Acute allergic skin reaction

The allergen-specific skin reaction was measured after injection of the specific protein in the ear pinnae. Non-sensitized mice were injected i.d. in the left ear with 20 µL casein (0.5 mg/mL in PBS) and in the right ear whey (0.5 mg/mL in PBS) to determine non-specific skin reactions. The casein- or whey-sensitized mice were injected in both ears with casein or whey respectively, whereas sham-sensitized mice were injected with casein in the left ear and whey in the right ear. Ear thickness of mice was measured in duplicate using a digital micrometer (Mitutoyo, Veenendaal, The Netherlands), at t=0 and 1 hour after challenge. The ear swelling is expressed as µm.

Passive transfer with spleen supernatants

Spleen supernatants were concentrated using vivaspin 20 spin filters (Millipore). Concentrated fractions were 0.2 µm filtered and transferred i.v. (100 µL, approximately 400 µg protein/mL) in naïve recipient mice. Sham mice received PBS as a control. After half an hour skin reaction tests were performed as described above.

Antagonist studies

In the oral sensitized mice as well as in mice passively transferred with spleen supernatants of sensitized mice, F991, an IgLC inhibitor (13, 20), or PBS as a control was administered i.v. (100 µL, 0.2 mg/mL F991) to determine whether the ear swelling was IgLC dependent or not. Half an hour after F991 administration the ear swelling test was performed as described above.

Cow's milk allergic subjects

Infants aged equal or less than 12 months suspected of CMA were recruited from the Baby Health Clinics in the region of Amsterdam, The Netherlands. Baby Health Clinics are involved in the prevention and early detection of diseases in infancy and early childhood, in growth and development, and in nutritional advice. Ninety-eight percent of the newborns are regularly controlled in this manner. Baby Health Clinic physicians are often the first to be confronted with symptoms of CMA. Infants suspected of CMA according to standardized criteria were referred to the Emma Children's Hospital Academic Medical Center, Amsterdam, The Netherlands for evaluation of symptoms and diagnostic work up. The following symptoms related to ingestion of CMP were considered suspect for CMA: skin symptoms (atopic dermatitis, urticaria, angioedema, erythema), gastrointestinal symptoms (colic, vomiting, diarrhoea), respiratory symptoms (rhinitis, cough, wheeze and dyspnoea) and general symptoms (inconsolable crying, refusing food, and failure to thrive). Atopic dermatitis was diagnosed according to criteria from Hanifin and Rajka (21). The extent and intensity of atopic dermatitis was estimated by using the objective SCORing Atopic Dermatitis (SCORAD) index (22, 23).

Diagnostic procedure

CMA was diagnosed according to a strict protocol based on elimination of CMP from the diet, a DBPCFC with CMP, and re-elimination of CMP from the infants diet according to international recommendations (24).

The elimination of CMP from the infant's diet was performed with a minimum duration of one week and a maximum of six weeks. To eliminate CMP from the infant's diet an infant formula based on a mixture of free amino acids (Neocate®, SHS International, Liverpool, UK) was provided to bottle-fed infants. CMP was excluded from the mothers diet in breastfed infants by strict elimination of CMP, soy and hen's egg. If symptoms suspected of CMA did not improve during the elimination phase the infant was excluded from the study. Otherwise, if symptoms disappeared or improved significantly a DBPCFC with CMP was conducted.

The active and placebo arms of the DBPCFC were conducted in a random order on two separate days with an interval of 2-7 days. A randomization database (www.randomization.com) was used to assess the order of active and placebo arms. During

active challenges skimmed milk powder (Institute of Food Research, Norwich, UK) dissolved in Neocate® was given in the following subsequent doses: 0.2 ml (0,003 mg), 2 ml (0,03 mg), 20 ml (0,3 mg) 0.2 ml (3 mg), 2 ml (30 mg), 6 ml (100 mg), 20 ml (300 mg), 60 ml (1000 mg) and 200 ml (3000 mg). Placebo challenges were performed by administrating the same volume of 100% Neocate. The time interval between each dose was 20 min. The challenge was stopped if clinical symptoms were observed or the highest dose was reached. The infants were observed for two hours after each challenge. A challenge was defined as positive if objective clinical reactions were observed. The objective SCORAD index was used to assess the severity of atopic dermatitis before and two hours after each challenge. Clinical reactions after discontinuation or within 2 hours after the highest dose were defined as early reactions, thereafter as late reactions. A standardized sheet was used for documenting administered doses and clinical reactions.
Elimination of CMP from the infant's diet was continued at the days between the active and placebo arms of the DBPCFC until one week (re-elimination) after the last challenge. One week after the last challenge the code was broken and the outcome of the DBPCFC was assessed. CMA was diagnosed by a positive DBPCFC. Subjects with a negative DBPCFC and cow's milk specific IgE levels in the normal range were included as non-allergic controls.

Serum immunoglobulin and plasma IgLC analysis
Peripheral blood samples were collected before challenge at the first day of the DBPCFC. Cow's milk specific IgE was determined by CAP System FEIA (Pharmacia Diagnostics, Uppsala, Sweden). In addition total levels of IgLC kappa and lambda were determined as described previously (12, 25).

Ethical Consideration
The study (MEC 05/254) was approved by the medical ethical committee of the Academic Medical Center, Amsterdam. Parental informed consent was obtained for all subjects.
Infants at high risk for allergy; serum Immunoglobulin kappa- and lambda-LC levels
Term born infants with a parental history of atopic eczema, allergic rhinitis or asthma in either mother or father were eligible for an prebiotic intervention study, which was described before (26, 27). The study protocol was approved by the Ethical Committee of the Macedonio Melloni Maternity Hospital. In a subpopulation plasma immunoglobulin levels were measured as described by Van Hoffen *et al.* (CMP-IgE, IgG_1, total- IgE, IgG_1 and IgG_2) (27). In total 25 infants had mild symptoms of AD (SCORAD 5.2 – 24.7) and 49 infants did not develop AD at the age of 6 months. For this study total levels of IgLC kappa and lambda were determined in plasma as described previously (12, 25).

Statistical analysis

Ear swelling data and murine IgLC data were analyzed using one way ANOVA and post hoc Dunnett's test and all other serological data were analyzed using two-tailed Mann-Whitney test. Statistical analyses were conducted using GraphPad Prism software (version 4.03). * means a p-value of <0.05, ** p<0.01 and *** p<0.001. Murine data are represented as mean ⁺ SEM, whereas clinical data are represented as median.

Results

Serum specific immunoglobulin levels

Serum levels of casein-specific IgE were not increased in mice orally sensitized with casein when compared to control mice (4.7 ± 1.6 vs 6.0 ± 3.5 AU, respectively) (Figure 1A). In contrast, casein-specific IgG_1 levels were enhanced (404.8 ± 217.5 vs 0 ± 0 AU), although this was not significant due to two non-responders (Figure 1B). In whey-sensitized mice both specific IgE (239.4 ± 123.6 AU; p<0.05) and IgG_1 (2671 ± 910 AU; p<0.01) were significantly increased compared to sham-sensitized mice (2.5 ± 1.1 and 14.0 ± 11.2 AU, respectively) (Figure 1C and D).

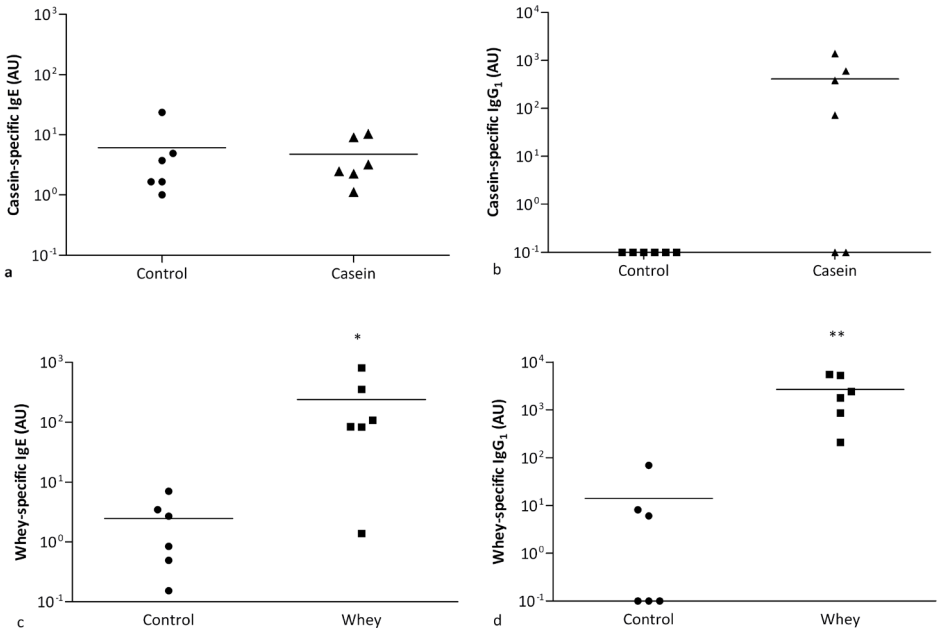

Figure 1. *Specific serum immunoglobulin levels in casein- and whey-sensitized mice. In the casein-sensitized mice no increase in casein-specific IgE was found (A), but IgG_1 (B) was increased in 4 out of 6 animals. Whey-sensitization induced levels of specific IgE (C) and IgG_1 (D). * p<0.05, ** p<0.01 and n=6.*

IgLC levels in sensitized mice

Next, it was examined if sensitized mice had enhanced total levels of IgLC. Sera of non-, casein- and whey-sensitized mice were analyzed by immunoblotting for the presence of kappa IgLC. The concentrations of kappa IgLC in casein-sensitized mice were significantly increased compared to non- and whey-sensitized animals ($p<0.05$; non-sensitized: 66.6 ± 5.5; whey 64.2 ± 8.7 and casein 92.3 ± 9.5 OD\mm2, Figure 2A).

To analyze production of IgLC in local lymph nodes, cells from mesenteric lymph

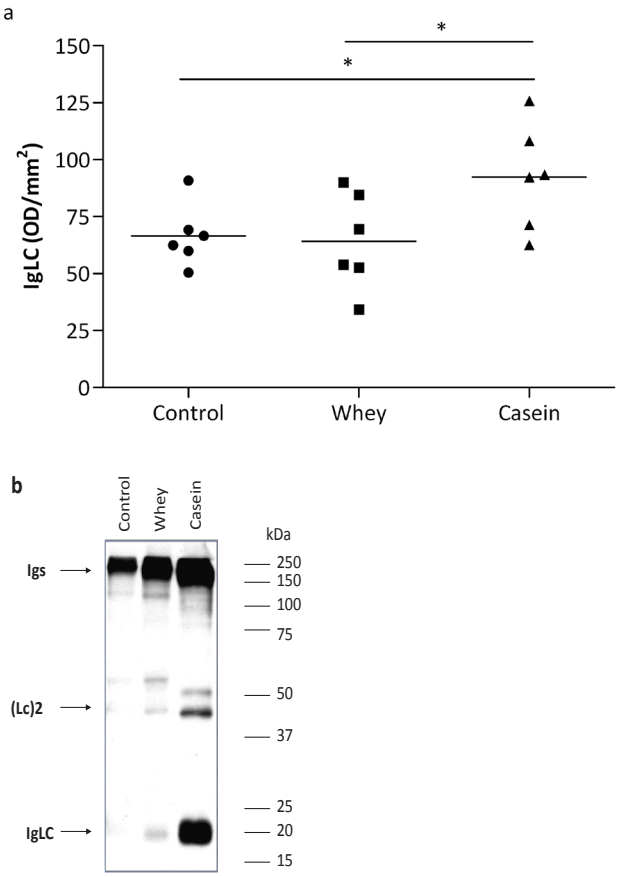

Figure 2. *Densitometric analysis of kappa IgLC in serum and supernatant from local mesenteric lymph nodes (MLN) revealed IgLC levels to be increased. (A) Analysis of kappa IgLC in serum from individual mice. In casein-sensitized mice, levels of IgLC were significantly increased in comparison to control- or whey-sensitized mice. (B) Representative Western-blot of IgLC levels in supernatants of MLN of control-, casein- and whey-sensitized mice. Igs are total immunoglobulins (IgA, IgD, IgE, IgG and IgM), (Lc)2 are IgLC dimers and IgLC are the monomers. * p<0.05 and n=6.*

nodes (MLN, n=6 pooled) were cultured for 24 hours and supernatants were analyzed for kappa IgLC. Samples fractionated by SDS-PAGE under non-reducing conditions showed complete immunoglobulins (about 180-200 kDa), IgLC dimers (about 45 kDa) and IgLC monomers (20 kDa). The MLN of casein-sensitized mice produced higher levels of IgLC compared to the control and whey-sensitized animals (Figure 2B).

