Exploring immunological mechanisms in cow's milk allergy

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GENERAL INTRODUCTION

FOOD ALLERGY

Definition

Hippocrates, the ancient Greek physician better known as "the father of medicine", is thought to be the first person who described adverse reactions to food.[1] More then 2000 years ago a detailed description of food allergy was reported in his writings:

> *"But there are persons who cannot readily change their diet with impunity; and if they make any alteration in it for one day, or even a part of day, they are greatly injured thereby. Such persons, provided they take dinner when it is not their wont, immediately become heavy and inactive, both in mind and body, and are weighed down with yawning, slumbering, and thirst; and if they take supper in addition, they are seized with flatulence, termini, and diarrhoea, and to many this has been the commencement of serious disease, when they have merely taken twice in a day the same food which they have been in of the custom of taking once."*

From these noble words written by Hippocrates we learn today that the clinical symptoms of adverse reaction to food have not changed in the millennia since he lived. In the last century the disparate use of definitions to describe adverse reactions to food often led to a great deal of controversy. In order to bring more uniformity to the nomenclature of adverse reactions to food, in 1995 a subcommittee of the European Academy of Allergy and Clinical Immunology (EAACI) published a position paper in which a mechanistic classification of adverse reactions to food was proposed.[2] This position paper was revised in 2001 by an EAACI task force and updated by the Nomenclature Review committee of the World Allergy Organization (WAO) in 2003.[3;4] The term *hypersensitivity* is proposed to be used to describe both allergic and non-allergic reactions to food (figure 1). Hypersensitivity is defined as follows: "Hypersensitivity causes objectively reproducible symptoms or signs initiated by exposure to a defined stimulus (e.g. food) at a dose tolerated by normal persons". *Allergic hypersensitivity* is defined as a hypersensitivity reaction initiated by specific immunologic mechanisms. Furthermore, allergic immune responses are presumed to be either antibody or cell mediated. Therefore, a*llergic hypersensitivity* is further subdivided into IgE-mediated and non-IgE mediated allergic hypersensitivity. In addition, hypersensitivity reactions which cannot be proven to be based on immunological mechanisms, as in hypersensitivity to aspirin,[5] the term *non allergic hypersensitivity* is described.

Following the position statements provided by the committees of the EAACI and the WAO, food allergy should be defined as hypersensitivity to food which is initiated by specific immunological mechanisms. When the hypersensitivity is

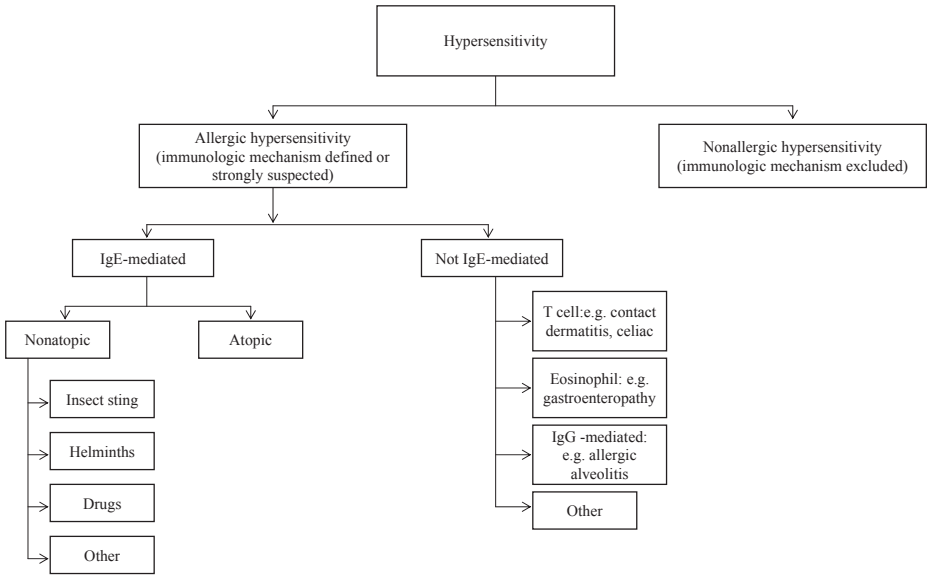

Figure 1. Nomenclature of food allergy. Hypersensitivity is classified into allergic and non-allergic hypersensitivity. Allergic hypersensitivity is further subdivided into IgE-mediated and Non-IgE-mediated hypersensitivity. Figure adapted form Johansson et al. Allergy 2001: 56: 813-824.

