The adaptive immune response to cow's milk proteins in allergy and tolerance

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The adaptive immune response to cow's milk proteins in allergy and tolerance

De adaptieve immuunrespons tegen koemelkeiwitten in allergie en tolerantie (met een samenvatting in het Nederlands)

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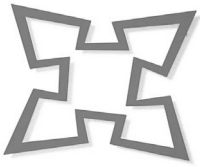

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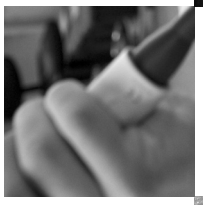

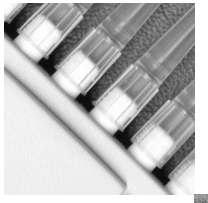
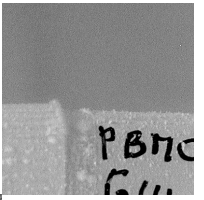
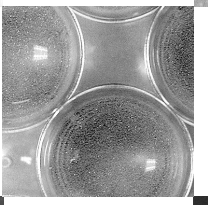
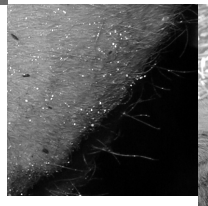
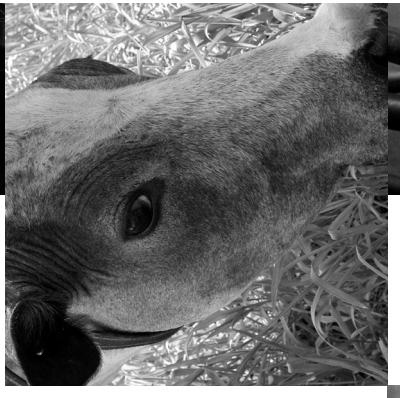
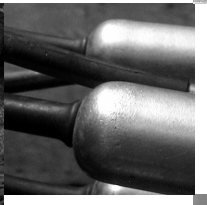
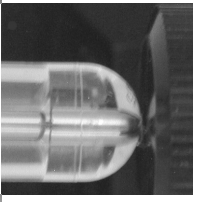
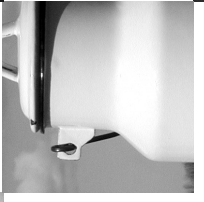
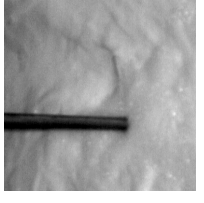

[1] General introduction

1.1 ALLERGY

Allergy is best described as an inappropriate immune response against innocuous antigens. Most of these antigens are proteins and are either airborne, or present in insect venom or in food. Although the human body encounters a wide variety of foreign proteins in substantial amounts each day, only a small proportion is known for their capacity to elicit an allergic response. The most allergenic airborne proteins originate from grass, grain and tree pollen, house dust mite, cockroach and pets such as cats and dogs.[1,2] Bee and wasp venom are the most important sources of insect venom allergens.[3] Cow's milk (CM), hen's egg, peanut, tree nuts, fish and shellfish contain the most allergenic food proteins.[4]

The key event in the elicitation of an allergic response is the production of allergen-specific immunoglobulin E (IgE), which binds to the allergenic protein with its antigen-specific Fab part. A unique feature of the IgE antibody is its capacity to bind to mast cells and basophils, which express the high affinity receptor for the Fc part of IgE (FcεRI). These cells degranulate and release mediators when the allergenic protein is encountered. It is these mediators that are responsible for the allergic reaction. At best, the symptoms of an allergic reaction are merely inconvenient, but at worst these can be life-threatening. In food allergy, symptoms can be diverse, ranging from cutaneous (urticaria, angioedema, atopic dermatitis), oropharyngeal (itch, lip swelling), gastro-intestinal (nausea, abdominal pain, diarrhoea), and respiratory (wheezing, asthma), to systemic anaphylaxis (collapse due to hypotension).[4] In this thesis, the word "allergy" will be used to indicate IgE-mediated allergy, as opposed to non-IgE-mediated allergy, which implies intolerance caused by an aberrant immunological response without the production of allergen-specific IgE.[5]

1.2 COW'S MILK ALLERGY

Allergy towards cow's milk proteins (CMPs) is the most common allergy in infancy. CM is usually the first food antigen that is introduced into an infants diet, and cow's milk allergy (CMA) is often the first presentation of the atopic constitution of an individual.[4] A unique aspect of CMA is its transient nature in most of the infants: Of the 1.5% of infants with IgE-mediated CMA, 70% develops clinical tolerance within three years of age. Only 15% is still allergic by the age of nine years.[6,7] Although CMA in childhood is in most cases a transient and relatively mild type of allergy, children with CMA are at greater risk for developing other atopic disorders than those without CMA, and this risk is most pronounced in children with persistent CMA.[7,8]

The first symptoms of CMA can also appear later in life, as is observed in the majority of adult subjects with CMA. In this group, CMA is less frequent than in children, but tends to persist longer.[9]

Allergy to CM can be diagnosed by a skin prick test with CMPs, the determination of CMP-specific IgE levels in serum, and the double-blind placebo-controlled food challenge (DBPCFC).[4,10] The latter procedure has been described as the "gold standard" for food allergy

diagnosis and uses increasing doses of allergen concealed in a food matrix, as well as portions of the food matrix without the allergen (placebos), to enable an objective diagnosis of symptoms of food allergy. The protein fraction in CM is comprised of several potential allergens, of which the casein proteins αs1-, αs2-, β-, and κ-casein, and the whey proteins α-lactalbumin and β-lactoglobulin are the most abundant. These proteins comprise 32%, 10%, 28%, 10%, 5% and 10% of total CMP, respectively.[11] The IgE response in individuals with CMA is generally directed to all six proteins, although the casein fraction appears to be more allergenic than the whey proteins in both children and adults.[9,12] Regarding the individual casein proteins, αs1-casein was observed to be the most potent allergen and κ-casein the least allergenic.[13]

1.3 DIGESTION AND UPTAKE OF FOOD AND PHYSIOLOGICAL IMMUNE RESPONSES TO FOOD PROTEINS

The gastrointestinal tract consists of an enormous mucosal surface area (200-400 m^2), which facilitates the uptake of food proteins, lipids, carbohydrates and other nutritional substances. These nutrients are degraded by pepsin in combination with low pH in the stomach. After digestion in the stomach, protein-derived peptides and free amino acids (AAs) enter the duodenum. Here the peptides are prone to further enzymatic degradation, before the AAs and oligopeptides are absorbed via the mucosal membrane and serve for nutrition. The entire small bowel is involved in the uptake of digested protein.[14] Oligopeptides below a length of 8 AAs are not presented or recognized by immune cells and are therefore immunologically ignored.[15] However, a small part of the food protein content is not fully degraded and can elicit immune responses after absorption.[16] This part is estimated to be 2% in adults and is likely to be higher in young children, due to immaturity of the digestive system. The gut mucosal permeability is higher in this age group, whereas the digestive enzymatic activity is lower.[17] Immunologically active food protein fragments are recognized as foreign by the immune system and induce immune responses in both children and adults.

To ensure a proper uptake of nutrients, it is essential to avoid pathophysiological immune responses to food proteins. Therefore, the normal response to food proteins is the induction of local and systemic immunological tolerance, known as oral tolerance. Key players in this process appear to be prostaglandin E_2 (PGE_2), TGF-β and perhaps IL-10, which result in the partial maturation of dendritic cells (DCs) residing in the gut-associated lymphoid tissue (GALT). These DCs present the antigen to naive $CD4^+$ T cells, which differentiate into regulatory T cells producing IL-10, IFN-γ and TGF-β. The immunological consequences are suppression or modulation of potential Th1- or Th2-skewed responses, systemic tolerance, local immune homeostasis and local IgA production.[18] This immunoglobulin (Ig) isotype is excreted in large amounts into gut mucosal secretions and serves as a first line of defense by inhibiting adhesion of pathogens and preventing absorption of intact proteins.[19]

1.4 THE ANTIGEN-SPECIFIC T CELL RESPONSE

1.4.1 T CELL SUBSETS AND FUNCTIONING. T cells, together with B cells and DCs, are key players in the adaptive immune response. Progenitor T cells originate from the bone marrow and move to the thymus, where they are subject to positive selection (recognition of the antigen-presenting major histocompatibility complex, MHC) and negative selection (recognition of self antigens). T cells that survive the selection procedure recognize MHC-restricted epitopes of foreign antigens, which will be encountered in the periphery. These epitopes are short protein-derived peptides, which are presented to T cells by antigen-presenting cells (APCs).[15]
In food allergy, $CD4^+$ T cells (T helper (Th) cells) are most important. This subset of T cells is activated upon recognition of epitopes of mainly extracellular pathogen-derived proteins or other foreign antigens, which are presented on MHC II molecules by APCs such as DCs, B cells and macrophages. Th cells require two signals to become efficiently stimulated. The first signal is provided by the peptide-MHC II complex, which interacts with the antigen-specific T cell receptor (TCR) and the co-receptor CD4. The TCR is associated with the CD3 complex that signals to the interior of the cell upon antigen binding. The second (co-stimulatory) signal is provided by the interaction of CD80 and CD86 on the APC with CD28 on the T cell.[20]
Upon antigen recognition, $CD4^+$ T cells can differentiate into various types of Th cells.
The response of Th1 cells is mainly directed to intracellular bacteria and small extracellular pathogens, which can be phagocytosed by macrophages and neutrophils. Th1 cells produce proinflammatory cytokines such as IFN-γ, which activate the microbicidal properties of macrophages, and induce pathogen-specific B cells to make strongly opsonizing IgG (IgG1/IgG3) antibodies. The Th2 cell response is principally directed to extracellular antigens and large pathogens such as parasitic worms. Th2 cells initiate the humoral immune response by activating naive antigen-specific B cells to produce IgM antibodies. Subsequently, Th2 cells can stimulate the production of IgA, IgE, and IgG4, which is a neutralizing and only weakly opsonizing IgG isotype. Due to their IgE-inducing properties, Th2 cells are the most important effector Th cells in allergy. The cytokines IL-4, IL-5 and IL-13 are secreted by Th2 cells and mediate the effector functions of these cells.[20]
Besides Th1 and Th2 cells, which initiate and continue the adaptive immune response to foreign antigens, naive $CD4^+$ T cells can differentiate into regulatory T cells (Tregs) that have an immunomodulatory or suppressive function.[21,22] Tregs can be divided into various subtypes. Naturally occurring $CD4^+$ $CD25^{high}$ Tregs express the transcription factor Foxp3 and are mainly produced in the thymus. These Tregs appear to suppress other T cells in a cell contact-dependent and cytokine-independent manner. Other types of Tregs are induced in the periphery, such as Tr1 cells and Th3 cells, which produce IL-10 and TGF-β, respectively. These cytokines enable Tr1 and Th3 cells to modulate or suppress effector T cell responses. Moreover, IL-10 and TGF-β influence Ig responses by promoting the respective production of IgG4 and IgA.[23,24]
The differentiation of Th cells is strongly influenced by the antigen-presenting DCs.[25] For example, it was observed that active IL-12-producing DCs, in combination with high antigen

doses, induce Th1 cells, whereas less active, exhausted DCs and low antigen doses give rise to a Th2 response.[26] Naturally occurring $CD4^{+}CD25^{high}$ Tregs appear to arise in the thymus upon interaction with medullary DCs during the process of negative selection.[27] A variety of characteristics have been reported for DCs that induce Tr1 and Th3 cells.[28] These Tregs appear to be mainly activated by mucosal or cutaneous DCs, as well as by DCs producing IL-10, TGF-β, or providing suboptimal costimulation.

1.4.2 THE CMP-SPECIFIC T CELL RESPONSE IN CMA AND TOLERANCE. T cell responses to CMPs in humans have mainly been investigated using T cells isolated from peripheral blood mononuclear cells (PBMCs), because T cells located in the gut mucosa are difficult and invasive to obtain. The frequency of allergen-specific T cells in peripheral blood is very low (in the order of $1{:}10^{4}$-$5{:}10^{4}$),[29] and CMPs are weakly stimulating antigens. Therefore, most studies on T cell responses specific to CMP (and other allergens) use T cell lines (TCLs), which are selected polyclonal populations of specific T cells, or T cell clones (TCCs), which are populations of identical T cells, derived from one specific progenitor T cell.[30]
Children with CMA and tolerant children have been observed to differ in their CMP-specific T cell responses. Using TCCs, it was shown that CMP-specific T cells of infants with CMA produced high levels of the Th2-associated cytokines IL-4, IL-5 and IL-13. In contrast, specific T cells of atopic infants without CMA were Th1-skewed, producing high IFN-γ, and T cells of non-atopic subjects displayed a Th0-like phenotype, secreting balanced and relatively low levels of IL-4 and IFN-γ. Upon spontaneous development of tolerance in children with CMA, the specific T cell response initially changed into a Th1-type and was expected to differentiate into a Th0 response at later age, which was also assumed for the T cell response in atopic children without CMA.[31]
Whereas this study focussed on Th1- and Th2-associated cytokines, the role of IL-10 was later shown in tolerance to CM. In a group of older children, CMP-specific TCCs of subjects with persistent CMA produced high levels of IL-4, IL-13 and to a lesser extent IFN-γ. Interestingly, specific TCCs in children with a food allergy but without CMA (atopic controls) were less Th2-skewed, and produced high levels of IL-10. Non-atopic children had low levels of all these cytokines, indicative of a Th0 response. These results raised the hypothesis that individuals with an atopic predisposition need an increased IL-10 response to maintain tolerance to CM.[32]

1.5 PRESENTATION AND RECOGNITION OF T CELL EPITOPES IN ALLERGENS

1.5.1 T CELL EPITOPE RECOGNITION IN ALLERGENIC PROTEINS. Antigen-specific Th cells recognize protein-derived epitopes when these are bound to MHC II molecules on APCs. The presented peptides typically have a length of 12-26 AAs. These peptides include a sequence of 9 AAs, known as the binding core segment, which interacts with the peptide binding groove of the MHC II and is bound by the TCR. The residues flanking this segment increase peptide-binding

affinity for the MHC II or can also serve as direct TCR contact residues.[15]
Numerous studies have characterized T cell epitopes in food proteins and other allergens. However, only few groups have compared T cell epitopes in allergens between allergic and tolerant subjects. T cell epitopes in the major birch pollen allergen Bet v 1 and the major cat allergen Fel d 1 were found to be similar in allergic and non-allergic subjects.[33,34] In the bee venom allergen PLA2, epitopes differed only slightly between allergic individuals and non-allergic beekeepers, the allergic subjects having T cell responses restricted to a larger number of epitopes.[35] In contrast, epitopes in the wasp venom allergen Ves v 5 differed considerably between allergic and non-allergic individuals. T cell lines of allergic patients not only recognized different epitopes, but also showed a broader epitope specificity and a higher stimulation index (i.e. the ratio of antigen-specific to non-specific T cell proliferation).[36] With regard to CM allergens, T cell epitopes were identified in αS1-casein and β-lactoglobulin, in low numbers of subjects with CMA.[37-40] No comparisons were made between allergic and tolerant subjects in these studies.

1.5.2 GENES ENCODING MHC II AND THEIR ROLE IN ALLERGY. MHC II molecules are encoded by the human leucocyte antigen (HLA) genes, which are divided into three groups: HLA-DP, -DQ, and -DR. To enable the immune system to mount a response against a large variety of potentially harmful antigens, MHC II molecules are designed to present a broad scala of different peptides. Hence, the HLA genes show great diversity in the population and are the most polymorphic genes known, as these are under strong evolutionary pressure. The repertoire of T cell and B cell receptors is even far more diverse than that of MHC molecules, but most variations in these molecules originate from DNA recombination in the T cells and B cells and are not encoded in the genomic DNA. MHC II molecules consist of an α-chain and a β-chain, of which the α_1- and β_1-domains form the peptide-binding groove. These domains determine which peptides are bound. Particularly the β_1-domains in the MHC II are highly polymorphic. Studies on associations of HLA genes with allergy have therefore mainly focussed on the β_1-domains, which are encoded by the HLA-DPB1, -DQB1, and -DRB1 genes.[20]
Conflicting data have been published about the association between HLA genotypes and allergies. Positive associations were described for e.g. bee venom allergy and peanut allergy.[41,42] However, a lack of association has also been reported for e.g. house dust mite allergy and (again) peanut allergy.[43,44] The correlation between an HLA genotype and sensitization to an allergen appears to be strongest if the allergen contains a single immunodominant T cell epitope, as shown for the major mugwort pollen allergen Art v 1.[45] Allergy to birch pollen and cross-reactive foods was found to be positively associated with HLA-DRB1*04 and DRB1*07 genotypes,[46] but negatively linked to DRB1*01, DQA1*0101 and DQB1*0501.[47] Thus, HLA genotypes can either predispose or protect for the development of allergy. In the only study on HLA associations with CMA so far, Italian children with CMA were reported to have a higher frequency of HLA-DQB1*0301/0304 (the HLA-DQ7 serotype) than age-matched healthy controls.[48]

To estimate the functional relevance of an association between an HLA genotype and an allergic phenotype, it is necessary to determine which HLA types encode the MHC II molecules that present the most prominent T cell epitopes in the allergen(s). The T cell recognition of a peptide in the context of a certain type of MHC molecule in humans is known as HLA restriction. Numerous studies have shown that T cell epitopes in both inhalant and food allergens are predominantly restricted to HLA-DR.[39,49-51] This may be explained by the fact that HLA-DR molecules are most abundantly present on APCs, followed by HLA-DP, whereas expression of HLA-DQ is lowest.[52]

1.6 THE ANTIGEN-SPECIFIC B CELL RESPONSE

1.6.1 B CELL FUNCTIONING. Similar to naive T cells, naive B cells originate from the bone marrow. B cells migrate to the peripheral lymphoid tissue where they become activated. Similar to T cells, B cells need two signals for activation. The first signal is provided by B cell receptors, which are Ig molecules bound to the B cell membrane, that have the same antigen-specificity as the Igs that are to be produced when the B cell is activated. The B cell receptors (BCRs) bind antigenic molecules, which leads to receptor cross-linking when these molecules are large enough to bind to at least two receptor molecules. Upon BCR crosslinking, the antigen is internalized, degraded, and peptides of the antigen are presented on MHC II molecules. These peptides are recognized by Th cells that are specific for epitopes within the antigenic molecule. Subsequently, the Th cell provides the second activation signal by costimulation through CD40L on the T cell, which binds to CD40 on the B cell. Cytokine release by T cells, in combination with BCR and CD40 signalling, leads to B cell proliferation and Ig production.[20] The type of produced Ig is dependent on the cytokines that are secreted by the responding Th cells. IL-4 stimulates production of IgG1, IgG3 and IgG4, as well as IgE.[53,54] IgG4 and IgE are also induced by IL-13, independently of IL-4.[54,55] The Th1-associated cytokine IFN-γ stimulates release of IgG1 and IgG3 and appears to reciprocally inhibit Th2-related cytokines like IL-4 and IL-13.[20] Regarding the immunoregulatory cytokines, IL-10 increases production of IgG1, IgG2, IgG3, IgG4 and IgA, but decreases IgE.[23,56] TGF-β stimulates IgA production,[24] and inhibits release of IgG4 and IgE.[57]

1.6.2 B CELL RESPONSES TO CMP IN ALLERGY AND TOLERANCE. The most important difference between food-allergic and tolerant subjects is the presence and level of food-specific IgE.[58-60] Besides, food proteins induce Ig responses of other isotypes, in both allergic and non-allergic subjects. For example, peanut-allergic individuals not only have peanut-specific IgE, but also specific IgG1, IgG4 and IgA, which correlate in magnitude with the IgE response. Non-allergic subjects were found to have lower levels of these Igs, which reached significance for IgG1 and IgG4.[58] In subjects with CMA, CMP-specific IgG1, IgG4 and IgA were abundantly present as well, and correlated with specific IgE levels. Also in this case, allergic subjects were found to have significantly higher levels of all CMP-specific Ig isotypes than individuals without CMA or food

allergy.[59] Still, CMP- and peanut-specific Igs other than IgE were well-detectable in non-allergic subjects, indicating that these Igs are part of a physiological immune response to food proteins.

1.6.3 B CELL RESPONSES TO INDIVIDUAL CMPS AND B CELL EPITOPE RECOGNITION. The casein fraction in CM has been shown to be more allergenic than the whey fraction in both children and adults.[9,61,62] Upon testing individual casein and whey proteins, α- and β-casein were found to be more allergenic and immunogenic than α-lactalbumin and β-lactoglobulin.[59] The only study investigating IgE responses to all four casein proteins showed that αs1-casein was the most allergenic casein protein in children.[13]
In the search for prognostic laboratory tests that can facilitate the prediction of the clinical outcome of young children with CMA, the focus has mainly been on the CMP-specific IgE response. Transiently and persistently allergic children do not seem to differ in the IgE response to the individual CMPs, although the overall IgE levels are higher in the last group. Possible differences between children with transient and persistent CMA have therefore been investigated for the IgE-binding epitopes in the most abundant CMPs.
Using overlapping synthetic peptides spanning the AA sequence of the CMPs, IgE-binding epitopes could be detected in αs1-casein,[63] αs2-casein,[64] β- and κ-casein,[65] α-lactalbumin and β-lactoglobulin.[66] Children who were persistently allergic had an IgE response directed to more different epitopes than children who would outgrow CMA. Some epitopes were only bound by IgE of children becoming persistently allergic. Additional studies compared these predictive epitopes and concluded that the presence and levels of specific IgE against the five most distinctive epitopes (present in αs1-, αs2, and κ-casein) could be used as a marker of persistent CMA.[67,68]

1.7 ROLES OF IGE IN ALLERGY

1.7.1 ACTIVATION OF MAST CELLS. The key process in the IgE-mediated allergic reaction is the activation of mast cells. These cells line the body surfaces and their physiological role is to guard against invading pathogens, such as parasites. However, in allergy these cells provoke the reactions to innocuous antigens that are the origin of the clinical symptoms. IgE antibodies are stably bound to the high affinity IgE receptor (FcεRI), located on the mast cell membrane. Binding of pathogen-derived antigens or allergens to receptor-bound IgE molecules leads to cross-linking of these receptors and triggers rapid degranulation of the mast cells. Pre-formed histamine, chemokines and cytokines such as TNF-α are released, which cause a local increase in blood flow and vascular permeability, and the attraction of various leukocytes. In allergy, these actions give rise to both the immediate symptoms within a few minutes after the encounter of allergen, as well as the late-phase response, which is mediated by the recruited lymphocytes and occurs approximately eight hours after allergen exposure.[4,20]

1.7.2 IGE-FACILITATED ALLERGEN PRESENTATION. The prominent role of IgE in the allergic response is not restricted to its function in mast cell degranulation. Allergen-specific IgE also facilitates the uptake and presentation of allergen by APCs, via the high-affinity (FcεRI) and low-affinity (FcεRII) IgE receptors. FcεRI is present on DCs and monocytes, as well as on Langerhans cells in patients with atopic dermatitis.[69-71] FcεRII (CD23) exists in two isoforms, CD23a and CD23b. Both forms are expressed on B cells, whereas macrophages only express CD23b, which does not internalize efficiently.[72] Hence, B cells appear to be the primary APCs in IgE-facilitated allergen presentation (IgE-FAP) via CD23.[73] After entering the body, allergen binds to FcεRI-bound IgE or forms complexes with circulating specific IgE. The multiple IgE molecules in these complexes give rise to sufficient binding avidity for complex binding to the low-affinity receptor CD23. Subsequently, the allergen is internalized, processed and presented to allergen-specific T cells.[69-71,73] It has been demonstrated that IgE-FAP via CD23 enhances specific T cell responses, resulting in an increase of specific IgE production by B cells.[74] This suggests that the mechanism of IgE-FAP leads to a vicious circle in the allergic response: Once a specific IgE response is established, the amount of allergen that is sufficient to induce Th2 cell activation and perpetuate the allergic response is gradually decreasing.[73,74]

1.8 THERAPY FOR CMA. Most infants with CMA develop spontaneous tolerance to CM within a few years. However, 10 - 15% of children retain their allergy into the second decade of life, and are thus deprived of an important source of protein in the diet. Moreover, CMA may develop at later age as well, and extend over a long period. Therefore, a therapy to induce tolerance to CM in allergic subjects would be desirable. Although numerous medications exist to relieve allergic symptoms, no therapies have as yet been designed to actually cure food allergy. Elimination of the offending allergen thus remains the only way of preventing an allergic reaction.[10]

To date, the only therapy that has been proven to induce clinical tolerance in allergic subjects, is allergen-specific immunotherapy (SIT).[75] This therapy has been applied in inhalant and venom allergies and consists of a series of either subcutaneously or orally administered amounts of allergen. The mechanisms by which SIT exerts its tolerogenic effect will be dealt with in the general discussion (chapter 8).

In food allergy, the risk/benefit ratio of conventional SIT was to date considered unacceptable, due to serious side effects caused by the administered allergen, which can still elicit IgE-mediated responses. However, the use of allergen peptides in SIT may decrease these side effects and prove useful in treating food allergy, as peptides are too small to cross-link IgE receptors, whereas modulation of T cell responses is still possible.[76]

Alternatively, SIT with digested allergens or modified recombinant allergens may be safe therapeutical options.[77] A different approach to inhibit the allergic response is to supplement the diet with prebiotics or probiotics.[78,79] These therapies enhance or supplement the intestinal microflora and may hereby strengthen its immunomodulatory capacity.[80] Both prebiotics and probiotics have been shown to reduce the incidence of atopic dermatitis.[78,79] Whether

these treatments can reduce the chance of development of other atopic diseases, remains to be investigated.

1.9 OUTLINE OF THIS THESIS

CM and dairy products are prominently present in the diet of Dutch citizens. CMA seriously hampers the development of infants and quality of life of older affected subjects, which underlines the relevance of studying CMA. At present, we are only beginning to understand the immunological mechanism underlying CMA. This thesis aims to expand the basic knowledge of CMA by investigating responses of T cells and B cells to CMPs in subjects with CMA, atopic individuals without CMA and non-atopic subjects.

The immune response towards CMP is initiated by APCs, which present CMP-derived epitopes that are recognized by specific T cells. In **chapter 2**, T cell epitopes were investigated in αS1-casein, the most abundant CMP, using polyclonal TCLs. Epitope recognition was compared between cow's milk allergic, atopic and non-atopic children. Cytokine profiles of IL-10, IL-13 and IFN-γ in response to the four casein proteins and to the various epitopes in αS1-casein were studied as well. The presentation of T cell epitopes in αS1-casein was investigated in **chapter 3**. TCCs of the same three subject groups as in chapter 2 were tested for αS1-casein epitope specificity and HLA restriction. In addition, MHC II-encoding HLA genotypes were investigated in the children and in an adult subject group, to study the possible relation between genetic factors that influence antigen presentation, and allergy or tolerance to CM.

Whereas CMA is well studied in infants and children, little information is available on CMA in adults. The immunological background of CMA may be different in adult patients, as these subjects mainly develop CMA in adulthood. **Chapter 4** deals with the cellular immune response to CMPs in adults. In this study, PBMCs were used to obtain an optimal polyclonal response. These cells were derived from adults with CMA, adults sensitized to CM but without CMA, and non-atopic adults. Proliferation and cytokine responses (IL-10, IL-13, IFN-γ, TNF-α, and TGF-β) were measured to whole CMP and the six most abundant individual CMPs.

CMP-specific T cells initiate the production of specific antibodies by B cells. Specific IgE is the key factor in the immediate allergic symptoms in CMA, but other Ig isotypes may also have a role in CMA and tolerance. In **chapter 5**, plasma levels of Igs (IgE, IgG1, IgG4, and IgA) specific to whole CMP and the six individual CMPs were compared between allergic, atopic and non-atopic subjects of three different age groups. Furthermore, CMA-specific Ig levels were compared with ovomucoid- and house dust mite-specific responses.

Allergen-specific IgG has been shown to block binding of IgE to allergen, which may interfere with IgE-mediated mechanisms that enhance the allergic response, such as IgE-facilitated allergen presentation (IgE-FAP). In **chapter 6**, the functional effect of allergen-specific IgG in binding of complexes to CD23 on B cells was investigated. Plasma of subjects with three different allergies, characterized by high specific IgG (CMA), moderate specific IgG (peanut allergy) and

low specific IgG (birch pollen allergy), was used to study differences in the efficiency of complex binding to B cells, as the first step of IgE-FAP.
The immune system of infants is immature and Th2-skewed at birth, which may increase the risk of development of CMA, especially in infants with an atopic predisposition. **Chapter 7** describes an approach to modulate the immune response in infants at risk for atopy. The effect of a prebiotic mixture of oligosaccharides supplemented to CM formula was analyzed on total, CMP-specific and DTP-specific levels of IgE, and different IgG isotypes.