IgLC inhibitor F991 blocks the acute allergic skin reaction in casein-sensitized mice
To analyze whether the increase in IgLC in casein-sensitized animals was functional relevant regarding the allergic reaction, the effect of the antagonist F991 was tested on the allergen-induced ear swelling. F991 greatly reduced the skin reaction in the casein-sensitized mice (147.4 ± 16.0 µm without and 83.3 ± 8.3 µm with F991, $p<0.05$, Figure 3A). As expected, the IgLC antagonist did not affect the ear swelling of whey-sensitized mice (Figure 3B, 145.0 ± 9.66 and 148.5 ± 13.9 µm). Also non-specific ear swelling (irritant reaction) in control mice was not affected by the F991. Casein did not provoke an ear swelling in whey-allergic mice and vice versa (data not shown).

IgLC responsible for passive transfer of allergic sensitization to casein
In the previous experiments, it was shown that local lymphoid organs from casein-sensitized mice produced significantly more IgLC compared to sham- and whey-sensitized animals. To investigate the involvement of IgLC as a humoral factor in the induction of the acute allergic skin reaction, naïve recipient mice were injected intravenously (i.v.) with concentrated spleen supernatants (or PBS as a control), obtained from allergic mice, in presence or absence of F991 as previously described (14). Subsequently, the cutaneous swelling was measured at one hour after intradermal (i.d.) allergen challenge. For recipient mice receiving spleen supernatants of casein-sensitized mice the allergen challenge induced a significant ear swelling (110.9 ± 10.2 µm). F991 inhibited the casein-induced ear swelling highly significantly (57.1 ± 5.7 µm, $p<0.001$, Figure 4). The IgLC antagonist did not affect the ear swelling of recipient mice receiving spleen supernatants of whey-sensitized mice (105.9 ± 6.6 and 105.0 ± 5.1 µm), confirming that IgLC did not play a significant role in the induction of the acute allergic skin reaction against whey protein. Casein did not provoke an ear swelling in whey spleen supernatant transferred mice and vice versa (data not shown).

Immunoglobulin levels and IgLC levels in infants with cow's milk allergy
Sixteen infants (age: 2.5 – 8.9 months, median 5.4) with a positive DBPCFC and seventeen infants (age: 3.1 – 7.3 months, median 4.0) with a negative DBPCFC and cow's milk specific IgE levels in the normal range of age were included in this study.

Both IgLC kappa (median (IQR) 5.3 (3.1) vs. 10.1 (5.2); p=0.0042; Figure 5A) as well as IgLC lambda (6.8 (5.7) vs 8.6 (6.0); p=0.0222; Figure 5B) concentrations were significant enhanced in children suffering from CMA. IgLC kappa or IgLC lambda levels did not differ in the subgroups of CMA infants with detectable and not detectable IgE levels (data not shown).

Immunoglobulin levels and IgLC levels in infants at high risk for allergy

In order to extend the clinical relevance of the current findings, immunoglobulin levels and IgLC levels were also measured in plasma from children at risk of developing allergic disease from a double-blind, randomized, placebo controlled prebiotic

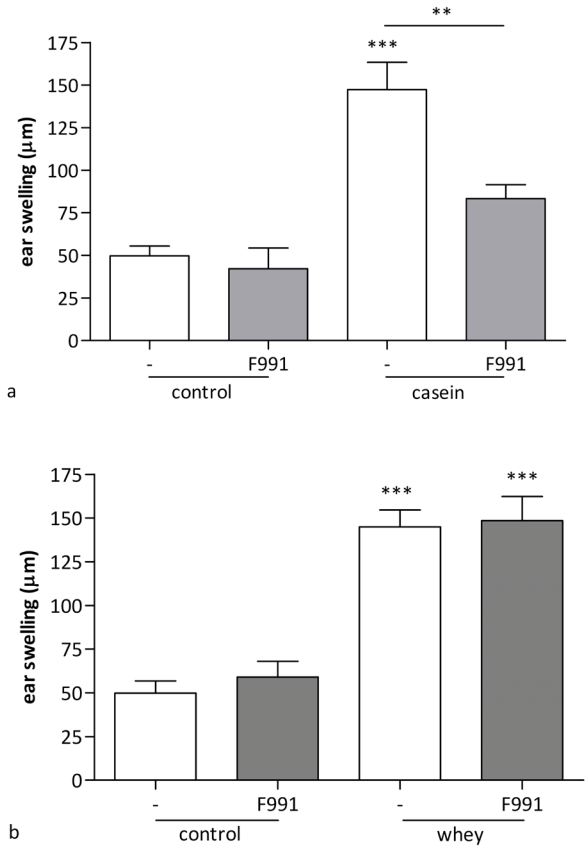

Figure 3. *Effect of F991 on the induction of an acute allergen-specific skin reaction in (A) casein- and (B) whey-sensitized mice. F991 decreased casein-induced ear swelling significantly but whey-induced ear swelling was unaffected. Ear swelling (µm) is calculated as the increase in ear thickness induced by the corresponding antigen at one hour after challenge. ** p<0.01, *** p<0.001 and n=6.*

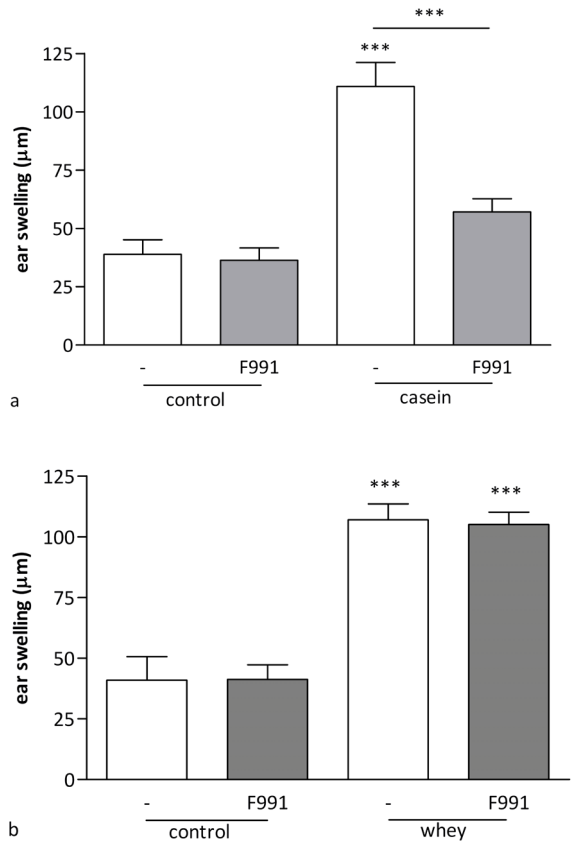

Figure 4. *Effect of F991 on the induction of an acute allergic skin reaction in naïve mice injected (i.v.) with spleen supernatants or PBS as a control. Passive transfer with spleen supernatants of (A) casein and (B) whey allergic mice resulted in acute allergic skin reaction upon i.d. allergen challenge. Only in casein recipients the acute allergic skin reaction was abrogated by F991. *** p<0.001 and n=6.*

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intervention study (26, 27). These infants were six months of age and developed AD (SCORAD between 5.2 and 24.7) or remained negative for AD (SCORAD -). No differences were found between the two groups for total levels of IgE, IgG_1, IgG_2, cow's milk-specific IgE and IgG_1 (Table I).

In support to our findings in the preclinical and the clinical studies, IgLC kappa levels were found be increased in the infants that developed AD (n=25) when compared to infants without AD (n=49) (p=0.0075) IgLC lambda levels showed similar tendency (p=0.14).

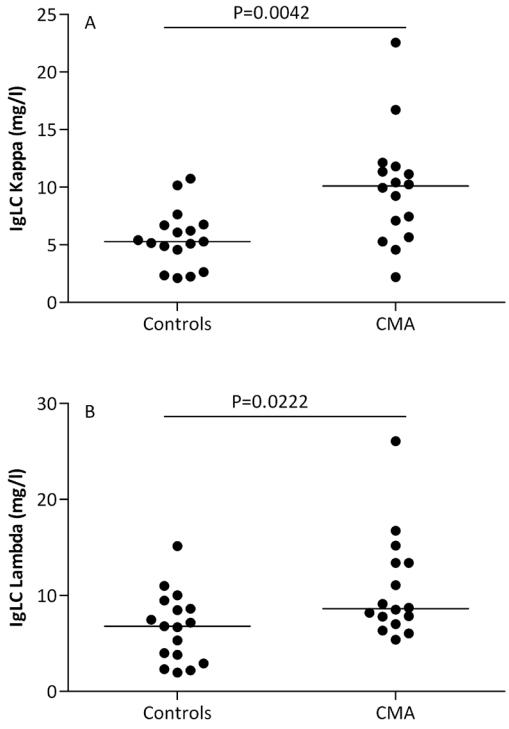

Figure 5. *Plasma IgLC levels in non-allergic controls (n=17) and infants with CMA (n=16). Both (A) kappa IgLC levels (p=0.0042) and (B) lambda IgLC levels (p=0.0222) were enhanced in CMA infants.*

Table I. *Immunoglobulin levels in infants at high risk for allergy, divided in healthy (SCORAD -) and AD (SCORAD $^{+}$) groups.*

		Atopic dermatitis		
		SCORAD -	**SCORAD $^{+}$**	
	Number of infants	n=49	n=25	
	Median (IQR)	0.0 (0)	6.9 (6.5)	
Serum antibody	**Isotype**	**Median (IQR)**	**Median (IQR)**	**p-value**
Total	IgE (kU/L)	4.50 (9)	6.00 (22)	p=0.30
	IgG_1 (g/L)	2.69 (1.9)	2.95 (2.5)	p=0.15
	IgG_2 (g/L)	0.78 (0.4)	0.91 (0.6)	p=0.41
	Ig-fLC κ (µg/mL)	6.1 (4.0)	7.4 (3.0)	p=0.0075
	Ig-fLC λ (µg/mL)	6.1 (4.1)	7.7 (5.6)	p=0.14
CMP-specific	IgE (ng/mL)	1.95 (3.1)	2.90 (3.2)	p=0.10
	IgG_1 (AU/mL)	1.50 (6.5)	3.25 (7.7)	p=0.27

Discussion

In previous studies, it was shown that the allergic skin response in mice sensitized with casein or whey, both major allergens responsible for CMA, is substantially different (10, 11, 28). Whey-sensitized mice show a classical type I allergic skin reaction combined with high levels of specific IgE. However, in casein-sensitized mice acute allergic responses were elicited in the absence of casein-specific IgE. Here, new data provide evidence that casein-sensitization of mice results in the induction of an immunoglobulin free light chain (IgLC) dependent acute hypersensitivity response. In addition, plasma IgLC concentrations were found to be enhanced in CMA patients as well as patients with AD implying a possible contribution of IgLC in the pathophysiology of clinical allergic disease.

Food allergy is thought to be mediated by IgE and non-IgE dependent mechanisms. IgE mediated food allergy is a classical type I allergy with symptoms occurring within a few hours. Non-IgE mediated food allergy usually is defined as a delayed-type hypersensitivity with enhanced T-helper 1 activity and the symptoms are noticeable after one or more days while cellular mechanisms are involved (29-33). However, of interest is a subgroup of patients which have symptoms of an immediate type hypersensitivity response, while lacking detectable levels of IgE (3, 5, 6). In contrast to the murine model, in humans caseins can elicit strong IgE responses (3). The mice were sensitized for either casein or whey, while in infants hypersensitivity is raised against whey and casein derived from whole cow's milk. Direct comparison between mice and human with regard to the sensitizing capacities for casein and whey are therefore difficult to perform. However, the concept of IgLC being involved in the pathophysiology of human disease has been described for inflammatory as well as allergic asthma (12, 14, 17, 18, 34, 35).