based on IgE-mediated mechanisms the appropriate term is *IgE-mediated food allergy*. Food hypersensitivity caused by other immunological mechanisms is best defined as *Non-IgE-mediated food allergy.*

Prevalence

In the last decades food allergy has become an emerging health problem, which is caused by a worldwide increase in prevalence and a considerable rise in fatal or near-fatal allergic reactions.[6-14] To date the prevalence of food allergy in Europe is estimated to be greater in young children (5-8%) then in adults (1-2%).[15-17] With an estimated prevalence of 2-3 % cow's milk allergy (CMA) is the most common food allergy in young children,[18] followed by hen's egg (0-2.5%),[19] peanut (1%),[20] wheat (0.4%), soy (0.4%), tree nuts (0.2%), fish (0.1%) and shellfish allergy (0.1%).[21] Most children with allergy to cow's milk proteins (CMP), hen's egg, wheat and soy become tolerant to the food allergen in early childhood. However, children with food allergy are approximately 2 to 4 times more likely to have related conditions such as asthma, atopic dermatitis and respiratory allergies compared with children without food allergy.[10] Other allergies such as peanut, tree nuts and seafood allergies tend to be persistent. Therefore, adults are more likely to have allergies to shellfish (2%), peanut (0.6%), tree nuts (0.5%) and fish (0.4%).[22] In addition, new data on the prevalence of food allergy in young children will be provided by the Europrevall study in the near future. The Europrevall study is a large multinational

birth cohort study, which aims to investigate the prevalence, costs and basis of food allergy in young children across Europe.[23]

Pathogenesis

The gastro-intestinal tract encompasses the largest surface area in the human body (200-400 m^2). Its main function is to process ingested food that can be absorbed and used for energy and growth. At the same time, luminal and brush border enzymes, bile salts, and extremes of pH serve to destroy pathogens and render antigens less immunogenic. To accomplish these different functions the gut compromises both a physiochemical barrier and immunological components. The physiological barrier consists of a single-layer of columnar intestinal epithelial cells which form intrinsic tight junctions to prevent paracellular passage of antigenic particles.[24] The immunological components present in the gut can be divided in innate and adaptive immune systems. The innate immune system consists of immune cells which can non-specific attack pathogens, for example natural killer cells, basophils, neutrophils and macrophages. In contrast, the adaptive immune response compromises cells which are both able to recognize and remember specific antigens. T-cells, together with B-cells and dendritic cells (DCs) are key players in the adaptive immune response. The immaturity of both the physiological barrier and the immunological components of the gut in infants and young children is presumed to play an important role in the increased prevalence of food allergies in the first years of life.

In allergic individuals an adaptive immune response is generated towards a harmless antigen. In patients with CMA immunity instead of tolerance is raised towards cow's milk protein (CMP). In healthy, non-allergic individuals a pathophysiological immune response to food proteins is prevented by the development of oral tolerance. Tolerance to food antigens is generated in the gut associated lymphoid tissue (GALT). Peyer's patches and mesenteric lymph nodes (MLN) are the most important parts of the GALT: here immunity or oral tolerance is induced. Food proteins are taken up by DCs in the peyer's patches or the lamina propria.[25] Consecutively, the DCs migrate to the MLN, process the protein into smaller peptides, and present them on major histocompatibility complex II (MHC II) molecules to naïve T-cells. In food and inhalation allergies, $CD4^+$ T-cells (T helper (Th) cells) are most important in steering this immune response.[26] Naïve T-cells require three signals to become fully activated.[27] The first signal is provided by the peptide-MHC II complex, which interacts with antigen-specific T-cell receptor and the co-receptor CD4. The second signal is provided by the interaction of the costimulatory molecules CD80 and CD86 on the DC with CD28 on the T cell. Once the two signal activation is complete the CD4+ T-cells will proliferate and depending on DC-derived signal 3, the T-cells will differentiate into various types of Th-cells, including effector T-cells, such as Th1, Th2 or Th17 cells or regulatory T-cells.[26] Many factors influence the differentiation of naïve Th-cells, including the nature and degree of costimulation and the cytokine milieu surrounding