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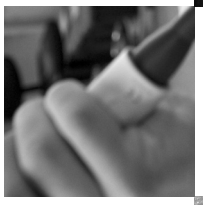

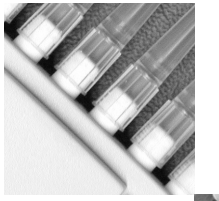
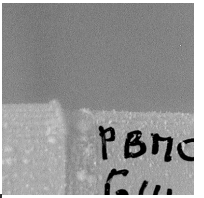
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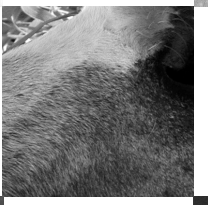
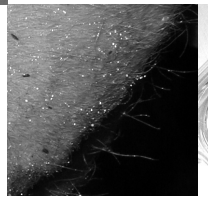
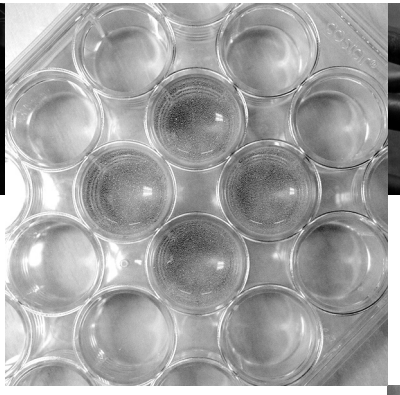
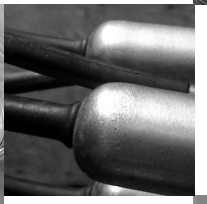
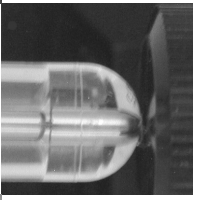
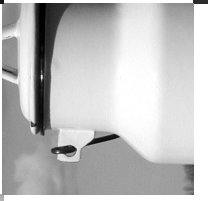
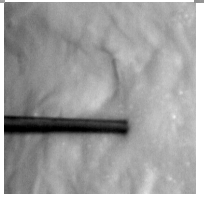

[2] Characterization of T cell epitopes in αs1-casein in cow's milk allergic, atopic and non-atopic children

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ABSTRACT

BACKGROUND. One to two percent of infants suffer from IgE-mediated allergic reactions against cow's milk proteins. Most children develop clinical tolerance, but approximately 15% are still allergic by the age of 10 years. Little is known about the T cell epitopes in individual cow's milk proteins in relation to allergy and tolerance.
OBJECTIVE. To identify T cell epitopes in αs1-casein, the most abundant milk protein, and to investigate T cell responses toward these epitopes in allergic, atopic and non-atopic children.
METHODS. Allergen-specific T cell lines (TCLs) were derived from peripheral blood mononuclear cells (PBMCs) of 11 cow's milk allergic, nine atopic and nine non-atopic children. T cell responses were measured to αs1-casein and to overlapping peptides (18-mers), spanning the αs1-casein molecule. Proliferation was determined by incorporation of ^{3}H-thymidine, and cytokine production (IL-10, IL-13 and IFN-γ) was measured by ELISA.
RESULTS. Four main regions (amino acid (AA) residues 43-66, 73-96, 91-114 and 127-180) in the αs1-casein molecule were immunogenic to T cells, among which the AA residues 133-156 spanned the immunodominant part. Only subtle differences were found in peptide recognition between the subject groups. Some of the peptides induced slightly Th1- or Th2-skewed cytokine responses. The increased levels of IL-10 in response to αs1-casein observed in TCLs from atopic children appeared not to be linked to recognition of specific IL-10-inducing epitopes.
CONCLUSION. The immunodominant sequence in αs1-casein is spanned by AA residues 133-156. Tolerance towards αs1-casein in atopic children may be mediated by an overall induction of IL-10 and not by recognition of certain T cell epitopes. The identified T cell epitopes in children with cow's milk allergy may be useful targets in developing peptide immunotherapy.

INTRODUCTION

Cow's milk allergy (CMA) is the most common food allergy in young children, affecting 1-2% of all infants. Most children develop clinical tolerance to cow's milk before the age of 3 years. However, approximately 15% of these infants retain their CMA into the second decade of life.[1] Casein is one of the major allergens in cow's milk. Cow's milk casein consists of four proteins: αs1-casein, αs2-casein, β-casein and κ-casein, representing 32%, 10%, 28% and 10% of the total milk protein, respectively.[2] αs1-Casein, a single-chain linear phosphoprotein of 199 AA residues, is the most abundant protein in cow's milk and is thought to be the most potent among all casein proteins in inducing a specific IgE response.[3] This protein has only a small amount of secondary structure, such as α-helices or β-sheets, and lacks disulfide bonds, resulting in a reduction of tertiary interactions.[4] IgE- and IgG-binding regions have been identified in αs1-casein. Interestingly, Chatchatee et al. showed differences in epitopes recognized by IgE of children with persistent CMA compared to children with transient CMA.[5] Inversely, IgG-binding epitopes revealed no discrimination between these groups. Differences in IgE-binding epitopes were also found for other casein proteins and were suggested to be a diagnostic tool to screen for persistent CMA.[6]

The isotype switching of antigen-specific B cells to IgE is controlled by T cells that play a key role in the initiation of allergic symptoms, as well as in tolerance induction. The difference in IgE-binding epitope recognition between children with persistent and transient CMA, as well as immunological differences between CMA and tolerant subjects, may therefore be reflected in the T cell responses. So far, T cell epitopes in αs1-casein have only been described in allergic individuals. Nakajima-Adachi et al. used synthetic peptides spanning the αs1-casein molecule to find T cell epitopes recognized by five T cell lines (TCLs) from two subjects with CMA.[7] Elsayed et al. used a similar approach, but found different epitopes.[8] However, these data were limited to only one TCL.

Besides T cell epitope recognition *per se*, also cytokine production in response to αs1-casein-derived epitopes may be different between CMA and non-CMA subjects. Our group has demonstrated previously that T cell clones isolated from infants with CMA produce higher amounts of IL-4, IL-5, and IL-13 upon stimulation with cow's milk proteins than T cells from atopic infants without CMA.[9] Tiemessen et al. showed the same Th2-skewed phenotype in T cell clones from children with persistent CMA.[10] Surprisingly, T cells from atopic children without CMA produced higher levels of IL-10 after cow's milk-specific stimulation than T cells from children with persistent CMA and non-atopic controls. This suggests that T cells from predisposed children need to upregulate IL-10 production to create a tolerogenic microenvironment and thereby maintain tolerance to cow's milk proteins. It is not known whether this increased IL-10 production is induced by distinct T cell epitopes.

The goal of the present study was to define immunodominant T cell epitopes in αs1-casein and to evaluate whether specific epitope recognition and cytokine production are linked to allergy or tolerance. T cell responses were investigated in children with CMA, atopic children who were

tolerant to cow's milk, and non-atopic children. Polyclonal casein-specific TCLs were used to determine T cell proliferation and cytokine production in response to whole αs1-casein, and to overlapping synthetic peptides spanning the entire αs1-casein molecule.

METHODS

ALLERGIC, ATOPIC AND NON-ATOPIC SUBJECTS. Eleven children with CMA (age: 0.3-14 years, mean 4.6), nine atopic children without CMA (age: 0.5-9.6 years, mean 3.8) and nine non-atopic controls (age: 0.5-10.1 years, mean 4.1) were included in this study. Most of these children have been described previously.[9,10] CMA was diagnosed by a positive skin prick test, a positive double-blind placebo-controlled food challenge with cow's milk and positive serum IgE levels specific for cow's milk, as determined by CAP System FEIA (Pharmacia Diagnostics, Uppsala, Sweden). Moreover, positive serum IgE levels specific for αs1-casein in children with CMA were detected by enzyme-linked immunosorbent assay (ELISA). Five children in the CMA group were allergic at the time of blood sampling, but later became tolerant to cow's milk. These children were therefore considered transiently cow's milk allergic. The other six children in this group were still allergic by the age of 6 years and were considered persistently allergic. In the atopic group, four children had a history of CMA but had become tolerant to cow's milk before the blood sample was taken that was used in this study. The remaining five children had a diagnosed food allergy, but no history of CMA. The non-atopic controls had no elevated serum IgE levels and no clinical or family history of atopy or allergy. Informed consent was obtained, and the study was approved by the Medical Ethical Committee of the University Medical Center, Utrecht.

PREPARATION AND CULTURE OF COW'S MILK-SPECIFIC TCLS. As in PBMCs proliferative and cytokine responses to purified casein proteins and synthetic peptides are generally below detection levels, allergen-specific T cells were enriched in polyclonal TCLs. Cow's milk-specific TCLs were generated as described previously.[9] The TCLs were maintained in culture by restimulation every 14 days with irradiated, autologous Epstein Barr-virus transformed B cells (EBV-B cells) as antigen-presenting cells. The EBV-B cells were preincubated overnight with whole casein (50 μg/ml, a gift from Dr. R. Floris, NIZO Food Research, Ede, the Netherlands). TCLs were cultured in RPMI-1640 medium (Gibco, NY, USA), supplemented with 10% pooled human recalcified AB plasma and 50 IU/ml IL-2 and IL-4. In the lymphocyte stimulation tests (LSTs), IL-2 and IL-4 were omitted from the medium. All media were supplemented with penicillin (100 IU/ml), streptomycin (100 mg/ml) and glutamin (1 mM) (Gibco).

PEPTIDE SYNTHESIS. T cell epitope mapping was performed with 36 C-amidated peptides with a length of 18 AAs (Thermohybaid, Ulm, Germany). The peptides were synthesized using the 9-fluorenyl-methoxycarbonyl (Fmoc) technique and purified to > 95% by HPLC, according to the manufacturer's instruction. Of the 36 peptides, 32 peptides had a 12 AAs overlap and spanned

the sequence of the most common variant B of the αs1-casein molecule (199 AAs). The post-translational phosphorylation on positions 46, 48, 64, 66, 67, 68, 75 and 115 was included. The remaining four peptides were non-phosphorylated versions of the peptides that covered the AA sequence 49-84 (Fig. 1).

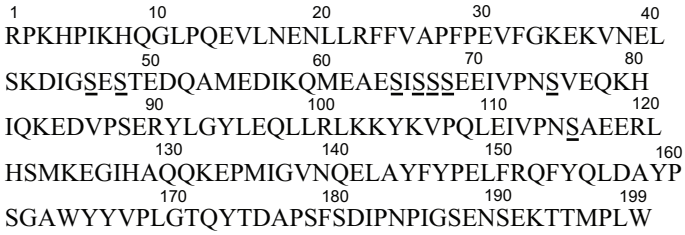

Figure 1.
Amino acid (AA) sequence of the most common variant B of αs1-casein. Underlined are the serine residues phosphorylated in the native protein. The serine residues at positions 64, 66, 67, 68 and 75 were present in the synthetic peptides in both phosphorylated and non-phosphorylated forms.

T CELL PROLIFERATION AND CYTOKINE RELEASE. To determine casein- or peptide-specific T cell proliferation and cytokine release, LSTs were performed in triplicate in 96-well U-bottom plates (Greiner, Frickenhausen, Germany) at 37°C in a humidified 5% CO_2 atmosphere. These tests were carried out at the start of a restimulation-cycle of the T cells in culture. Each well contained 3 x 10^4 T cells and 4 x 10^4 irradiated autologous EBV-B cells that had been preincubated overnight with either total casein, αs1-casein, αs2-casein, β-casein, κ-casein (all provided by Dr. R. Floris, NIZO Food Research) or a synthetic peptide. All caseins and peptides were free of LPS-contamination and were tested at a final concentration of 1 μM. Control cultures of TCLs and EBV-B cells without antigen were prepared in parallel to determine background levels of proliferation and cytokine production. After 24 hours of culture, supernatants of TCL cultures were collected and stored at -20°C. Tritiated thymidine ([^{3}H]-TdR, 1 μCi/well; Amersham, Aylesbury, UK) was added to measure proliferation, and the cells were harvested after 18 hours. [^{3}H]-TdR-incorporation was determined by a 1205 Betaplate counter (Wallac, Turku, Finland) and is expressed as counts per minute (cpm). Cytokine levels were measured in TCL culture supernatants by means of ELISA, according to the manufacturer's recommendations (IL-10, IL-13 and IFN-γ; Sanquin, Amsterdam, the Netherlands). The detection limit was 2.3 pg/ml for IL-10, 1.2 pg/ml for IL-13, and 3.1 pg/ml for IFN-γ.

STATISTICAL ANALYSIS. Background values were subtracted from antigen-specific proliferation and cytokine data, except for calculation of the stimulation indices (SIs). The SI of T cell proliferation to αs1-casein and the synthetic peptides was calculated as ratio between cpm measured in T cell cultures stimulated with αs1-casein or peptide (antigen-specific proliferation) and cpm determined in T cell cultures without αs1-casein or peptide (background proliferation). A SI >

1.5 was considered positive, because antigen-specific proliferation measured in triplicate was in these cases significantly higher than background proliferation. Cytokine data were normalized by ^{10}log-transformation. Differences in variance between groups were tested using the Fisher test. A two-tailed Student's t-test was subsequently used to analyse differences in proliferation and cytokine levels. Differences associated with p-values < 0.05 were considered to be significant.

RESULTS

PROLIFERATION OF TCLS TO THE CASEIN PROTEINS. Casein-specific TCLs from 29 subjects were tested for proliferation in response to whole casein and the separate casein proteins, αs1-casein, αs2-casein, β-casein and κ-casein (Fig. 2A). αs1-Casein was shown to induce higher proliferation levels than β-casein ($p < 0.01$) and αs2-casein ($p < 0.05$). No differences in proliferation levels were found between children with CMA, atopic and non-atopic children (data not shown).

CYTOKINE PRODUCTION INDUCED BY WHOLE CASEIN AND αS1-CASEIN. The levels of IL-10, IL-13 and IFN-γ secreted by TCLs in response to whole casein and αs1-casein are shown per subject group in Fig. 2B. Whereas levels of IL-13 and IFN-γ were comparable in the CMA, atopic and non-atopic group, a difference was observed in the levels of IL-10. TCLs from atopic children produced higher levels of IL-10 in response to αs1-casein than TCLs from the CMA ($p < 0.01$) and non-atopic ($p < 0.05$) groups.

PROLIFERATION OF TCLS TO SYNTHETIC PEPTIDES SPANNING αS1-CASEIN. The TCLs were subsequently tested for αs1-casein epitope specificity using a panel of 32 overlapping peptides. Figure 3 shows the recognized peptides per TCL and per subject group. In total, 10 TCLs could be tested from the CMA group, eight TCLs from the atopic group and five TCLs from the non-atopic group. Of the transiently cow's milk allergic subjects no. 7 and 8, TCLs were propagated at both the allergic and tolerant timepoints. These subjects were therefore included in both the CMA and atopic group.

The magnitude of the SI's induced by αs1-casein and the peptides was comparable between the subject groups. On average, a TCL responded to three peptides, varying from one to six peptides. Immunogenic peptides spanned AA residues 43-66, 73-96, 91-114, and 127-180. These peptides were recognized by at least three TCLs (> 10% of the total number of TCLs). The immunodominant region in αs1-casein was defined by AA residues 133-156; in total, 12 TCLs recognized epitopes in this region (52% of all tested TCLs). Three TCLs responded only to AA residues 133-150 and three TCLs to AA residues 139-156. Six TCLs were positive for both peptides and likely recognized epitopes in the overlapping part. Although T cell epitopes were found to be spread over the major part of αs1-casein, the AA sequence at the carboxyterminus (AA residues 175-199) was not recognized by any TCL.

A subtle difference in epitope recognition could be detected between children with and

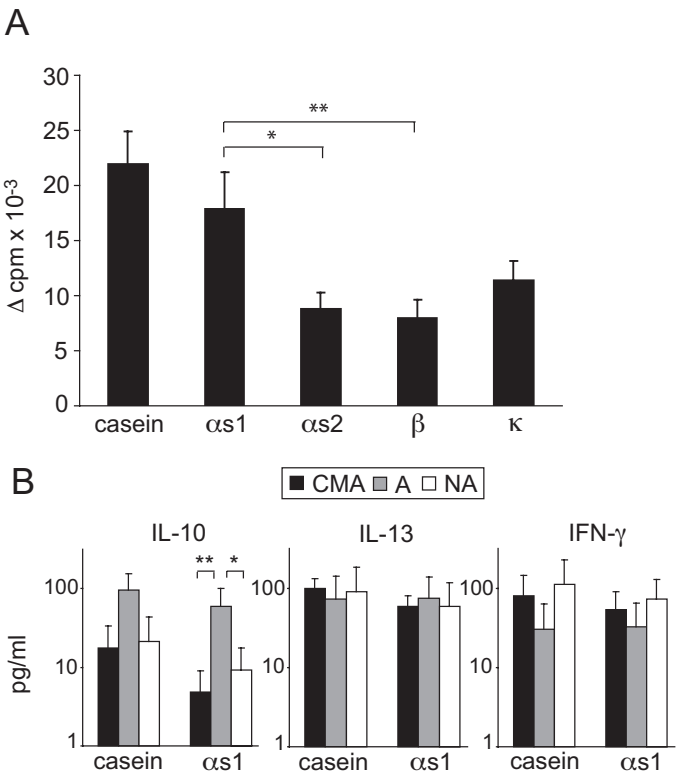

Figure 2.
Proliferation and cytokine responses of T cell lines (TCLs) to casein proteins.
A. Proliferation of TCLs (n = 29) in response to whole casein and the four casein proteins. Δcpm = antigen-specific proliferation - background proliferation (shown as mean + SEM).
B. Cytokine production of TCLs in response to whole casein and αs1-casein. The amounts of IL-10, IL-13 and IFN-γ were measured in culture supernatants of TCLs from 11 children with CMA (CMA), nine atopic children (A) and nine non-atopic children (NA). Data were normalized using ^{10}log transformation and are shown as mean + SEM. Significant differences are indicated in the graphs (Student's t-test, * $p < 0.05$, ** $p < 0.01$).

without CMA: the region spanned by AA residues 55-96 was only recognized by TCLs from children tolerant to cow's milk (atopic and non-atopic). No major differences in epitope recognition were found between persistently (subject no. 1-6) and transiently (no. 7A-10) allergic children in the CMA group. In the atopic group, TCLs from children who had previously become tolerant to cow's milk (no. 7B-11) and children who had a diagnosed food allergy other than CMA (no. 12-16) recognized corresponding epitopes.
A part of the αs1-casein sequence (AA residues 64-68) is highly phosphorylated in the native

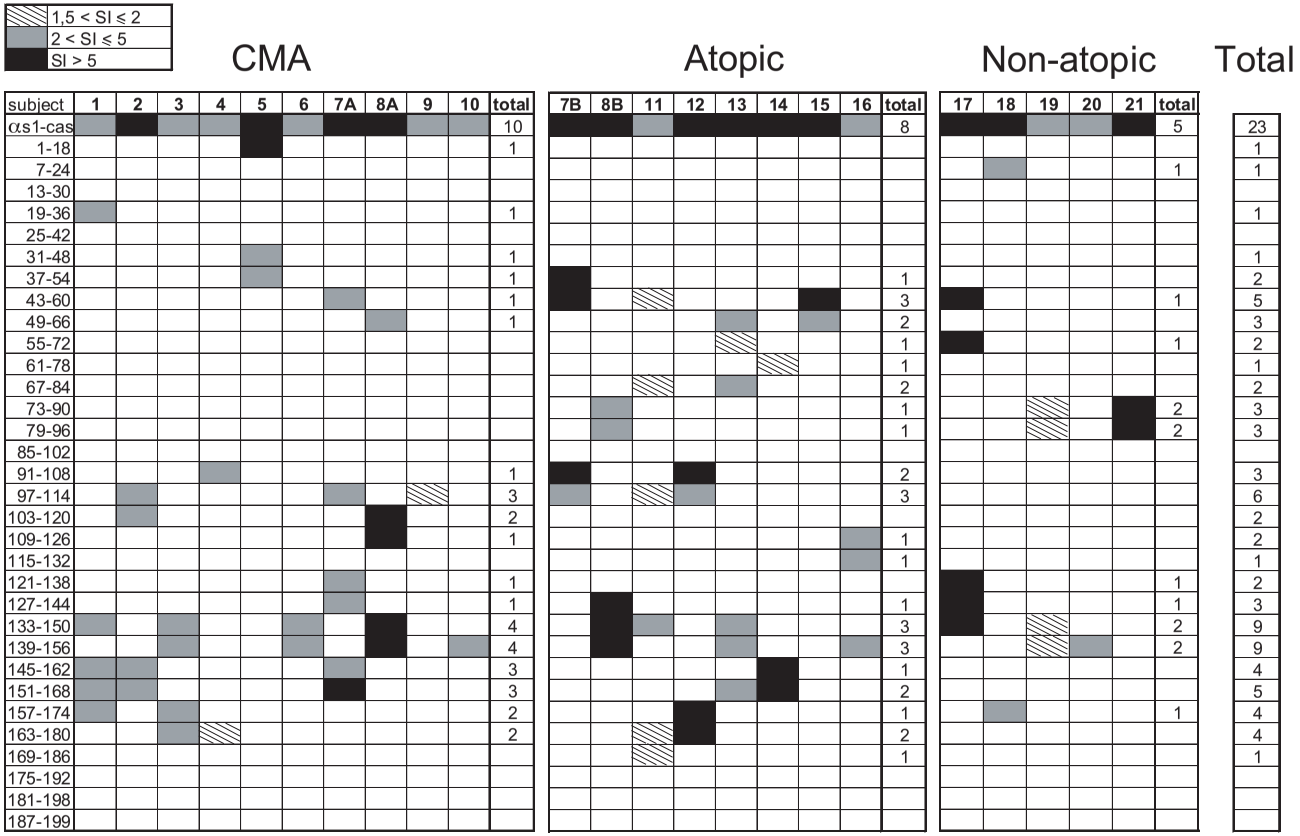

Figure 3.
Proliferation of T cell lines (TCLs) to overlapping synthetic peptides, spanning the αs1-casein molecule. TCLs from 10 children with CMA, eight atopic and five non-atopic children were assessed for their proliferation to αs1-casein and the peptides (18-mers). Peptides inducing a SI > 1.5 are indicated.

protein (see Fig. 1). Both phosphorylated and non-phosphorylated forms of the peptides spanning AA residues 49-84 were tested. The state of phosphorylation did not have a consistent effect on T cell recognition. Therefore, only data obtained with phosphorylated peptides were included in Fig. 3.

CYTOKINE PRODUCTION INDUCED BY PEPTIDES SPANNING αS1-CASEIN. To investigate whether higher levels of IL-10 secreted by TCLs from atopic children in response to whole αs1-casein were induced by certain parts of the αs1-casein protein, the levels of IL-10 were measured after stimulation with either αs1-casein or the peptides that induced proliferation in at least three TCLs. In addition, levels of IL-13 and IFN-γ were determined (Fig. 4A). To evaluate the variation in cytokine levels *per se* induced by the peptides, no differentiation was made between the subject groups. In general, the immunodominant region AA residues 133-156 induced the highest cytokine levels. Despite subtle differences, no particularly high levels of IL-10 could be detected for any peptide. The peptide with AA residues 43-60, which was recognized by TCLs of a relatively large proportion of atopic children (see Fig. 3), induced the highest ratios of IL-10/IL-13 and IL-10/IFN-γ. Regarding the levels of IL-13 and IFN-γ, most peptides evoked a Th0-like response (IL-13 ≈ IFN-γ). Although the data varied between the TCLs, peptides with AA residues 43-60, 49-66 and 163-180 seemed to generate a slightly Th2-skewed response (IL-13 > IFN-γ). Two peptides (AA residues 91-108 and 127-144) elicited a Th1-skewed response (IFN-γ > IL-13). IFN-γ levels induced by the peptides with AA residues 43-60 and 49-66 were lower than those evoked by AA residues 91-108 and 127-144 ($p < 0.01$).

Figure 4B shows the mean levels of peptide-induced proliferation in the same TCLs as shown in Fig. 4A, which allows comparison between proliferative and cytokine responses. Cytokine levels did not always correlate with the magnitude of proliferation. For example, the peptides covering AA residues 91-114 induced relatively low proliferation, but cytokine levels were comparable to those evoked by more immunogenic peptides.

The immunodominant region with AA residues 133-156 was recognized by three or more TCLs from each subject group, which made it relevant to compare cytokine levels between the subject groups (Fig. 5). Levels of IL-10 and IL-13 in response to this region were not different between the three groups. TCLs from the atopic group tended to produce lower levels of IFN-γ than TCLs from the CMA and non-atopic groups ($p = 0.10$).

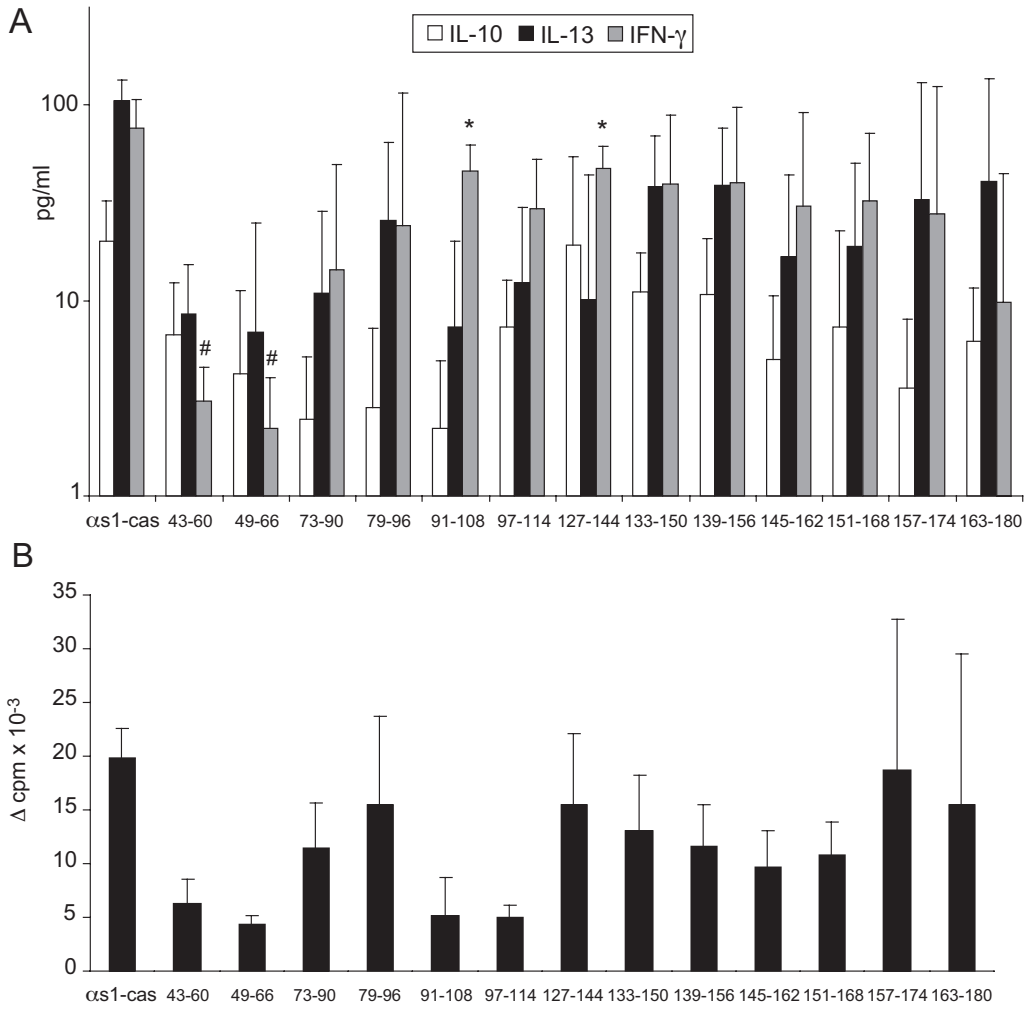

Figure 4

Cytokine responses and proliferation of T cell lines (TCLs) to the most immunogenic synthetic peptides.

A. Cytokine production in response to synthetic peptides that were recognized by at least three TCLs, irrespective of the subject group. The levels of IL-10, IL-13 and IFN-γ, induced by αS1-casein and by the peptides, were determined in culture supernatants. Data were normalized using ^{10}log transformation and are expressed as mean + SEM. Significant differences are indicated in the graph (Student's t-test, * vs. #: $p < 0.01$).

B. Proliferation of these TCLs in response to αS1-casein and to the most immunogenic peptides. Δ = antigen-specific proliferation - background proliferation (shown as mean + SEM).

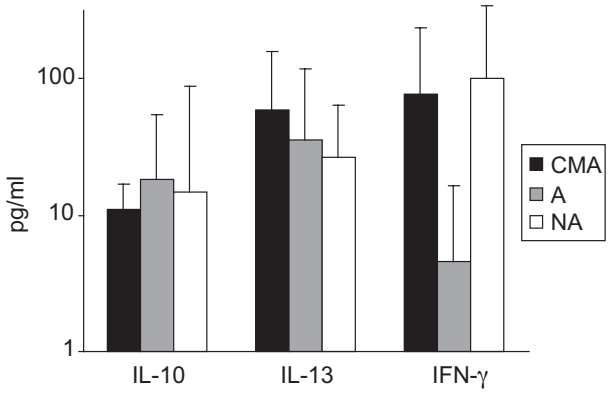

Figure 5.
Cytokine production of T cell lines (TCLs) in response to the peptides spanning AA residues 133-156. Five TCLs from children with CMA, four TCLs from atopic children and three TCLs from non-atopic children were assessed for their production of IL-10, IL-13 and IFN-γ in response to the peptides spanning AA residues 133-150 and 139-156. Data were normalized using [10]log transformation and are shown as mean + SEM.