Several lines of evidence suggest the critical involvement of IgLC in the induction of casein hypersensitivity in mice. First, casein-sensitization results in increased levels of IgLC in serum, while local lymph nodes of casein-sensitized mice show enhanced production of IgLC *ex vivo*. Secondly, inhibition of IgLC induced hypersensitivity using F991 prevented the acute allergic skin response in casein sensitized mice. A minor part of the ear swelling in casein-sensitized mice was not affected by F991, which could suggest a residual role for IgG_1 or for IgE. In several studies it has been shown that F991 is specific for IgLC and does not affect IgE nor IgG mediated skin responses. Furthermore, F991 prevents local inflammation in different hypersensitivity models (12, 14, 20, 36). Thirdly, allergic sensitivity to casein could be transferred to naïve animals by i.v. injection of culture supernatant from splenocytes of casein-sensitized mice and was found to be dependent on IgLC. Together these data indicate a prominent role for IgLC in the induction of casein allergy in mice. In another study (data not shown) mice were sensitized with both casein and whey and mice were

subsequently challenged intradermally with either casein or whey. Both casein and whey challenge provoked an acute allergic skin response, whereas casein-specific IgE levels remained below detection.
Other studies using murine models for CMA indicates various, sometimes conflicting, results. Oral sensitization with α-casein resulted in an increase in α-casein-specific IgE and IgG_1 titers in C3H mice (37). On the other hand, Lara-Villoslada *et al.* (38) only detected whey and casein specific IgG_1, while IgE was not detectable in a CMA model induced by simultaneous oral sensitization with both casein and whey. The differences in route of sensitization, intraperitoneal vs oral, mouse strains and variation in allergen fractions e.g. α-casein vs complete casein fraction or combined whey and casein, might explain the contrasting outcome in these and the present studies. The fundamental different mechanisms of allergy observed for casein and whey may be due to the differences in digestive speed, dietary, chemical, physical and physiological features of the proteins (39-42). Therefore, these differences between the proteins influence not only the intestinal physiology, but likely the response of the immune system as well, which is a topic for further studies.

In previous studies, it has been shown that IgLC can mediate immediate hypersensitivity-like responses. Mast cells are the primary target cells for IgLC and subsequent cross-linking of cell surface-bound IgLC by cognate antigen results in mast cell activation and the induction of a local inflammatory response (14). Polyclonal IgLC levels are enhanced in chronic inflammatory diseases like inflammatory bowel disease (43), rheumatoid arthritis (16), Sjögren's syndrome (18), multiple sclerosis (17, 44) and systemic lupus erythematosus (34). Also in allergic and non-allergic asthma, IgLC levels are increased when compared to healthy controls (12). It is estimated that in one third of the children suffering from AD the skin symptoms are triggered by sensitization to food (45, 46). Notably, children with severe AD have higher levels of lambda and kappa light chain in serum (47). Furthermore, in non-IgE mediated rhinitis patients both lambda and kappa IgLC levels are found to be enhanced in nasal secretions (35). The present study confirms that increased IgLC may be associated with development of CMA. In sera from DBPCFC confirmed CMA patients both kappa as well as lambda IgLC concentrations were significantly enhanced compared to controls. IgLC concentrations did not differ between IgE and non-IgE mediated CMA infants, indicating that IgLC might contribute to the allergic symptoms in the non-IgE patients. A larger cohort of patients with non-IgE mediated CMA is required to elucidate this. In order to extend the evidence of a possible role of IgLC in allergic disease developed during early infancy, plasma from infants at high risk for allergy who developed mild AD were investigated as well. Neither enhanced CMP-specific serum IgE and IgG_1 nor enhanced total IgE, IgG_1 and IgG_2 concentrations were observed in these children. However, serum levels of kappa

IgLC were elevated significantly and lambda IgLC showed a similar increment. These clinical data suggest that measurement of total- and ultimately, specific-levels of IgLC may have relevance in the clinical diagnosis for CMA and AD.

In summary, sensitization with CMP can lead to both IgE-dependent and IgE-independent allergic hypersensitivity responses. In this study, evidence is provided indicating that the IgE-independent allergic response to casein is mediated at least in part by specific IgLC. An increase in IgLC levels may be of relevance in CMA and AD with or without noticeable involvement of IgE. Further research is warranted to determine the clinical significance of assaying IgLC for the diagnosis of allergic diseases.

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Immunoglobulin free light chains play a possible role in induction of tolerance to food proteins

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Abstract

Introduction Preventing cow's milk allergy by rendering the immune system hyporesponsive to antigen after oral feeding (oral tolerance) is preferred above avoidance strategies. Besides IgE specific for food allergens, immunoglobulin free light chain (IgLC) can elicit an acute allergic response and it has been shown recently that these mechanisms may be interchangeable. Modulation of IgLC production could therefore be a mechanism relevant in the induction of tolerance to food allergens.

Objective It was investigated whether modulation of IgLC may contribute to oral tolerance induction to whey proteins in a mouse model for cow's milk allergy.

Methods: Mice were sensitized orally with whey once a week for five consecutive weeks using cholera toxin as an adjuvant. Prior to sensitization mice were treated orally with 50 mg whey (day-7 till day -2) in order to induce tolerance. To block IgLC, at day -1 mice were injected intraperitoneally (i.p.) with F991, a specific inhibitor of IgLC. In addition, a group of mice was injected intravenously (i.v.) with IgLC in the absence of oral pre-treatment with whey, one day prior to whey sensitization. The acute allergic skin response was measured 1 hour after intradermal whey challenge. Whey-specific antibodies and IgLC levels were analyzed in serum.

Results The acute allergic skin response and serum levels of whey-specific IgE were increased in whey-sensitized mice compared to non-sensitized mice which was prevented by whey feeding prior to sensitization. Interestingly, IgLC serum levels were enhanced in these tolerized mice. F991 reduced the protective effects of whey pre-treatment, indicating that IgLC might be involved in oral tolerance induction to whey. Moreover, i.v. administration of IgLC prior to whey sensitization reduced the acute allergic skin response compared to regular sensitized mice.

Conclusion In this study it was demonstrated that IgLC may contribute to the mechanism of oral tolerance induction for food derived proteins.

Introduction

Cow's milk allergy is one of the leading causes of food allergy in children. Clinical symptoms of cow's milk allergy affect the skin, respiratory and gastrointestinal tract and are mostly caused by immunoglobulin (Ig)E-induced hypersensitivity. Binding of IgE to the high affinity receptor FcεR1 on mast cells or basophils followed by subsequent cross-linking of the receptors by the allergen provokes degranulation and the release of mediators such as histamine, leukotriens and cytokines causing the clinical symptoms. Mast cells have been implicated in the pathogenesis of IgE-mediated and non-allergic hypersensitivity responses in gastrointestinal disorders (1-4). Cross-linking of the high-affinity IgE receptor is an important mechanism for antigen-specific activation of mast cells, but also other mechanisms such allergen cross-linking of IgLC can elicit acute allergic symptoms in the absence of IgE antibodies (4-7). Recently, it was described that IgLC play a crucial role in casein-induced food allergy in mice and increased serum IgLC was found in cow's milk allergic children as well (8).

Oral tolerance to food proteins is a key feature of the immune system to actively induce T-cell and B-cell unresponsiveness to food allergens and most likely involves the induction of regulatory T-cells (9, 10). In addition, humoral factors, like IgA, IgG_1, and IgG_4 can affect IgE responses and they most likely play a supportive role in specific immunotherapy (11-14). In addition, other suppressive factors have been described to inhibit allergic immune responses (15-17). In a previous study, it was demonstrated that depletion of CD25-positive lymphocytes switched an IgE-driven cow's milk allergy into an IgLC-dependent response (18). In this study it was investigated whether modulation of IgLC would contribute to tolerization to whey proteins in a mouse model for cow's milk allergy.

Methods

Reagents and antibodies

Whey was obtained from DMV International, Veghel, the Netherlands. Cholera toxin was purchased from Quadratech Diagnositcs, Epsom, UK. Biotin labeled rat anti-mouse IgE was obtained from BD Biosciences, Alphen a/d Rijn, the Netherlands. All other chemicals were obtained from Sigma-Aldrich, Zwijndrecht, the Netherlands. F991 was synthesized by Fmoc Chemistry (Ansynth, Roosendaal, the Netherlands). Oxazolon-specifc IgLC was manufactured as described previously (7).

Animals

Three- to 4-week-old pathogen-free female C3H/HeOuJ mice were purchased from Charles River Laboratories (Maastricht, the Netherlands), maintained on cow's milk protein free standard mouse chow (AIN-93G, Research Diet Services, Wijk bij Duurstede, the Netherlands) and housed in the animal facility at the Utrecht

University. Animal care and use were performed in accordance with the guidelines of the Dutch Committee of Animal Experiments.

Oral tolerance induction, oral sensitization and challenge of mice

Mice were sensitized with whey as described previously. In short, mice were sensitized orally on day 0, 7, 14, 21 and 28 with 20 mg of whey per mouse homogenized in PBS (0.5 ml, Cambrex Bio Science, Verviers, Belgium) mixed with 10 µg cholera toxin. Non-sensitized mice received cholera toxin in PBS only. Five days after the last sensitization on day 33 the acute allergic skin response (ear swelling at 1 hour) after intradermal whey challenge was measured. Mice were sacrificed and blood samples were collected, centrifuged for 15 minutes at 20,000 g and stored at -70°C.

To induce oral tolerance, prior to whey sensitization mice were pre-treated orally (daily; day -7 until day -2) with 50 mg whey or PBS (sham), using a blunt needle (gavage). To inhibit IgLC, at day -1 mice were injected with F991 (50 µg in 100 µl, i.p), a specific inhibitor of IgLC (6). Another group was injected with IgLC (5 µg i.v. in 100 µl per mouse) one day prior to whey sensitization at day -1 without prior whey pre-treatment.

Measurement of whey-specific IgE immunogobulins

Whey-specific IgE levels were measured in serum using an ELISA as described earlier (19). Microlon plates (Greiner, Alphen aan de Rijn, the Netherlands) were coated with 20 µg of whey in coating buffer (Sigma) for 18 hours at 4°C. Plates were washed and blocked for 1 hour with buffer containing 50 mM Tris, 2 mM EDTA en 137 mM NaCl /0.05% Tween and 0.5% BSA. Serum samples were incubated for 2 hours at room temperature. Plates were washed and incubated with 1 µg biotin labelled rat anti-mouse IgE (Pharmingen, Alphen a/d Rijn, the Netherlands) for one hour at room temperature. After washing the plates were incubated with streptavidin-horse radish peroxidase (Sanquin, Amsterdam, the Netherlands) for one hour, washed and developed with o-phenylendiamine (Sigma). The reaction was stopped with 4M H_2SO_4 and absorbance was measured at 490 nm on a Benchmark microplate reader (Biorad, Veenendaal, the Netherlands) and results were expressed as arbitrary units (AU).

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Measurement of IgLC in serum

Serum samples were precipitated as described (18). In short, 20 µl of serum was depleted from albumin by resuspension in 80 µl ice-cold 10% TCA/acetone followed by incubation for 90 minutes at -20 °C. Samples were centrifuged for 20 minutes at 15,000g at 4 °C. Supernatant was removed and pellet was washed with 1 ml ice-cold acetone and dissolved in Laemmli buffer. A small volume was run on a 12% SDS-PAGE gel followed by electro transfer to PVDF (Bio-Rad). Membranes were blocked

o/n in PBS-T with 2% non-fat dry milk. IgLC were detected with anti-mouse kappa light chain mAb conjugated to horse radish peroxidase (Southern Biotech, Alabama, USA). Immunoreactive bands were visualized with ECL and X-Ray Film (Pierce, Etten-Leur, the Netherlands). Films were scanned and analyzed on a GS710 Calibrated Imaging Densitometer equipped with Quantity One v. 4.0.3 software (Bio-Rad, Veenendaal, the Netherlands).

Acute allergic skin response

An acute allergen-specific ear swelling in whey sensitized mice was determined 1 hour after intradermal challenge with 10 µg total whey protein in the ear pinnae as described previously (19). As a negative control non-sensitized mice were challenged in the ear with similar whey protein. Ear thickness was measured in duplicate using a digital micrometer (Mitutoyo, Veenendaal, the Netherlands). The allergen-specific net ear swelling was calculated by correcting the allergen-induced increase in ear thickness with the non-specific ear swelling due to local injection in the non-sensitized mice. The ear swelling is expressed as delta µm.