the differentiating cells.[28] Th1 cells produce proinflammatory cytokines such as IFN-γ and TNF-α, which activate macrophages to kill bacteria they have engulfed and stimulate proliferation of CD8+ T-cells. IFN-γ also inhibits the production of cytokines such as IL-4, which is an important cytokine of the Th2 response. Allergic immune responses are attributed to Th2 cells that produce IL-4, IL-5 and IL-13. Th2 cells and their associated cytokines IL-4 and IL-13 play a crucial role in promoting host survival during infection with parasitic helminthes. In allergic reactions, Th2 cytokines (primarily IL-4), regulate isotype switching and differentiation of B-cells into plasma cells that produce and secrete immunoglobulines (Ig). Immunoglobulines consist of a heavy chain (IgG, IgA, IgM, IgD and IgE) and a light chain (kappa or lambda) linked together. These Ig bind to Fc receptors on mast cells and after a second allergen exposure, receptor cross linking results in mast cell activation and degranulation. Mediators released by mast cells such as histamine cause immediate allergic symptoms, such as urticaria, angioedema, asthma and in some cases life-threatening anaphylaxis. In parallel to complete antibody production, a surplus of free Ig light chains (Ig-fLC) is generated and secreted by plasma cells.[29] The immunological function of Ig-fLC has long been open to debate; however in the last decade increasing evidence has been generated which demonstrates that Ig-fLC possess antigen specific binding activity and can activate mast cells leading to degranulation and immediate hypersensitivity reactions.[30] Th17 cells are thought to play a key role in autoimmune diseases.[31;32] A more natural role for Th17 cells is suggested by studies that have demonstrated preferential induction of IL-17 in cases of host infection with various bacterial and fungal species.[33] The role of Th17 in food allergy is still largely unresolved. To date three members of the IL-17 cytokine family, IL-17A, IL-17E (IL-25), and IL-17F, have been best characterized both in vitro and in vivo.[34] Although IL-25 is structurally related to IL-17, previous studies have shown that its biological effects differ from that of IL-17 and other members of the IL-17 family.[34] Several studies indicate that IL-25 may be an important mediator of type 2 immune-pathologies by inducing and enhancing Th2 responses.[35-37] A possible role of IL-25 in food allergy remains to be investigated. Besides effector T-cells, regulatory T-cells play a central role in the development of tolerance.[38;39] In contrast to Th1 and Th2 cells, which initiate and continue the adaptive immune response to antigens, regulatory T-cells have an immuno-modulatory or suppressive function.[40] Several types of regulatory T cells have been described both in mice and men. Natural regulatory T cells express the transcription factor foxp3 and are directly derived from the thymus, while adaptive or induced regulatory T cells acquire a regulatory phenotype after contact with an antigen. Regulatory T-cells can modulate and suppress effector T-cell responses through several mechanisms among which the production of cytokines (for example IL-10 and TGF-β, respectively).Once antigen-specific T-cells have been activated, they leave the MLN, travel subsequently through the bloodstream and home to

their target organs, where they initiate an allergic inflammation by IgE synthesis of plasma cells and eosinophil recruitment.

COW'S MILK ALLERGY

Natural history

Allergy towards CMP is the most common allergic disease in infancy. Cow's milk is usually the first food antigen that is introduced into an infant's diet, and hypersensitivity reactions to CMP can evoke a wide spectrum of clinical symptoms. A unique aspect of CMA is that the majority of children regain clinical tolerance to cow's milk protein. Most studies show that the prognosis of developing tolerance is good, with the majority outgrowing their allergy by age three years.[14;41] However, more recent studies have found less optimistic results, with persistency of IgE-mediated CMA in 15% - 58% of children by age 9 years.[42;43] This increase in prevalence of persistent CMA is particular alarming, whereas children with IgE mediated and/or persistent CMA are of greater risk for the development of additional allergic disorders later in childhood than those without cow's milk allergy or non-IgE mediated CMA, and this risk is most pronounced in children with IgE-mediated CMA and persistent CMA.[10;42;44]

Genetic allergic predisposition has been dedicated to play an important role in the development of allergic disorders. Children with a positive family history of allergy have been shown to be at risk for the development of CMA.[45-47] Children with a single parental history of allergy have a risk of 20-40% to develop an allergic disorder, whereas children with a double parental history of allergy this risk increased towards 50-70%.[41]

Clinical presentation

Allergic reactions to CMP can cause a wide spectrum of clinical reactions and are commonly classified in immediate and delayed hypersensitivity reactions.[41] Immediate hypersensitivity reactions occur directly after ingestion of a food allergen and can cause diverse symptoms ranging from cutaneous (urticaria, angioedema), gastrointestinal (nausea, vomiting, diarrhoea), respiratory (wheezing, asthma) to life-threatening systemic reactions (anaphylaxis). Delayed hypersensitivity reactions are best described as clinical reactions which develop more then 2 hours after ingestion of a food allergen and mostly result in cutaneous (atopic dermatitis) and/or gastrointestinal (diarrhoea) symptoms. General symptoms like inconsolable crying, refusing food, failure to thrive, and irritability occur frequently, but mostly in combination with other symptoms.[41]