DISCUSSION

This is the first study that investigated T cell epitopes in αs1-casein, using substantial numbers of polyclonal TCLs from cow's milk allergic, atopic and non-atopic children. Four main regions (AA residues 43-66, 73-96, 91-114 and 127-180) in the αs1-casein molecule were immunogenic to T cells. The immunodominant part (AA residues 133-156) was recognized by 12 out of 23 TCLs (52%). The immunogenic AA sequences reported in the studies of Nakajima-Adachi et al. (AA residues 31-50, 76-125, 136-170) and Elsayed et al. (AA residues 1-18, 136-155) evoked responses in at least one TCL in the present study, and included the immunodominant sequence.[7,8] In contrast to these reports, the large number of TCLs enabled us to define more and less immunogenic regions in αs1-casein. No striking differences were found in epitope recognition between the three subject groups. This is in accordance with other studies defining T cell epitopes in allergens in allergic and non-allergic individuals.[11-13] However, T cell epitopes in Ves v 5, a major allergen from wasp venom, differed between vespid-allergic and non-allergic subjects.[14] In the present study, the region covered by AA residues 55-96 was only recognized by T cells from children tolerant to cow's milk (atopic and non-atopic subjects). Although larger groups are needed to confirm these data, this sequence might play a tolerogenic role.
Interestingly, some of the peptides that were not or only slightly immunogenic to TCLs, spanned regions in αs1-casein that contain highly immunogenic IgE-binding epitopes. IgE- and IgG-binding epitopes in αs1-casein were previously identified by Chatchatee et al., using sera from 24 cow's milk allergic children.[5] Although these subjects were different from the children included in the present study, it is interesting to compare the data obtained in these groups of children.

Whereas T cell epitopes in subjects with CMA in our study are mainly located in the sequence spanned by AA residues 133-168, IgE-binding regions seem to be more diverse. The AA residues 17-36, 69-78, 109-120, and 173-194 contain hardly any T cell epitopes, but strong IgE-binding epitopes. Two of these regions, namely AA residues 69-78 and 173-194, were only bound by IgE from children with persistent CMA and not by IgE from younger children who were likely to outgrow their allergy.[5] In contrast to these findings, it was not possible to define AA sequences that were differentially recognized by T cells from children with persistent CMA as compared to those with transient CMA in our study.

Although no clear differences were found in epitope recognition between the subject groups, cytokine responses could well be discriminative. Therefore, levels of IL-10, IL-13 and IFN-γ were measured in TCLs in response to the different casein proteins and to the peptides spanning αs1-casein. IL-13 was preferred to IL-4 as a representative Th2-cytokine, because it acts not only synergistically with IL-4, but also independently in regulation of IgE synthesis.[15] The cytokine data reported in this study confirm and extend the findings of Tiemessen et al.[10] In the present polyclonal system, αs1-casein induced higher levels of IL-10 in the atopic group compared to the CMA and non-atopic group. Nevertheless, the higher levels of IL-10 in the atopic group could not be related to recognition of certain IL-10-inducing peptides. Cytokine responses to the region with AA residues 73-96, which was only recognized by T cells from children tolerant to cow's milk, were neither Th1-skewed nor demonstrated high IL-10 levels. Overall, most peptides seemed to elicit a Th0-like cytokine pattern (IFN-γ ≈ IL-13), comparable to the responses evoked by whole casein and αs1-casein. Two peptides induced a Th1-skewed response (IFN-γ > IL-13) and three peptides generated a slightly Th2-skewed pattern (IL-13 > IFN-γ). Despite these subtle differences between cytokines relative to each other, no particularly high levels of IL-10, IL-13 or IFN-γ were found for any peptide. Reefer et al. observed that of two peptides spanning the most immunogenic region in the major cat allergen (Fel d 1), one peptide induced strikingly high levels of IL-10 and the other evoked high IFN-γ levels.[16] These responses were seen in PBMCs from both cat-allergic and non-allergic subjects. This discrepancy with our data might be due to intrinsic differences between the inhalation allergen Fel d 1 and the food allergen αs1-casein. However, since the cytokine levels in the present study showed large variation between the TCLs and because the numbers of TCLs that responded per peptide were limited, unequivocal conclusions cannot be drawn. Future studies testing proliferative and cytokine responses to the peptides in PBMCs of larger subject groups may reveal differences in cytokine levels induced by distinct peptides. The data discussed above suggest that tolerance to αs1-casein in atopic children is mediated by a general induction of IL-10 and not by recognition of distinct IL-10-inducing T cell epitopes. In concordance with these findings, PBMCs from bee venom-allergic subjects receiving immunotherapy and from non-allergic beekeepers secreted more IL-10 in response to the major allergen (phospholipase A) than PBMCs from allergic patients.[17] Despite differential IL-10 levels, T cells from these three subject groups recognized the same epitopes in the bee venom allergen.[11] With respect to the induction of tolerance in CMA, immunotherapy with short peptides (12-18

AA residues in length) could be considered. Peptide immunotherapy has the advantage that it is not limited to the use of parts of the allergen that do not contain IgE-binding epitopes, since short peptides are not able to cross-link IgE-receptors on mast cells.[18] In humans, immunotherapy with peptides has been reported in bee venom allergy and in cat allergy.[19,20] The latter study showed that peptide immunotherapy is associated with increased IL-10 secretion in PBMCs upon antigen-specific stimulation. The possibility of inducing tolerance to αs1-casein with an immunodominant peptide has already been shown in mice.[21] Therefore, multiple dosing with the peptides that were found in this study to be most immunogenic to T cells from CMA subjects, may induce tolerance by an overall enhancement of antigen-specific secretion of IL-10.
In conclusion, T cell epitopes were identified in αs1-casein, the most immunogenic casein protein. The sequence spanned by AA residues 133-156 was found to be immunodominant. No major differences were observed in epitope recognition between allergic and tolerant subjects. T cells from atopic children who are tolerant to cow's milk produce more IL-10 in response to αs1-casein than T cells from allergic or non-atopic children. However, this could not be linked to specific T cell epitopes. The information obtained in this study may be useful for the development of peptide immunotherapy to induce tolerance towards αs1-casein, a major cow's milk allergen.

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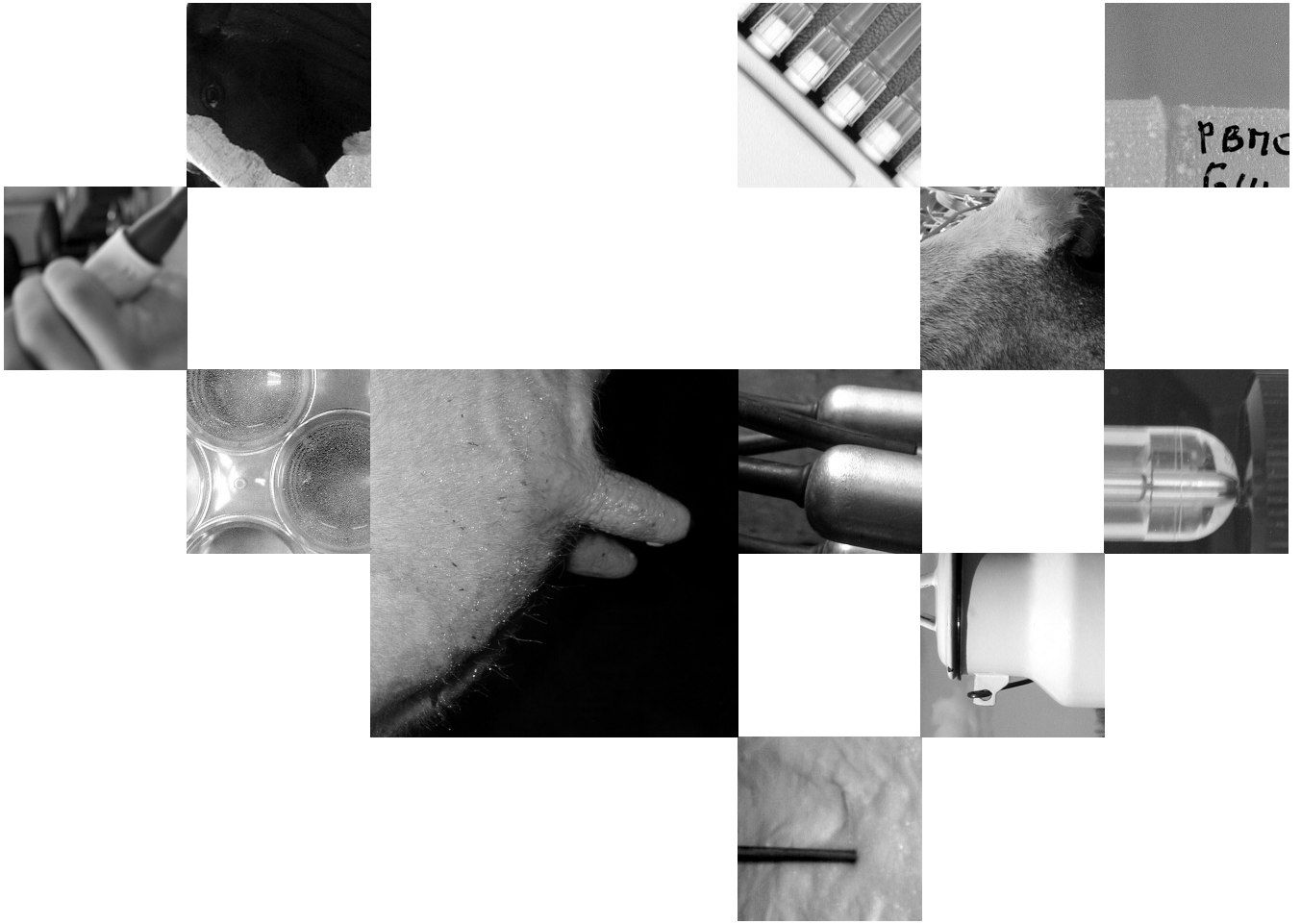

[3] Role of human leucocyte antigen-DQ in the presentation of T cell epitopes in the major cow's milk allergen αs1-casein

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ABSTRACT

BACKGROUND. Little is known about the association between human leucocyte antigen (HLA) and cow's milk allergy (CMA).
OBJECTIVE. The aim of the present study was to determine the HLA-restriction of T cell clones (TCCs) specific for αs1-casein, the most abundant milk protein, and to study possible HLA class II allele associations with CMA.
METHODS. αs1-Casein-specific TCCs were derived from six children with CMA, nine atopic children without CMA, and five non-atopic children. T cell epitope specificity was defined by stimulation with overlapping peptides, spanning the αs1-casein molecule. HLA-restriction was determined in proliferation assays using antibodies blocking either HLA-DP, -DQ, or -DR. HLA genotyping was performed in 32 subjects with CMA, 23 atopic and 22 non-atopic individuals.
RESULTS. Ten TCCs were restricted to HLA-DQ, six TCCs to HLA-DR and four TCCs to HLA-DP. The sequence in αs1-casein that was most immunogenic to T cells from children with CMA contained T cell epitopes restricted to DQB1*0201, DPB1*0401 and DRB1*1501. The DQB1*0501 allele frequency was lower in children with CMA than in non-atopic children, but this difference could not be confirmed in an additional group of subjects with and without CMA.
CONCLUSION. HLA-DQ plays a substantial role in the presentation of T cell epitopes in αs1-casein. However, HLA class II allele frequencies do not show major differences between cow's milk allergic, atopic and non-atopic subjects. T cell epitopes in the most immunogenic region are presented by various abundantly present HLA genotypes. This sequence may therefore be a suitable target for peptide immunotherapy.

INTRODUCTION

Human leucocyte antigen (HLA) genotypes determine the repertoire of presented peptides to T cells. The HLA-DP, -DQ and -DR genes encode major histocompatibility complex II (MHC II) molecules which are present on the membrane of antigen-presenting cells (APCs). MHC II molecules present peptides from proteins that are internalized by APCs, such as allergens. HLA-DR molecules are generally known to be most abundantly present on the APC membrane, followed by HLA-DP, whereas expression of HLA-DQ is even lower.[1] In the early 1980s, Marsh et al. observed an association between an MHC II-encoding HLA genotype and the immune response to the short ragweed pollen allergen Amb a 5 (Ra5).[2] Since then, several HLA genotypes have been shown to be associated with respiratory and food allergies.[3-6] The correlation between an HLA genotype and sensitization to an allergen is strongest if the allergen contains a single immunodominant T cell epitope, as shown for the major mugwort pollen allergen Art v 1.[7] To date, only one study reported an association of a particular HLA genotype with cow's milk allergy (CMA), being HLA-DQB1*0301/0304 (the HLA-DQ7 serotype).[8] To estimate the functional relevance of an association between an HLA genotype and an allergic phenotype, it is necessary to determine the HLA restriction of the most prominent T cell epitopes in the allergen(s). Previously, a predominant HLA-DR restriction was found for T cell epitopes in the respiratory allergens cat dander and grass pollen (Phl p 5), as well as in the food allergens ovalbumin, peanut, and β-lactoglobulin, a cow's milk allergen.[9-13]

The most abundant protein in cow's milk is αs1-casein, which represents 32% of total milk protein.[14] αs1-Casein is also the most allergenic casein protein.[15] Allergen-specific T cells play a key role in either an allergic or tolerant response towards cow's milk. Recently, we defined T cell epitopes in αs1-casein in polyclonal T cell lines from children with CMA, atopic children who were tolerant to cow's milk, and non-atopic children.[16] So far, it is not known which HLA genotypes determine the presentation of these epitopes. Therefore, the aim of the present study was to assess the individual contribution of HLA-DP, -DQ and -DR to the presentation of T cell epitopes in αs1-casein, and to define CMA-associated HLA genotypes. αs1-Casein-specific T cell clones (TCCs) of children with CMA, atopic children without CMA and non-atopic children were tested for epitope specificity and HLA restriction. HLA genotyping was performed of these children, as well as adults with CMA, atopic adults sensitized to cow's milk but without allergic symptoms, and non-atopic adults.

METHODS

SUBJECTS. A group of children and a group of adults were included in this study. The group of children consisted of eight children with persistent CMA (age: 6-14 years, mean 8.9), nine children with transient CMA (age: 0.4-1 year, mean 0.7), 13 atopic children without CMA (age: 0.4-9.6 years, mean 2.9), and 10 non-atopic children (age: 0.6-10.1 years, mean 4.3). Most of these children have been described previously.[16-18] The children in the transient CMA group

became tolerant to cow's milk before the age of 4 years. The atopic children without CMA had a diagnosed allergy, but no history of CMA or sensitization to cow's milk. The group of adults was comprised of 15 subjects with CMA (age: 26-68 years, mean 42), 10 atopic subjects sensitized to cow's milk but without CMA (age: 23-54 years, mean 40), and 12 non-atopic adults (age: 23-59 years, mean 31). CMA was diagnosed by a positive skin prick test (SPT) with cow's milk and/or positive serum IgE levels specific for cow's milk, as determined by CAP System FEIA (Pharmacia Diagnostics, Uppsala, Sweden), and was confirmed by a positive double-blind placebo-controlled food challenge (DBPCFC). All children and adults with CMA also had positive plasma IgE levels specific for αs1-casein, as determined by ELISA (unpublished observations). Adults sensitized to cow's milk but without CMA had a positive SPT and/or positive specific serum IgE levels, but a negative DBPCFC. The non-atopic controls had no elevated serum IgE levels and no clinical or family history of allergy or atopy. Informed consent was obtained before venous blood samples were taken, and the study was approved by the Medical Ethical Committee of the University Medical Center, Utrecht.

PREPARATION AND CULTURE OF αS1-CASEIN-SPECIFIC T CELL CLONES. TCCs specific to αs1-casein were generated and cultured as described previously. Moreover, cytokine profiles and surface markers of the TCCs were determined in previous studies.[17-19] Most of the TCCs described in this study were derived from the cow's milk-specific polyclonal T cell lines that were used previously to define T cell epitopes in αs1-casein.[16] Multiple αs1-casein-specific TCCs could be isolated from all children of whom a TCC is mentioned here. However, TCCs from an individual subject mostly recognized the same synthetic peptides, indicating that the TCCs were likely derived from the same progenitor. Therefore, only one αs1-casein-specific TCC is described per child.

T CELL PROLIFERATION TO SYNTHETIC PEPTIDES AND BLOCKING OF ANTIGEN PRESENTATION. Synthetic peptides with a length of 18 amino acid (AA) residues (Thermohybaid, Ulm, Germany), which spanned the sequence of the αs1-casein molecule, were used to screen the epitope-specificity of the αs1-casein-specific TCCs. The peptides and this procedure have been described previously.[16] T cell proliferation tests were performed in triplicate in 96-well U-bottom plates (Greiner, Frickenhausen, Germany) at 37°C. Each well contained 4×10^4 irradiated autologous Epstein Barr-virus transformed B cells (EBV-B cells) as antigen-presenting cells, which had been pre-incubated overnight with 1 μM of αs1-casein (NIZO Food Research, Ede, the Netherlands) or peptide. To determine the type of MHC II molecules that presented the individual peptides, the EBV-B cells were subsequently incubated for 1 h with a 1:5 dilution of hybridoma culture supernatants containing murine mAbs binding to either HLA-DP (B7/21), -DQ (SPV L3) or -DR (B8.11.2) (a gift from Dr. G. van Bleek, University Medical Center, Utrecht, the Netherlands), which have been shown to block antigen presentation.[9] Subsequently, T cells were added (2×10^4/well). After 24 h of culture, tritiated thymidine ([^{3}H]-TdR, 1 μCi/well; Amersham,

Aylesbury, UK) was added to measure proliferation, and the cells were harvested after 18 h. [^{3}H]-TdR incorporation was determined by a 1205 Betaplate counter (Wallac, Türkü, Finland) and is expressed as counts per minute (cpm). Control cultures of TCCs and EBV-B cells without antigen were prepared in parallel to determine background levels of proliferation, which ranged from 105 to 2930 cpm (median 260).

SEQUENCING-BASED TYPING OF HLA-DPB1, -DQB1, AND -DRB1. Genomic DNA was isolated from PBMCs of all subjects using the QIAmp blood mini kit (Qiagen, Hilden, Germany). Sequencing-based typing of the loci encoding the β-chain of HLA class II (HLA-DPB1, -DQB1, and -DRB1) was performed according to previously described protocols.[20-22] The HLA-DPB1, -DQB1, and -DRB1 loci were genotyped in the children group. The only difference in allele frequency between children with CMA and non-atopic children was observed for a HLA-DQB1 genotype. Therefore, only HLA-DQB1 was genotyped in the adult group. Sequence data were analysed using SBT Engine® software (Genome Diagnostics, Utrecht, the Netherlands).

DATA ANALYSIS. Statistical analyses regarding the HLA allele frequencies were carried out using a two-sided Fisher's exact test. P-values < 0.05 were considered significant.

RESULTS

HLA-RESTRICTION AND EPITOPE SPECIFICITY OF αS1-CASEIN-SPECIFIC T CELL CLONES.

A panel of 32 overlapping synthetic peptides, which spanned the AA sequence of the αs1-casein molecule, was used to determine the epitope specificity and HLA-restriction of 20 αs1-casein-specific TCCs. TCCs specific to this protein were generated previously from six children with CMA, nine atopic children without CMA, and five non-atopic children.[17,18] In the CMA group, subjects 1-4 were persistently allergic, whereas subjects 5-6 had transient CMA. For every TCC, the stimulation index (antigen-specific proliferation/background proliferation) induced by the recognized peptide(s) was at least 7. HLA-blocking antibodies reduced proliferation of the responding TCC by at least 50%. Data from a representative experiment are shown in Fig. 1. Antibodies against HLA-DQ, but not HLA-DP or -DR, inhibited proliferation of this TCC by 75%, indicating that the TCC was restricted to HLA-DQ.

Figure 2 summarizes the recognized peptides and HLA restriction per TCC and per subject group. Ten TCCs responded to a peptide presented by HLA-DQ, six TCCs recognized an HLA-DR- and four TCCs an HLA-DP-bound peptide. No major differences in HLA restriction and peptide recognition were observed between the subject groups. The sequence spanned by AA residues 133-156 was most frequently recognized by the TCCs. In general, the peptide recognition pattern of the TCCs was comparable to that of the T cell lines tested previously.[16]

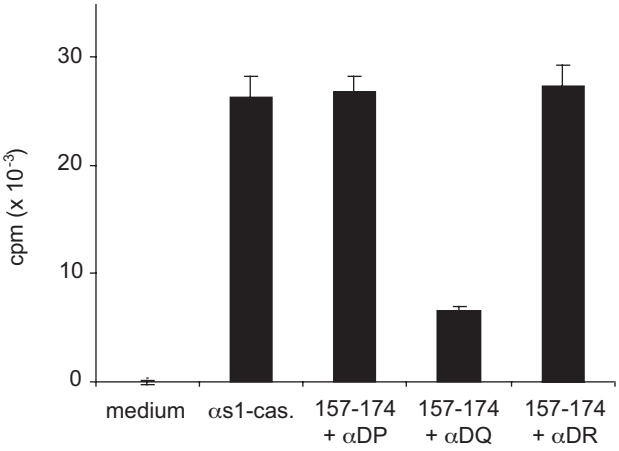

Figure 1.
Antibodies against HLA-DQ inhibit proliferation of an αs1-casein-specific TCC. The TCC, derived from subject 13, proliferated to whole αs1-casein and to a synthetic peptide spanning AA residues 157-174 of this protein. Antibodies against HLA-DQ, but not HLA-DP or HLA-DR, reduced recognition of the peptide by the TCC and thereby inhibited proliferation (shown as mean cpm + SEM).

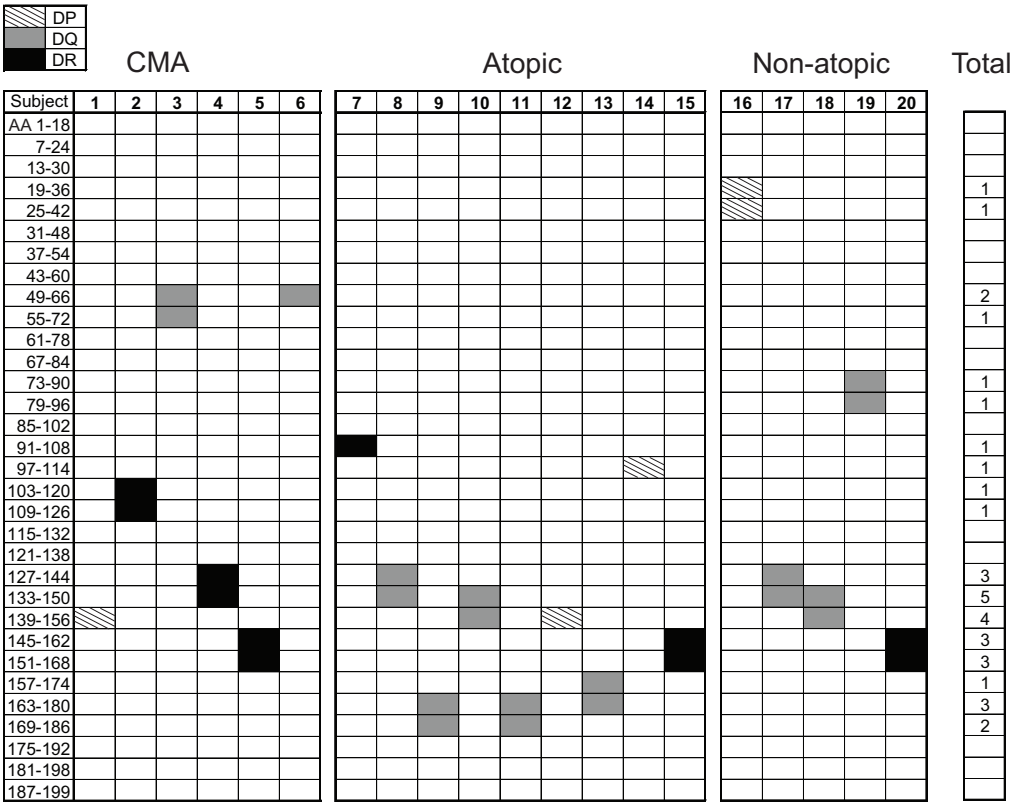

Figure 2.
Epitope specificity and HLA restriction of 20 αs1-casein-specific TCCs. TCCs from six children with CMA, nine atopic children without CMA, and five non-atopic children were assessed for proliferation to overlapping synthetic peptides (18-mers), spanning the AA sequence of αs1-casein. Four TCCs responded to peptides presented by HLA-DQ, ten TCCs were restricted to HLA-DQ and six TCCs to HLA-DR.

HLA RESTRICTION OF TCCS IN RELATION TO HLA GENOTYPES. The data above indicated that HLA-DQ plays an important role in presentation of T cell epitopes in αs1-casein. This led us to focus on the HLA genotypes of our children group. Sequencing-based typing of the loci encoding the β-chains of HLA class II (HLA-DPB1, -DQB1, and -DRB1) was performed using genomic DNA from eight children with persistent CMA, nine children with transient CMA, 13 atopic children, and 10 non-atopic children. These groups included the children of whom an αs1-casein-specific TCC was tested. The epitope specificity and HLA restriction of the TCCs and the HLA genotypes of the corresponding children are summarized in Table 1. Because subjects 6 and 8 were homozygous for DQB1*0201, it could be concluded that T cell epitopes present in AA residues

49-66 and 133-144 (the overlapping sequence between the peptides spanning AA residues 127-144 and 133-150) were restricted to this genotype (Table 1, Fig. 2). Also the region spanned by AA residues 139-150 likely contained DQB1*0201-restricted epitopes. This sequence was recognized by TCCs from subjects 10 and 18, who were both heterozygous for this genotype. Moreover, a DPB1*0401-restricted epitope was present in AA residues 139-156, which was recognized by TCCs from the homozygous subject 12 and the heterozygous subject 1. Probable HLA restrictions could further be found for T cell epitopes in AA residues 169-180 (DQB1*0202, subjects 9 and 11), and 151-162 (DRB1*1501, subjects 15 and 20).

Table 1. Epitope recognition and HLA restriction of the TCCs and HLA genotypes of the corresponding children

Subject [1]	AA	HLA genotypes [2]
HLA-DP-restricted TCCs		
16	25-36	DPB1*0101/0301
14	97-114	DPB1*0101/0401
1	**139-156**	DPB1*0202/**0401**
12	**139-156**	DPB1***0401/0401**
HLA-DQ-restricted TCCs		
6	**49-66**	DQB1***0201/0201**
3	55-66	DQB1*0201/0604
19	79-90	DQB1*0201/0501
8	**133-144**	DQB1***0201/0201**
17	133-144	DQB1*0302/0603
10	**139-150**	DQB1***0201**/0602
18	**139-150**	DQB1***0201**/0501
13	163-174	DQB1*0301/0302
9	**169-180**	DQB1*0201/**0202**
11	**169-180**	DQB1***0202**/0503
HLA-DR-restricted TCCs		
7	91-108	DRB1*1301/0401
2	109-120	DRB1*0301/0701
4	133-144	DRB1*1301/0301
5	151-162	DRB1*1301/0401
15	**151-162**	DRB1*0101/**1501**
20	**151-162**	DRB1***1501**/0801

[1] Subject numbers correspond to those in Fig. 1B

[2] (Probable) HLA restrictions are given in bold

HLA-DQB1 ALLELE FREQUENCIES IN COW'S MILK ALLERGIC, ATOPIC AND NON-ATOPIC CHILDREN . The frequencies of the HLA-DQB1 alleles in the entire children group and in two reference populations are shown in Table 2. The reference populations had been described in literature and consisted of 2440 Dutch blood donors (RP1) and 1899 European-American bone marrow donors (RP2), who were nonselected with respect to CMA.[23,24] The HLA genotype frequencies had been determined by serological methods in RP1 and by sequence-specific DNA typing in RP2. Although numbers of subjects were relatively small in our children group, a significant difference was found in the occurrence of the DQB1*0501 genotype in children with CMA compared to non-atopic children, but not to atopic children. This genotype was negatively associated with CMA (persistent CMA vs non-atopic: $p = 0.04$; persistent + transient CMA vs non-atopic: $p = 0.02$). The DQB1*0501 allele frequency in children with CMA also tended to be lower than in RP2 ($p = 0.07$), and the total frequency of DQ5 alleles was lower in children with CMA than in RP1 ($p = 0.006$). No significant differences were found between the subject groups for the other HLA-DQB1 alleles. Moreover, allele frequencies of HLA-DPB1 and HLA-DRB1 showed no differences between children with CMA, atopic and non-atopic children (data not shown).

Table 2. HLA-DQB1 allele frequencies (%) in 17 cow's milk allergic, 13 atopic and 10 non-atopic children, as well as in two reference populations

Subjects	Pers. CMA	Trans. CMA	Atopic	Non-atopic	RP1	RP2
Alleles (2n)	16	18	26	20	4880	3798
0201 (DQ2)	25.0	22.2	15.4	20.0	20.8	13.2
0202 (DQ2)	6.3	11.1	7.7	0		11.2
0301 (DQ7)	18.8	27.8	11.5	5.0	15.1	17.9
0302 (DQ8)	12.5	11.1	15.4	10.0	10.7	10.5
0303 (DQ9)	0	0	3.8	0	4.0	4.5
0401 (DQ4)	0	0	0	0	1.6	nd
0402 (DQ4)	0	0	0	10.0		2.4
0501 (DQ5)	0*	5.6*	11.5	25.0*		11.6
0502 (DQ5)	0	0	3.8	0	19.1	1.3
0503 (DQ5)	0	0	3.8	0		2.1
0602 (DQ6)	18.8	5.6	19.2	15.0		14.3
0603 (DQ6)	12.5	11.1	7.7	15.0	29.4	5.9
0604 (DQ6)	6.3	0	0	0		3.4

Serotypes are given in parentheses. Serotype frequencies are shown for RP1.
n.d. = not determined
* p = 0.02, combined CMA groups versus non-atopic children.