Statistics

All data were analyzed using one way ANOVA and posthoc Dunnett's test. A probability value of $p<0.05$ was considered significant. Statistical analyses were conducted using GraphPad Prism software. Data are represented as mean or individual values ± SEM of 6 mice per group.

Results

Whey pre-treatment abolished the production of whey-specific IgE

In sensitized mice, whey-specific IgE levels were increased in serum when compared to non-sensitized mice (figure 1; 324.0 ± 81.4 AU vs 84.7 ± 24.8 AU; $p<0.05$). As expected, oral tolerization with whey prior to sensitization abolished the induction of whey-specific IgE significantly compared to the whey sensitized control (figure 1; 66.2 ± 27.8 AU; $p<0.05$).

Increased IgLC serum levels in whey pre-treated mice

It was examined whether oral tolerance induction for whey proteins was associated with alterations in serum IgLC levels. Immunoblot analysis showed that IgLC protein expression was strongly increased in serum of tolerized mice, while no increase in IgLC levels was found in the whey-sensitized animals as compared to the non-sensitized controls (figure 2).

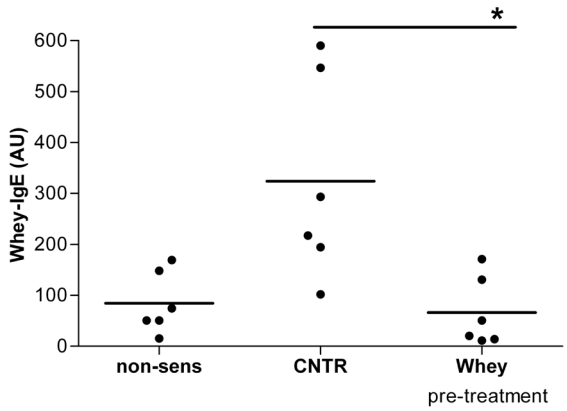

Figure 1. *Whey-specific IgE levels were measured in serum of non-sensitized and whey sensitized mice pre-treated with PBS (CNTR) or whey (whey pre-treatment) for 6 consecutive days (day -7 to -2) prior to whey sensitization. Data are expressed as individual values in AU of 6 animals/group; *p<0.05. Data are representative for 2 independent experiments.*

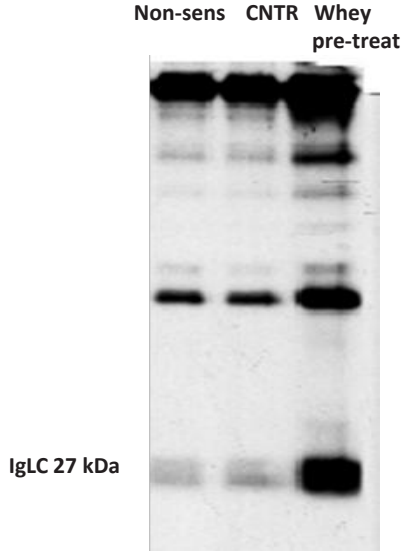

Figure 2. *At day 33, IgLC levels were measured in serum of non-sensitized mice and whey sensitized mice treated with PBS (CNTR) or whey (Whey) prior to whey sensitization. Representative blots are shown from 2 independent experiments. Gels were run under non-reducing conditions. IgLC = monomer Immunoglobulin free light chain.*

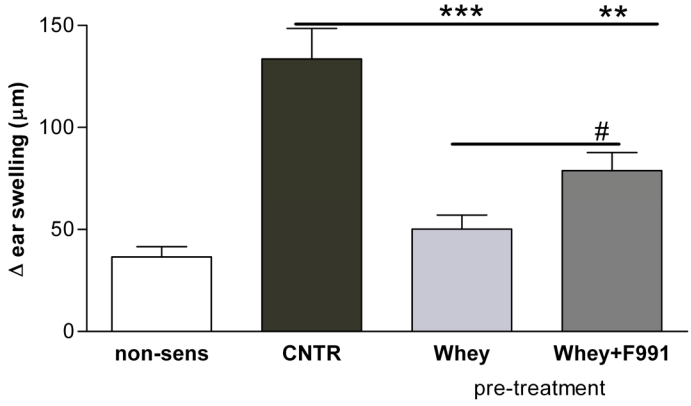

Figure 3. *The acute allergic skin response was measured 1 hour after intradermal whey challenge in the ears of non-sensitized and whey sensitized mice. Whey sensitized mice were pre-treated with PBS (CNTR) or whey (Whey) for 6 consecutive days (day -7 to -2) before start of the sensitization with whey. One day before start of the sensitization with whey (day -1) one group of mice were injected with PBS or F991 (Whey+F991), as specific inhibitor of IgLC. Data are expressed as mean values of 6 animals/group ± SEM; #p<0.05, **p<0.01, ***p<0.001.*

Whey pre-treatment reduced the acute allergic skin response

In previous studies IgLC were found to contribute to the acute allergic skin response (8, 18). Therefore the acute allergic skin response was determined in the whey tolerized mice with low serum whey-specific IgE and enhanced IgLC levels. A significant ear swelling at 1 hour in whey sensitized animals was observed compared to non-sensitized mice (figure 3; 133.5 ± 15.1 vs 36.5 ± 5.1 μm; $p<0.05$). In tolerized mice the acute allergic skin response was significantly reduced (figure 3; 50.2 ± 7.0; $p<0.001$).

To further investigate if IgLC produced during induction of oral tolerance was of functional importance in down regulation of the allergic response, circulating IgLC were inhibited with F991 at 1 day before start of sensitization. F991 partly blocked induction of oral tolerance indicating that increased IgLC before start of the sensitization may be important in allergen-specific tolerance induction (figure 3; 78.8 ± 8.9; $p<0.05$).

Role of IgLC in desensitization to whey-induced allergy

F991 partly blocked the induction of oral tolerance indicating that increased IgLC before start of the sensitization might play a role in whey induced tolerance (figure 3).

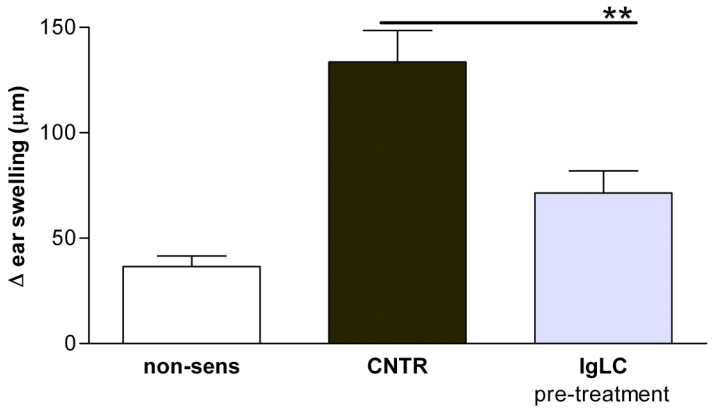

Figure 4. *At day 33, the acute allergic skin response was measured 1 hour after intradermal whey challenge in the ears of non-sensitized and whey sensitized mice. Whey sensitized mice were injected i.v. with PBS (CNTR) or oxazolon-specific IgLC (IgLC) one day before start of the sensitization with whey (day -1). Data are expressed as mean values of 6 animals/group ± SEM; **p<0.01*

To investigate whether the effect of oral tolerization could be mimicked by IgLC injection only, mice were pre-treated with non-specific IgLC (7) one day prior to whey sensitization. In IgLC pre-treated mice, the acute allergic skin response was greatly reduced compared to the control pre-treated group (figure 4; 71.4 µm ± 10.6 µm vs 133.5 µm ± 15.1 µm; $p<0.01$).

Discussion

Oral tolerance to harmless food proteins is a pivotal default mechanism of the immune system. Diverse populations of regulatory T-cells but also humoral factors contribute to oral tolerance induction (9, 12-14, 20-23). In this study it is shown that IgLC are enhanced after oral tolerization in a food allergy mouse model. Moreover, we show that presence of IgLC prior to sensitization inhibits the allergic sensitization to the cow's milk protein whey. This study suggests that IgLC may contribute to the development of oral tolerance to food allergens.

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Oral tolerance to harmless food proteins is an essential task of the immune system to actively induce T-cell and B-cell unresponsiveness to food allergens and most likely involves the induction of regulatory T-cells (9). Regulatory and effector T-cells play a crucial role in balancing the immune system and a compromised function of $CD4^+CD25^+$ T-cells may provide an explanation for the failure to establish oral tolerance. Recently it was shown that depletion of CD25-positive lymphocytes caused a shift from an IgE-mediated towards an IgLC-mediated food allergic response (18).

This suggests that $CD25^+$ T-cells may balance the immunoglobulin production by B lymphocytes and determine whether sensitization results in an IgLC-dependent allergic response.

In the current study, it is shown that the time of IgLC production may be of crucial importance for the outcome of the allergic response since the described data suggest that if IgLC are present prior to the sensitization, allergic sensitization is reduced. It was shown that oral tolerization with whey coincided with increased IgLC levels in mice sensitized to whey which was partly reversed after antagonizing the circulating IgLC with F991 before sensitization. The effect of F991 was not complete, which could be caused by the short half life of the peptide or may indicate that other mechanisms play an additional role. Moreover, non-specific oxazolon-specific IgLC administered prior to whey sensitization reduced the acute allergic immune response also. These data support the findings that increased IgLC levels in tolerized mice may contribute to desensitization of the acute allergic effector response.

In animal models employed to study cutaneous and lung delayed type hypersensitivity (DTH) responses it was already shown over 20 years ago that a suppressive factor derived from spleen cells of mice sensitized via the skin to picryl chloride exerted protective effects. Injection of the spleen derived factor prior to skin sensitization inhibited the early, mast cell dependent phase of a DTH response in the skin (24) and lungs (25). These factors could inhibit the onset of other hypersensitivity reactions in a non-specific manner, called isotype suppression (16, 24, 26). Interestingly, in later studies it was shown that IgLC was one of the constituents of this factor (7). Hence, these studies suggest that IgLC might exert immune modulating effects in allergic immune responses.

The induction of tolerance by IgLC in relation to food allergens is a novel finding not described in literature. The mechanism by which IgLC exerted the observed protective effects is yet to be explored. Induction or re-establishment of oral tolerance is currently one of the most promising strategies for the treatment of food allergic symptoms (22, 27-31). Further studies are needed to investigate the nature of the modulating effect of IgLC and whether IgLC-therapy might show its benefit in inducing oral tolerance to food proteins.

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Summarizing Discussion

In newborns the mucosal barrier is still immature for optimal absorption of nutrients provided by breastfeeding. The immature status of the immune system combined with a "leaky" immature gut is considered a risk factor to develop immune reactivity to food antigens when introduced too early in life (1-3). On the other hand, antigens provided via the oral route are important for the maturation and training of the mucosal immune system (4, 5). Hence, a delicate balance occurs between avoidance of proteins to prevent early sensitization and the need for proteins to develop oral tolerance. Recent results indicate that avoidance of dietary components fails to prevent food allergy (6-8). Moreover, the prevalence of peanut allergy is significantly lower in countries were peanut is consumed at an early age (9, 10) indicating that prevention of food allergy initiated after oral exposure to food antigens might be beneficial above avoidance strategies (11).

Hypoallergenic cow's milk (HA) formulas play an important role in the prevention of cow's milk allergy in high risk children as well as in treatment strategies. As outlined in chapter 1, this thesis describes the development of a murine model for cow's milk allergy to analyze the residual allergenicity and sensitizing capacities of cow's milk protein hydrolysates. Safety testing of new HA infant formulas in animal models is indispensable to confirm the absence of allergenic peptides in the HA infant formulas. Prevention of cow's milk allergy with hydrolysed proteins is mainly based on avoidance of the allergen but can also be a result of oral tolerance induction (see also review chapter 2). Therefore, the potency of these hydrolysates to induce oral tolerance and the mechanisms behind these protective effects were topic of investigation described in this thesis.