In most children with CMA the onset of symptoms is closely related to the time of introduction of cow's milk protein based formulas.[48;49] However, the first symptoms of CMA can also appear in children who are exclusively breastfed.[50] The majority of children develop the first symptoms of CMA before three months of age.[41] Furthermore, it has been shown that most children with CMA present with

two or more symptoms, and with symptoms from two or more organ systems.[41] In addition it has been shown that CMA plays a pathogenic role in approximately 35% to 40% of infants with severe atopic dermatitis.[51;52]

Diagnosis

An accurate diagnosis of CMA is of critical importance because a wrong diagnosis of the disease will lead to unnecessary avoidance of the suspected food allergen form the patients diet, which may result in dietary deficiencies, severe allergic reactions[53] and has been shown to have a major impact on the quality of life of the patient and their family.[54]

To date the gold standard for the diagnosis of CMA is the in 1976 introduced double-blind placebo-controlled food challenge (DBPCFC).[55] The DBPCFC is part of a diagnostic procedure which consists of three phases: (1) elimination of CMP from the patient's diet, (2) an oral food challenge (preferably a DBPCFC) and (3) re-elimination of CMP. The DBPCFC has its limitations; it is a time consuming and costly test which can only performed by well-trained personnel in specialized facilities, the interpretation of a DBPCFC may be difficult due to the occurrence of subjective symptoms and placebo reactions,[56] and the performance of a DBPCFC always involves the risk of a life-threatening allergic reaction (anaphylaxis). In daily clinical practice facilities to perform a DBPCFC often do not exist and consequently to establish the diagnosis CMA the clinician needs to rely on other diagnostic tests which possess less sensitivity, specificity and positive predictive accuracy. The use of open food challenges instead of DBPCFCs is still broadly accepted, probably because these are easier to conduct and are less expensive, although it is commonly well known that the number of patients incorrectly diagnosed with food allergy by the performance of open food challenges is greater then 25%.[57;58]

Next to food challenges other tests often used in clinical practice to diagnose CMA include allergen-specific IgE tests and skin prick tests (SPT). Various studies have demonstrated the limited value of food-specific IgE levels for the diagnosis of food allergy as over 50% of children who display raised food specific IgE levels or increased food specific wheal diameters do not have food allergy.[59;60] The usefulness of diagnostic decision points or cut-off levels of specific IgE and SPT wheal size to predict food allergy has been studied thoroughly. So far, the cut-off levels of allergen-specific IgE and skin prick test wheal diameter demonstrated to be predictive for food allergy (e.g. peanut specific IgE levels higher than 14.0 kU/L and cow's milk protein induced wheal diameters bigger than 12.5 mm)[61;62] are unequivocally high and thus are only applicable for a small proportion of the population.[63;64] However even the use of the cut-off levels described in several studies is open to debate as for specific food allergen vary remarkably[56] and high specific IgE-levels have been reported in non-allergic individuals.

Diagnostic tests less commonly used and available for the diagnosis of CMA are atopy patch tests (APT) and *in vitro* tests such as basophile activation tests and lymphocyte proliferation tests. Whereas SPT are aimed at the diagnosis of

immediate reactions, IgE-mediated reactions to CMP, APT are focussed on delayed, non-IgE mediated reactions to CMP. Although the APT shows promise,[65-67] there are currently no standardized reagents, methods of application, or interpretations, and the additional information in some studies appears marginal.[68;69] Until today, basophil activation tests or lymphocyte proliferation tests have not demonstrated an acceptable sensitivity and/or specificity in the diagnosis of CMA.[70;71] In addition, recently several studies have described the potential usefulness of micro-array based IgE detection for the diagnosis of food allergy.[72;73] These studies have shown that the advantage of micro-array analysis is that far less blood is necessary than the current standard (*in vitro* IgE antibody measurement) to test multiple antigens. However, because currently the knowledge of the diagnostic and prognostic value of positive results to many proteins is limited, interpretation of the result is difficult.

In conclusion, the DBPCFC, remains the gold standard for the diagnosis of CMA.[74] None of the available laboratory methods have proved to be indicative of clinical disease, or reaches sufficient sensitivity, specificity, and positive predictive accuracy. Recently, recommendations for clinical guidelines for the diagnosis of CMA have been published in several position papers and review articles.[74-77] In these guidelines the DBPCF remains the gold standard to diagnose CMA. Open food challenges are recommended to be used if the performance of a DBPCFC is not feasible, to help identify which foods are causing an allergic reaction or for rejecting the diagnosis CMA if the child is likely developing tolerance to CMP. Skin prick tests or measurement of IgE are recommended if the performance of a food challenge is not feasible[75] or for identifying other foods that may provoke allergic reactions.[74] Improved or new testing methodologies are needed for determining the presence of CMA and the prognosis of young children diagnosed with CMA. The development of a diagnostic method which can identify the CMA-infant at risk for persistent CMA and/ or the development of other allergic disorders later in childhood may provide tools for clinicians to decide in which patient therapeutic and preventive strategies are needed.