HLA-DQB1 ALLELE FREQUENCIES IN COW'S MILK ALLERGIC, ATOPIC AND NON-ATOPIC ADULTS. To investigate the apparent negative association of DQB1*0501 with CMA more thoroughly, the HLA-DQB1 loci were genotyped in DNA from 15 adults with CMA, 10 atopic adults sensitized to CMA but without clinical symptoms, and 12 non-atopic adults. Table 3 summarizes the HLA-DQB1 allele frequencies in our adult group and in the two reference populations. No difference was found between the adult subject groups for any HLA-DQB1 genotype. Four heterozygous adults with CMA carried the DQB1*0501 allele, whereas it was not present in children with persistent CMA. The DQB1*0501 allele frequency in adults with CMA was not different from that in RP2, and the total frequency of DQ5 was not significantly lower than in RP1.

Table 3. HLA-DQB1 allele frequencies (%) in 15 cow's milk allergic, 10 atopic and 12 non-atopic adults, as well as in two reference populations

Subjects	CMA	Atopic	Non-atopic	RP1	RP2
Alleles (2n)	30	20	24	4880	3798
0201 (DQ2)	13.3	15.0	12.5	20.8	13.2
0202 (DQ2)	6.7	5.0	0		11.2
0301 (DQ7)	13.3	30.0	12.5	15.1	17.9
0302 (DQ8)	10.0	5.0	4.2	10.7	10.5
0303 (DQ9)	0	5.0	12.5	4.0	4.5
0401 (DQ4)	3.3	0	0	1.6	nd
0402 (DQ4)	3.3	10.0	8.3		2.4
0501 (DQ5)	13.3	0	8.3		11.6
0502 (DQ5)	0	0	0	19.1	1.3
0503 (DQ5)	0	5.0	8.3		2.1
0602 (DQ6)	16.7	5.0	12.5		14.3
0603 (DQ6)	6.7	5.0	8.3	29.4	5.9
0604 (DQ6)	10.0	5.0	4.2		3.4

Serotypes are given in parentheses. Serotype frequencies are shown for RP1.
n.d. = not determined

DISCUSSION

For a better comprehension of the immune response towards allergens, it is essential to determine the genetic background underlying this response. To date, little is known about the role of genetic factors in allergy towards cow's milk proteins. In the present study, 20 αs1-casein-specific TCCs, derived from children with CMA, atopic and non-atopic children, were tested for epitope specificity and HLA-restriction. The peptides recognized by the TCCs were comparable to those that were immunogenic to the polyclonal T cell lines tested earlier, and were not markedly different between the subject groups.[16] Ten TCCs were restricted to HLA-DQ, six TCCs to HLA-DR and four TCCs to HLA-DP. To our knowledge, this is the first time that a predominant HLA-DQ-restriction is shown for T cell epitopes in an allergen, using TCCs from a substantial number of

different subjects. This finding is interesting, because the expression of HLA-DQ in APCs is at least 3-fold and 5-fold lower than that of HLA-DP and HLA-DR, respectively.[1] Previously, expression of HLA-DQ in intestinal epithelium cells was shown to be even lower than in conventional APCs.[25] This renders it unlikely that the predominant HLA-DQ-restriction of αs1-casein epitopes is caused by alternative HLA expression at the site of allergen take-up. T cell epitopes in other (food) allergens were previously found to be predominantly restricted to HLA-DR.[9-13] In contrast, the food antigen gliadin is well-known for its HLA-DQ-restricted epitopes. Gliadin plays a key role in the pathogenic response leading to celiac disease.[26] This disease is strongly correlated with the presence of HLA-DQB1*0201, DQB1*0202 and DQB1*0302 genotypes, which are involved in the presentation of gliadin-derived T cell epitopes. However, celiac disease is not an IgE-mediated disorder, and is therefore not fully comparable with food allergy.

HLA genotyping of our children group showed that the DQB1*0501 allele was less frequently present in children with persistent or transient CMA than in non-atopic children, which suggested a protective effect (Table 2). In line with these findings, DQB1*0501 was reported earlier to be negatively associated with hazelnut allergy.[6] Nevertheless, HLA genotyping in adult subjects revealed no differences in DQB1*0501 allele frequency between adults with CMA and non-atopic adults. This discrepancy between the two age groups is unlikely to be caused by a different mechanism underlying CMA in children and adults, because two of the four adult CMA subjects carrying the DQB1*0501 allele had become allergic before the age of three years. These subjects could therefore be considered comparable to children with persistent CMA. Moreover, a difference in age does not influence the results of genotyping. Although our subject groups were relatively small, these data suggest that CMA is not significantly associated with certain HLA genotypes. The previously reported positive correlation of CMA with HLA-DQB1*0301/0304 (DQ7) could not be confirmed in our study.[8] This association was found by determining population frequencies of HLA-DQ genotypes. In contrast, allele frequencies of HLA-DQB1 genotypes were calculated in the present study, which correct for homo- or heterozygosity. Although a slightly higher DQB1*0301 allele frequency was present in children with CMA compared to RPI ($p = 0.07$), this effect was completely absent in adults with CMA.

The protein fraction in cow's milk is comprised of at least six different antigens, which are all potentially allergenic in individuals with CMA.[14] Like αs1-casein, the other milk proteins probably contain several different T cell epitopes, which can be assumed to be presented by a broad range of HLA-types. In this light, the influence of a single DQB1 haplotype on the maintenance of tolerance towards cow's milk can be expected to be moderate. Therefore, a significant association with certain HLA genotypes is less likely for CMA than for allergies that are caused by proteins containing few T cell epitopes, such as the major mugwort pollen allergen.[7]

Thinking of potential therapeutic approaches, it could be considered to induce tolerance towards αs1-casein in subjects with CMA by immunotherapy (IT) with short peptides (12-18 AA residues in length). Peptide IT has been successfully applied in subjects allergic to bee venom and cat allergens.[27,28] To develop cow's milk-specific peptide IT, it is necessary to identify the epitopes

recognized by T cells of subjects with CMA, and to assess which of these epitopes are suitable for treating a population of allergic subjects with different HLA genotypes. In our previous work and in the present study, we showed that T cells from children with CMA most frequently recognized αs1-casein epitopes in the region spanned by AA residues 133-168.[16] This sequence contained various T cell epitopes that were presented by several HLA genotypes (Fig. 2, Table 1), a.o. DQB1*0201 (AA 133-144, AA 139-150), DPB1*0401 (AA 139-156), and DRB1*1501 (AA 151-162). These genotypes were abundantly present in children with CMA (23.5%, 38.2%, and 14.7%, resp., data not shown), as well as in the normal population.[24,29] Therefore, peptides spanning the αs1-casein AA residues 133-168 seem promising for application of peptide IT in the majority of subjects with CMA. A combination of peptides spanning the most immunogenic sequences of αs1-casein and other important milk allergens, like β-lactoglobulin, may well be effective in inducing tolerance to cow's milk in CMA patients.[13]

In conclusion, we found that T cell epitopes in αs1-casein, a major allergen in cow's milk, were predominantly restricted to HLA-DQ. However, no major differences could be found in allele frequencies of HLA genotypes between subjects with CMA, atopic individuals without CMA, and non-atopic subjects. The sequence spanned by AA residues 133-168 in αs1-casein is highly immunogenic to T cells from allergic children. Epitopes in this region are presented by various HLA genotypes, which are likely to be abundantly present in subjects with CMA in general. This sequence may therefore be a suitable target for peptide IT.

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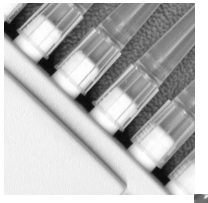
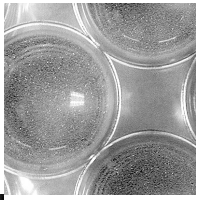
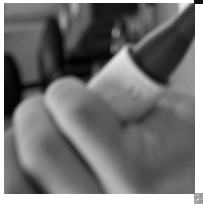
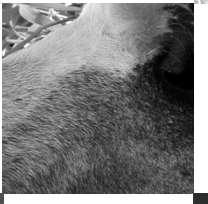

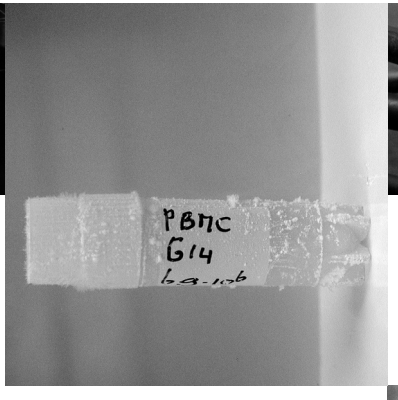
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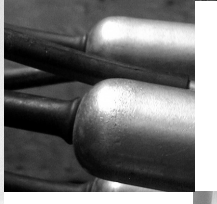
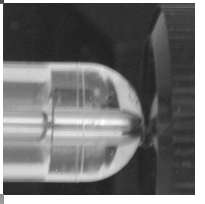
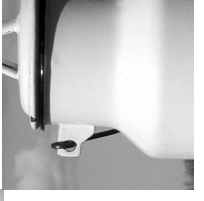
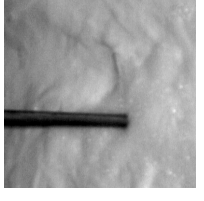

[4] Cow's milk-specific mononuclear cell responses in cow's milk allergic, sensitized and non-atopic adults

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ABSTRACT

BACKGROUND. Cow's milk allergy (CMA) is less frequently diagnosed in adults than in infants and children. The majority of adult patients develop CMA in adult life. Little is known about the specific immune response to cow's milk protein (CMP) in adults with CMA and tolerant individuals.

OBJECTIVE. To compare CMP-specific PBMC responses and levels of specific IgE and IgG4 between adult patients with CMA, subjects sensitized to cow's milk but without clinical symptoms of CMA, and non-atopic individuals.

METHODS. PBMCs and plasma were derived from 14 adults with CMA, 10 sensitized subjects without CMA, and 14 non-atopic adults. Proliferation and cytokine responses (IL-13, IFN-γ, TNF-α, IL-10, TGF-β) were determined in PBMC cultures upon stimulation with whole CMP and the six most abundant individual CMPs. CMP-specific IgE and IgG4 were measured in plasma.

RESULTS. CMP-specific IgE was highest in subjects with CMA and higher for casein than for whey proteins. Specific IgG4 was highest in sensitized adults without CMA. Production of IL-13 in CMP-stimulated PBMCs and the ratio of IL-13/IFN-γ was higher in subjects with CMA than in non-atopic individuals. CMP-induced production of IL-10 and TNF-α were higher in sensitized subjects without CMA than in non-atopic adults. Casein proteins predominantly induced IL-13 responses, whereas whey proteins induced mainly IFN-γ, TNF-α and IL-10.

CONCLUSION. CMA in adults is characterized by specific Th2 responses. Differences in specific IgE for casein and whey proteins are reflected in the specific PBMC responses. CMP-induced IL-10 may play a role in tolerance to cow's milk in sensitized subjects without CMA, by counter-regulating specific IgE and IgG4.

INTRODUCTION

Cow's milk allergy (CMA) is mainly observed in infants and young children. It affects about 2% of all infants and is the most common food allergy in this age group.[1,2] Most infants with CMA develop clinical tolerance to cow's milk before the age of 3 years. However, CMA persists after 8 years of age in approximately 15%.[3] CMA is less common in adults, affecting about 0.3% of the adult population.[4] In the majority of adult patients, the onset of CMA is in adult life, which indicates that the number of subjects with persisting youth-onset CMA is relatively low.[5]

Current knowledge on the aberrant immune response associated with CMA is mainly based on studies in children. Cow's milk protein (CMP)-specific proliferation of peripheral blood mononuclear cells (PBMCs) was observed to be higher in subjects with CMA than in children tolerant to cow's milk.[6-8] Moreover, previous studies from our group demonstrated that CMP-specific T cell clones (TCCs), derived from children with CMA, produced more IL-4 and IL-13 upon antigen-specific stimulation than TCCs from tolerant children.[6,9] These findings were substantiated by the examination of cell-surface markers on the TCCs, which indicated that CMP-specific T cells from children with CMA are highly activated and Th2-skewed.[9,10] Interestingly, clinical tolerance to cow's milk in atopic children, who have a diagnosed allergy but no CMA, was found to be characterized by increased production of IL-10 by CMP-specific TCCs.[9]

So far, the CMP-specific cellular immune response has scarcely been investigated in adults. PBMC responses to cow's milk casein were previously found to be increased in adults in which atopic dermatitis (AD) worsened after milk ingestion, compared to patients with non-milk-responsive AD.[11,12] A study conducted within our group demonstrated that the CMP-specific T cell response was equally suppressed by $CD4^+CD25^+$ regulatory T cells in adult subjects with CMA and healthy controls, indicating that regulatory T cells were not functionally impaired in adults with CMA.[13] Previously, we observed that CMP-specific IgG4 levels were found to be higher in atopic adults without CMA than in non-atopic subjects, whereas specific IgA tended to be lower in cow's milk allergic than in non-atopic individuals (manuscript in press). Antigen-specific production of IgG4 and IgA has been demonstrated to be enhanced by the immunoregulatory cytokines IL-10 and TGF-β, respectively.[14,15] Hence, we hypothesized that these cytokines may influence CMP-specific immunoglobulin (Ig) responses. The present study is the first to investigate cellular CMP-specific proliferation and cytokine responses in adult patients with CMA. PBMC responses were studied to whole CMP, as well as to the six most abundant individual allergens in cow's milk.[16] These were compared with PBMC responses in atopic adults who were sensitized to CMP, but were clinically tolerant to cow's milk. In addition, a control group of non-atopic subjects was included.

METHODS

SUBJECTS. Thirty-eight adult subjects were included in this study, divided into three groups. The first group comprised 14 subjects with CMA (age: 26-68 years, median 41), of whom four individuals had early-onset CMA (< 3 years of age), and 10 subjects had developed CMA after the age of 16 years. The second group consisted of 10 subjects who were sensitized to cow's milk, but without CMA (age: 23-54 years, median 41). The third group comprised 14 non-atopic adults (age: 23-59 years, median 28). The clinical characteristics of the individual patients with CMA and the sensitized subjects without CMA, as well as pooled data of the non-atopic individuals, are shown in Table 1. CMA was diagnosed by a positive skin prick test (SPT) with CMP and/or positive serum IgE levels specific for CMP, as determined by CAP System FEIA (Pharmacia Diagnostics, Uppsala, Sweden), and was confirmed by a positive double-blind placebo-controlled food challenge (DBPCFC). SPT was measured according to Aas and Belin.[17] All subjects with CMA had immediate symptoms during DBPCFC, and had a cow's milk elimination diet. Adults in the second group all had a history of atopic disease (atopic dermatitis, asthma and/or rhinoconjunctivitis), and were included on the basis of a history of sensitization to CMP (positive SPT and/or positive specific serum IgE levels). Three subjects were no longer sensitized at the time of blood sampling for the current study.
All (previously) sensitized sensitized subjects in this group included cow's milk and related products in their diet. The non-atopic subjects had no elevated serum IgE levels and no clinical or family history of allergy or atopy. After informed consent was obtained, a heparinized venous blood sample was taken. Plasma was separated by centrifugation, and PBMCs were isolated by density gradient centrifugation with Ficoll (Amersham, Uppsala, Sweden) and cryopreserved until use. The study was approved by the Medical Ethical Committee of the University Medical Center, Utrecht.

DETERMINATION OF CMP-SPECIFIC IG LEVELS. Plasma IgE and IgG4 levels specific to CMP were determined using ELISA. Whole CMP (kindly provided by R. Floris, PhD, NIZO Food Research, Ede, the Netherlands) was coated in duplicate at 20 μg/ml (1 μg/well) in PBS in 96-well microtiter plates (Nunc Maxisorp, Roskilde, Denmark), and incubated overnight at 4°C. In addition, duplicate wells were left uncoated to determine background levels of Ig binding. All subsequent incubations were performed at 37°C. Plates were washed with PBS containing 0.05% Tween 20 (PBS-T) and blocked with 2% HSA in PBS-T for 1 hour. Subsequently, plates were emptied and diluted plasma samples were added. Plasma dilutions were made in PBS-T + 0.5% HSA and ranged from 1:5-1:250 (IgE) and 1:5-1:500 (IgG4). High optical density (OD) values were avoided, in order to remain in the slope area of quantitative assessment. Plates were incubated for 1 hour. After washing, horseradish peroxidase (HRP)-conjugated goat-anti-human IgE (KPL, Gaithersburg, MD, USA) or HRP-conjugated mouse-anti-human IgG4 (Sanquin, Amsterdam, the Netherlands) was added in 1:10.000 and 1:30.000 dilution, resp. Plates were incubated for 1 hour and washed. All plates were developed using TMB peroxidase substrate (KPL), which was

Table 1. Characteristics of the included subjects

Subjects	Age (yr) (median)	Gender	Onset CMA (yr)	SPT with CMP	CAP CMP (kU/l)	CAP cas (kU/l)	CAP α-lac (kU/l)	CAP β-lac (kU/l)
CMA *								
1	28	F	0-3	+++	> 100	> 100	37.0	40.0
2	26	M	0-3	++	< 0.35	< 0.35	< 0.35	< 0.35
3	40	F	0-3	+++	1.3	< 0.35	< 0.35	0.5
4	42	M	0-3	+++	84.0	87.0	17.5	24.6
5	41	F	>16	-	< 0.35	< 0.35	< 0.35	< 0.35
6	45	F	>16	++	5.6	9.1	0.4	0.8
7	68	F	>16	++	37.0	43.0	< 0.35	< 0.35
8	65	F	>16	++	2.0	2.2	< 0.35	< 0.35
9	58	M	>16	+++	4.5	7.8	< 0.35	< 0.35
10	27	F	>16	-	22.3	3.8	< 0.35	37.0
11	38	F	>16	+++	11.3	12.4	< 0.35	4.6
12	39	F	>16	-	< 0.35	< 0.35	< 0.35	< 0.35
13	41	F	>16	+++	> 100	> 100	> 100	> 100
14	32	F	>16	++	2.1	2.3	< 0.35	< 0.35
Sensitized								
15	23	F	-	++	< 0.35	< 0.35	< 0.35	< 0.35
16	54	M	-	++	7.2	< 0.35	< 0.35	< 0.35
17	36	M	-	-	0.4	< 0.35	< 0.35	0.5
18	42	M	-	-	15.1	0.7	0.5	0.4
19	48	F	-	++	6.5	9.2	0.4	0.5
20	54	F	-	-	< 0.35	< 0.35	< 0.35	< 0.35
21	38	F	-	-	< 0.35	< 0.35	< 0.35	< 0.35
22	27	F	-	-	< 0.35	< 0.35	< 0.35	< 0.35
23	53	M	-	-	5.6	1.2	6.5	< 0.35
24	39	M	-	-	7.8	1.4	1.6	3.4
Non-atopic								
25 - 38	23 - 59 (28)	9F/5M	-	-	< 0.35	-	-	-

* All subjects with CMA had immediate symptoms during DBPCFC.

incubated for 10 minutes at RT before the reaction was stopped with 1M H_3PO_4 and OD-values were measured at 450 nm.

CMP-SPECIFIC PROLIFERATION AND CYTOKINE RELEASE. After thawing, PBMCs were cultured in triplicate (2 x 10^5 cells/well) for 6 days in 96-well U-bottom culture plates (Greiner, Frickenhausen, Germany) in serum-free Ultra Culture medium (Cambrex, Verviers, Belgium), supplemented with penicillin (100 IU/ml), streptomycin (100 mg/ml), and glutamine (1 mM) (Gibco, New York, NY, USA). Culturing was performed in the absence or presence of whole CMP, or the individual CMPs αs1-casein, αs2-casein, β-casein, κ-casein, α-lactalbumin, and β-lactoglobulin, at a concentration of 50 μg/ml (NIZO Food Research, Ede, the Netherlands). The CMPs

were > 95% pure, as determined by reversed-phase HPLC. After 6 days of culture, supernatants were collected and stored at -20°C for cytokine measurements. Tritiated thymidine ([^{3}H]-TdR, 1 μCi/well, Amersham, Aylesbury, UK) was added to the cultures, and the cells were harvested after 18 hours. Proliferation was determined by measuring incorporation of [^{3}H]-TdR, using a 1205 betaplate counter (Wallac, Turku, Finland). Cytokines were measured by means of ELISA, according to the manufacturer's recommendations (IL-10, IL-13, IFN-γ and TNF-α: Sanquin, Amsterdam, the Netherlands; TGF-β: R&D Systems, Minneapolis, MN, USA). The detection limit was 2.3 pg/ml for IL-10, 1.2 pg/ml for IL-13, 3.1 pg/ml for IFN-γ, 3.1 pg/ml for TNF-α, and 15.6 pg/ml for TGF-β.

DATA ANALYSIS AND STATISTICS. The nonparametric Wilcoxon signed ranks test was used to assess significant differences between nonspecific and antigen-specific proliferation and cytokine production in the PBMC cultures. The nonparametric Mann-Whitney U test was applied to determine significant differences between the subject groups for PBMC responses and specific Ig levels. P-values < 0.05 were considered significant. For the Ig ELISA, the OD values in CMP-coated wells were corrected for those in non-coated wells and compared with standard curves, which were generated with a pool plasma containing high levels of CMP-specific IgE and IgG4. Specific IgE and IgG4 levels were determined in this poolplasma by RAST, as described previously.[18] Quantitative data could therefore be obtained for CMP-specific IgE (ng/ml) and IgG4 (μg/ml). Subsequently, data were multiplied by the plasma dilution to calculate absolute specific Ig levels per subject.

RESULTS

CMP-SPECIFIC IGE AND IGG4 LEVELS. Plasma and PBMCs were obtained from 14 adults with CMA, 10 atopic adults who were sensitized to cow's milk but without clinical symptoms of CMA, and 14 non-atopic individuals (Table 1). Levels of CMP-specific IgE and IgG4 were determined in plasma by ELISA. As expected, CMP-specific IgE in subjects with CMA was higher than in non-atopic individuals, and tended to be higher than in sensitized individuals without CMA ($p = 0.07$) (Fig. 1). On the contrary, specific IgG4 was highest in sensitized subjects without CMA and tended to be higher than the IgG4 levels in non-atopic individuals ($p = 0.07$). The ratio of specific IgE/IgG4 was higher in patients with CMA (median 0.34×10^{-3}, range $0.00 - 8.89 \times 10^{-3}$) than in sensitized subjects without v (median 0.02×10^{-3}, range $0.00 - 0.62 \times 10^{-3}$; $p < 0.05$). At the time of diagnosis, specific IgE was determined by CAP-FEIA for whole CMP, as well as for whole casein, α-lactalbumin and β-lactoglobulin. In subjects with CMA, specific IgE was higher for casein than for α-lactalbumin ($p < 0.01$) and β-lactoglobulin ($p < 0.05$) (Table 1).

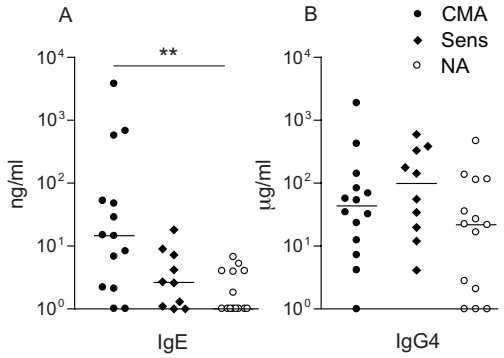

Figure 1.
Levels of CMP-specific IgE (**A**) and IgG4 (**B**) in plasma of 14 subjects with CMP (CMP), 10 atopic subjects sensitized to cow's milk but without clinical symptoms of CMP (Sens), and 14 non-atopic individuals (NA). Significant differences are indicated in the graph (** $p < 0.01$).

CMP-SPECIFIC PROLIFERATION AND CYTOKINE PRODUCTION IN PBMCS. PBMCs were cultured in the presence or absence of CMP and proliferation, as well as cytokine production was determined. In general, levels of proliferation and production of particularly IL-13 and TGF-β in unstimulated PBMCs were relatively high in the culturing conditions used. CMP-specific proliferation of PBMCs was higher than nonspecific proliferation in all three groups, and tended to be higher in subjects with CMA, compared to non-atopic individuals ($p = 0.09$) (Fig. 2A). Only in the CMA group, production of IL-13 upon stimulation with CMP was higher than nonspecific IL-13 release (Fig. 2B). CMP-specific production of IFN-γ seemed highest in non-atopic individuals, but levels were not significantly different between the subject groups (Fig. 2C). The ratio of IL-13/IFN-γ in CMP-stimulated PBMCs was higher in subjects with CMA (median 0.072, range 0.009-0.342) than in non-atopic subjects (median 0.016, range 0.002 - 0.114; $p < 0.01$). PBMCs from sensitized adults without CMA released more TNF-α and IL-10 upon stimulation with CMP than those from non-atopic subjects (Fig. 2D+E). Production of TGF-β in CMP-stimulated cultures was not different from nonspecific release in all groups.

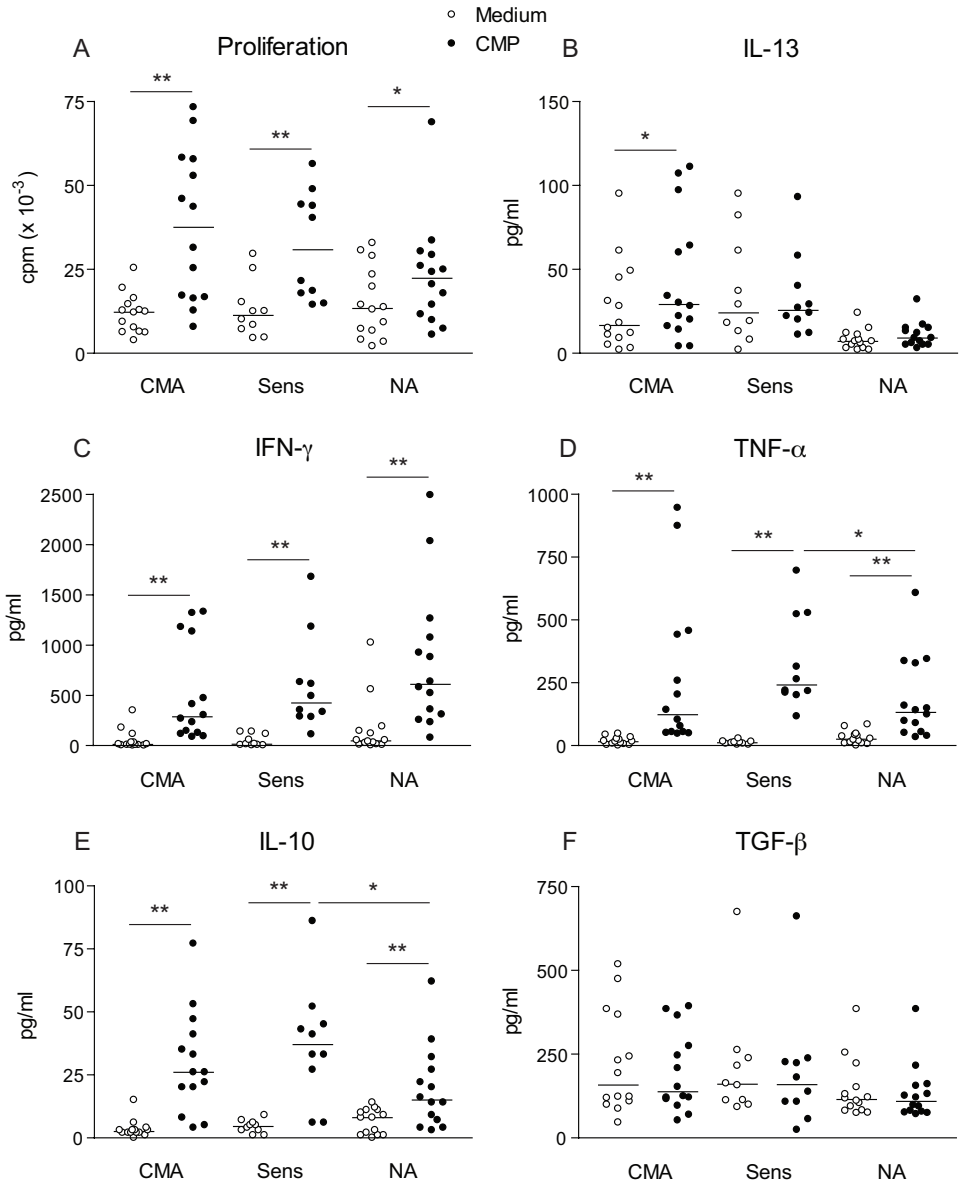

Figure 2.

Spontaneous and CMP-specific proliferation and cytokine production in PBMCs of 14 subjects with CMA (CMA), 10 atopic subjects sensitized to cow's milk but without clinical symptoms of CMA (Sens), and 14 non-atopic individuals (NA). Proliferation of PBMCs (**A**) and production of IL-13 (**B**), IFN-γ (**C**), TNF-α (**D**), IL-10 (**E**) and TGF-β (**F**) are shown in unstimulated cultures (Medium) and in cultures stimulated with CMP (CMP). Individual data and median levels are shown. Significant differences are indicated in the graphs (* $p < 0.05$, ** $p < 0.01$).