Mouse model for cow's milk allergy and safety testing of whey hydrolysates

In **chapter 3** a mouse model for whey or casein induced allergy using the oral route of sensitization is described. The model has been developed based on existing models for whole cow's milk and peanut proteins (12-14). Cholera toxin was used as a mucosal adjuvant to circumvent oral tolerance induction normally occurring upon oral administration of food proteins to mice (15). Although it is unknown which mucosal factors contribute to the development of food allergy in human, bacterial derived components may be involved in the Th2 polarization of the immune response. Cholera toxin has been shown to have strong Th2 polarizing capacities and is used to break tolerance in mice (16). Although cholera toxin is not the most likely candidate to be involved in the induction of food allergy in human, Staphylococcus aureus derived enterotoxins are a common cause of food contamination indicating that adjuvants are not necessary artificial in relation to the human situation. In addition, these enterotoxins have comparable adjuvant capacities in mouse models for food allergy when compared to cholera toxin (17). Both casein and whey proteins

were able to sensitize the mice as determined after an oral and/or intradermal allergen challenge. The challenge evoked an antigen specific acute allergic skin response, mucosal mast cell degranulation and increased water levels in the feces, an indicator of diarrhea. These parameters are a reflection of the effector phase (secondary encounter) of the allergic response. Interestingly, the immunological response upon sensitization was different for casein and whey. In contrast to whey, no allergen-specific IgE levels were detectable in casein sensitized mice indicating that additional mechanisms contributed to the acute allergic response observed in casein sensitized mice. Casein and whey proteins differ with regard to physical properties. Casein proteins are gathered in three dimensional aggregates (micelles) in contrast to whey proteins which form linear structures of mostly monomers and dimers (18). As a consequence, these differences in physicochemical characteristics may have influenced the immunological response for either protein. The nature of the acute allergic symptoms in absence of IgE was further unraveled in the murine model for casein allergy which is discussed later in this chapter.

Animal models are essential for assessing the safety of HA infant formulas before their efficacy can be demonstrated in humans. For safety reasons, the hypoallergenicity of hydrolysed infant formulas needs to be determined by showing that the hypoallergenic formulas are not able to sensitize animals to the protein source they are derived from (19). In Europe most HA infant formulas are based on hydrolysed whey proteins. Therefore the whey based mouse model for orally induced cow's milk allergy was positioned for the assessment of residual allergenicity and sensitizing capacity of partial whey hydrolysates (WH) (**chapter 4, 5 and 6**). Guinea pigs sensitized by the oral route are used as the common model for the identification of the residual sensitizing capacity of new hypoallergenic formulas due to their innate responsiveness (20). A main disadvantage of the guinea pig model, in contrast to the mouse model described in this thesis, is the generation of an IgG_{1a} subclass anaphylactic antibody response instead of an IgE antibody mediated responses, which is the main physiological antibody response in allergic humans. This makes the suitability of the guinea pig model questionable with regard to the potential extrapolation to the human situation, although this model was frequently used due to the absence of other suitable and validated oral sensitization models. In this thesis in **chapter 4 and 5** it is shown that partial WH lost the putative capacity to sensitize animals thereby preventing the induction of clinical related symptoms upon challenge with the native whey protein. This indicates the feasibility of this model to asses the residual sensitizing capacity of partial WH before used in humans. In addition to the residual sensitizing capacity, the residual capacity of the partial WH to cross-link human IgE antibodies on RBL- cells transfected with the human FcεRI receptor was investigated. The degranulation assay, which may serve

as a model for the effector phase of the allergic response (as a reflection for clinical effects in allergic children), is considered a highly relevant *in vitro* model for the human situation because of predictive value of recognition of residual proteins by human immune system. The combination of both assays covering the two phases of the allergic response (figure 1, chapter 1) provide a good strategy for the screening of new hypoallergenic formulas aimed at preventing sensitization in atopic children and avoiding clinical symptoms in infants suffering from cow's milk allergy.

The importance of an adequate and reliable *in vivo* model for scientific as well as safety purpose is evident, therefore a ring trial was carried out. In this study the mouse model for cow's milk allergy (whey) was simultaneously performed in four research facilities as described in **chapter 6**. Although minor differences in results were observed between the research facilities it was concluded that the *in vivo* model was successfully transferred to four different independent research facilities. Sensitization to whey (enhanced levels of whey-specific IgE/IgG_1 and IgG_{2a}) was observed in all research facilities and was associated with enhanced mucosal mast cell derived mMCP-1 concentrations after oral whey challenge. In addition, an acute allergic skin response and anaphylactic shock reactions after intradermal whey challenge were observed in three out of four research facilities. The multi-phase ring trial was aimed to validate the mouse model. To position this model as a new *in vivo* test for efficacy and safety testing of new HA infant formulas, recently phase two of this ring trial was finalized in which an extensive whey hydrolysate was included and body temperature measured as an additional parameter. Currently, data are collected and being analyzed.

Oral Tolerance induction by whey hydrolysates

In genetically predisposed children who are unable to be completely breast fed, there is support for a modest long-term preventive effect of HA infant formulas on allergic manifestations in children at high risk to develop food allergies (21-23). Ten years of clinical studies show that both partial and extensive hydrolysed formulas are effective in preventing cow's milk allergy in these high risk children (23-27). Clinical studies addressing the effectiveness of HA infant formulas in preventing cow's milk allergy were mainly designed as avoidance strategies to prevent sensitization and are limited in answering whether the beneficial effects are due to avoidance of the allergic epitopes or a result of oral tolerance induction. It is evident that strategies aiming at inducing oral tolerance without the risk of inducing sensitization are of huge interest for genetically predisposed children. Although animal studies have been used intensively to study oral tolerance to intact dietary proteins, few studies with antigen fragments or digest have been performed (28-31). In **chapter 7** it was shown using *in vivo* and *in vitro* assays that incubation of whey with the enzymes

used to manufacture partial and extensive HA infant formulas, time dependently reduced the capacity to cross-link cell-bound IgE without affecting human T-cell responses. By selecting the epitopes with T-cell reactivity the tolerance inducing capacity of these peptides can be investigated and ultimately being used as specific immunotherapy in establishing oral tolerance to whey proteins. Recently it has been shown that peptide immunotherapy reduced the allergic response in mice sensitized with hen's egg (32). From this point of view, also cow's milk hydrolysate derived peptides might be considered for specific immunotherapy in cow's milk allergic infants.

This thesis provides information that partial and not extensive WH retained tolerizing capacities in mice when provided orally prior to the induction of cow's milk allergy in these mice. This might be interesting for both prevention as well as treatment of whey allergies. The tolerizing capacity of partial and extensive WH was already described in animal models using systemic rather than oral sensitization (31, 33-35). Children are most likely sensitized via the oral route. Therefore animal models using the oral route of sensitization are considered a beneficial tool to gain more insight in the tolerizing mechanisms of partial WH. In **chapter 8** it was shown that partial and not extensive WH possessed the putative capacity to reduce the acute allergic effector response. These protective effects induced after gavage feeding of the partial WH to naïve mice prior to oral whey sensitization is most likely a result of active immune suppression. In the mesenteric lymph nodes (MLN) partial WH enhanced $Foxp3^+$ regulatory T-cell while whey prefeeding enhanced the relative numbers of Th1 cells, both tended to decrease the relative numbers of activated Th2-cells (see figure 1 this chapter). In addition, the tolerogenic effect could be adoptively transferred when MLN cell suspensions were injected in naïve recipient mice prior to whey sensitization. These findings are in line with human data confirming that regulatory T-cells are associated with tolerance induction to cow's milk proteins (36, 37). Moreover, children born with dysfunctional Foxp3, a key gene for development of $CD25^+$ regulatory T-cells, develop severe dermatitis with high levels of IgE and occasionally eosinophilia which suggests a suppressive role for $Foxp3^+$ regulatory T-cells in allergic immune reactions in humans (38, 39). The observation that the tolerizing effects of digested whey proteins are associated with $Foxp3^+$ regulatory T-cells has not been described in literature yet and might be of interest for strategies aiming at improving oral tolerance. Although partial WH reduced the acute effector response in mice it did not prevent initial sensitization to whey as indicated by unaffected whey-specific IgE serum levels. One of the possible explanations of a reduced effector response in the presence of whey-specific IgE/IgG_1 may be that IgE receptor signaling of mast cells is diminished by active suppression involving regulatory T-cells which can be mediated via cell-cell contact

or mediator release (40, 41). Future experiments are needed to determine whether prolonged feeding of mice will suppress IgE levels.

Infant milk formulas supplemented with non-digestible oligosaccharides containing neutral short-chain galacto oligosaccharides (scGOS) and long-chain fructo oligosaccharides (lcFOS) in a ratio of 9:1 and scGOS/lcFOS combined with pectin derived acidic oligosaccharides (AOS) in a ratio of 9:1:1 (GFA-mixture) have been designed to structural and functional mimic some of the health and immune promoting properties of human milk oligosaccharides. Previous studies have indicated that the GFA-mixture generates functional suppression involving the contribution of regulatory T-cells (42, 43). Since tolerogenic dendritic cells (DC) form the bridge between the innate and adaptive immune system and induce these regulatory T-cells also intestinal DC were topic of investigation. Several subclasses of DC with regulatory properties have been identified in the intestine. The conventional myeloid $CD11c^{+}CD11b^{+}$ DC and the $CD11c^{+}B220^{+}$ plasmacytoid DC have unique regulatory functions in the intestine and may contribute to oral tolerance induction (44, 45). A substantial proportion of the DC present in the intestinal lamina propria and MLN express the integrin subunit CD103 (46, 47) and are likely to mark migratory DC arriving from the lamina propria. Both mice and human $CD103^{+}$ DC promote the differentiation of $Foxp3^{+}$ regulatory T-cells by mechanisms involving TGF_{β}, the dietary component retinoic acid (47-50) and their role in tolerance induction has been suggested recently (51). It was hypothesized, based on animal data and human studies (42, 43, 52-61), that non-digestible oligosaccharides may fulfill a supportive role in oral tolerance induction to harmless food antigens like cow's milk proteins and anticipated that the GFA-mixture would support the development of tolerogenic DC and regulatory T-cell populations.

In **chapter 9** it was demonstrated that a diet containing the GFA-mixture enhanced the efficacy of tolerance induction by a partial WH. This resulted in an abolished acute allergic skin response to whey, prevention of mucosal mast cell degranulation and a tendency towards reduced IgE serum levels. Interestingly, our study showed that the protective effect of the partial WH coincided with increased percentages of $Foxp3^{+}$ regulatory T-cells and $CD103^{+}$ DC. Typically the relative number of $CD103^{+}$ DC further increased in mice fed the GFA-mixture in the presence of a partial WH. From these results it was concluded that the GFA-mixture changed the environmental conditions in the gut leading to increased percentages of $CD103^{+}$ DC in the MLN thereby contributing to partial WH induced oral tolerance to whey proteins, most likely via the in situ production of $Foxp3^{+}$ regulatory T-cells in the MLN. It has recently been demonstrated that the intestine is a preferential site for de novo production of $Foxp3^{+}$ regulatory T-cells after antigen presentation by mucosal DC to naïve T-cells (50) (see figure 1 in this chapter). DC trafficking from the lamina propria to the

MLN have been shown to be essential for the induction of oral tolerance (62-64) indicating that migratory $CD103^+$ DC might play an important role in oral tolerance induction to food proteins. Migratory $CD103^+$ DC are most likely representing a mature population of DC. However, also immature or semi-mature DC in the absence of maturation signals might contribute to tolerance induction as well (65). Most likely, the function of intestinal DC depends on the environment they reside and is controlled by interplay with other local cells such as epithelial cells and their secretion products (66-68).