Therapy

Currently, treatment of CMA is based on elimination of CMP from the infant's diet and initiating therapy in case of accidental ingestion. Patients and caregivers should be educated in label reading, avoidance of cross-contact of foods with CMP during meal preparation, care in obtaining foods from restaurants and taught in recognizing allergic symptoms and using emergency medication and activating emergency services in case of anaphylaxis. The strict diet and risks of accidental ingestion have a major impact on the quality of life of patients and their families.[54]

Various medications can be prescribed for the treatment of allergic symptoms. Antihistamines are useful for relieve of mild IgE-mediated skin symptoms. In case of anaphylaxis, prompt intramuscular administration of epinephrine is the

key treatment. Adjunctive therapies for the treatment of anaphylaxis include antihistamines, glucocorticoids and bronchodilators.

Future therapies for food allergy which are currently investigated include sublingual/ oral immunotherapy[78;79] injection of anti-IgE antibodies,[80] cytokine/ anticytokine therapies,[81] Chinese herbal therapies,[82] and novel immunotherapy's utilizing engineered proteins and strategic immunomodulators.[83;84] The approach undergoing the most current research in cow's milk allergy is oral immunotherapy (OIT), in which doses of CMP are given in gradually increasing amounts toward a maintenance dose.[85-88] OIT has been attempted at least a decade, with mixed success. In 2008 the results of the first double-blind trial of milk OIT showed that the median dose eliciting a clinical response increased from 40 mg CMP to approximately 5 mg in the treated group but was unchanged in the placebo group.[78] In some studies OIT has been supplemented with IFN-γ.[89;90] These studies have shown that OIT can increase the threshold of reactivity to CMP in about 80% of patients with CMA. However, milde adverse reactions are very common, and occasionally more severe reactions occur. Although OIT is presumed to restore or induce a tolerance state towards CMP, it should be noticed that a distinction must be made between desensitization, in which the allergen is ingested without symptoms during treatment but requires daily ingestion, and tolerance, in which the food may be ingested without allergy symptoms despite periods of abstinence. The importance of this distinction is emphasized by a milk OIT study which showed that in children treated by OIT for approximately two years, after discontinuation of daily therapy for 2 months the percentage of children which continued to have true tolerance was 36%, which was a percentage that matched tolerance achieved in untreated control subjects.[79]

In conclusion, albeit OIT may be a promising future therapy for the treatment of CMA, more studies are needed to assess safety, efficacy and mechanisms. To date, the primary treatment of CMA remains avoidance of CMP from the child's diet.

CMP-SPECIFIC T-CELL RESPONSES

Immunogenicity of CMPs

The protein fraction of cow's milk compromises at least 20 proteins and in principle, all can act as antigens. Cow's milk consists of 80% casein proteins (αs1-, αs2-, β-, and κ-casein) and 20% whey proteins (β-lactoglobulin a, α-lactalbumin and bovine serum albumine).[91;92] Of those, αs1-casein is proposed to possess the most immunogenic properties, followed by α-lactalbumin and β-lactoglobulin.[93] Based on this immunogenic character, most studies have described T cell responses against αs1-casein and β-lactoglobulin.

CMP-specific T-cell proliferation and cytokine production

CMP-specific T-cell responses in children with CMA have been shown to be different from children without CMA. Conflicting data have been published about differences

in CMP-specific T-cell proliferation between children with and without CMA. Several studies have reported significant higher CMP-specific T-cell proliferation in children with CMA than in children without CMA.[70;94-96] However, other studies found no differences in CMP-specific proliferation between both groups.[97-99] In contrast to these controversial reports on CMP-specific T-cell proliferation, it has been clearly demonstrated that CMP-specific T-cell cytokine production in children with CMA is different from children without CMA. By using both blood derived lymphocytes[99] and mucosal lymphocytes from the gastrointestinal tract[100] it has been shown that CMP-specific T-cells of children with CMA produce high levels of Th2 derived cytokines IL-4, IL-5 and IL-13. In contrast, CMP-specific T-cell responses of children without CMA produce higher levels of Th1 and T-regulatory cytokines than children with CMA, such as IFN-γ and IL-10[99;101] Moreover, mitogen-induced lymphocyte production of IFN-γ and TNF-α has been shown to be lower in infants with CMA than in healthy children.[102;103]