PBMC RESPONSES TO THE INDIVIDUAL CMPS. PBMCs of a selection of subjects from each group were used to investigate proliferative and cytokine responses to the six most abundant individual CMPs: αs1-, αs2, β- and κ-casein, α-lactalbumin and β-lactoglobulin.[16] No differences were observed in the recognition per se of the individual CMPs between patients with CMA and tolerant subjects. Therefore, data are only shown for the CMA group, in which responses to the individual CMPs were determined in nine subjects (Fig. 3). Stimulation with αs2-casein, β-casein, or β-lactoglobulin induced higher proliferation than background levels (Fig. 3A). Production of IL-13 upon stimulation with αs2-, β-, and κ-casein was higher than non-specific release (Fig. 3B). In contrast, production of IFN-γ, TNF-α and IL-10 was most pronounced in response to α-lactalbumin and to a lesser extent β-lactoglobulin (Fig. 3C-E).

DISCUSSION

CMA is less frequently diagnosed in adults than in young children. Interestingly, the majority of affected adults were previously observed to develop CMA in adult life, which was confirmed in our study population (Table 1).[5] The difference in age of onset between infants and adults with CMA may point towards a different causative immunological mechanism. However, to date the CMP-specific cellular immune response has not been investigated in adults with IgE-mediated CMA.

In the present study, PBMC responses to CMP were compared between adult subjects with CMA, atopic adults sensitized to cow's milk but without clinical symptoms of CMA, and non-atopic adults. Only PBMCs from subjects with CMA produced higher levels of IL-13 in response to CMP than in unstimulated cultures. Moreover, the ratio of IL-13/IFN-γ in CMP-stimulated PBMCs was significantly higher in patients with CMA than in non-atopic adults, which indicated a Th2-skewed response in CMA and a Th1-polarized response in non-atopic subjects. These findings underscore the importance of CMP-induced Th2 responses in CMA, as was previously shown in infants and children with CMA by our group,[6,9] and recently by other investigators.[19]

Previously, it was demonstrated that PBMCs from infants with mainly non-IgE-mediated CMA produced relatively high levels of TNF-α. The increased amounts of TNF-α were shown to decrease the barrier capacity of intestinal epithelial cells, and were therefore hypothesized to be an important factor in the intestinal symptoms of CMA.[20] However, CMP-specific production of TNF-α in our adult group was not different between adults with CMA and non-atopic subjects. Therefore, our data may suggest that CMP-specific induction of TNF-α does not play a major role in the pathogenesis of IgE-mediated CMA in adults.

In sensitized adults without CMA, the CMP-specific production of both TNF-α and IL-10 was significantly higher than in non-atopic subjects. Release of TNF-α is mainly associated with proinflammatory and Th1 responses, whereas IL-10 is a regulatory cytokine.[14,21] CMP-specific production of these cytokines appeared to be correlated, as was also observed in the cytokine response to the whey proteins. In sensitized subjects without CMA, the upregulation of TNF-α

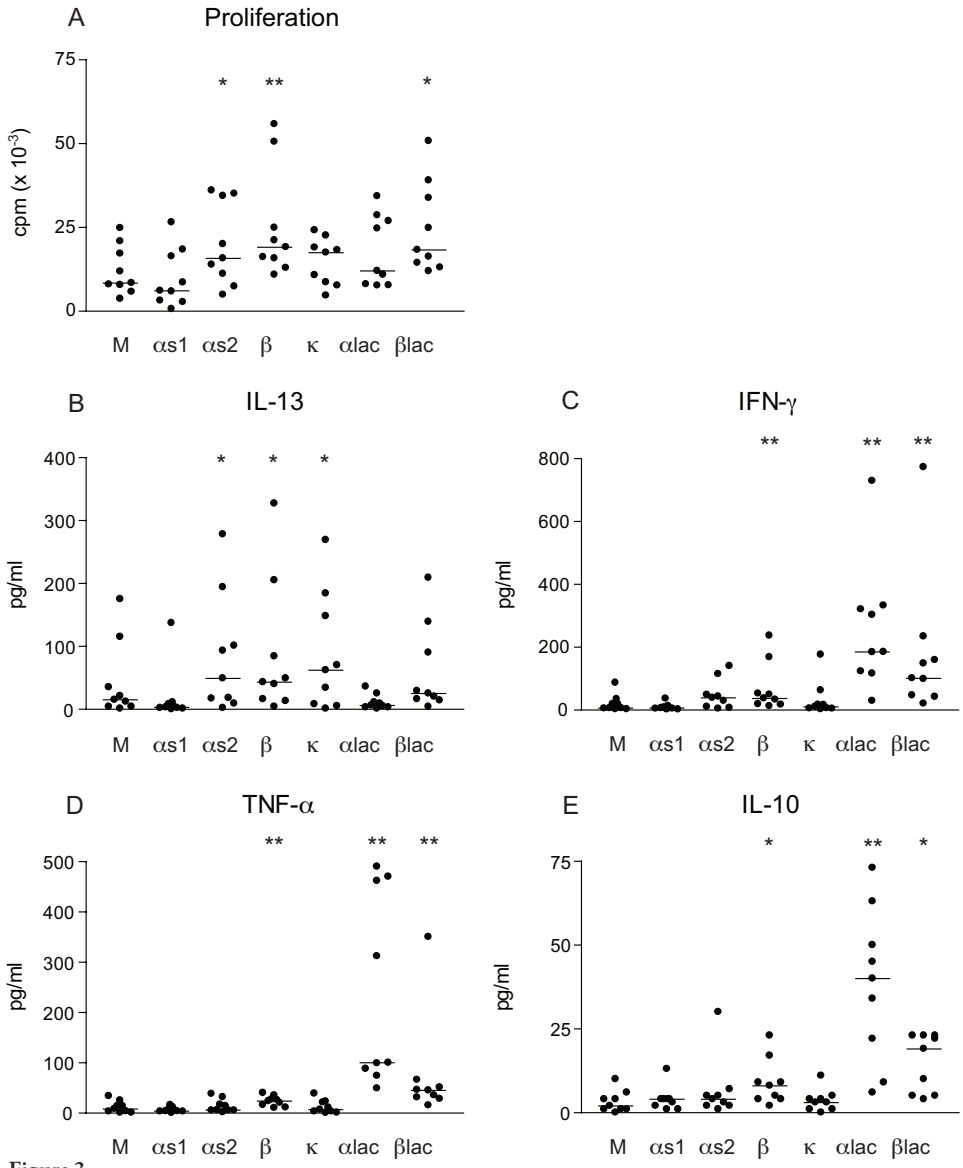

Figure 3.

Proliferation and cytokine production of PBMCs in response to the six most abundant individual CMPs. PBMCs from nine subjects with CMA were assessed for proliferation and cytokine production in unstimulated cultures (M), as well as in cultures stimulated with αs1-casein (αs1), αs2-casein (αs2), β-casein (β), κ-casein (κ), α-lactalbumin (αlac), or β-lactoglobulin (βlac). Individual data and median levels of proliferation (**A**), IL-13 (**B**), IFN-γ (**C**), TNF-α (**D**), and IL-10 (**E**) are shown. Specific proliferative and cytokine responses that were significantly higher than responses in unstimulated cultures are marked in the graphs (* $p < 0.05$, ** $p < 0.01$).

and IL-10 may be needed to balance the CMP-specific immune response in an atopic, Th2-skewed environment. The higher CMP-specific production of IL-10 in sensitized subjects without CMA, as compared to non-atopic individuals, is in line with a previous study from our group.[9] In this study, production of IL-10 was demonstrated to be higher in CMP-stimulated TCCs of atopic children without CMA, as compared to non-atopic children. Nevertheless, it should be noted that the sensitized adults in the present study differed from the atopic children with respect to the presence of CMP-specific IgE and/or a positive CMP-specific SPT. Interestingly, the IL-10 responses in PBMCs ran parallel to the CMP-specific IgG4 levels in plasma, which tended to be higher in sensitized subjects without CMA than in non-atopic subjects. Previous studies demonstrated that allergen-specific IL-10 responses and IgG4 are upregulated after specific immunotherapy, and that IL-10 directly increases production of specific IgG4 and decreased IgE synthesis by allergen-specific B cells.[14,22] The increased CMP-specific IL-10 response in sensitized adults without CMA compared to non-atopic adults may have a similar tolerogenic function.
High production of TGF-β was observed in unstimulated PBMCs in all three groups, which is in line with previously published observations.[23] However, no CMP-specific release of TGF-β could be detected in any group. Previously, a general reduction of TGF-β was observed in the intestine of children with predominantly non-IgE-mediated CMA.[24] This may indicate that local constitutive production of TGF-β is more important in the modulation of potentially allergenic CMP-specific immune responses than peripheral spontaneous or CMP-stimulated TGF-β release.
In addition to whole CMP, also the six most abundant individual CMPs were tested for the induction of PBMC responses. Interestingly, strikingly different responses were observed to the individual CMPs, particularly with regard to cytokine production. Whereas the casein proteins αs2-, β-, and κ-casein induced significant IL-13 responses, the whey proteins β-lactoglobulin and in particular α-lactalbumin induced high levels of IFN-γ, TNF-α and IL-10. These data suggest that even within a potentially allergenic food protein mixture, the individual allergens can induce widely varying immune responses. The pronounced Th2 response induced by three of the four casein proteins, versus the more Th1-skewed response towards the whey proteins, is in accordance with the observation that specific IgE in the subjects with CMA was significantly higher for casein than for each of the two whey proteins. A comparable predominantly casein-specific IgE response in adults with CMA was reported previously.[5] Remarkably, PBMC responses to αs1-casein were generally very low, whereas αs1-casein-specific T cell responses could well be detected by means of CMP-specific T cell lines and TCCs in our previous studies.[9,25] Moreover, we observed that αs1-casein induced the highest IgE response of all individual CMPs in the adults with CMA described in this study (manuscript in press). An explanation for the discrepancy between αs1-casein-specific responses in PBMCs and specific IgE levels in plasma may be that low T cell stimulation by αs1-casein could enhance the specific IgE response. Previously, it was observed that T cells with low T cell receptor affinity develop predominantly into Th2 cells.[26] One could speculate that T cells specific for αs1-casein may also have relatively low T cell receptor affinities. This may result in a low T cell response upon encounter of antigen, which could hamper detec-

tion in unselected mononuclear cells. Yet, the low affinity may enhance Th2 polarization, and may render these T cells particularly capable of inducing αs1-casein-specific IgE responses. The present study is the first to compare CMP-specific PBMC responses between adult subjects with CMA and tolerant individuals. CMP-specific Th2 responses were most pronounced in the CMA group, whereas a Th1-skewed response was observed in non-atopic subjects. Casein proteins predominantly induced IL-13 responses, and whey proteins induced mainly IFN-γ, TNF-α and IL-10. Accordingly, specific IgE was higher for casein than for the whey proteins in CMA. The CMP-specific immune response in adult subjects sensitized to cow's milk but without clinical symptoms of CMA appears to be modulated by upregulation of IL-10, which may increase the concentrations of allergen-specific IgG4, and decrease IgE.

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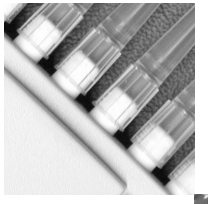
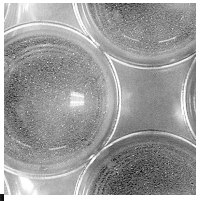
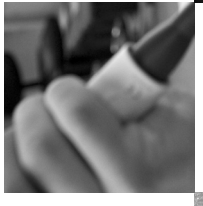
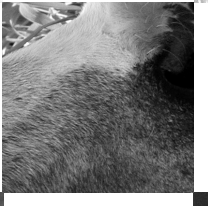
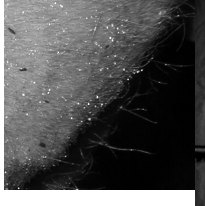

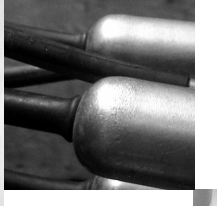
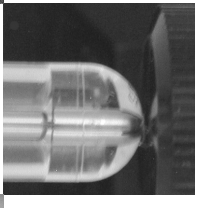
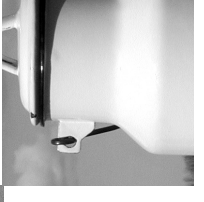
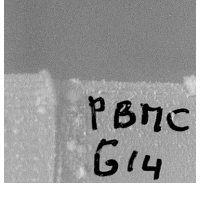
PBMC
G14

[5] Maintenance of tolerance to cow's milk in atopic individuals is characterized by high levels of specific IgG4

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ABSTRACT

BACKGROUND. The central role of specific IgE in cow's milk allergy (CMA) is well documented. However, less is known about the function of other immunoglobulin isotypes in allergy and tolerance to cow's milk proteins (CMPs).
OBJECTIVE. To determine differences in the antibody responses that are associated with allergy and tolerance to cow's milk in allergic, atopic and non-atopic individuals of different age groups.
METHODS. Nineteen infants (< 1 year), 18 children (6-14 years) and 41 adults (21-68 years) were included. Each age group was comprised of subjects with CMA, atopic individuals without a history of CMA, and non-atopic subjects. Levels of specific IgE, IgG4, IgG1 and IgA to whole cow's milk and the six most abundant individual CMPs were determined in plasma by ELISA. For comparison, specific IgE and IgG4 were measured to ovomucoid and house dust mite in individuals allergic for the respective allergens, and in atopic and non-atopic subjects without allergy.
RESULTS. In infants and children with CMA, αs1-casein and β-lactoglobulin induced the highest specific IgE response, whereas αs1-casein was the most allergenic CMP in adult patients. Specific IgG4 and IgG1 responses were highest to αs1-casein and β-lactoglobulin in all age groups, while κ-casein and α-lactalbumin induced the highest levels of IgA. CMP-specific IgG4 was higher in atopic children and adults without CMA, as compared to non-atopic individuals. A similar difference between atopic and non-atopic subjects was observed for IgG4 specific to ovomucoid, whereas house dust mite-specific IgG4 was not detectable in these subjects.
CONCLUSION. Maintenance of tolerance to cow's milk in atopic children and adults without CMA is associated with elevated levels of specific IgG4, in combination with low specific IgE. The upregulation of specific IgG4 in tolerant atopic individuals may be related to the type of allergen and its regular dose of exposure.

INTRODUCTION

Cow's milk allergy (CMA) is the most common food allergy in young children, affecting about 2% of all infants.[1] Most children develop clinical tolerance to cow's milk before the age of 3 years. However, CMA persists after 8 years of age in approximately 15%.[2] CMA is less common in adults than in children, and is rarely documented.[3]

The majority of clinical symptoms of food allergy is caused by food-specific IgE antibodies.[4,5] Although the central role of IgE is well investigated, little is known about the function of other immunoglobulin (Ig) isotypes in allergy and tolerance to food proteins. For certain respiratory and insect venom allergies, immunological changes have been shown in allergic individuals who became tolerant after allergen-specific immunotherapy (IT). For example, IT with bee venom resulted in an increase in specific IgG4 and a decrease in specific IgE.[6] The decrease in specific IgE/IgG4 ratio was shown to be mediated by an increase in allergen-induced IL-10, which is involved in the counter-regulation of these Ig responses.[7] Correspondingly, birch pollen IT induced an increase in IgG4 specific to the major birch pollen allergen, as well as the crossreactive apple allergen.[8] Food allergens differ from respiratory and venom allergens with respect to the route of entry and the dose of exposure, which is generally higher and may increase the levels of food allergen-specific IgG4.[9,10] Although specific IgG4 appears to play a role in the induction of tolerance by allergen-specific IT, conflicting data have been reported for the role of IgG4 in non-IgE-mediated intolerance to cow's milk proteins (CMPs).[11,12] Hence, it would be of interest to compare CMP-specific IgG4 responses between subjects with IgE-mediated CMA and tolerant individuals. In addition, CMP-specific IgG1 and IgA responses may be informative, as specific Igs of these isotypes are produced in both food-allergic and non-allergic subjects.[13] Total serum IgA concentrations have been observed to be lower in atopic than in non-atopic infants.[14] This difference may be reflected in the levels of food-specific IgA. Regarding specific IgG1 responses, no consistent differences have yet been reported between food-allergic and tolerant subjects.

Previously, our group has demonstrated that clinical tolerance to cow's milk in atopic children, who have a diagnosed allergy but no CMA, is characterized by increased production of IL-10 by CMP-specific T cells.[15] In analogy with its effect in IT, elevated IL-10 may well result in increased levels of CMP-specific IgG4, which prompted us to compare Ig responses of atopic subjects without CMA with those of cow's milk allergic and non-atopic subjects.

The very young age of exposure to cow's milk enables comparison of specific immune responses between a wide range of age groups. In the present study, plasma levels of IgE, IgG4, IgG1 and IgA specific to whole CMP, as well as the six most important purified cow's milk allergens,[16] were determined in infants, children and adults. Our aim was to study differences in the Ig responses that are associated with allergy and tolerance to cow's milk in allergic, atopic and non-atopic individuals.

METHODS

SUBJECTS. Nineteen infants (age 0.4-1.0 years), 18 children (6-14 years) and 41 adults (21-68 years) were included in this study. After informed consent was obtained, a heparinized venous blood sample was taken and plasma was separated from the cellular fraction by centrifugation. All three age groups were comprised of subjects with CMA, atopic individuals without a history of CMA, and non-atopic subjects. The clinical characteristics of all individual subjects with CMA, and the pooled data of the atopic and non-atopic individuals, are shown in Table 1. The group of infants with CMA included two subjects with persistent CMA and six with transient CMA. The infants with persistent CMA were still allergic by the age of six years, whereas the subjects with transient CMA became tolerant to cow's milk before the age of four years. Of the 15 adults with CMA, four subjects had early-onset CMA (< 3 years of age), whereas 11 adults developed CMA after the age of 16 years.

CMA was diagnosed by a positive skin prick test (SPT) and/or positive serum IgE levels specific for cow's milk as determined by RAST (infants) or CAP System FEIA (children and adults) (Pharmacia Diagnostics, Uppsala, Sweden), and was confirmed by a positive double-blind placebo-controlled food challenge (DBPCFC). SPT was measured according to Aas and Belin.[17] All subjects with CMA had immediate symptoms (angioedema and/or urticaria in infants, and oral allergy syndrome, urticaria, rhinoconjunctivitis, and/or dyspnoe in adults) during DBPCFC, and had a cow's milk elimination diet. The atopic subjects had a diagnosed inhalant and/or food allergy, but no history of CMA and no sensitization to cow's milk. The non-atopic individuals had no elevated serum IgE levels and no clinical or family history of allergy or atopy. The specific antibody responses in CMA were compared with those in other allergies. Allergy to hen's egg-white and house dust mite was defined by a suggestive history and positive allergen-specific serum IgE levels as determined by CAP-FEIA. The study was approved by the Medical Ethical Committee of the University Medical Center, Utrecht.

ELISA FOR DETERMINATION OF ALLERGEN-SPECIFIC IG LEVELS. Plasma IgE, IgG4, IgG1 and IgA antibody levels specific to whole CMP, αs1-casein, αs2-casein, β-casein, κ-casein, α-lactalbumin, and β-lactoglobulin (kindly provided by R. Floris, PhD, NIZO Food Research, Ede, the Netherlands) were determined using ELISA. The individual CMPs had been separated by liquid chromatography and were > 95% pure, as determined by reversed-phase HPLC. For comparison, plasma IgE and IgG4 levels specific to ovomucoid (T2011, Sigma Aldrich, Steinheim, Germany) and house dust mite extract (HAL, Haarlem, the Netherlands) were determined. All allergens were coated in duplicate at 20 μg/ml (1 μg/well) in PBS in 96-well microtiter plates (Nunc Maxisorp, Roskilde, Denmark), and incubated overnight at 4°C. In addition, duplicate wells were left uncoated to determine background levels of Ig binding. All subsequent incubations were performed at 37°C. Plates were washed with PBS containing 0.05% Tween 20 (PBS-T) and blocked with 2% HSA in PBS-T for 1 hour. Subsequently, plates were emptied and diluted plasma samples were added. Plasma dilutions were made in PBS-T + 0.5% HSA and ranged from 1:5-1:250 (IgE),

Table 1. Clinical characteristics of the included subjects.

Subject group	Subject no.	Age (yr) (median)	CMP-specific IgE (kU/l)	SPT with CMP
Infants				
CMA *	1	0.5	< 0.35	-
	2	1.0	0.7	+++
	3	0.4	< 0.35	++
	4	0.5	2.0	++
	5	0.8	36.0	+
	6	0.7	0.8	+
	7	0.8	< 0.35	++
	8	0.8	> 100	+++
Atopic	9 - 14	0.4 - 0.6 (0.6)	< 0.35	-
Non-atopic	15 - 19	0.6 - 0.8 (0.8)	< 0.35	nd
Children				
CMA *	20	14.0	44.0	++
	21	6.0	3.0	+++
	22	12.3	11.6	+++
	23	9.1	9.2	+++
	24	10.7	30.0	++
	25	7.1	3.8	++
	26	12.8	51.0	+++
Atopic	27 - 32	6.2 - 9.6 (7.7)	< 0.35	-
Non-atopic	33 - 37	6.4 - 10.1 (7.9)	< 0.35	nd
Adults				
CMA *	38	41	< 0.35	-
	39	28	> 100	+++
	40	45	5.6	++
	41	26	< 0.35	++
	42	40	1.3	+++
	43	42	84.0	+++
	44	37	> 100	++
	45	68	37.0	++
	46	65	2.0	++
	47	58	4.5	+++
	48	27	22.3	-
	49	38	11.3	+++
	50	39	< 0.35	-
	51	41	> 100	+++
	52	32	2.1	++
Atopic	53 - 62	21 - 54 (38)	< 0.35	-
Non-atopic	63 - 78	23 - 59 (30)	< 0.35	nd

* All subjects with CMA had immediate symptoms during DBPCFC.

nd = not determined

1:5-1:500 (IgG4), 1:5-1:150 (IgG1), and 1:5-1:70 (IgA). High optical density (OD) values were avoided, in order to remain in the slope area of quantitative assessment. Plates were incubated for 1 hour. After washing, horseradish peroxidase (HRP)-conjugated goat-anti-human IgE (KPL, Gaithersburg, MD, USA), HRP-conjugated mouse-anti-human IgG4 or mouse-anti-human IgG1 (Sanquin, Amsterdam, the Netherlands), or biotin-conjugated mouse-anti-human IgA (BD Pharmingen, San Diego, CA, USA) was added in 1:10.000, 1:30.000, 1:20.000 and 1:20.000 dilution, resp. Plates were incubated for 1 hour and washed. The plates with anti-IgA were subsequently incubated with streptavidin-HRP (Sanquin) in 1:15.000 for 30 min and washed. All plates were developed using TMB peroxidase substrate (KPL), which was incubated for 10 min at RT before the reaction was stopped with 1M H_3PO_4 and OD-values were measured at 450 nm.

DATA ANALYSIS AND STATISTICS. The OD values in allergen-coated wells (ranging from 0.1-2.0) were corrected for those in non-coated wells (always below 0.1) and compared with standard curves, which were generated with a pool plasma containing high levels of CMP-specific IgE, IgG4, IgG1 and IgA. Specific IgE and IgG4 levels were determined in this pool plasma by RAST as described previously.[18] Quantitative data could therefore be obtained for CMP-specific IgE (ng/ml) and IgG4 (μg/ml), whereas levels of specific IgG1 and IgA were calculated as arbitrary units (AU/ml). Subsequently, data were multiplied by the plasma dilution to calculate absolute specific Ig levels per subject. Similar methods were applied for measurement of Igs to house dust mite and ovomucoid. Levels of Igs binding to CMP, ovomucoid and house dust mite were normalized by ^{10}log-transformation, pooled and averaged per subject group. To determine the relative Ig response to the six individual CMPs, the untransformed levels of Igs bound to these proteins were added up for each Ig isotype and set at 100% for each subject. Responses to the individual proteins were calculated as a percentage of the total response to all six proteins. These percentages were pooled and averaged per protein for each subject group, and compared with the expected mean percentage of Ig-binding (100/6 = 16.7%). The two-tailed Student's t-test was used to analyse differences between groups. Nonparametric Spearman rank test was used to determine correlation using untransformed data. P-values < 0.05 were considered significant.

RESULTS

CORRELATION BETWEEN CMP-SPECIFIC IGE LEVELS MEASURED BY CAP-FEIA AND ELISA. To verify the reliability of the data obtained with ELISA, the levels of CMP-specific IgE in plasma of children and adults with CMA as measured by CAP System FEIA at diagnosis were compared to the values determined by ELISA. A strong positive correlation was observed ($r^2 = 0.92$, $p < 0.001$), and absolute values measured by the two techniques were comparable (data not shown). This indicated that the ELISA yielded reliable and quantitative data.

ALLERGENICITY AND ANTIGENICITY OF THE INDIVIDUAL CMPS . The clinical data of all subjects with CMA, atopic individuals without a history of CMA, and non-atopic subjects are shown in Table 1. Antibody responses to the individual CMPs were analyzed to determine the allergenicity (defined by binding of IgE) and antigenicity (defined by binding of IgG4, IgG1, and IgA) of each protein in the different subject groups. Levels of specific IgE for the individual CMPs were only analyzed in the subjects with CMA. Specific IgG4, IgG1 and IgA were determined in all subjects. Infants and children had similar Ig responses to the individual CMPs. Moreover, the antigenicity of these proteins was comparable in allergic, atopic and non-atopic subjects within the same age group (data not shown). Therefore, data were pooled for all infants and children (Fig. 1A), as well as for all adults (Fig. 1B). To correct for differences in absolute specific Ig levels between subjects, the Ig responses to individual CMPs were calculated as a percentage of the total response to all six proteins, per Ig isotype and per subject (see Methods). In infants and children, αs1-casein and β-lactoglobulin were the most allergenic CMPs, since these proteins induced a higher IgE response than average ($p < 0.05$ and $p < 0.01$, resp.). In adults, αs1-casein was the most allergenic protein ($p < 0.01$). Specific IgG4 was highest for β-lactoglobulin in infants and children ($p < 0.01$), and for αs1-casein and β-lactoglobulin in adults ($p < 0.01$ for both). The IgG1 response was most pronounced to αs1-casein and β-lactoglobulin in infants and children ($p < 0.01$ for both), and to αs1-casein in adults ($p < 0.01$). Interestingly, the levels of specific IgA showed a different pattern, being highest for α-lactalbumin in infants and children ($p < 0.01$), and for κ-casein and α-lactalbumin in adults ($p < 0.01$ for both). In all age groups, the response to κ-casein was remarkably lower than average for IgE and IgG4 ($p < 0.01$), but not for IgA.

CMP-SPECIFIC IG LEVELS IN THREE DIFFERENT AGE GROUPS OF COW'S MILK ALLERGIC AND TOLERANT SUBJECTS. Relative specific Ig responses to the individual CMPs were comparable between subjects of the same age. Therefore, whole CMP (a mix containing equal amounts of the six individual CMPs) was used in the ELISA to study differences in the absolute levels of specific IgE, IgG4, IgG1 and IgA between subjects with CMA, atopic subjects without a history of CMA, and non-atopic individuals. CMP-specific IgE in infants with CMA was not distinctly higher than in atopic and non-atopic infants (Fig. 2A). However, two infants within the CMA group that would become persistently allergic had higher specific IgE than the other six infants who would outgrow CMA (data not shown). In children and adults, specific IgE was highest in subjects with CMA (Fig. 2B+C). Levels of CMP-specific IgE in children and adults with CMA correlated significantly with specific IgG4 ($r^2 = 0.62$, $p < 0.001$) and IgG1 ($r^2 = 0.56$, $p < 0.001$) (data not shown). Interestingly, CMP-specific IgG4 tended to be higher in atopic children without a history of CMA than in non-atopic children, and was significantly higher in atopic than in non-atopic adults ($p < 0.05$). Although specific IgG1 was higher in children with CMA than in atopic and non-atopic children, no consistent differences were found between any other subject groups of the same age. In general, CMP-specific IgE and IgG1 were observed to decrease with age. On the contrary, specific IgG4 increased with age in young atopic children with and without CMA, but not in non-

atopic children. Levels of specific IgG4 were significantly higher in atopic children than in atopic infants ($p < 0.01$) and slightly higher in children with CMA than in infants with CMA ($p = 0.06$). After childhood, no further increase in specific IgG4 was found. Levels of specific IgA remained constant with age and showed no significant differences between the subject groups.