Immunoglobulin free light chains (IgLC)

In **chapter 10** it was demonstrated that besides IgE, IgLC can elicit an acute hypersensitivity response (see figure 1 in this chapter), and IgLC are responsible for the observed casein induced allergic response in mice as described in **chapter 3**. In addition, we showed that these two mechanisms resulting in acute allergic symptoms may be interchangeable as described in **chapter 11**. Human studies confirmed the presence of increased IgLC levels in serum of infants with proven cow's milk allergy and atopic dermatitis, suggesting a significant role for IgLC in allergic disease. In addition to the acute allergic response as described above, the intestinal motility using *ex vivo* carbachol induced muscle contraction was investigated in mice sensitized to either whey or casein (**chapter 3**). Intestinal symptoms occurring in a subgroup of food allergic patients are generally not associated with enhanced levels of specific serum IgE (71) and these adverse effects involve motility disturbances (72, 73). We observed decreased colon motility in casein sensitized mice that had high levels of IgLC and almost no specific IgE and showed that the motility of the colon was not affected in mice sensitized to whey that had high specific IgE and low levels of IgLC. Interestingly, in **chapter 9** it was demonstrated that IgLC is linked to hyper-responsiveness of the colon in whey sensitized mice after depletion of $CD25^+$ lymphocytes. This suggests that a compromised function of $CD4^+CD25^+$ T-cells may contribute to the mechanism underlying non-IgE mediated motility disorders of the intestine. In this regard it would be interesting to speculate that intestinal symptoms are particular found in patients with enhanced IgLC levels, however no studies to confirm this hypothesis are available in the current literature concerning food allergic patients. Interestingly, in patients with eosinophilic gastroenteritis, which is a mixed IgE and non-IgE mediated disease commonly linked to food allergy (74), preliminary data show IgLC serum levels to be elevated in patients suffering from eosinophilic gastroenteritis with abdominal pain as one of the characteristics (unpublished observations). This indicates that enhanced IgLC levels may underlie the intestinal symptoms in these patients. Overall these data suggest that IgLC might be a promising new biomarker in the early diagnoses of allergic diseases like food allergy. More studies are needed to define the relation between IgLC and allergic

disease.

Besides the role for IgLC in inducing an allergic effector response the possible protective role of IgLC in tolerance induction was investigated since it has been shown that humoral factors, like IgA, IgG_1, and IgG_4 produced by plasma-cells can down regulate IgE mediated allergic responses (75-78). It was already shown over 20 years ago that a factor derived from spleen cells of mice sensitized via the skin exerted protective effects inhibiting the early, mast cell dependent phase of a DTH response in the skin (79) and lungs (80). In later studies it was shown that IgLC was one of the constituents of this factor (81). Based on the above described findings it was hypothesized that IgLC might affect IgE mediated allergic responses to food proteins. Interestingly, as described in **chapter 12**, IgLC levels were enhanced in mice tolerized to whey protein. In addition, we showed that injection of non-specific IgLC prior to sensitization with whey mimicked the observed effects after oral tolerance induction with whey feeding in naïve mice. Our results indicate that, although IgLC can induce acute hypersensitivity response, if IgLC is present prior to sensitization

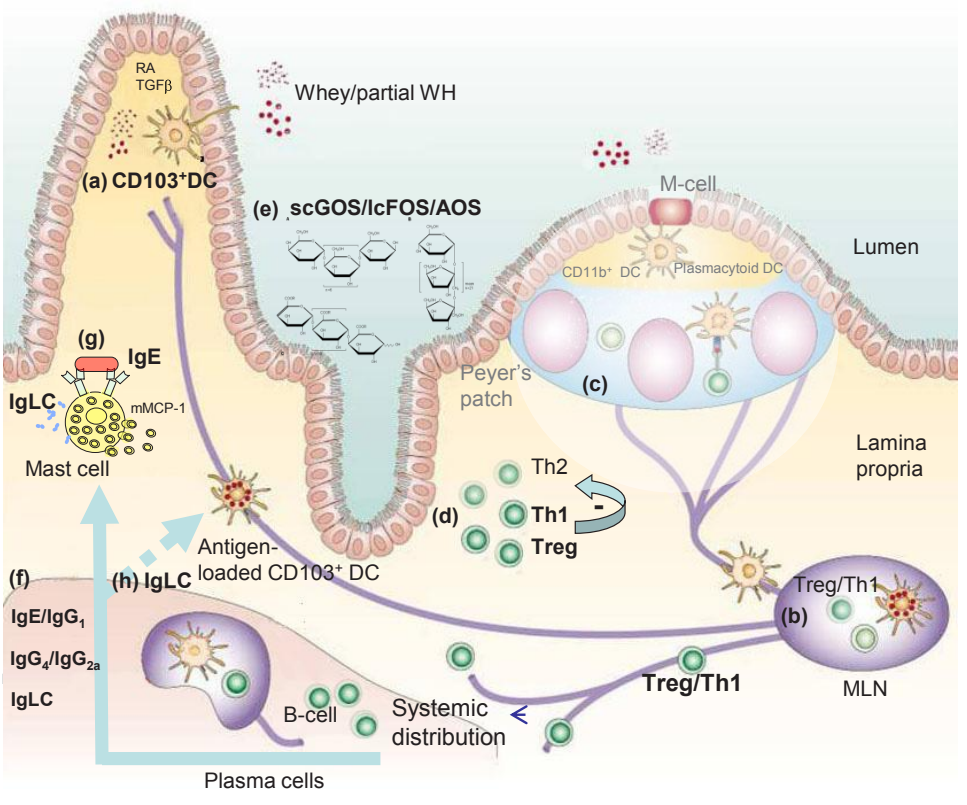

Figure 1. *Concluding figure (adapted from Mowat et al, Nat Rev Immunol; 2003).*
Prevention of cow's milk allergy by restoring oral tolerance is of interest for infants at risk for developing allergic disease. The hypothesis of this thesis concerns the mechanisms underlying oral tolerance induction by whey proteins and especially partially hydrolysed whey proteins (partial WH). Food proteins might enter the lamina propria and are sampled by e.a. tolerogenic CD103$^+$ dendritic cells (DC) in the lamina propria or the lumen ***(a)*** *and antigen-loaded CD103$^+$ DC traffic to the mesenteric lymph nodes (MLN)* ***(b)****. Alternatively, antigens are taken up through the microfold (M) cells in the Peyer's patches and transferred to local DC* ***(c)****. In this thesis the MLN cell phenotypes were studied in particular. Antigen is presented in the MLN to naïve T cells that will subsequently proliferate and differentiate or render them anergic (69). Oral tolerance generated by partial WH induced regulatory T-cells (Treg) or Th1 cells (this thesis,* ***chapter 8****) and suppressed the relative numbers of Th2 cells in the MLN, hence skewing away from the allergic phenotype. Treg and effector T-cells are known to home to the lamina propria where they can participate in the effector immune response generated towards the specific antigen* ***(d)*** *(70). Partial WH induced tolerance was associated with enhanced percentages of Foxp3$^+$ Treg and CD103$^+$ DC in the MLN (this thesis* ***chapter 9****). Tolerogenic DC like CD103$^+$ DC are known for their capacity to induce Foxp3$^+$ Treg, suppressors of effector cell activation, after interaction with naïve T-cells. A specific mixture of non-digestible oligosaccharides which structurally and functionally mimics properties of non digestible carbohydrates abundantly present in breast milk, improved partial WH induced tolerance* ***(e)*** *(this thesis* ***chapter 9****). Epithelium derived mediators like retinoic acid (RA) and TGF$_\beta$ might indirectly contribute to these effects since these are known to induce CD103$^+$ DC (67, 68). In case of allergic sensitization and/or upon tolerization, plasma-cells produce allergen-specific IgE and IgG$_1$ (human + mouse), IgG$_4$ (human), IgG$_{2a}$ (mouse) or IgLC* ***(f)****. Opsonization of mast cells with IgE, IgG$_1$ and/or IgLC results in degranulation of mast cells and the induction of clinical symptoms in cow's milk allergic mice upon a secondary encounter to the same allergen* ***(g)****. IgLC was found to evoke acute allergic symptoms in casein sensitized mice and enhanced IgLC serum levels were measured in infants with proven cow's milk allergy (this thesis* ***chapter 11****). Functional depletion of Treg and in part effector T-cells using an in vivo anti-CD25 treatment resulted in a switch from IgE- to IgLC mediated acute responses in whey allergic mice (this thesis* ***chapter 10****). Hence a compromised adaptive immune response towards a specific allergen may underlie IgLC mediated allergic disease. Intervention with IgLC prior to sensitization can result in tolerization most likely generated at the level of the DC* ***(h)****. This novel discovered phenomenon may be involved in oral tolerance induction since IgLC serum levels were enhanced in mice orally tolerized with whey protein (this thesis* ***chapter 12****).*

it may contribute to the development of oral tolerance to food allergens. The role of IgLC in the induction of tolerance in relation to food allergens is a novel finding and not described in literature. The mechanism by which IgLC exert the observed protective effects is yet to be explored. Although regulatory T-cells most likely play a role in oral tolerance to harmless food proteins, our data indicate that increased levels of IgLC prior to initial sensitization also contribute to the mechanisms underlying oral tolerance to whey proteins.

To conclude (see also figure 1)

In this thesis we positioned the whey based mouse model for cow's milk allergy, as a new *in vivo* model, for safety testing of HA infant formulas. We started to validate the mouse model in a ring trial with four independent research facilities. Using this model, the studies described in this thesis have shown that a partial WH with limited sensitizing capacities was capable to induce oral tolerance to whey proteins in mice and that this protective effect can be enhanced using non-digestible oligosaccharides. Most likely $CD11c^{+}CD103^{+}$ and $Foxp3^{+}$ regulatory T-cells contribute to these protective effects. The partial WH induced protection could be adoptively transferred using MLN cells of tolerized mice when provided to naïve recipient mice prior to whey sensitization. This indicates the importance of MLN in the transfer of local to systemic tolerance. It was observed that sensitization with cow's milk proteins can result in IgLC-dependent acute allergic and intestinal hypersensitivity responses indicating that IgLC is a promising new biomarker for food allergy. Besides its role in the induction of cow's milk allergy it was demonstrated that IgLC might contribute in the oral tolerance induction to whey proteins as well.

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Nederlandse samenvatting

Tijdens de afgelopen decennia blijkt er een toename te zijn in het ontstaan van allergische aandoeningen, inclusief voedselallergie. Ongeveer 2 tot 3% van de kinderen ontwikkelt een allergie tegen koemelkeiwitten, met caseïne en wei als de twee belangrijkste eiwitten. De allergische reactie kan plaatsvinden in de huid, de darmen, de luchtwegen en in het ernstigste geval zelfs leiden tot een anaphylactische shock reactie. In jonge kinderen is het immuunsysteem en ook de darmwand nog niet volledig ontwikkeld, waardoor er een verhoogde kans is op het ontwikkelen van een allergie tegen voedselcomponenten. Ongeveer 80% van de kinderen die lijden aan koemelkallergie groeit uiteindelijk over de allergie heen. Onderzoeksresultaten laten echter zien dat er een mogelijk verband is tussen koemelkallergie en allergieën later in het leven, zodat deze kinderen vaak een overgevoeligheid ontwikkelen tegen andere allergenen zoals huisstof of pollen. Dit is een belangrijke reden waarom behandelingen gericht op het voorkomen van koemelkallergie van groot belang zijn.

Kinderen van allergische ouders hebben een vergrote kans op het ontwikkelen van een allergie, variërend van 50% als één ouder allergisch is tot 70% als beide ouders allergisch zijn. Borstvoeding wordt aangeraden als beste bescherming tegen het ontwikkelen van allergische aandoeningen. Als er gekozen wordt voor aanvullende voeding zijn er formula voedingen op de markt die veelal koemelk bevatten. Voor erfelijk belaste kinderen zijn speciale babyvoedingen, die geen allergische reactie kunnen uitlokken, een goed alternatief voor borstvoeding. Deze hypoallergene (HA) babyvoedingen bestaan in Europa meestal uit gehydrolyseerde (gedeeltelijk verteerde) wei-eiwitten, waaraan onder andere de oligosacchariden, die lijken op de oligosacchariden uit moedermelk, worden toegevoegd. De gehydrolyseerde wei-eiwitten worden geproduceerd door de wei-eiwitten te verhitten en te behandelen met enzymen gevolgd door een filtratie, waarbij de eiwitten met een bepaalde grootte verwijderd worden. Door de enzymen worden de wei-eiwitten geknipt wat resulteert in een babyvoeding, die nog wel de juiste voedingswaarden bevat maar niet meer kan leiden tot een allergische reactie. Deze gehydrolyseerde wei-eiwitten kunnen verdeeld worden in twee groepen, de partiële en de extensieve hydrolysaten. Het belangrijkste verschil tussen deze twee is dat de partiële hydrolysaten grotere fragmenten wei-eiwit bevatten dan de extensieve hydrolysaten.