CMP-specific T-cell responses in older children with CMA who had developed tolerance to CMP have been compared with children with persistent CMA. It has been shown that CMP-specific T-cells of children with CMA who developed tolerance to CMP produce high levels of immunosuppressive cytokines, such IL-10.[101] In contrast, CMP-specific T-cells of children with persistent CMA have been shown to secrete high levels of Th2 cytokines, including Il-4 and Il-13. These data indicate that the induction of tolerance to CMP is accompanied by an increase in IL-10 production, most likely by regulatory T-cells. In agreement with these results, it has been observed that after CMP challenge *in vivo,* children who developed tolerance to CMP had an increase in circulating CD4+CD25+ T-cells, whereas in children with persistent CMA the number of circulating CD4+CD25+ T-cells was significantly reduced.[104] Furthermore, after depletion of CD25+ T-cells *in vitro* a five fold increase in T-cell proliferation to CMP in the tolerant group was found compared to a two fold increase in the persistent group.[104] These results were confirmed in another study, which showed that after depletion of CD4+CD25+ regulatory T-cells CMP-specific proliferation was significantly increased in patients who developed tolerance to CMP, whereas no increase in proliferation was found in patients with persistent CMA.[105] In contrast to theses studies, a recent study reported that persistent CMA was characterized by a combined T regulatory cell and Th2 profile, while the development of tolerance was not characterized by activation of circulating regulatory T-cells.[106]

In addition, it should be noted that studies on CMP-specific T-cell responses are difficult to compare, because of differences in the CMP proteins used as antigens, the concentrations of antigens and differences in study populations (e.g. IgE-mediated or non-IgE mediated CMA). The importance of distinguishing between the CMP proteins used as antigens is emphasized by a study which reported that no difference in T-cell proliferation to casein was found between children with persistent CMA and children who had outgrown CMA, while in the

same study a significant difference was found in T-cell proliferation against whey protein between both groups.[95]

CMP-specific T-cell epitopes

To further unravel the T-cell mediated immune response to CMP, several studies have been done aimed at the identification of CMP-specific T-cell epitopes. All studies have been done in relatively small populations of children with CMA and T-cell epitopes have been identified at β-lactoglobulin and alpha(s)1-casein only. Inoue et al. observed that β-lactoglobulin-specific T-cell lines of four patients with CMA recognized seven different epitopes on β-lactoglobulin.[107] Sakaguchi et al reported that four out of six T-cell clones specific for β-lactoglobulin derived from five patients with CMA recognized one specific epitope on β-lactoglobulin, BLGp97-117.[108] In a subsequent study the proliferative responses of two of the T-cell clones specific for BLGp97-117was investigated against single amino acid substitutions in BLGp97-117. It was shown that the minimum essential region in BLGp97-117 is BLGp102-112, and two single aminoacid substitutions in this core epitope lead to decreased proliferative responses and cytokine production.[84] Nakajima-Adachi et al. determined the specificities of seven alpha(s)1-casein-specific T-cell lines established form two patients with CMA and found that all T-cell lines had different specificities to alpha(s)1-casein. Recently, Ruiter et al. compared the responses of alpha(s)1-casein-specific T-cell lines established to overlapping peptides (18-mers), spanning the alphas1-casein molecule between patients with CMA, atopic and non-atopic controls. Interestingly, next to the identification of an immunodominant sequence recognized by all three groups, a specific region on α1-casein was reported which was only recognized by T cells from children tolerant to CMPs and not by T-cells of children with CMA.[98]

In conclusion, the CMP-specific T-cell response is presumed to play an important role both in the presence or absence of clinical reactivity to CMP and in the development of tolerance to CMP. Several studies have shown promising results, which in the future may lead to the development of preventive, therapeutic and new diagnostic methods.

However, current studies have primarily been focussed on the CMP-specific T-cell response in CMA-children older than 1 year of age with CMA, while the majority of children develop the first symptoms of CMA before three months of age.[41] Furthermore, open food challenges instead of DBPCFCs have been used in most studies for diagnosis of CMA, which are known to render a large percentage of false positive results.[57;58] Therefore, prospective follow-up studies are needed which investigate the CMP-specific T-cell response in CMA-infants in relation to development of tolerance and the development of other allergic disorders later in childhood are warranted. In the end, early identification of the child at risk for CMA and the allergic march may help clinicians in deciding in which patient preventive and therapeutic strategies are needed.