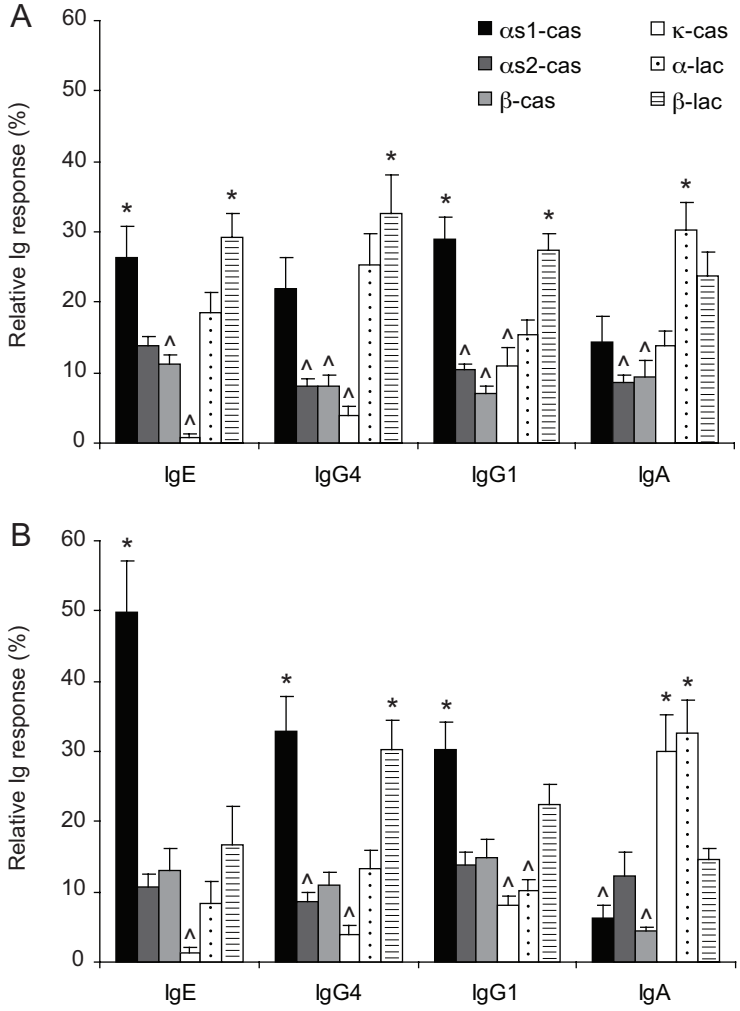

Figure 1.
Allergenicity and antigenicity of the individual CMPs in infants and children (**A**) and adults (**B**). Levels of specific IgE to the six most abundant CMPs were determined by ELISA in all subjects with CMA (8 infants + 7 children, and 15 adults), and specific IgG4, IgG1 and IgA were measured in the entire subject group (19 infants + 18 children, and 41 adults). To correct for differences in absolute Ig levels between subjects, relative values were calculated (see Methods). * $p < 0.05$ (higher than average), ^ $p < 0.05$ (lower than average).

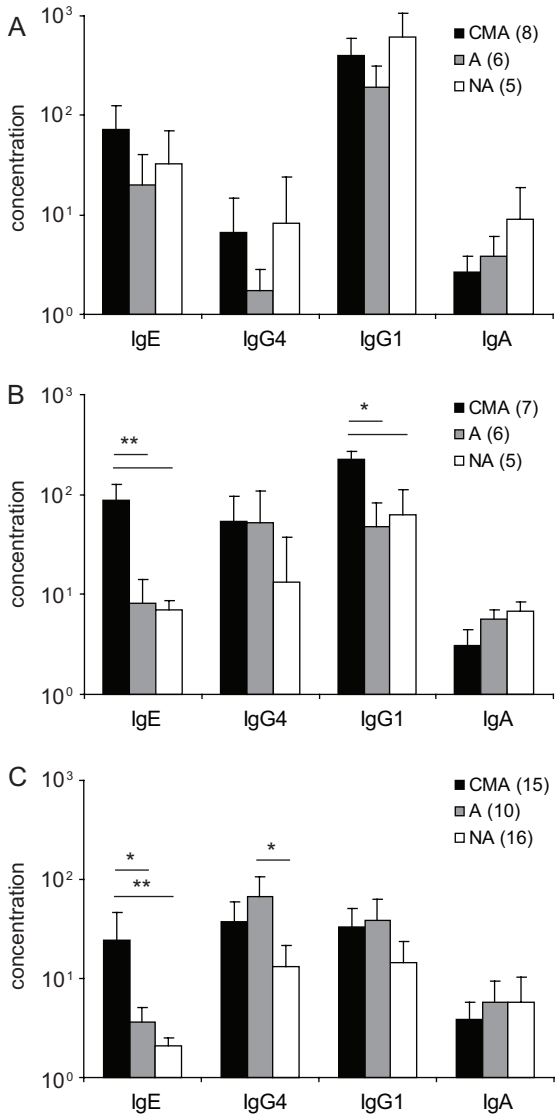

Figure 2.

CMP-specific Ig levels in three different age groups of cow's milk allergic, atopic and non-atopic subjects. Levels of specific IgE (ng/ml), IgG4 (μg/ml), IgG1 (AU/ml) and IgA (AU/ml) to CMP were measured by ELISA in infants (**A**), children (**B**), and adults (**C**). Each age group was comprised of subjects with CMA (CMA), atopic individuals without a history of CMA (A), and non-atopic subjects (NA). Numbers of subjects per group are between brackets. * $p < 0.05$, ** $p < 0.01$.

SPECIFIC IGE AND IGG4 FOR OVOMUCOID AND HOUSE DUST MITE ALLERGEN. To determine whether differences in IgE and IgG4 between our subject groups were specific for cow's milk, levels of specific IgE and IgG4 for the food allergen ovomucoid (present in hen's eggwhite) and the inhalant allergen house dust mite were measured. Responses were determined in subjects (age 3-68 years) who were either allergic to these allergens, atopic but tolerant to these allergens, or non-atopic. In most of these subjects, also CMP-specific Ig levels were determined, as described above. As expected, specific IgE levels to both ovomucoid (Fig. 3A) and house dust mite extract (Fig. 3B) were higher in allergic than in atopic and non-atopic subjects. Ovomucoid-specific IgG4 in atopic individuals without allergy to hen's egg was significantly higher than in non-atopic subjects ($p < 0.05$), and tended to be higher than in hen's egg-allergic subjects ($p = 0.07$). The IgG4-response to house dust mite extract was only detectable in house dust mite-allergic subjects.

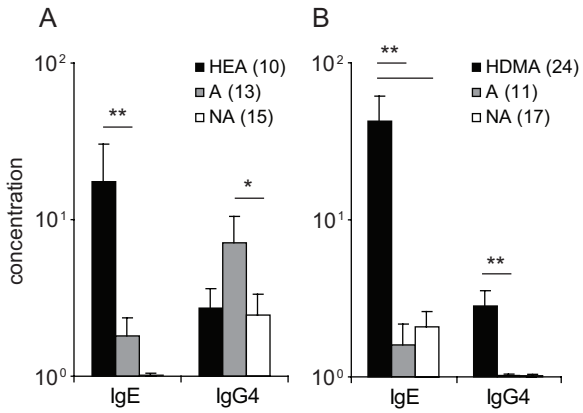

Figure 3.
Specific IgE and IgG4 to ovomucoid (**A**) and house dust mite allergen (**B**) in hen's egg-allergic (HEA), house dust mite-allergic (HDMA), atopic (A) and non-atopic (NA) subjects. The levels of specific IgE (ng/ml) and IgG4 (μg/ml) to ovomucoid and house dust mite allergen extract were measured by ELISA in children and adults. Numbers of subjects per group are between brackets. * $p < 0.05$, ** $p < 0.01$.

DISCUSSION

The IgE response to CMPs is the main determinant of clinical symptoms in CMA.[2] The present study is the first to compare allergenicity and antigenicity of the six most abundant CMPs on the level of specific Ig responses, in subjects with CMA, atopic individuals without a history of CMA, and non-atopic subjects of different age groups. In infants and children, αs1-casein and β-lactoglobulin induced the highest specific IgE response, whereas αs1-casein was the single most allergenic CMP in adults. These data correspond to previously reported results with regard

to the allergenicity of the four casein proteins in children with CMA,[19] and the IgE response to the casein and whey fraction in allergic adults.[3] In general, the most allergenic CMPs also induced the highest levels of specific IgG4 and IgG1. This positive correlation was described previously in a combined group of children and young adults with CMA,[11] and was even more pronounced in the present study. However, CMP-specific IgA levels appeared not to be correlated with specific IgE ($r^2 = 0.02$, data not shown). This observation is slightly different from the weak positive correlation that was reported by Shek et al.[11] In addition, these investigators observed that specific Ig responses to α-lactalbumin and β-lactoglobulin were lower than to α- and β-casein. In the entire group of subjects with CMA (n = 30) in our study, specific Ig responses were somewhat different, as these were highest to αs1-casein and β-lactoglobulin. Although the study group of Shek et al. was considerably larger than the group with CMA presented here, our data suggest that particularly in children with CMA the importance of the whey proteins should not be underestimated.

Previously, total serum IgA levels in severely atopic infants were reported to be lower than in non-atopic infants.[14] Moreover, upregulation of specific IgA, together with IgG4 and IgG1, was observed in house dust mite-allergic subjects after treatment with mite-specific IT.[20] These data may suggest a tolerogenic role for allergen-specific IgA. In the present study however, levels of CMP-specific IgA were only slightly lower in individuals with CMA than in non-atopic subjects (p = 0.11 when data of all three age groups were pooled). An important issue in this context is whether serum levels of specific IgA reflect the IgA response at the mucosal sites, particularly the gastro-intestinal tract where exposition to cow's milk takes place. A previous study that compared Ig responses in subjects with and without celiac disease, demonstrated that β-lactoglobulin-specific IgA levels in the jejunum were reasonably reflected in the serum.[21] This could indicate that the decrease in specific IgA in subjects with CMA as compared to non-atopic individuals may have some clinical relevance, although the difference was marginal. For specific IgG1, no consistent differences were found between cow's milk allergic and tolerant subjects.

Specific IgG is assumed to block binding of IgE to the allergen, which can have multiple beneficial effects, such as inhibition of mast cell degranulation, and a decrease in IgE-facilitated allergen presentation to specific T cells.[22,23] Via these mechanisms, specific IgG may be responsible for the induction of tolerance in allergic subjects who received IT.[6,8,24] Interestingly, children and adults with CMA who had high CMP-specific IgE, also had high levels of specific IgG4 and IgG1. This polyisotypic Ig response in subjects with CMA was reported earlier, and appears to be the result of a more robust overall immune response towards cow's milk.[11] The ratio between CMP-specific plasma IgE and IgG4 in children and adults with CMA is approximately 1:1000. Still, the concentration of specific IgG4 is apparently not high enough to prevent an allergic response. A possible explanation is that the amount of specific IgE bound to mast cells in the affected tissues, which is probably the most relevant in allergic reactions, may be poorly reflected in the plasma IgE concentration.[25] Alternatively, the affinity of CMP-specific IgE may be higher than that of specific IgG4. Indeed, ragweed-allergic subjects were previously reported to have specific IgE

with a significantly higher affinity compared to IgG4 and IgG1.[26] Taken together, CMP-specific IgE appears to "overrule" high levels of specific IgG4 and IgG1, and plays a decisive role in allergy or tolerance to cow's milk.

High CMP-specific IgG4 in combination with low specific IgE was observed in atopic children and adults without a history of CMA. Specific IgG4 levels tended to be higher in atopic children without CMA, and were higher in atopic adults without CMA, than in non-atopic individuals of the same age. Accordingly, specific IgG4 to β-lactoglobulin was previously observed to be higher in infants with atopic dermatitis (age 18 months) than in non-atopic infants, although this difference had disappeared at 8 years of age.[27] A former study from our group showed that CMP-specific T cells of atopic children without a history of CMA produced more IL-10 than those of children with CMA and non-atopic children.[15] Similar to its effect in bee venom IT,[6,7] IL-10 may upregulate the production of CMP-specific IgG4 and decrease specific IgE. This suggests that in atopic subjects without CMA, induction of specific IgG4 in combination with low specific IgE is involved in the maintenance of tolerance to cow's milk. Previously, birch pollen-allergic patients treated with allergen-specific IT were demonstrated to have increased specific IgG titers as compared to untreated allergic subjects, and higher affinity of specific IgG1 and IgG4 correlated with less allergic symptoms in treated patients.[28] Correspondingly, the potentially blocking effect of CMP-specific IgG4 in atopic subjects without CMA may be attributable to both an increase in specific IgG4 titers and a higher affinity as compared to specific IgG4 in individuals with CMA.

Further evidence for a tolerogenic role of IgG4 was provided by our preliminary observation that in four of the six infants with transient CMA mentioned in this study, specific IgG4 increased upon development of tolerance, whereas IgE decreased (data not shown, no second plasma sample was obtained from the other two subjects). It should be noted that the increase in IgG4 was probably partly age-related, since we observed that specific IgG4 increased with age in young atopic children with and without CMA. On the other hand, a recent study demonstrated that β-lactoglobulin-specific IgG4 was lower in children who had outgrown non-IgE-mediated CMA than in children with persistent CMA.[12] The authors therefore suggested a possible pathological role for IgG4 in non-IgE-mediated CMA. However, levels of specific IgG4 were compared at one timepoint between allergic and tolerant subjects, rather than before and after development of tolerance in children with transient CMA. Moreover, although our study focussed on IgE-mediated CMA, the observation that IgG4 levels were highest in atopic subjects who were tolerant to cow's milk suggests that CMP-specific IgG4 is rather tolerogenic than pathogenic.

The upregulation of specific IgG4 in tolerant atopic individuals may be related to the type of allergen. Ovomucoid-specific IgG4 was also highest in atopic subjects, who were tolerant to hen's egg. In contrast, IgG4 specific to house dust mite allergen was only detectable in subjects allergic to house dust mite. The induction of specific IgG4 appears to be allergen dose-dependent, as was previously shown for bee venom allergen (PLA) and cat allergen (Fel d 1).[9,29] Food allergens, which are taken up in relatively high amounts compared to inhalation allergens like house dust mite, may therefore be more potent inducers of IgG4. Remarkably, high cat allergen-specific IgG

and IgG4 in children who were highly exposed to cats was associated with decreased sensitization to this allergen,[29] which corresponds well with our data.
In summary, the present study shows that the individual CMPs differ in allergenic and antigenic properties, with αs1-casein and β-lactoglobulin being the most important allergens. In subjects with an atopic constitution, maintenance of tolerance to cow's milk may be mediated by elevated specific IgG4, in combination with low specific IgE.

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PBMC
G/4

[6] Role of IgE and IgG in binding of allergen-immunoglobulin complexes to CD23 in allergy to cow's milk, peanut or birch pollen

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Manuscript in preparation

ABSTRACT

BACKGROUND. IgE-facilitated allergen presentation (IgE-FAP) to T cells enhances the specific T cell response at low allergen concentrations, and may therefore be important in the maintenance of the allergic immune response. The efficiency of IgE-FAP is reduced by allergen-specific IgG, as is observed after specific immunotherapy.
OBJECTIVE. To determine the influence of allergen-specific IgG in three different allergies on the efficiency of allergen-Ig complex binding to B cells, as a first step of IgE-FAP.
METHODS. Plasma samples were derived from 15 subjects with cow's milk allergy (CMA), 15 with peanut allergy (PA), and 15 with birch pollen allergy (BPA). Levels of allergen-specific IgE, IgG4 and IgG1 were determined by ELISA. Binding of complexes to EBV-B cells was determined in 4 subjects from each group, using purified major allergens. Levels of specific IgE, IgG4, IgG1 and allergen bound in complexes, and the role of FcεRII (CD23) and FcγRII (CD32) in complex binding to B cells, was measured by flow cytometry.
RESULTS. Plasma levels of allergen-specific IgE were comparable between the three groups. In contrast, specific IgG was highest in CMA and lowest in BPA. The optimal allergen concentration for complex binding to B cells in CMA was 100-fold higher, and in PA 10-fold higher, than in BPA. For all allergens, IgE, IgG4 and IgG1 could be detected in allergen-Ig complexes bound to B cells. Complex binding was inhibited by anti-CD23, but not by anti-CD32.
CONCLUSION. In food allergy, allergen-specific IgG appears to compete with IgE for binding of allergen in allergen-Ig complex formation. This is most prominent for CMA, and to a lesser extent also for PA. Hereby it reduces CD23-mediated complex binding to B cells at low allergen concentrations. As a consequence, feedback enhancement of the specific Th2 response by IgE-FAP may be more prominent in inhalation allergy than in food allergy.

INTRODUCTION

Upon cross-linking of allergen-specific IgE bound to the high-affinity IgE receptor on mast cells and basophils by an allergen, these cells release inflammatory mediators, resulting in an immediate hypersensitivity reaction.[1] In addition to its direct pathologic role, IgE has been observed to be involved in the presentation of allergen to Th2 cells, which are key players in the allergic immune response as well.[2] In the process of IgE-facilitated allergen presentation (IgE-FAP), allergen is captured by IgE and internalized by antigen-presenting cells (APCs) via the high-affinity IgE receptor FcεRI or the low-affinity receptor FcεRII, also known as CD23.[3] Monocytes and dendritic cells are APCs that express FcεRI, whereas CD23 can be expressed by B cells and macrophages, of which B cells are thought to be the most important in IgE-FAP. The first step in IgE-FAP via CD23 is binding of circulating specific IgE to allergen, resulting in the formation of immune complexes at a ratio of IgE/allergen that favors multimeric binding. These complexes bind to CD23, and are subsequently internalized and processed. Eventually, T cell epitopes derived from the allergen are presented to Th cells.[3] The capture of allergen by IgE and its receptor-mediated uptake causes T cell activation by IgE-FAP to occur at very low allergen doses. In vitro data showed that by means of IgE-FAP, 100- to 1000-fold lower allergen concentrations are required to fully activate specific T cells.[2] Later, it was demonstrated in a mouse model that IgE-FAP induces a 10-fold stronger specific T cell response.[4] The high efficiency of IgE-FAP may explain why pronounced allergen-specific T cell responses are maintained in allergic subjects, even if the dose of allergen exposure is very low.[3,5]

In birch pollen-allergic subjects receiving specific immunotherapy (SIT), it was demonstrated that allergen-specific IgG, which was induced by the treatment, inhibits IgE-FAP.[6] Specific IgG was suggested to block binding of IgE to the allergen, leading to a decrease of IgE-FAP at low allergen concentrations. This mechanism resulted in significantly higher allergen threshold levels to activate specific T cells, and may therefore add to the tolerizing effect of SIT. Similar observations were later reported in grass pollen-allergic subjects receiving SIT.[7]

Previously, we found that levels of cow's milk-specific IgG4 in subjects with cow's milk allergy (CMA) were approximately 10-fold higher than house dust mite (HDM)-specific IgG4 in HDM-allergic subjects, whereas allergen-specific IgE levels were comparable (manuscript in press). We hypothesized that variations in allergen-specific IgG in subjects with either food or inhalant allergies could influence the efficiency of IgE-FAP. In the present study, binding of allergen-immunoglobulin (Ig) complexes to B cells was investigated as the first step of IgE-FAP. This was performed using plasma of subjects with CMA, individuals with peanut allergy (PA), and patients with birch pollen allergy (BPA). To enable an accurate comparison of the effects of allergen-specific IgE and IgG levels on complex binding, the purified major allergens αs1-casein, Ara h 2, and Bet v 1 were used, rather than the whole allergenic protein mixtures or extracts. Presence of IgE, IgG4, IgG1 and allergen in complexes, as well as the role of CD23 and the low-affinity IgG receptor FcγRII (CD32) in complex binding, was determined by flow cytometry.

METHODS

SUBJECTS. Forty-five adult subjects were included in this study. Fifteen subjects had CMA (age 26-68, mean 42), 15 individuals had PA (age 20-37, mean 25), and the remaining 15 subjects had BPA (age 18-60, mean 40). CMA, PA and BPA were defined by a suggestive history and diagnosed by a positive skin prick test with cow's milk protein, peanut extract, or birch pollen extract, and/or positive serum IgE levels specific for these allergens, as determined by CAP System FEIA (Pharmacia Diagnostics, Uppsala, Sweden). CMA and PA were confirmed by a positive double-blind placebo-controlled food challenge (DBPCFC). After informed consent was obtained, a heparinized venous blood sample was taken and plasma was separated from the cellular fraction by centrifugation. The study was approved by the Medical Ethical Committee of the University Medical Center, Utrecht.

DETERMINATION OF ALLERGEN-SPECIFIC IG LEVELS. Levels of IgE, IgG4 and IgG1 specific for cow's milk protein (CMP), crude peanut extract (CPE), and birch pollen extract (BPE), as well as specific IgE, IgG4, and IgG1 for αs1-casein, Ara h 2 and Bet v 1, were determined in plasma using ELISA. CMP and αs1-casein were kindly provided by R. Floris, PhD (NIZO Food Research, Ede, the Netherlands). CPE and Ara h 2 were obtained from TNO Quality of Life (Zeist, the Netherlands), and BPE and Bet v 1 were from ALK-Abelló (Hørsholm, Denmark). All allergens were coated in duplicate at 20 µg/ml (1 µg/well) in PBS in 96-well microtiter plates (Nunc Maxisorp, Roskilde, Denmark), and incubated overnight at 4°C. Background levels of Ig binding were determined in uncoated wells. All subsequent incubations were performed at 37°C. Plates were washed with PBS containing 0.05% Tween 20 (PBS-T) and blocked with 2% HSA in PBS-T for 1 hour. Subsequently, plates were emptied and diluted plasma samples were added. Plasma dilutions were made in PBS-T + 0,5% HSA and ranged from 1:10-1:100. High optical density (OD) values were avoided, to remain in the slope area of quantitative assessment. Plates were incubated for 1 hour. After washing, horseradish peroxidase (HRP)-conjugated goat-anti-human IgE (KPL, Gaithersburg, MD, USA), HRP-conjugated mouse-anti-human IgG4 or mouse-anti-human IgG1 (Sanquin, Amsterdam, the Netherlands), was added in 1:10.000, 1:30.000, and 1:20.000 dilution, resp. Plates were incubated for 1 hour and washed. All plates were developed using TMB peroxidase substrate (KPL), which was incubated for 10 min at RT before the reaction was stopped with 1M H_3PO_4 and OD-values were measured at 450 nm.

DETERMINATION OF ALLERGEN, IGE, IGG4 AND IGG1 BOUND IN COMPLEXES TO B CELLS. Complex binding to B cells was investigated using plasma samples of 12 subjects (4 patients from each subject group). The plasma samples were incubated with 0, 0.01, 0.1, 1, or 10 µg/ml of αs1-casein, Ara h 2, or Bet v 1, for 1 hour at 37°C to form allergen-Ig complexes. Similar incubations were performed in parallel with biotinylated αs1-casein, Ara h 2, and Bet v 1 (biotinylation kindly performed by J.H. Akkerdaas, PhD, AMC Amsterdam, the Netherlands). Epstein Barr Virus (EBV)-transformed B cells obtained from a subject with atopic dermatitis were then added

at 1.5 x 10^5/sample and incubated for 1 hour at 37°C to allow complexes to bind to the B cells. To investigate the role of CD23 and CD32 in complex binding, B cells were pre-incubated with mouse-anti-human CD23-FITC (clone MHM6), CD19-FITC (clone HD37) (DakoCytomation, Glostrup, Denmark) or CD32 (clone IV.3) (a kind gift from J.H. Leusen, PhD, UMC Utrecht, the Netherlands), in a 10 μg/ml dilution in FACS buffer (PBS + 2% FCS + 0.5 x 10^{-3} % NaN_3) for 30 min and washed, before adding the cells to the plasma containing allergen-Ig complexes. Subsequently, the B cells were washed with cold FACS-buffer. The B cells bearing complexes with unbiotinylated allergens were incubated for 30 min on ice with biotinylated mouse-anti-human IgE, -IgG4, or -IgG1 (BD Pharmingen, San Diego, CA, USA), which were diluted 1:250 in FACS buffer. The B cells bearing complexes with biotinylated allergens were incubated with goat-anti-human IgE-FITC (KPL, Gaithersburg, MD, USA) and streptavidin-PE (Strep-PE, BD Pharmingen), in a 1:40 and 1:300 dilution, resp. Cells were washed with FACS buffer and the B cells bearing complexes with unbiotinylated allergens were subsequently incubated with Strep-PE for 30 min on ice. Finally, B cells were washed and fluorescence was measured by flow cytometry (FACS-Calibur, BD Biosciences, San Jose, CA, USA).

DATA ANALYSIS AND STATISTICS. Regarding the ELISA, the OD values in allergen-coated wells (ranging from 0.1-2.0) were corrected for those in non-coated wells (always below 0.1) and compared with standard curves. Subsequently, data were multiplied by the plasma dilution to calculate specific Ig levels per subject in arbitrary units (AU/ml). The nonparametric Mann-Whitney U test was applied to determine significant differences in specific Ig levels between the subject groups. P-values < 0.05 were considered significant.

RESULTS

ALLERGEN-SPECIFIC IGE, IGG4 AND IGG1 IN PATIENTS WITH CMA, PA AND BPA. Plasma levels of allergen-specific IgE, IgG4 and IgG1 were measured in 15 adult subjects with CMA, 15 adults with PA and 15 with BPA (Fig. 1). Levels of IgE, IgG4 and IgG1 specific for cow's milk protein (CMP) were determined in the CMA group. In subjects with PA, specific Igs were measured for crude peanut extract (CPE), and specific Igs for birch pollen extract (BPE) were analyzed in individuals with BPA. Levels of allergen-specific IgE were not different between the subject groups. In contrast, specific IgG4 was highest in the CMA group, and differed significantly from IgG4 in subjects with PA and BPA ($p < 0.01$). Moreover, specific IgG4 in patients with PA was higher than in BPA ($p < 0.01$). Allergen-specific IgG1 was not different between subjects with CMA and PA, but was higher in these groups than in BPA ($p < 0.01$).

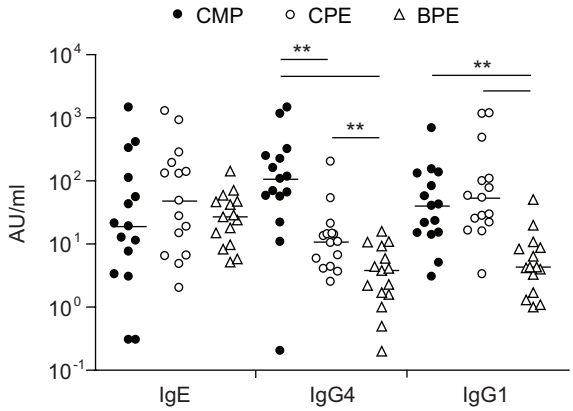

Figure 1.
Allergen-specific IgE, IgG4 and IgG1 in subjects with CMA, PA and BPA. Plasma levels were determined of specific IgE, IgG4 and IgG1 for cow's milk protein (CMP) in 15 subjects with CMA, for crude peanut extract (CPE) in 15 individuals with PA, and for birch pollen extract (BPE) in 15 subjects with BPA. All specific Igs were determined by ELISA and are shown in AU/ml. ** $p < 0.01$.

LEVELS OF IGE, IGG4 AND IGG1 SPECIFIC FOR THE PURIFIED MAJOR ALLERGENS. Four patients from each group were selected for the subsequent experiments. Allergen-specific IgE was high in the selected subjects with CMA and PA, and intermediate in the subjects with BPA. In plasma samples of these 12 subjects, specific IgE, IgG4 and IgG1 were measured for the respective purified major allergens from cow's milk (αs1-casein), peanut (Ara h 2), and birch pollen (Bet v 1). Ratios of specific IgE/IgG4 and IgE/IgG1 were determined as well (Table 1). Levels of IgE specific for αs1-casein in subjects with CMA and for Ara h 2 in patients with PA were slightly higher than Bet v 1-specific IgE in subjects with BPA ($p = 0.07$). Specific IgG4 for αs1-casein in CMA was higher than Bet v 1-specific IgG4 in BPA ($p < 0.05$). As a consequence, the ratio of αs1-casein-specific IgE/IgG4 was lower than Bet v 1-specific IgE/IgG4 ($p < 0.05$). Allergen-specific IgG1 was higher in subjects with CMA and PA than in patients with BPA ($p < 0.05$). Although measured in a small subject group, these data show that the Ig response to the major allergens was representative for the response to the whole protein mixture or extract (Fig. 1).

LEVELS OF SPECIFIC IGE, IGG4 AND IGG1 IN ALLERGEN-IG COMPLEXES BOUND TO B CELLS. Subsequently, plasma of the 12 subjects was used to investigate binding of allergen-Ig complexes to B cells, which was measured by flow cytometry. This procedure has been described previously, and was slightly adjusted.[7] One EBV-transformed B cell line, which expressed high CD23 and moderate CD32, was used in all experiments. Binding of allergen-Ig complexes was determined at four different allergen concentrations. The levels of bound IgE, IgG4 and IgG1 in the complexes were analyzed (Fig. 2). Levels of IgE in complexes bound to B cells are shown in Fig. 2A (subjects with CMA), 2B (PA), and 2C (BPA). In the CMA group, increased binding of IgE-allergen com-

Table 1. Levels (AU/ml) and ratios of specific Igs for the purified allergens in CMA, PA and BPA.

αs1-cas	IgE	IgG4	IgG1	IgE/IgG4	IgE/IgG1
CMA1	21.7	14.4	10.6	1.5	2.0
CMA2	34.6	130.0	5.6	0.3	6.2
CMA3	16.3	9.6	2.1	1.7	7.6
CMA4	160.0	63.2	11.6	2.5	13.8
median	28.2	38.8*	8.1+	1.6*	6.9

Ara h 2	IgE	IgG4	IgG1	IgE/IgG4	IgE/IgG1
PA1	14.9	16.2	1.1	0.9	13.9
PA2	75.2	3.0	15.5	25.0	4.9
PA3	8.0	0.1	1.4	80.2	5.6
PA4	140.0	1.6	19.7	88.3	7.1
median	45.0	2.3	8.5+	52.6	6.4

Bet v 1	IgE	IgG4	IgG1	IgE/IgG4	IgE/IgG1
BPA1	5.3	0.10	0.10	53.2	53.2
BPA2	18.0	0.48	0.60	37.6	29.9
BPA3	4.0	0.05	0.10	80.9	40.5
BPA4	3.5	0.75	0.51	4.7	6.9
median	4.7	0.29*	0.30+	45.4*	35.2

* $p < 0.05$, CMA vs BPA
+ $p < 0.05$, CMA vs BPA, PA vs BPA

plexes to B cells, as compared to background levels in incubations without allergen, was only observed in two of the four subjects. Besides, background levels of IgE binding were highest in this group. Three of the four subjects with PA showed increased IgE binding upon incubation with allergen, whereas it was found in all subjects with BPA. In subjects with CMA, levels of αs1-casein-specific IgE in complexes started to rise at an allergen concentration of 0.1 μg/ml and were optimal at 1-10 μg/ml. In the PA group, Ara h 2-specific IgE in complexes was also highest at 1-10 μg/ml, but already showed an increase at 0.01 μg/ml. In contrast, Bet v 1-specific IgE in complexes in subjects with BPA was highest at 0.01 μg/ml.