In internationaal onderzoek is aangetoond dat HA babyvoeding effectief is in het voorkomen van koemelkallergie. Deze voeding wordt daarom veelvuldig gebruikt in vele Europese landen ter voorkoming van allergie bij kinderen. Deze studies

geven echter geen duidelijkheid of de allergie wordt voorkomen doordat het immuunsysteem niet in aanraking komt met koemelk allergeen (eliminatie), of dat de bescherming optreedt doordat het immuunsysteem getraind wordt. Bij eliminatie wordt ervan uitgegaan dat de geknipte eiwitten geen overgevoeligheidsreactie meer kunnen opwekken, het immuunsysteem reageert dan niet. Bij het trainen van het immuunsysteem hebben deze geknipte eiwitten nog wel de capaciteit om een beschermende immuunreactie op te wekken, de zogenaamde orale tolerantie.

We vroegen ons af of het mogelijk is om tolerantie te induceren met door enzymen in stukjes geknipte wei-eiwitten, zonder dat deze stukjes wei-eiwit zelf een allergische reactie uitlokken. Borstvoeding bevat heel veel verschillende componenten die het immuunsysteem kunnen beïnvloeden. Eén van deze componenten omvat niet-verteerbare koolhydraten (een soort suiker), de zogenaamde oligosacchariden. Daarom is onderzocht of een speciaal dieet van oligosacchariden (2% scGOS/lcFOS/pAOS) de orale tolerantie capaciteit van partiële hydrolysaten kan versterken. Verder hebben we onderzocht of specifieke cellen uit het immuunsysteem, de zogenaamde tolerogene dendritische cellen en regulatoire T-cellen, hierbij een rol spelen.

Hoe ontstaat een allergie?
Het immuunsysteem (afweer) dat het lichaam beschermt tegen schadelijke indringers zoals virussen en bacteriën, zorgt er ook voor dat er in het lichaam geen afweerreactie optreedt tegen onschadelijke eiwitten, die voorkomen in voeding (tolerantie). Het immuunsysteem van de darm levert hierbij een belangrijk aandeel. Bij een voedselallergie is deze immunologische tolerantie niet voldoende ontwikkeld en treedt er een overgevoeligheid van het immuunsysteem op gericht tegen een specifiek eiwit in voeding. De eiwitten waartegen deze overgevoeligheidsreactie optreedt, noemt men allergenen. Het immuunsysteem is opgebouwd uit een netwerk van cellen (zoals dendritische cellen, T-cellen, B-cellen en mestcellen) die met elkaar communiceren, elk heeft een specifieke taak. Dendritische cellen in de darm zijn antigeen-presenterende cellen. Ze nemen eiwitten/allergenen op en presenteren een klein deel hiervan op een speciale manier aan T-cellen, dit gebeurt onder andere in de lymfeknopen van de darm. Deze T-cellen zijn uniek, omdat elke T-cel een ander eiwitdeel (peptide) herkent. De T-cellen die het allergeen herkennen delen zich en produceren zogenaamde cytokinen (zie figuur 1 in hoofdstuk 1). B-cellen gaan daardoor immunoglobuline (Ig)E maken, een molecuul dat bestaat uit twee lichte en twee zware ketens, die met elkaar zijn verbonden. Het IgE is specifiek gericht tegen het allergeen en bindt aan IgE receptoren op mestcellen. Deze fase van de allergische reactie noemt men sensibilisatie (gevoelig maken voor). Bij allergische personen is vaak de concentratie IgE in het bloed verhoogd en in de kliniek is dit samen met de huidpriktest de belangrijkste uitleesparameter voor allergie. Als een

eenmaal gesensibiliseerd persoon opnieuw het allergeen binnenkrijgt, leidt dit tot een snelle overgevoeligheids- ofwel allergische reactie. Het allergeen komt dan namelijk in contact met het specifieke IgE dat aan de mestcel gebonden zit en zal de IgE moleculen verbinden, wat leidt tot activatie en degranulatie. De in de mestcel opgeslagen stoffen, zoals o.a. histamine, komen hierbij vrij en leiden tot de acute klinische symptomen van voedselallergie.
Orale tolerantie is de natuurlijke reactie van het immuunsysteem op onschuldige componenten uit voeding. Ook hierbij spelen dendritische cellen (antigeen presenterende cel), T- en B-cellen een sleutelrol. Vooral de regulatoire T-cellen (cellen die een allergische reactie kunnen onderdrukken) spelen een belangrijke rol bij orale tolerantie.

Diermodellen helpen bij het onderzoek naar de betrokken mechanismen die leiden tot koemelkallergie, waardoor betere therapieën voor voedselallergie ontwikkeld kunnen worden. In dit proefschrift beschrijven we een muismodel voor koemelkallergie. Hierbij zijn de muizen via de orale route gesensibiliseerd (gevoelig gemaakt) voor caseïne- of wei-eiwitten. De allergische oortest is geïntroduceerd om de allergie te kunnen meten. Hiervoor wordt caseïne en/of wei in het oor van gesensibiliseerde muizen gespoten. Een allergische reactie (mestcel-degranulatie) leidt tot een oorzwelling in deze muizen en deze verdikking wordt gemeten (hoofdstuk 3 en 4). Zowel in caseïne- als wei-gesensibiliseerde muizen werd een oorzwelling gemeten wat aangeeft dat de muizen allergisch zijn geworden. Er waren echter ook aanzienlijke verschillen tussen deze twee koemelkeiwitten. In een orgaanbadopstelling werd het samentrekken van de darmspieren gemeten na stimulatie met carbachol. De caseïne allergie ging gepaard met een verminderde samentrekking van de darm. Dit zou kunnen betekenen dat caseïne eiwitten bijdragen aan darmproblemen bij koemelkallergische kinderen en volwassenen. Natuurlijk is er nog veel onderzoek nodig om deze hypothese te onderbouwen. Een ander belangrijk verschil tussen caseïne en wei betreft de productie van immunoglobulines. In tegenstelling tot wei, werd geen specifiek IgE geproduceerd in caseïne-gesensibiliseerde muizen. We weten uit eerder dieronderzoek dat er naast IgE ook immunoglobuline vrije lichte ketens (IgLC) kunnen binden aan de mestcel, wat kan leiden tot allergeen specifieke zwelling van het oor. In hoofdstuk 11 hebben we aangetoond dat de allergische reactie in caseïne-gesensibiliseerde muizen veroorzaakt wordt door IgLC. Om de vertaling te kunnen maken naar de mens hebben we ook het bloed van een groep koemelkallergische kinderen onderzocht. In deze kinderen werd een verhoogde hoeveelheid IgLC gemeten, wat kan betekenen dat naast IgE ook IgLC een nieuwe uitleesparameter zou kunnen zijn om te bepalen of er sprake is van een allergische aandoening. Er zijn namelijk een groot aantal kinderen met overgevoeligheidsklachten na het drinken van koemelk die geen of

weinig IgE in hun bloed hebben. Meer onderzoek is nodig om de verschillen in de immunologische respons tegen caseïne en wei te begrijpen.

Het is van groot belang dat de partiële en extensieve wei hydrolysaten, die worden toegevoegd aan HA babyvoedingen, op eventueel nog aanwezige allergenen worden getest. In dit promotieonderzoek is het wei-model voor koemelkallergie geïntroduceerd als een nieuw muismodel om de veiligheid van HA babyvoeding te testen (hoofdstuk 4 en 5). Naast het meten van de oorzwelling en IgE zijn in deze studies ook klinische symptomen, zoals lichaamstemperatuur en shockverschijnselen gemeten. Om het muismodel te kunnen gaan toepassen, om de veiligheid van deze producten te testen, is het van belang dat het muismodel gevalideerd wordt. De eerste fase van dit traject is beschreven in dit proefschrift. Daarvoor hebben we in vier onafhankelijke laboratoria de muizen allergisch gemaakt voor het koemelkeiwit wei en de resultaten met elkaar vergeleken (hoofdstuk 6). Over het algemeen bleek er een vergelijkbare allergische reactie op te treden als het muismodel werd toegepast in de verschillende laboratoria, er zaten echter wat nuanceverschillen in de gemeten parameters. Vervolgonderzoek zal uitwijzen of deze bevindingen reproduceerbaar zijn. In de volgende fase wordt de sensibilisatie-capaciteit van de wei-hydrolysaten gemeten op elke onafhankelijke lokatie.

Naast het diermodel is ook een speciale mestcel met de humane IgE receptor gebruikt om te onderzoeken of er nog eiwitten aanwezig zijn in de HA babyvoeding, die de mestcel kunnen “cross-linken”. Door het gebruik van de humane IgE receptor in deze cellijn benadert deze test de menselijke situatie. Resultaten zoals beschreven in hoofdstuk 4 en 7 laten zien dat partiële en extensieve hydrolysaten en wei-eiwitten die 15 minuten gehydrolyseerd zijn, niet meer in staat zijn om de mestcel gebonden IgE moleculen te verbinden. Hierdoor kunnen deze wei hydrolysaten geen reactie meer uitlokken in allergische kinderen. Dit 15 minuten hydrolysaat kan echter nog wel T-cellen activeren (hoofdstuk 7) die mogelijk bij kunnen dragen aan de orale tolerantie (bescherming) tegen wei-eiwitten.

In hoofdstuk 8 en 9 hebben we daarom onderzocht of partiële en extensieve hydrolysaten, die niet meer kunnen sensibiliseren, nog wel de capaciteit hebben om orale tolerantie te induceren. Er is gevonden dat alleen partiële hydrolysaten deze capaciteit bezitten. Dit werd gemeten als een verminderde oorzwelling en mMCP-1 (product uit mestcellen) in het bloed. Dit ging gepaard met een toename van het aantal regulatoire T-cellen en een afname van het aantal Th2-cellen (belangrijk bij ontstaan allergie) in de lymfeknopen. Om aan te tonen dat regulatoire cellen verantwoordelijk zijn voor het beschermende effect van het partiële hydrolysaat zijn de cellen van de lymfeknopen ingespoten in onbehandelde dieren. Hieruit is

gebleken dat de cellen uit de lymfeknopen van de tolerante dieren kunnen zorgen voor een bescherming tegen overgevoeligheid voor wei.

Uit de resultaten, zoals beschreven in hoofdstuk 9, bleek dat de oligosacchariden de tolerogene capaciteit van een partiële hydrolysaat kunnen versterken. Dit was geassocieerd met een verhoogd aantal $CD103^+$ dendritische cellen in de lymfeknopen. Uit deze resultaten hebben we geconcludeerd dat een speciaal type dendritische cel ($CD103^+$) mogelijk bijdraagt aan een betere tolerantie tegen wei-eiwitten als de muizen voor de sensibilisatie het partiële hydrolysaat in combinatie met een oligosacchariden-dieet toegediend krijgen. Vermoedelijk kunnen de $CD103^+$ dendritische cellen de functie van regulatoire T-cellen in de darm ondersteunen. Zeer waarschijnlijk wordt de tolerogene functie van dendritische cellen in de darm versterkt door de omgeving waarin ze verblijven waarbij het samenspel met locale cellen zoals epitheelcellen van de darm erg belangrijk is. Verder onderzoek zal gericht zijn op de communicatie tussen deze cellen.

Zoals eerder genoemd hebben we in hoofdstuk 11 laten zien dat IgLC een acute allergische reactie kan veroorzaken in caseïne-gesensibiliseerde muizen. In hoofdstuk 10 laten we zien dat ook wei-sensibilisatie kan leiden tot een IgLC-afhankelijke acute oorzwelling na inspuiten van wei in het oor. Dit treedt op als bepaalde T-cellen uit het immuunsysteem, via antilichamen, onwerkzaam worden gemaakt (depletie). Na de depletie van deze '$CD25^+$ T-cellen' is namelijk gebleken dat niet IgE maar IgLC verhoogd aanwezig was in het bloed. De oorzwelling was IgLC-afhankelijk, omdat deze tegengegaan kon worden door een IgLC-remmer (F991). Dit is een extra aanwijzing dat IgLC verantwoordelijk kan zijn voor de koemelkallergische reacties tegen de twee belangrijkste koemelkeiwitten, caseïne en wei.
Naast de duidelijke rol voor IgLC in de allergische respons voor koemelkeiwitten is in hoofdstuk 12 onderzocht of IgLC ook een rol zou kunnen spelen in de tolerogene reactie (bescherming) op koemelkeiwitten. Deze gedachte is gedeeltelijk gebaseerd op het feit dat immuunglobulines ook allergische reacties kunnen onderdrukken. Het bleek, dat in muizen die tolerant waren gemaakt voor wei, een verhoogde hoeveelheid IgLC werd gemeten. Verder konden we het beschermende effect van wei nabootsen door voor sensibilisatie de muizen in te spuiten met IgLC. Hierdoor werd een verminderde allergische oorzwelling gemeten na wei-sensibilisatie. Het mechanisme waardoor het effect van IgLC werd veroorzaakt moet nog verder worden onderzocht. Deze resultaten kunnen echter betekenen dat er een ondersteunende rol is weggelegd voor IgLC tijdens de normale tolerogene reactie van het lichaam op voedseleiwitten.