CMP-SPECIFIC B-CELL RESPONSES

Immunogenicity of CMPs

Similar to CMP-specific T-cell response, the major immunogenic proteins in CMP recognized by B-cells are the casein and whey fractions[109;110] Of those, it has been shown that αs1-casein is most immunogenic, followed by β-casein, β-lactoglobulin and α-lactalbumin.[111]

CMP-specific IgE

Immunoglobulin (Ig) E has been generally accepted as the key player in food-allergic B-cell mediated immune responses. Several studies have compared the presence and levels of CMP-specific IgE between children with and without CMA. In large population based studies it has been shown that more than 50% of children with CMA have detectable CMP-specific IgE levels in serum,[41] and that children with CMA have significantly higher levels of CMP-specific IgE than those without CMA. Similar to the CMP-specific T-cell responses observed in children without CMA, enhanced serum CMP-specific IgE levels have also been found in children without CMA. These results have illustrated that the presence of sensitization to CMP is not directly related to clinical hypersensitivity, and indicate that immunoglobulines are part of a physiological response. Next to IgE, the CMP-fraction is able to induce Ig responses of other isotypes, including IgG1, IgG4 and IgA. In concordance with CMP-specific IgE, these other Ig isotypes have been determined in children with and in children without CMA, and higher levels of these isotypes have been detected in the serum of children with CMA.[111]

Several studies have related the presence and levels of serum CMP-specific IgE in children with CMA with the development of tolerance or persistency of CMA later in childhood. It has been shown that infants with non-IgE mediated CMA develop tolerance to CMP earlier in childhood than infants with IgE mediated CMA.[41;42;112] Furthermore, higher levels of CMP-specific IgE level have been associated with persistent CMA and the development of additional allergic disorders later in life.[42;43] In contrast, a decrease in CMP-specific IgE levels over time has been shown to be prognostic for the development of tolerance to CMP.[113;114] In addition, a limited number of studies have investigated the relation between CMP-specific IgG4 and the development of tolerance. High levels of CMP-specific IgG4 have been associated with the development of tolerance to CMP in food allergic children[115] and the maintenance of clinical tolerance to CMP in atopic children and adults without CMA.[116] In a recent study it has been showed that IgE and IgG4 antibodies recognize similar epitopes on the various CMP.[114] Therefore it has been suggested that IgG4 induces tolerance by blocking the binding of specific IgE to allergen.[117;118]

CMP-specific B-cell epitopes

In the last decade, numerous studies have been done which were aimed at the identification of IgE-binding epitopes and have shown promising results. IgE binding epitopes have been identified on the following major CMPs: αS1-casein,[119]

αS2-casein,[120] β-and κ-casein,[121] α-lactalbumin and β-lactoglobulin.[122] IgE-binding patterns have been shown to be different at diagnosis in children with CMA who developed tolerance to CMP than those who had persistent CMA later in childhood. Beyer et al. have shown that from the known IgE-binding regions five IgE-binding epitopes (2 on αS1-casein, 1 on αS2-casein, and 2 on κ-casein) were not recognized in children who had developed tolerance (n=10) but showed binding by the majority of the patients with persistent CMA (n=10).[123] These data have been confirmed in a larger population of children with CMA (n=74).[124] In contrast, a more recent study reported that at diagnosis IgE-binding patterns of children with persistent CMA were similar to children who had outgrown their allergy, and thus did not provide prognostic information. In addition, this study described that children with persistent CMA recognized IgE-peptide regions with greater intensity and more stable over time than children who developed tolerance to CMP. Interestingly, in children who developed tolerance the signal of IgG4 binding to peptides increased and that of IgE decreased over time.[114]

Immunoglobulin free light chains

Immunoglobulin free light chains (Ig-fLC) have been shown to possess antigen specific binding activity and elicit mast cells degranulation which resulted in immediate skin and asthma-like hypersensitivity reactions in mice.[30;125] This Ig-fLC-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. Previously, two preclinical models for CMA have been introduced in which mice were sensitized orally for whey or casein.[126] In these models the acute allergic skin reaction was monitored as a possible equivalent of the SPT. In both models it was shown that 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 IgG1. 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 IgG1 this was found not to correlate quantitatively with the skin reaction.[126] Possibly, Ig-fLC play an important role in the acute allergic skin reaction upon local allergen challenge in casein sensitized mice. So far data on Ig-fLC in human models of allergy are limited. Concentrations of total Ig-fLC have been demonstrated to be significant higher in the sera of patients with allergic asthma[125] and allergic rhinitis[127] as compared to healthy non-allergic controls. To our knowledge no data are available which describe concentrations of total Ig-fLC in CMA-individuals. .