In addition to specific IgE, complexes were found to contain specific IgG4 and IgG1, even in subjects with BPA. Figures 2D-F show levels of IgG4 in complexes in subjects with CMA (D), PA (E), and BPA (F). Figures 2G-I illustrate levels of specific IgG1 in complexes in the CMA (G), PA (H), and BPA (I) group. In general, the presence of IgG4 and IgG1 correlated with IgE in complexes. At the optimal allergen concentration, complex-bound IgG4 was higher than IgG1 in CMA and BPA, whereas IgG1 was predominant in PA.

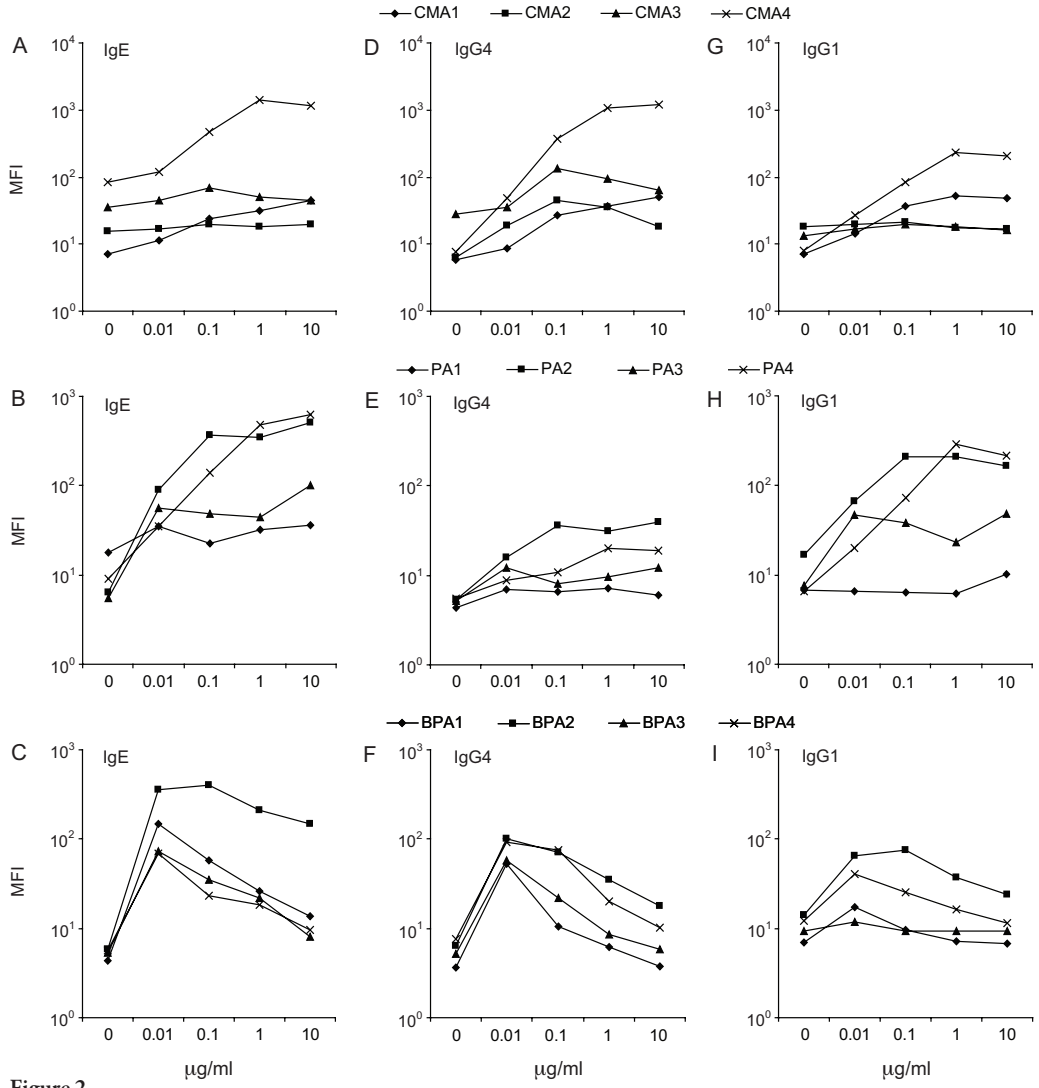

Figure 2.

Specific IgE, IgG4, and IgG1 in complexes bound to B cells. Levels of allergen-specific IgE, IgG4, and IgG1 in allergen-Ig complexes bound to EBV-B cells were determined by flow cytometry at increasing allergen concentrations. IgE levels in complexes were measured in 4 subjects with CMA (**A**), 4 with PA (**B**) and 4 with BPA (**C**). Moreover, IgG4 and IgG1 levels in complexes are shown in CMA (**D, G**), PA (**E, H**), and BPA (**F, I**). All levels are shown as geometric mean fluorescence intensity (MFI).

ROLE OF CD23 AND CD32 IN BINDING OF ALLERGEN-IG COMPLEXES TO B CELLS. The combined presence of IgE and IgG in complexes raised the possibility that complexes would not only bind to the low-affinity IgE receptor CD23, but also to the low-affinity IgG receptor CD32 on B cells. To investigate the role of CD23 and CD32 in binding of allergen-Ig complexes, B cells were incubated with anti-CD23, anti-CD32, or both, and complex binding was determined by measuring bound IgE, IgG4 and IgG1 (Fig. 3). Blocking of CD23 resulted in an average decrease in complex binding of > 95%, and complex-bound IgE, IgG4 and IgG1 were equally reduced. In contrast, blocking of CD32 had no effect on binding of complexes. This indicates that allergen-Ig complexes bind to B cells via CD23, whereas CD32 is not involved.

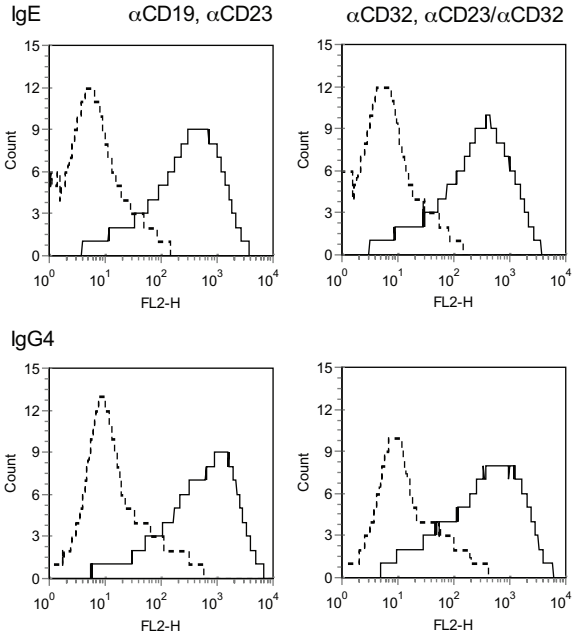

Figure 3.
Inhibition of complex binding to B cells by blocking CD23. Binding of allergen-Ig complexes to EBV-B cells, as determined by measuring bound IgE, IgG4 and IgG1, was reduced upon pre-incubation of B cells with anti-CD23, but not with anti-CD32. Graphs indicate the fluorescence intensity. B cells were pre-incubated with anti-CD19 (control Ab, solid line in left panels), anti-CD23 (dotted line in left panels), anti-CD32 (solid line in right panels) or anti-CD23 + anti-CD32 (dotted line in right panels), before incubation with allergen-Ig complexes. Data are shown for subject CMA4. Blocking of CD23 resulted in an average decrease in complex binding of > 95%, whereas blocking of CD32 had no effect. These experiments were performed for two subjects from each group, with similar results.

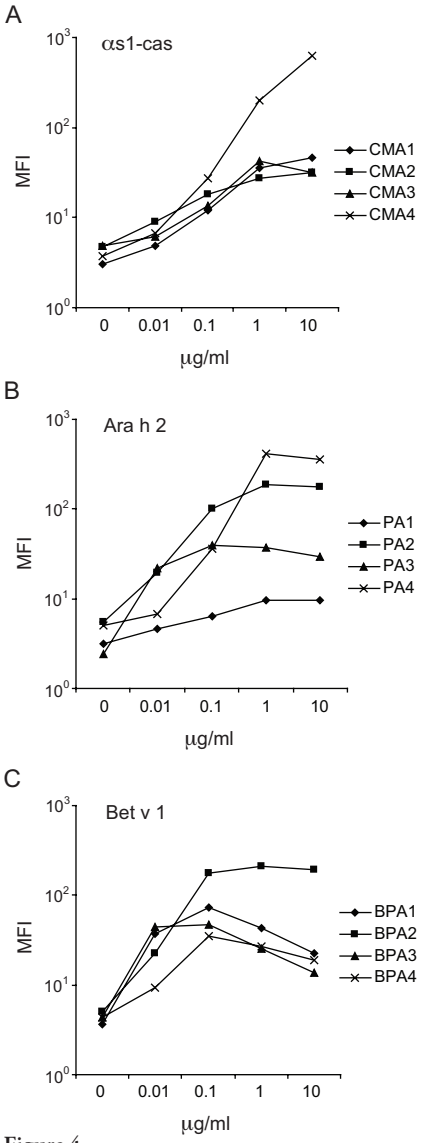

Figure 4.

Levels of allergen in complexes bound to B cells. The amounts of biotinylated major allergens in complexes bound to EBV-B cells were determined by flow cytometry and are shown for the 4 subjects with CMA (**A**), PA (**B**), and BPA (**C**). The biotinylated allergens were stained with Strep-PE. Data are presented as geometric mean fluorescence intensity (MFI).

AMOUNTS OF ALLERGEN IN ALLERGEN-IG COMPLEXES BOUND TO B CELLS. The varying levels of allergen-specific Igs in plasma and complexes may not only influence the optimal allergen concentration for complex formation, but also the amount of bound allergen in the complexes. Levels of allergen were therefore determined in the complexes, by means of biotinylated allergens (Fig. 4). Overall, the amounts of bound allergen were in line with IgE in complexes. An increase in bound allergen as compared to background levels was found in all subjects, also in those without an increase in complex-bound IgE (see Fig. 2A + B). Optimal binding of allergen in complexes was observed at an allergen concentration of 10 μg/ml in CMA (Fig. 4A), 1 μg/ml in PA (B), and 0.1 μg/ml in BPA (C). The optimal allergen concentration for binding of allergen in complexes was somewhat higher than the optimum for IgE-binding, particularly in the BPA group. Although the allergens varied widely with regard to specific Ig responses in plasma and characteristics of complex formation, the ratio between IgE and allergen in the complexes at the optimal allergen concentration was comparable between the subject groups (Table 2).

Table 2. Ratios of IgE/allergen in complexes at optimal allergen concentrations

IgE/αs1-cas, 10 mg/ml		IgE/Ara h 2, 1 mg/ml		IgE/Bet v 1, 0.1 mg/ml	
CMA1	0.45	PA1	2.82	BPA1	0.44
CMA2	0.76	PA2	0.50	BPA2	0.47
CMA3	0.77	PA3	0.66	BPA3	0.49
CMA4	0.34	PA4	0.34	BPA4	0.48
median	0.60	median	0.58	median	0.48

Ratios were calculated as MFI of anti-IgE-FITC/MFI of Strep-PE bound to biotinylated allergens.

DISCUSSION

The present study provides evidence that the initial step of IgE-FAP via CD23 occurs less efficiently in food allergic subjects, due to relatively high levels of allergen-specific IgG. In patients with CMA, levels of specific IgG for the tested major allergen were highest, whereas these were lowest in subjects with BPA. As a consequence, the optimal allergen concentration for binding of allergen-Ig complexes to CD23-expressing B cells in subjects with CMA was observed to be 10-fold higher than in PA and even 100-fold higher than in BPA. These data indicate that specific IgG inhibits binding of allergen-Ig complexes to B cells, particularly at low allergen concentrations. Interestingly, IgG4 and IgG1 could be detected in complexes in all three groups, even in subjects with BPA who had very low specific plasma IgG levels. Apparently, the presence of IgG in the complexes as such does not prevent binding via the low affinity IgE receptor CD23, as long as the ratio of IgE/IgG in the complexes is high enough. However, when specific IgG levels in plasma are high, the ratio of IgE/IgG in complexes may be too low for complex binding to CD23 at low allergen concentrations.

In the allergen-specific IgG response, the predominant isotypes are IgG1 and particularly IgG4, as has been shown after SIT.[8-10] It is assumed that specific IgG4 is the most important isotype in inhibiting allergen-Ig complex binding to B cells.[11] In accordance with these data, the lowest complex binding at low allergen concentrations was found in the CMA group and in subject PA1, in which specific IgG4 levels were highest. For subjects PA2 and PA4, who had low IgG4 but high IgG1, the optimal allergen concentration for complex binding was higher than for patients with BPA, indicating that also IgG1 slightly reduces complex binding to B cells at low allergen concentrations. Ultimate evidence for an inhibitory effect of specific IgG on binding of allergen-Ig complexes to CD23 can only be provided by means of IgG-depleted plasma samples. These experiments are currently being performed.

Whereas complex-bound IgG decreases IgE-FAP via B cells, it may enhance IgG-FAP via binding to the low affinity IgG receptor CD32. Although the contribution of this receptor in complex binding to B cells was observed to be minimal, other APCs can bind and internalize antigen-IgG complexes via CD32, resulting in enhanced T cell activation.[12,13] IgE-FAP via CD23 on B cells has been demonstrated to enhance the specific Th2 and IgE response.[14] In contrast, IgG-FAP appears to be mediated most efficiently by immature DCs, which have been shown to secrete IL-10 and pro-inflammatory cytokines upon cross-linking of CD32.[12,15,16] These cytokines may attenuate the allergen-specific Th2 response upon ligation of CD32 by allergen-Ig complexes and subsequent IgG-FAP.

Remarkably, we observed that the ratio between IgE and allergen in complexes at the optimal allergen concentration was similar for αs1-casein, Ara h 2, and Bet v 1. This ratio seems to be a constant factor, which is reached at a low allergen concentration in BPA and at a higher concentration in PA and CMA. Previously, we found that plasma levels of CMP-specific IgG4 are 1000-fold higher than specific IgE in patients with CMA (manuscript in press). In contrast, the affinity for allergen of specific IgE in allergic subjects appears to be approximately 1000-fold higher than that of specific IgG.[17] Based on these findings, we hypothesize that specific IgG almost completely blocks binding of IgE at low allergen doses, resulting in a ratio of complex-bound IgE/allergen that is too low to facilitate strong binding to CD23. As a consequence, binding of allergen-Ig complexes to B cells is hardly detectable. However, the IgG-rich complexes that are likely formed at low allergen concentrations may bind to CD32 on other APCs. At higher doses, the allergen is no longer fully captured by IgG, which increases the chance of specific IgE to bind. In this situation, the higher affinity of specific IgE as compared to IgG may compensate for the difference in plasma titers, leading to an increase in complex-bound IgE and in complex binding to CD23.

The efficiency of complex binding to B cells as the first step of IgE-FAP is high in BPA, moderate in PA and low in CMA, and is inversely related to plasma levels of allergen-specific IgG4. In previous studies, pronounced specific IgG4 responses were demonstrated to be induced by high exposure to allergen.[18,19] Comparatively, the high CMP-specific IgG4 response in subjects with CMA may be the result of exposure to relatively high doses of cow's milk before the onset of CMA.[20] In general, exposure to cow's milk quantitatively exceeds exposure to peanut and birch pollen,

which may explain the difference in specific IgG4 responses. These strong IgG4 responses may be part of a natural mechanism to reduce the chance of development of allergy and escalation of the allergic response by IgE-FAP. Such a mechanism would be particularly relevant for allergens that are normally encountered in high amounts. As a result of high CMP-specific IgG4, the minimal allergen concentration for substantial complex binding to B cells in CMA is not lower than for allergen presentation without IgE-FAP.[21] Therefore, the contribution of IgE-FAP to allergen-specific Th2 cell activation in CMA appears to be less relevant than in BPA. One could speculate that the low feedback level of specific IgE on the allergen-specific T cell response via CD23 influences the clinical characteristics of CMA. For example, CMA is unique for the fact that tolerance develops spontaneously in most infants with CMA.[22] Moreover, the prevalence of CMA in adults appears to be 20-fold lower than that of BPA.[23,24] Still, CMA occurs in adult subjects, despite high levels of CMP-specific IgG4. Whereas IgE-FAP via CD23 does not appear to be relevant in CMA, IgE-FAP might occur via the high affinity receptor FcεRI on DCs and monocytes, which may maintain the CMP-specific Th2 response in these subjects.[25,26]

In summary, the present study demonstrates that the efficiency of the inital step of IgE-FAP is high in BPA, moderate in PA and low in CMA. Our data suggest that allergen-specific IgG, particularly of the IgG4 isotype, inhibits binding of complexes to B cells via CD23 at low allergen concentrations. As a result, feedback enhancement of the allergen-specific T cell response by IgE may be less prominent in food allergies than in inhalant allergies. Complex-bound specific IgG may mediate IgG-FAP via CD32 and attenuate the specific Th2 response. These findings may provide an explanation for differences in prevalence as well as persistence between allergies.

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[7] A specific mixture of short chain Galacto-oligosaccharides and long chain Fructo-oligosaccharides induces a beneficial immunoglobulin profile in infants at high risk for allergy

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ABSTRACT

BACKGROUND. It has been suggested that human breast milk oligosaccharides play a role in the development of the immune system in infants, and may subsequently inhibit the onset of allergy. A specific prebiotic oligosaccharide mixture of short chain Galacto-oligosaccharides and long chain Fructo-oligosaccharides (GOS/FOS) has been shown to reduce the cumulative incidence of atopic dermatitis (AD) at six months of age in infants at risk for allergy. Therefore, it was hypothesized that GOS/FOS is able to modulate the immune response in infants.

METHODS. A prospective double-blind randomized placebo-controlled study with parallel groups was performed. If breastfeeding was not successful within two weeks after birth, infants received a hypoallergenic whey formula with either 8 g/l GOS/FOS in a 9:1 ratio (IMMUNOFORTIS™) or 8 g/l maltodextrine (placebo) for six months. Severity of AD was determined by SCORAD. At three months of age, children were vaccinated with Hexavac against diphtheria, tetanus and polio (DTP). At six months of age plasma samples were collected from 84 infants (verum group n = 41, placebo group n = 43). Levels of total immunoglobulins (Igs), as well as of cow's milk protein (CMP-) and DTP-specific Igs were measured.

RESULTS. Supplementation of GOS/FOS led to a significant decrease in the incidence of AD after 6 months. Furthermore, a significant reduction was observed in the plasma level of total IgE, IgG1, IgG2 and IgG3, but not of IgG4. CMP-specific IgG1 was significantly decreased. DTP-specific Ig levels were not affected by GOS/FOS supplementation.

CONCLUSION. This study shows that GOS/FOS, in addition to reducing the incidence of AD, induces a beneficial antibody profile. Furthermore, GOS/FOS supplementation specifically modulates the immune response towards food allergens, while leaving the response to pathogens intact. This suggests that oral GOS/FOS supplementation is a safe method to restrain the atopic march.

INTRODUCTION

The prevalence of allergic disorders has steadily increased during the last decades in developed countries.[1] A delayed maturation of the immune system has been associated with a higher risk of allergy in children. Normal immune maturation after birth has been characterized by down regulation of Th2 responses, that are involved in the induction of IgE mediated allergies. It has been hypothesized that breast milk plays an important role in immune maturation.[2] Furthermore, it has been suggested that breast-feeding reduces the incidence of allergic disorders in children.[3-5] One of the potential mechanisms by which breast-feeding protects the infant against atopic dermatitis (AD), is the prebiotic capacity of breast milk. Particularly the neutral oligosaccharides in breast milk stimulate the development of a complex intestinal microbiota dominated by bifidobacteria and lactobacilli.[6] These bifidobacteria and lactobacilli have been associated with protective effects against AD.[7,8] Based on molecular size, a specific mixture of short chain Galacto-oligosaccharides (GOS) and long chain Fructo-oligosaccharides (FOS) in a 9:1 ratio (IMMUNOFORTIS™) was developed.[9] Previously, it was shown that an infant milk formula supplemented with this mixture of GOS/FOS has prebiotic activities similar to breast milk.[10,11] Recently, new data indicated that GOS/FOS supplementation reduces the cumulative incidence of AD in high-risk infants.[12] Moreover, oral supplementation of GOS/FOS in mice was shown to stimulate antigen-specific Th1-mediated immunity upon vaccination, indicating that GOS/FOS can modulate both intestinal microbiota as well as a specific immune response.[13]

In the current study, it was hypothesized that GOS/FOS supplementation in infants would lead to a reduction of the allergen-specific immune response without loss of the specific immune response upon vaccination. Therefore, total antibody levels were measured, as well as levels of antibodies specific for cow's milk protein (CMP) and DTP antigens after Hexavac-vaccination. The plasma samples were obtained from the study described previously.[12]

METHODS

STUDY POPULATION. The study population has been described previously.[12] Briefly, the study population consisted of term infants with a parental history of atopy/allergy (i.e. mother or father with atopic eczema, allergic rhinitis or asthma). The parental atopy/allergy diagnoses were based on a documented physician's certification. Participating infants were born at the Macedonio Melloni Maternity Hospital in Milan, Italy. In total, 259 infants were included, of which 206 infants completed the study.[12] The main reasons for drop-out were continuation of mixed feeding with breast milk for more than six weeks, or reestablishment of breast feeding within the six weeks time period. Blood samples were obtained from 84 infants, for which the parents gave written informed consent. The establishment of the study population is depicted in Fig. 1. The study was approved by the Ethical Committee of the Macedonio Melloni Maternity Hospital in Milan, Italy.

STUDY DESIGN. The study was performed as a prospective double-blind, randomized, placebo-controlled study with parallel groups.[12] According to the hospital's practice, breast feeding was recommended to all mothers. Parents who started with formula feeding within two weeks post partum, were asked to contact the hospital and to participate in this study. Mixed feeding was accepted until the age of six weeks. The participating infants were randomly assigned to one of the two groups receiving a hypoallergenic formula (Aptamil HA, Milupa, Friedrichsdorf, Germany) based on an extensively hydrolyzed cow's milk whey protein, supplemented with either 8 g/l GOS/FOS (IMMUNOFORTIS™), or 8 g/l maltodextrines as placebo. Both formulas were fed ad libitum.

Children were examined one day before starting the formula feeding (visit 1, within two weeks after birth), and at the age of three (visit 2) and six months (visit 3) after birth. At visit 2, infants were vaccinated against diphteria, tetanus, polio, pertussis, hepatitis B and *Haemophilus influenzae* (Hexavac, Aventis Hasteur MSD, Roma, Italy). At each visit to the hospital, the skin of the infants was inspected on symptoms of AD, determined by SCORAD, as previously described.[12]

In a subgroup of 84 infants, blood samples (1 ml) were taken in EDTA at six months of age to investigate antibody levels and vaccination responsiveness of the infants. Blood samples were centrifuged and plasma was aliquoted and stored at −80°C. Total Ig levels were measured in 43 (placebo group) and 41 (GOS/FOS group) infants except for IgE, which was measured in 33 (placebo group) and 35 (GOS/FOS group) infants. Because of possible influence of infectious diseases or antibiotic use on Ig levels, blood samples were obtained only after a period of two weeks free of infectious symptoms and/or antibiotic treatment. Otherwise, it was allowed to postpone the sampling for a maximum of four weeks.

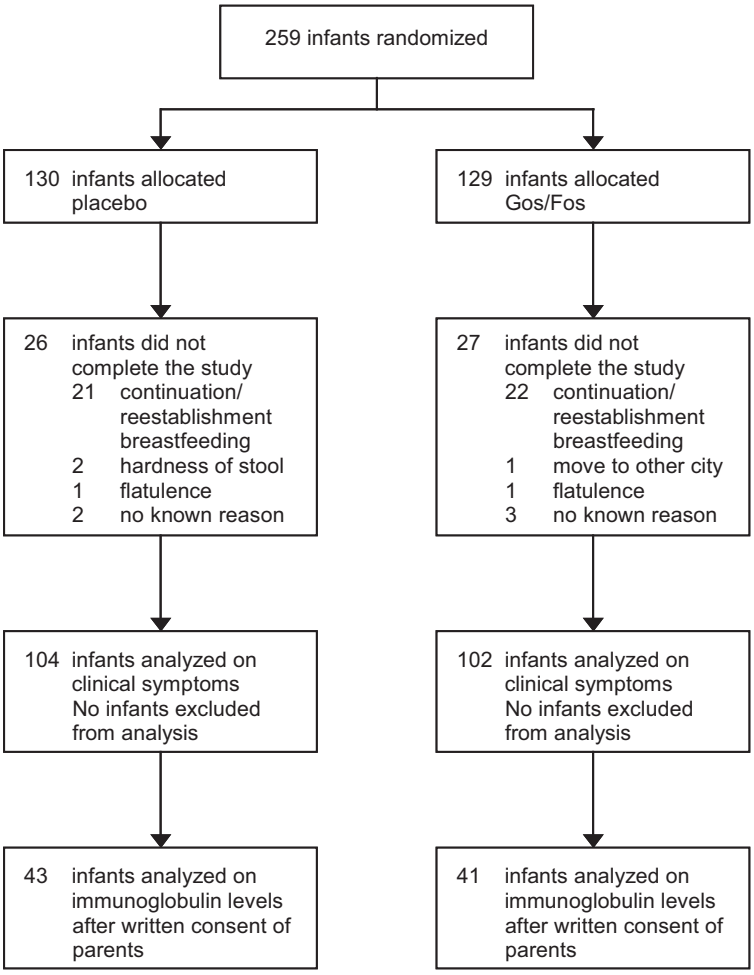

Figure 1.
Trial profile.

DETERMINATION OF TOTAL IMMUNOGLOBULIN LEVELS BY IMMUNOCAP AND ELISA. Total IgE was measured in plasma by ImmunoCap Technology (Phadia, Uppsala, Sweden), according to the manufacturers instructions.

Total IgG1, IgG2, IgG3, and IgG4 antibody levels in plasma were determined using ELISA procedures. Briefly, microplates (96-well flat-bottom, high binding EIA/RIA plates, Corning Life Sciences, Acton, MA, USA) were coated overnight at 4°C with mouse anti-human IgE Mab (2 µg/ml), mouse anti-human IgG1 Mab (4 µg/ml), mouse anti-human IgG4 Mab (1 µg/ml, all from BD Pharmingen, San Diego, CA, USA), mouse anti-human IgG2 Mab (2 µg/ml) or mouse anti-human

IgG3 Mab (2 µg/ml, both from Zymed Laboratories, Invitrogen, Carlsbad, CA, USA) in carbonate buffer (pH 9.6). Plates were washed with wash buffer (0.005% Tween-20 in PBS) and blocked with ELISA buffer (1% Bovine Serum Albumine (Fraction V, MP Biomedicals, Solon, OH, USA) in PBS). Subsequently, plates were washed and standard curve dilutions (Purified IgG1, IgG2, IgG3 or IgG4, all from Biogenesis, Nuclilab BV, Ede, the Netherlands), made in ELISA buffer were added in a range from 2000 pg/ml to 2.7 pg/ml. Plasma samples were added and incubated in 8 dilutions in ELISA buffer, ranging from 1:1000-1:128000 (IgG1 and IgG2), 1:500-1:64000 (IgG3) or 1:1.95-1:250 (IgG4). Afterwards, plates were washed and biotin-conjugated mouse-anti-human IgG1 Mab (2 µg/ml), IgG2 Mab (2 µg/ml), IgG4 Mab (1 µg/ml, all from BD Pharmingen, San Diego, CA, USA) or IgG3 Mab (4 µg/ml, from Zymed Laboratories, Invitrogen, Carlsbad, CA, USA) was added in ELISA buffer. Plates were incubated with horseradish peroxidase (HRP)-conjugated streptavidin (0.25 µg/ml, Biosource, Etten-Leur, the Netherlands), followed by TMB substrate (1-step ultra TMB, Perbio Science, Etten-Leur, the Netherlands). OD-values at 450 nm were measured to determine Ig-isotype concentrations.