Samenvattend hebben we een strategie gepresenteerd, waarin een nieuw muismodel is gepositioneerd voor het testen van de veiligheid en tolerogene capaciteit van wei-hydrolysaten die toepassing vinden in HA babyvoedingen. Er is een start gemaakt om het muismodel te valideren in een ringstudie met vier onafhankelijke onderzoekslaboratoria. Ook voor wetenschappelijke doeleinden is validatie van het muismodel voor koemelkallergie van belang. De studies in dit proefschrift hebben laten zien dat partiële hydrolysaten de capaciteit hebben om orale tolerantie (bescherming) tegen wei-eiwitten te induceren en dat deze beschermende effecten versterkt kunnen worden door niet-verteerbare oligosacchariden toe te voegen aan het dieet. Waarschijnlijk spelen regulatoire T-cellen en $CD103^+$ dendritische cellen hierbij een belangrijke rol. Er is gevonden dat sensibilisatie met koemelk eiwitten kan leiden tot zowel IgE- als IgLC-afhankelijke allergische reacties en dat naast de bijdrage van IgLC aan de koemelkallergische reactie, IgLC mogelijk ook een beschermende rol speelt bij de orale tolerantie voor voedseleiwitten.

Dankwoord

Wat een reis! Eindelijk tijd om het dankwoord te schrijven. Wat heb ik veel geleerd de afgelopen 5 jaar. Door de komst van Johan naar de fijne farmacologie familie (zoals gedoopt door Gert) is mijn blik verschoven van allergisch astma naar koemelkallergie. Een nieuw begin met nieuwe kansen. Graag wil ik iedereen bedanken die hieraan heeft bijgedragen.

Johan, mijn promotor, de man met het master-plan. Zonder jou was dit boekje er niet geweest. Jij hebt me het vertrouwen en niet te vergeten de kans gegeven om te promoveren. Je hebt me altijd gesteund in de keuzes die ik maakte in het onderzoek. Jouw stimulerende kracht en vooral je vertrouwen in je achterban is enorm. Na de EAACI 2004 is er veel veranderd!

Mijn co-promotoren,
Linette, jou ben ik zo gaan waarderen de afgelopen jaren. We waren het op wetenschappelijk vlak niet altijd met elkaar eens maar jouw steun en vooral je tomeloze energie om van mijn artikelen iets moois te maken heeft me geraakt. Zelfs in de tijd waarin je een grote persoonlijke wens in vervulling zag gaan had je nog tijd om desnoods midden in de nacht naar mijn stukken te kijken. Dank je wel! We zullen ons onderzoek zeker voortzetten.

Léon, even een korte sms, e-mail of telefoongesprek was genoeg om dingen met je te bespreken of kort te sluiten. Daar houd ik van. Toen ik in januari 2011 met mijn eindsprint begon bleef jij feilloos in mijn wiel. Ik heb vooral binnen Danone veel van je geleerd. Bedankt voor je vertrouwen, bovendien is het met jou altijd gezellig. Jij weet iedereen te mobiliseren om op congressen toch nog even te gaan dansen. Als ik het woord referentie lees of hoor denk ik aan jou!

En dan de Fijne Farmacologie Familie (FFF®),
Mijn kamergenoten Gerard en Bart.
Het is even wennen zonder mijn mannen op de kamer. Door de overgang naar het nieuwe gebouw zijn we gescheiden, dus reken er maar op dat ik vaak binnenloop!
Gerard, jij bent een van mijn paranimfen. De afgelopen jaren heb je me zoveel uit handen genomen. Niets was je teveel en je maakte altijd tijd waardoor ik meer ruimte kreeg voor alle andere zaken, die bij promoveren om de hoek komen kijken. Thanx!
Bart, met jouw enorme werklust en vrolijke noot heb je vele samples voor de light chain stukken gemeten. Vaak even snel tussendoor! Bedankt daarvoor. Er was altijd

een goede sfeer op de kamer en met jouw aanwezigheid heb ik vele letters op papier gezet.

Frank, bedankt voor je heldere schrijfstijl waardoor je belangrijke input hebt gegeven aan de immunoglobulin free light chain manuscripten.

Bastiaan, nu een Danoontje. Om jouw woorden te gebruiken. Wat had ik zonder jou gemoeten. Waar ik volgens jou onmisbaar was aan het begin van je promotie-onderzoek zo ben jij dat voor mij op het eind. Ik ben heel blij dat je de lay-out hebt gedaan voor mijn boekje.

Frans, Paul, Gert en Ferdi, jullie ken ik ondertussen al 25 jaar. Alleen in een FFF® hou je het zo lang vol op een afdeling.
Paul, bij jou liep ik stage. Vanuit Brabant naar de grote stad. Ik weet nog hoe zenuwachtig ik werd van je wenkbrauwen die omhoog gingen als ik je iets vroeg. Bij jou is het allemaal begonnen. Bedankt, het blijft leuk om met je bij te kletsen.
Gert, jij bent een apart verhaal en goud waard! Jij geeft altijd steun waar het nodig is en verliest nooit je positieve inslag. Je FFF® wordt door iedereen gebruikt en ook de BBB betekent echt wat voor me.
Ferdi, jij bent voor mij het geweten van de afdeling en hoewel we weinig contact hebben ben jij zo iemand op wie je altijd kunt rekenen.
Frans, een van je eerste worden waren: je hoeft geen U te zeggen hoor. Frans ik waardeer je en ben erg vereerd dat je “mijn voorzitter” wilt zijn bij de verdediging.

En dan de overige leden en ex-leden van de FFF®. Bedankt voor al jullie steun en gezelligheid. Tom bedankt voor je spontane en snelle hulp bij de humane IgLC bepalingen. Antoon, we hebben veel leuk onderzoek gedaan en de immunotherapie ligt me nog steeds na aan het hart. Voor degene die me alleen achter mijn laptop kennen, ik kan niet wachten om weer “echt” aan de slag te gaan. Astrid, ik vind het erg leuk dat we samen een nieuw “project” zijn gestart.

Via de A12 kom ik dan in Wageningen terecht bij mijn “andere vrienden” zoals ik jullie altijd noem. In 2008 heb ik een part-time aanstelling gekregen bij Danone Research. Een hele fijne werkplek waar ik enorm gestimuleerd word en met veel enthousiasme werk. Het is niet toevallig dat een aantal FFF ’ers hun weg hebben gevonden naar Danone.
Graag wil ik iedereen bedanken voor de goede sfeer in de babydivisie en het platform en de goede wetenschappelijke discussies tijdens de dinsdag-besprekingen. Ik beloof jullie dat ik er de komende jaren vaker zal zijn! Niki, met jou kan ik altijd weer lachen! Het is fijn om af en toe elkaars klankbord te kunnen zijn.

Raymond, Joost en Rob de Pinda-mannen van het IRAS. Jullie wil ik bedanken voor een fijne samenwerking in de ring-studie. Ray bedankt voor je input in hoofdstuk 5 en 6 van mijn proefschrift! Net als Léon heb je wel eens de schaatsen in het vet gelaten om mijn stukken van suggesties te voorzien. Ik hoop dat we de komende jaren meer samen kunnen gaan doen aan voedselallergie.

Via Terry ben ik in een groep warme mensen gevallen die elkaar vaak al vanaf de basisschool kennen. Met onze Nederlandse, Surinaamse, Marokkaanse, Indische en Nigeriaanse genen zijn we een kleurrijk gezelschap. Bedankt voor jullie interesse, kaarten, telefoontjes en vooral gezelligheid. De afgelopen wintersport bleek maar weer hoe gemakkelijk we met elkaar omgaan. Hoewel eten met 15 volwassenen en 13 kinderen aan een krappe tafel soms wel een uitdaging was.
Harold, bedankt voor de mooie voorkant!

Jeanette, sinds onze eerste fiets-vakantie (20 jaar geleden) toen we elkaar net een paar weken kenden ben jij mijn beste vriendin. Wat hebben wij gelachen. Als ik aan onze tent-opzet actie 's avonds laat en aan ons door de buren gepresenteerde ontbijtje denk moet ik nog steeds lachen. Je bent mijn paranimf en ik ben blij dat je ook daar achter me staat.

Papa en mama, jullie wil ik bedanken voor alle steun en onvoorwaardelijke liefde. Niets is jullie teveel. Altijd staan jullie voor ons klaar en ons tuintje wordt altijd weer gedaan! Will en Jan, mijn broers, we zijn een gezin van weinig woorden, maar het voelt altijd goed als we bij elkaar zijn en ook Marno is op afstand nog steeds bij ons. En dan alle andere van Esch'n: Angela, Heidi, Maaike en Nick, Manon, Jannus en de Lobato's: Roy, Petra, Ramon, Dustin, Amber en tot slot mijn schoonouders Els en Paul, allemaal bedankt voor jullie liefde en steun.

En dan de belangrijkste mensen, mijn lieffies.
Julia en Joël, ons "poppetje" en "my hero" wat ben ik blij met jullie. Julia, jij bent zo lief, zachtaardig en ijverig. Zoals papa laatst met je verjaardag geschreven had: "Ons poppetje verandert in een hele mooie vlinder".
Joël, jouw "respect" op een moeilijk moment betekende heel veel voor me. We kunnen niet wachten tot je als pop-ster bekend zult worden. Ik hou van jullie!

Terry, jij bent mijn allerliefste. Zonder jouw steun was dit niet gelukt. Het zijn vooral de kleine dingen die jou zo bijzonder maken en waarom ik zoveel van je hou!

Curriculum Vitae

Betty van Esch was born in Loon op Zand the 16th of May 1964. In 1982, after graduating from the St. Paulus Lyceum in Tilburg, she started her study at the Higher Laboratory School (HLO) in Etten-Leur.
During this study she performed a traineeship at the Department of Pharmaceutical Sciences, Faculty of Veterinary Sciences of the Utrecht University under the supervision of Dr. Paul Henricks.

In 1986, after graduating she started as a research technician at the same department headed by Prof. Dr. Frans Nijkamp under supervision of Dr. Paul Henricks.

From 1992 until 1995 she worked as an account manager at Omnilabo B.V.

Thereafter, the author continued her work as a research technician at the Division of Pharmacology, Department of Pharmaceutical Sciences, Faculty of Science at the Utrecht University under supervision of Prof. Dr. A.J.M. van Oosterhout. She worked on different projects financed by the Dutch Asthma Foundation. For many years she participated in a project entitled: 'Allergen specific immunotherapy in allergic asthma'.

In 2003 and 2004 she participated in a project financed by Curax B.V. in Utrecht under supervision of Prof. Dr. Gert Folkerts. In this year a mouse model for chronic asthma was developed.

The author started her PhD research project on cow's milk allergy under supervision of Prof. Dr. Johan Garssen in 2006. In addition, she participated in the PhD project of Dr. Ir. Bastiaan Schouten from 2005 until 2009, also under supervision of Prof. Dr. Johan Garssen, which resulted in the thesis entitled: 'Cow's milk allergy: immune modulation by dietary intervention'.

Since May 2008 she is part-time employed as a scientist in the Immunology team of Danone Research Centre for Specialised Nutrition. The combination of research performed at the Division of Pharmacology at the University of Utrecht and the Immunology team of Danone Research-Centre for Specialised Nutrition, resulted in this PhD thesis entitled: 'Cow's milk Allergy; Avoidance versus tolerance: new concepts for allergy management'.