In summary, the CMP-specific B-cell response has been dedicated to play a major role in the pathogenesis of CMA and the development of additional allergic disorders later in childhood. The role of CMP-specific IgE in CMA has been studied extensively. Furthermore, interesting data have been published which show that

determination of specific IgE-binding epitopes may provide increased diagnostic utility.[128] However, previous studies have shown that CMP-specific IgE levels are detectable in about 50% of children with CMA.[41] Therefore further research to explore the role of other mediators (such as Ig-fLC) which can elicit a CMP-specific B-cell response is warranted.

AIMS AND SCOPE OF THIS THESIS

This thesis aims to attain more insight in the immunological mechanisms which underlie clinical allergic hypersensitivity reactions to CMP in infancy and the development of tolerance or persistency of CMA later in childhood. In a prospective controlled follow-up study, responses of T-cells and B-cells to CMP were investigated in children with CMA in infancy and related with the presence of CMA and the development of tolerance or persistency of the disease in early childhood. In addition, diagnostic methods based on the variety of presenting clinical symptoms of CMA are presented in this thesis which may help clinicians to decide whether to refer an infant suspect for CMA to a specialized centre to perform a DBPCFC or to initially perform an open food challenge.

In **Chapter II** a detailed description of the recruitment and diagnostic procedures used in this prospective controlled-follow-up study are described. Furthermore, an overview of the baseline clinical characteristics of the study population is given.

In **Chapter III,** CMP-specific T-cell responses, CMP-specific IgE levels and clinical responses to CMP in infancy were related to the development of tolerance of CMP or persistent CMA in early childhood. Furthermore, a HLA-DR1-binding matrix based computer algorithm designed to identify pan-DR binding T-cell epitopes was used to identify CMP-specific T-cell epitopes on the cow's milk proteins αs1-casein, αs2-casein, β-casein, κ-casein, α-lactalbumin and β-lactoglobulin. In addition, an overview of the development of tolerance to CMP in our study population is given.

In **Chapter IV** a study is presented which aimed to develop a clinical triage model for clinicians to decide whether to refer an infant suspect for CMA to a specialized centre to perform a DBPCFC or to initially perform an open food challenge. The predictive value of clinical signs and symptoms for a positive DBPCFC to CMP in infants suspected of CMA was investigated.

In **Chapter V** the involvement of immunoglobulin free light chains (Ig-fLC) in clinical allergic responses to casein and whey was investigated. In a murine model acute allergen-specific skin responses in mice which were casein or whey sensitized were determined and serum immunoglobulin and Ig-fLC concentrations were measured. Ig-fLC dependency was validated by using the Ig-fLC blocker F991, a specific antagonist for the immunological actions of Ig-fLC. Furthermore,

Ig-fLC serum concentrations were measured in a cohort of infants with CMA and infants with atopic dermatitis.

In **Chapter VI** a study is presented in which plasma cytokine levels in food allergic children were compared with food tolerant children to attain more insight in the factors that contribute to a food allergic response and the development of tolerance. Plasma levels of children suspected of peanut allergy and CMA were compared to food tolerant children.

In **Chapter VII** we responded to a paper from Eigenmann that was published in Pediatric Allergy and Immunology.[129] In this educational review, Eigenmann proposed a diagnostic flow chart for the diagnosis of CMA on which we commented. The presented diagnostic flow chart showed that in children with symptoms suggestive of non-IgE mediated CMA a successful avoidance diet is sufficient to establish the diagnosis CMA. However, in previous studies it has been clearly demonstrated that only 64-81%of food challenges in children with symptoms suspected for food allergy are positive.[130;131] Therefore, we aimed to illustrate the importance of performing a DBPCFC to confirm the diagnosis CMA after a successful completion of an avoidance diet.

In **Chapter VIII** we commented on a paper by Van den Plas and colleagues that was published in Archives of Disease in Childhood.[132] In this paper, the authors presented guidelines for the diagnosis and treatment of CMA on which we commented. The guidelines implied that children suspected of CMA with initial immediate symptoms such as urticaria and angioedema do not necessarily need to perform a food challenge to CMP in a hospital setting. Because of the increased risk of anaphylaxis in this subset of children, we replied to this paper to describe the importance of performing a food challenge in a hospital with specialized facilities and experience in performing food challenges.

In **Chapter IX** the studies are being discussed against the background of international literature. Concluding remarks are made and suggestions for future studies are given.

In **Chapter X** an overall summary of the results of this thesis is provided.

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