DETERMINATION OF COW'S MILK SPECIFIC IMMUNOGLOBULIN LEVELS BY ELISA. CMP-specific Igs were measured in 41 (placebo group) and 35 (GOS/FOS group) infants. Plasma IgE, IgG1 and IgG4 antibody levels specific to purified CMP (kindly provided by Dr. R. Floris, NIZO Food Research, Ede, the Netherlands) were determined using ELISA. Microplates (96-well flat-bottom, Nunc Maxisorb, Roskilde, Denmark) were coated with CMP at a concentration of 20 µg/ml (1 µg/well) in PBS and incubated overnight at 4°C. All subsequent incubations were performed at 37°C. Plates were washed with PBS-T (0.05% Tween-20 in PBS) and blocked with 2% HSA in PBS-T. Subsequently, plates were emptied and standard curve dilutions (pooled plasma containing high levels of cow's milk specific IgE (ng/ml by RAST), IgG4 (µg/ml by RAST) and IgG1 (AU/ml) were added. Plasma samples, diluted in PBS-T/0.5% HSA were added in a range from 1:10-1:50 (IgE), 1:10-1:20 (IgG4), and 1:20-1:100 (IgG1) and plates were incubated for 1 hour. Afterwards, plates were washed and HRP-conjugated goat-anti-human IgE (1:10000, KPL, Gaithersburg, Maryland, USA), mouse-anti-human IgG4 (1:30000) or mouse-anti-human IgG1 (1:20000, both from Sanquin, Amsterdam, the Netherlands) was added for 1 hr. Subsequently, TMB substrate (KPL) was added, and OD-values at 450 nm were used to measure Ig concentrations.

DETERMINATION OF DTP-SPECIFIC IMMUNOGLOBULIN LEVELS BY ELISA. DTP-specific Igs were measured in 19 (placebo group) and 19 (GOS/FOS group) infants. Plasma IgE, IgG1, IgG2 and IgG3 antibody levels specific to the DTP vaccin (Multidose (10), national institute of public health and the environment, Bilthoven, the Netherlands) were determined using essentially the same ELISA procedure as for total Ig levels. Briefly, microplates were coated with diluted DTP vaccin (1:32 for IgE, 1:125 for IgG1, 1:4000 for IgG2 or 1:250 for IgG3) in carbonate buffer (pH 9.6) and incubated overnight at 4°C. Standard curve dilutions (Normal Human Serum, San-

quin), made in ELISA buffer, were added in a range from 1:1.95-1:250 (IgE), 1:31.25-1:4000 (IgG1), 1:500-1:32000 (IgG2) or 1:15.6-1:2000 (IgG3). Plasma samples were added in 8 dilutions, made in ELISA buffer, and ranged from 1:3.9-1:500 (IgE), 1:15.6-1:2000 (IgG1 and IgG3) or 1:7.8-1:1000 (IgG2). Plates were incubated with biotin-conjugated mouse anti-human IgE, IgG1, or IgG2 Mab (2 μg/ml), or mouse anti-human IgG3 Mab (4 μg/ml). Plates were subsequently incubated with Streptavidine-HRP and TMB substrate as described above, and OD values at 450 nm were used to determine Ig-isotype concentrations.

STATISTICAL ANALYSIS. Randomisation and blinding were previously described.[12] To test the contingency of AD at six months of age, a two-sided Fisher's exact test was used. Normally distributed Ig data were tested using an unpaired two-sided t-test, whereas data without a normal distribution were tested with an unpaired two-sided Mann-Whitney-U-test. It should be noted that the level of total IgE (6 placebo, 10 GOS/FOS) and IgG4 (13 placebo, 9 GOS/FOS) of some samples were below the detection limit of the assay. For IgE, the detection limit was 2 kU/L. To include these samples in the analyses, the negative samples were arbitrarily assigned the value 1.9 kU/L. For IgG4, the dilution factor of the samples multiplied by the detection limit was taken as a value for these samples (5.35 μg/L). P-values < 0.05 were considered significant.

ROLE OF FUNDING SOURCE. The sponsor of the study had no role in the study design, management and sample collection. However, scientists from the funding source did have a role in the analyses and data interpretation. The corresponding author had full access to all data from this study.

RESULTS

GOS/FOS SUPPLEMENTATION REDUCES THE INCIDENCE OF AD. To evaluate the immunomodulatory capacities of GOS/FOS, a prospective double-blind randomized, placebo controlled study was performed.[12] Infants received a hypoallergenic formula with either 8 g/l GOS/FOS or 8 g/l maltodextrine (placebo) for six months. No significant differences were found between the two groups regarding weight, length, gender and the parental history of allergic symptoms at the moment of inclusion.[12] Six months after treatment, the incidence of AD was significantly reduced in the GOS/FOS group (10/102), as compared to the placebo group (24/104). The severity of AD as measured by SCORAD was not significantly different.[12]

LEVELS OF TOTAL IGE, IGG1, IGG2, IGG3 ARE REDUCED AFTER GOS/FOS SUPPLEMENTATION Levels of total plasma Igs were measured after six months of treatment. Total IgE levels were significantly declined after GOS/FOS supplementation ($p = 0.008$, Fig. 2A). In addition, a significant reduction in total IgG1 levels ($p = 0.005$, Fig. 2B), as well as in IgG2 (median GOS/FOS 0.66 g/l (IQR 0.51-0.90), median placebo 0.99 g/l (IQR 0.61-1.26), $p = 0.0006$) and IgG3

(median GOS/FOS 1.04 g/l (IQR 0.64-1.85), median placebo 1.76 g/l (IQR 1.15-2.77), p = 0.001) (not shown) was observed. No significant differences in the levels of total IgG4 were observed between the two treatment groups (Fig. 2C). Within the subgroup of infants with AD, the Ig levels were not different from the infants without AD (data not shown).

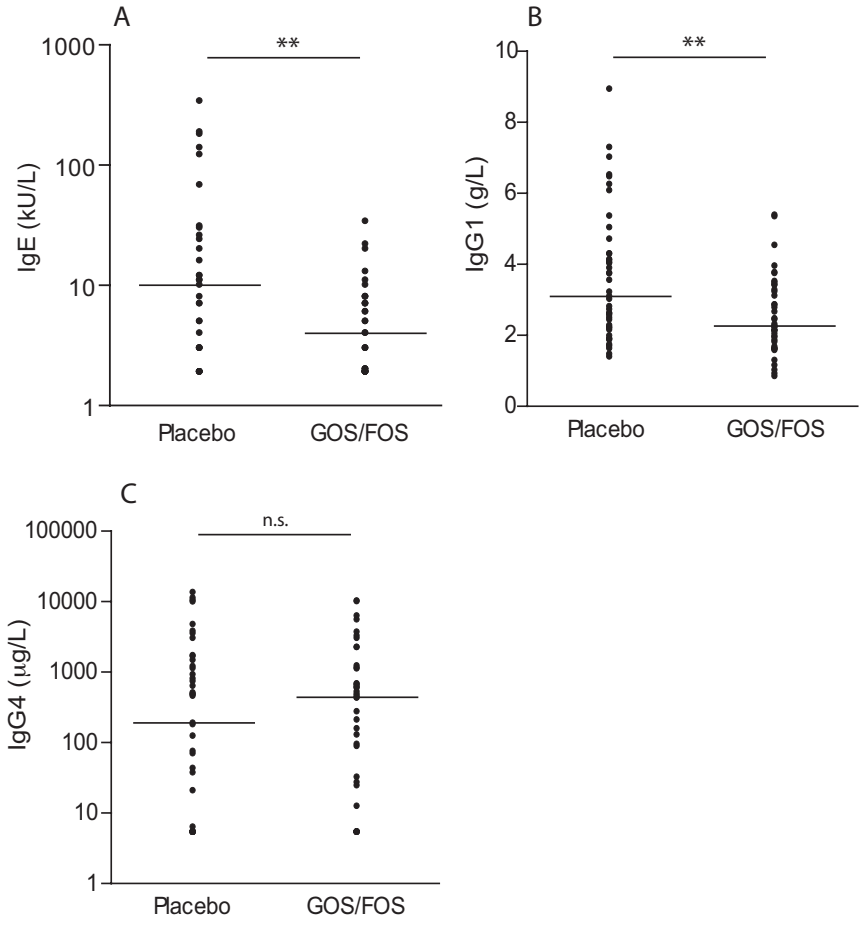

Figure 2.
Effect of **oral supplementation with a specific prebiotic mixture of** GOS/FOS to infants at high risk for allergy on the levels of total IgE (**A**), total IgG1 (**B**) and total IgG4 (**C**). Data represent individual results and median of the placebo group (IgE n = 33, other Igs n = 43) and GOS/FOS group (IgE n = 35, other Igs n = 41). ** p < 0.01; n.s., not significant

CMP-SPECIFIC IGG1 IS REDUCED AFTER GOS/FOS SUPPLEMENTATION. CMP-specific Igs were measured in 41 (placebo group) and 35 (GOS/FOS group) infants. Results are depicted in Fig. 3. Levels of CMP-specific IgE were very low, and no effect of GOS/FOS supplementation on CMP-specific IgE was observed. CMP-specific IgG4 was not detectable in these samples (data not shown). In contrast, CMP-specific IgG1 was significantly decreased in the GOS/FOS supplemented infants ($p = 0.01$, Fig. 3B).

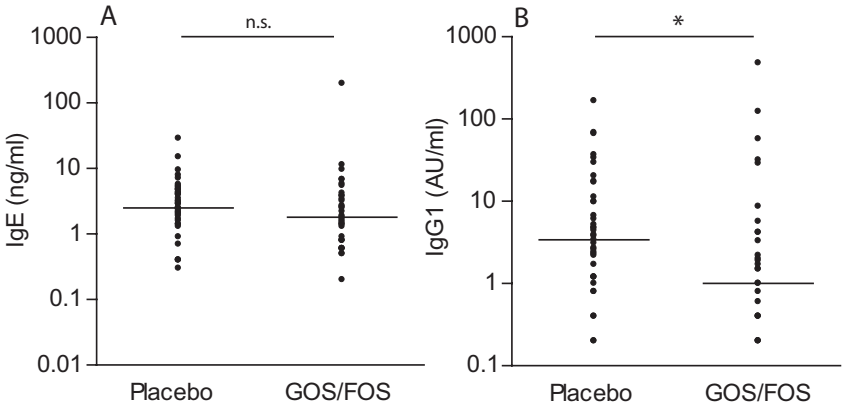

Figure 3.
Effect of oral supplementation with a specific prebiotic mixture of GOS/FOS to infants at high risk for allergy on the levels of CMP-specific IgE (**A**) and IgG1 (**B**). Data represent individual results and median of placebo group (n = 41) and GOS/FOS group (n = 35). Data from 2 patients (placebo group) and from 6 patients (GOS/FOS group) are lacking due to shortage of plasma. * $p < 0.05$; n.s., not significant

GOS/FOS SUPPLEMENTATION DOES NOT AFFECT THE VACCINATION RESPONSE. Vaccination responsiveness was analyzed by measuring DTP-specific Ig levels at 6 months of age in 19 infants from the GOS/FOS and 19 infants from the placebo group. No differences in any DTP-specific antibody isotype were observed in infants receiving GOS/FOS supplementation as compared to placebo supplemented infants, shown for IgE and IgG1 in Fig. 4.

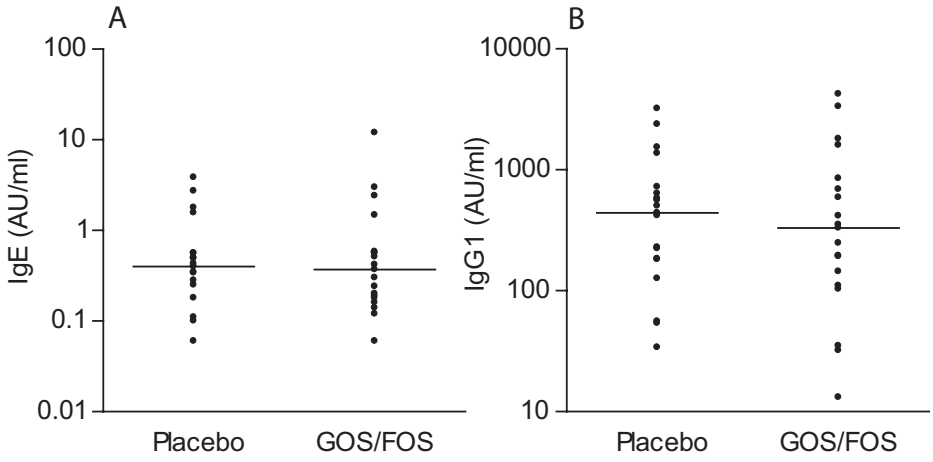

Figure 4.
Effect of oral supplementation with a specific prebiotic mixture of GOS/FOS to infants at high risk for allergy on DTP-specific IgE (**A**) and IgG1 (**B**) levels. Data represent individual results and median, obtained from a subgroup of the participants (placebo group n = 19; GOS/FOS group n = 19).

DISCUSSION

Recently, it was shown that oral supplementation with a specific mixture of GOS/FOS oligosaccharides (IMMUNOFORTIS™) results in a decreased incidence of AD in infants at risk for allergy of approximately 50%.[12] The present study was aimed to investigate whether the decline in the incidence of AD by GOS/FOS could be explained by modulation of the immune response in these high-risk infants.

Supplementation of 8 g/l GOS/FOS resulted in a significant decrease in total antibody levels for all isotypes, except for IgG4, suggesting a reduced immune activation. The lack of effect on total IgG4 levels may be due to the fact that IgG4 levels are very low at the age of 6 months.[14] Furthermore, compared to the other isotypes, the IgG4 levels observed in our study vary largely, ranging from 10 ng/ml to 10 µg/ml. This has been described before and may hamper statistical power.[15] The reduced Ig levels of the other isotyes, especially IgE, may be associated with the reduced incidence of AD in the GOS/FOS supplemented group.[12] This contrasts the study of Kalliomäki et al, who showed that reduction of the frequency of AD by *Lactobacillus rhamnosus* GG supple-

mentation was not accompanied by changes in total or specific IgE levels.[8] This may suggest that the prebiotic mixture of GOS/FOS has a stronger immunomodulatory potential than this specific probiotic strain. GOS/FOS supplementation has previously been shown to have a prebiotic effect, which induces a colon microbiota with characteristics that are very similar to that of breast-fed infants.[10,11,16] It has been suggested that probiotic strains, when daily provided, may acquire a 'tolerated' niche in the intestinal system after a certain period.[17] In contrast, GOS/FOS supplementation may promote a more diverse turnover of appropriate bacteria, thereby providing a stronger, continuous immune stimulation necessary to prevent atopic diseases. Importantly, preliminary data in a small group of exclusively breast fed infants at high risk for allergy in our study, that was not included in the analyses, showed no significant differences in plasma Ig levels compared to bottle fed GOS/FOS supplemented infants (data not shown). This suggests that the modulation of humoral immune responses observed by GOS/FOS supplementation is within normal physiological boundaries.

Remarkably, when analyzing the subgroups of infants with AD, no significant differences were observed in SCORAD values, nor in Ig levels between the verum and placebo group. This is in accordance with the data of Kalliomaki et al.[8] Apparently, although GOS/FOS is able to reduce the incidence of AD, GOS/FOS has no additional immuno-modulatory effect once AD is established. Because allergic responses in infants between 0 and 2 years of age are frequently directed towards CMPs, CMP-specific antibodies were analyzed. CMP-specific antibody levels were low compared to our observations in infants (manuscript in press), which might be due to the fact that an extensively hydrolyzed whey protein formula was used as basis for both study formulas, according to recent recommendations for the nutritional management of infants at risk for allergy.[18] Extensively hydrolyzed cow's milk formulas have been shown to induce lower specific IgG levels as compared to normal formulas.[19] CMP-specific IgE was too low to draw conclusions in the current study. This confirms previous data showing that the incidence of cow's milk allergy (CMA) in infants fed with an extensively hydrolyzed formula is very low.[20] Whether GOS/FOS supplementation would further reduce the incidence of CMA, should be investigated in other studies. Surprisingly, CMP-specific IgG1 was decreased significantly after GOS/FOS supplementation. CMP-specific IgG in infants has been shown to be of the IgG1 subtype.[21] Moreover, it was found that bottle fed infants have IgG1 with higher affinity towards CMP than breast fed infants.[22] Together, these data may suggest that lowering the level and affinity of CMP-specific IgG1 might be beneficial for infants, especially for those who are at risk for CMA.

The observed reduction in the level of total Igs after GOS/FOS supplementation could suggests a reduced capability of the immune system towards antigens in general. Because specific antibody responses towards pathogens are essential to preserve, the response towards vaccination antigens was investigated as well, as recommended by the WHO/ILSI.[23] Levels of DTP-specific Igs were determined after Hexavac vaccination. No differences between the GOS/FOS supplemented group and placebo supplemented group were detectable. This demonstrates that GOS/FOS left the vaccination response intact. Recently, it was demonstrated in a murine model that influenza vac-

cination was even enhanced.[13] Also in infants treated with probiotics in the presence of galacto-oligosaccharides, vaccination responses were shown to be at least as high as in placebo-treated infants.[24] The observation in our study that a food allergen-specific response is reduced, whereas a vaccination response remains intact, may suggest that GOS/FOS supplementation is specifically able to alter the response towards antigens that enter the body at the same site as the GOS/FOS, namely via intestinal exposure. However, preliminary unpublished clinical data from Guiarino et al. indicate that GOS/FOS feeding, in addition to reducing the incidence of AD, significantly reduces the incidence of upper respiratory tract infections, diarrhea, and antibiotics usage. This implies a broader range of potential immuno-modulatory effects than just towards antigens that enter via the intestinal route. Together, the data suggest that GOS/FOS supplementation supports proper regulation of the natural immune mechanisms, by down-regulating allergen-specific responses and promoting the defence towards infections, and that within this modulated environment, induction of a protective vaccination response is preserved.

In summary, the present study shows that GOS/FOS supplementation of children at risk for allergy reduces total plasma antibody levels, except for IgG4. CMP-specific IgG1 levels were decreased as well, whereas vaccination responsiveness as measured by DTP-specific Igs was not affected at all. This suggests that GOS/FOS, in addition to reducing the incidence of AD, can specifically suppress the immune response towards food allergens, while leaving the response to pathogens intact in children at risk for allergic disease. Since the Scientific Committee on Nutrition considered the studied GOS/FOS mixture as safe for infant nutrition up to a concentration of 8 g/l,[25] it implicates that supplementation of a specific mixture of short chain GOS and long chain FOS in a 9:1 ratio (IMMUNOFORTIS™) might be a safe method to restrain or down-modulate the atopic march.

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[8] General discussion

8.1 INTRODUCTION

Cow's milk (CM) and related products are an important source of protein and calcium in the diet, particularly at young age. Therefore, cow's milk allergy (CMA) can have serious implications on the development of young children, as well as on physiology at later age. In addition, CMA is inconvenient and potentially dangerous, and hereby affects the quality of life. Hence, knowledge of the immune response towards cow's milk proteins (CMPs) in CMA, as well as in tolerant individuals, is highly important and may provide insights for therapeutic strategies. In this thesis, the CMP-specific response of T cells and B cells is described in subjects with CMA, atopic individuals who had other allergies, but were tolerant to CM, and non-atopic subjects. The results presented in this thesis will be put into the perspective of current knowledge on CMA and allergy in general. After discussing T cell epitope recognition in CMA and tolerance in the context of other allergens in paragraph 2, the characteristics of CMP that may contribute to its allergenicity are discussed in paragraph 3. The most important features of the adaptive immune response to CMP in infants, children and adults with and without CMA are discussed in paragraph 4 and presented in a model in Fig. 1. Paragraph 5 elaborates on possible feedback mechanisms by which CMP-specific IgE and IgG can influence the specific T cell response in CMA. The role of these mechanisms may be influenced by the relatively high level of allergen-specific IgG in CMA, as compared to other food and inhalant allergies, which is discussed in paragraph 6. A detailed overview of the CMP-specific immune response in CMA is illustrated in Fig. 2. Lastly, paragraph 7 discusses possible therapies to induce tolerance to CM in allergic subjects, and to prevent the development of CMA in infants.

8.2 T CELL EPITOPE RECOGNITION IN CMA AND TOLERANCE

The initial steps in the adaptive immune response towards food proteins are the presentation of protein-derived peptides by APCs on MHC II molecules and the subsequent recognition of these epitopes by specific T cells. The protein content in CM is comprised of numerous different proteins, of which the casein proteins αs1-, αs2-, β-, and κ-casein, and the whey proteins α-lactalbumin and β-lactoglobulin are the most important. The concentration in CM of each of these proteins exceeds 1 g/l. The most abundant CMP is αs1-casein, which constitutes 12-15 g/l (32%) of total milk protein.[1]

In chapter 2 and 3 of this thesis, the recognition and HLA restriction of T cell epitopes in αs1-casein was investigated in children with CMA, atopic subjects without CMA, and non-atopic children. Apart from epitopes in the sequence spanned by amino acid 61-96, that were selectively bound by T cells from tolerant individuals, no major differences in epitope recognition were observed between the subject groups. These results were substantiated by the observation that MHC II-encoding HLA genotypes and HLA-restriction of T cell epitopes were not markedly different between cow's milk allergic and tolerant subjects. T cell epitopes in αs1-casein were mainly restricted to HLA-DQ (Fig. 2). This finding was unexpected, since expression of HLA-DR is

markedly higher than HLA-DQ in APCs, and T cell epitopes in other allergens are mainly HLA-DR-restricted.[2-5]
Previously, T cell epitopes in the major allergens PLA 2 (bee venom), Bet v 1 (birch pollen) and Fel d 1 (cat) were also demonstrated to correspond between allergic and tolerant subjects.[6-8] In contrast, for the wasp venom allergen Ves v 5, allergic individuals were observed to have a different and broader T cell epitope specificity than non-allergic subjects.[9] One of the immunodominant T cell epitopes in Ves v 5 appeared to be restricted to HLA-DRB3*0202, but the frequency of this genotype was not different between wasp venom allergic and non-allergic subjects. Interestingly, the T cell response to the major mugwort pollen allergen Art v 1 was observed to be dominated by one epitope.[10] Hence, the HLA-DRB1*01 genotype, which is the main restriction element for this epitope, is associated with mugwort pollen allergy.[11] Remarkably, αs1-casein and Ves v 5 contain far more T cell epitopes than Art v 1, whereas their molecular sizes are comparable. The broad diversity of T cell epitopes in these allergens may result in the less pronounced associations of HLA genotypes with the corresponding allergies (chapter 3).[9] In conclusion, T cell epitope specificity in patients with CMA is not strikingly different from tolerant subjects, and appears therefore not to be decisive in its pathogenesis.

8.3 FACTORS THAT INFLUENCE THE ALLERGENICITY OF CM

The phenotype of the antigen-specific immune response towards a foreign protein determines its allergenicity. The recognition of epitopes in CMPs by specific T cells does not appear to be different between patients with CMA and tolerant individuals. An obvious question is therefore, which factors determine the allergenicity of CM. As has been stated in the general introduction, only a limited number of proteins are potential allergens, and even if a protein is allergenic, only a small part of the population will develop allergy to that protein. Characteristics of CM that may increase its allergenicity include the relatively high amount of protein in CM, as well as the high number of potential linear IgE epitopes in CMPs.[12-16]

8.3.1 ROLE OF HIGH PROTEIN CONTENT AND HIGH EXPOSURE. The high protein content of CM and the high dose of exposure in young infants, for whom breast milk and CM are the only sources of nutrients in the first months of life, may partly be responsible for the fact that CMA is the most common food allergy in infants.[17] Due to immaturity of the gastro-intestinal tract, young children have increased gut mucosal permeability compared to older subjects, whereas digestive enzymatic activity and secretion of neutralizing IgA antibodies are lower.[18,19] Moreover, gastric acid secretion is relatively low in the first three months of life.[20] These factors may enhance the passage of undigested immunoreactive fragments from the gastro-intestine into the periphery, and hereby increase the risk of sensitization to CM in infants with an atopic predisposition. Upon gastro-intestinal maturation, which typically occurs in the first years of life,[19-21] CMPs will mainly be degraded into amino acids and peptides that are sufficiently small to be

immunologically inert. This could play an important role in the development of tolerance to CM, which is observed in the majority of infants with CMA before the age of three years.[22]
However, even in the mature gut, a small percentage of the ingested food proteins is absorbed in an immunologically intact form.[23] These protein fragments will induce immune responses in the gut-associated lymphoid tissue (GALT) and probably also in the periphery. This may explain why pronounced CMP-specific T cell and B cell responses could be detected in adult subjects (chapter 4 and 5), and why CMA also occurs in older children and adults. In addition to gastro-intestinal maturation, another factor that appears to enhance the development of tolerance to CM in young allergic children is maturation of the immune response. The immune system in infants is initially Th2-skewed, which is necessary for successful pregnancy, but may favor an allergic response to CM that declines with age in subjects with transient CMA.[24-27]
Paradoxically, high doses of orally ingested antigen are generally known to induce tolerance, whereas high exposure to CMP appears to increase the risk of CMA in infancy.[28-30] The initial Th2-skewed immune response in infants may provide an explanation for these contradictory observations. Another important factor is atopic predisposition, i.e. the hereditary tendency to become atopic. This enhances the chance of development of CMA both in infants and older subjects, despite the tolerogenic route by which CM is ingested.[30,31]

8.3.2 LINEAR AND CONFORMATIONAL IGE EPITOPES AND THE LINK WITH T CELL EPITOPES.

As has been pointed out in the general introduction, IgE epitopes have been identified in all six major CM allergens, by means of binding to overlapping synthetic peptides. The use of short peptides (~ 10 AA in length) for epitope mapping mainly detects linear IgE epitopes, which can be present in both the intact protein and in digested small fragments. This is opposed to conformational epitopes, which are only present in intact proteins in their native shape, as a result of their secondary and tertiary structure.[17]
Interestingly, children with persistent CMA have higher specific IgE to linear epitopes than children with transient CMA, and some of these epitopes in αs1-, αs2-, and κ-casein may be predictive for persistence of CMA.[15,32,33] IgE epitopes in children with transient CMA appear to be mainly conformational.[15] This has led to the hypothesis that enhanced digestion of CMPs as a result of gastro-intestinal maturation abrogates conformational IgE epitopes, and hereby contributes to development of tolerance.[13,15,32]
Two of the AA sequences in αs1-casein which contain IgE epitopes that may be predictive for persistent CMA (AA 69-78 and AA 173-194), appear to include very little T cell epitopes (chapter 2).[13,15] The sequence AA 69-78 was not bound by TCLs from subjects with CMA, whereas AA 173-194 was not immunogenic to any of the tested TCLs. It should be noted that epitope mapping for IgE and T cells was not performed in the same patient group, and that T cell and B cell epitopes within an antigen are not necessarily corresponding.[34] Nevertheless, the difference in recognition of these αs1-casein epitopes by T cells and immunoglobulins (Igs) is remarkable. Previous studies indicated that the presentation of T cell epitopes in tetanus toxin could be suppressed as well as

enhanced by antibodies that bound to the same domain.[35,36] This interesting observation may provide an explanation for the dichotomy between binding of T cells and IgE to the two αs1-casein sequences. The difference may be the result of suppressed presentation of T cell epitopes, induced by IgE or IgG that is bound to the same or adjacent epitopes. Hereby, antibodies binding to AA 69-78 and AA 173-194 may manipulate the T cell response away from these determinants.

8.4 T CELL AND B CELL RESPONSES TO CMP IN CMA AND TOLERANCE AT DIFFERENT AGES

Immune responses specific for CMP can be detected in both allergic and tolerant subjects, and in a wide variety of age groups. Previous studies from our group have focussed on the CMP-specific T cell response in infants and young children,[25,37,38] as well as in older children.[39] These studies were performed with CMP-specific T cell clones (TCCs). So far, CMP-specific immune responses were not investigated in adult allergic and tolerant subjects. Therefore, we studied CMP-specific PBMC responses in adults with CMA and non-atopic subjects, as well as in atopic adults who were sensitized to CM, but were clinically tolerant (chapter 4). We chose to use *in vitro* polyclonal cell populations to approach the systemic *in vivo* response as close as possible. Besides $CD4^+$ T cells (Th cells), also $CD8^+$ T cells, B cells and monocytes are present in PBMC populations. However, $CD4^+$ T cells appear to be predominant in the allergen-specific PBMC response.[40] The most important results of this study are combined with previous findings in our group on CMP-specific T cell responses, and are depicted in Fig. 1. In addition to T cell responses, this figure also shows results of our study on CMP-specific Ig levels in the three age groups, as described in chapter 5.

8.4.1 CMP-SPECIFIC IMMUNE RESPONSES IN SUBJECTS WITH CMA. As shown in Fig. 1, CMA in infants and children is characterized by a CMP-specific Th2 response, in which levels of IL-4 and IL-13 produced by specific TCCs are higher than IFN-γ.[37,39] CMP-specific Th2 responses as measured by production of IL-13 were also most pronounced in adults with CMA as compared to tolerant subjects, and the ratio of IL-13/IFN-γ in CMP-stimulated PBMCs was higher in CMA than in non-atopic adults (chapter 4). The Th2-skewed cytokine response in subjects with CMA was concomitant with a pronounced CMP-specific IgE response, as well as with relatively high specific IgG4 in children and adults (chapter 5).
Remarkably, the majority of adult subjects with CMA developed their allergy in adult life (chapter 4). The onset of CMA in adulthood has been reported earlier, and is diagnosed more often in female than in male subjects, whereas the majority of children with CMA appear to be boys.[31,41] This suggests that certain factors that are associated with the female gender may abrogate tolerance to CM at later age. Indeed, 35% of the women with CMA in the study of Stöger et al.[31] noticed their first symptoms of CMA during or soon after pregnancy. In addition, 16% had experienced a premenstrual aggravation of symptoms. These observations may implicate the in-

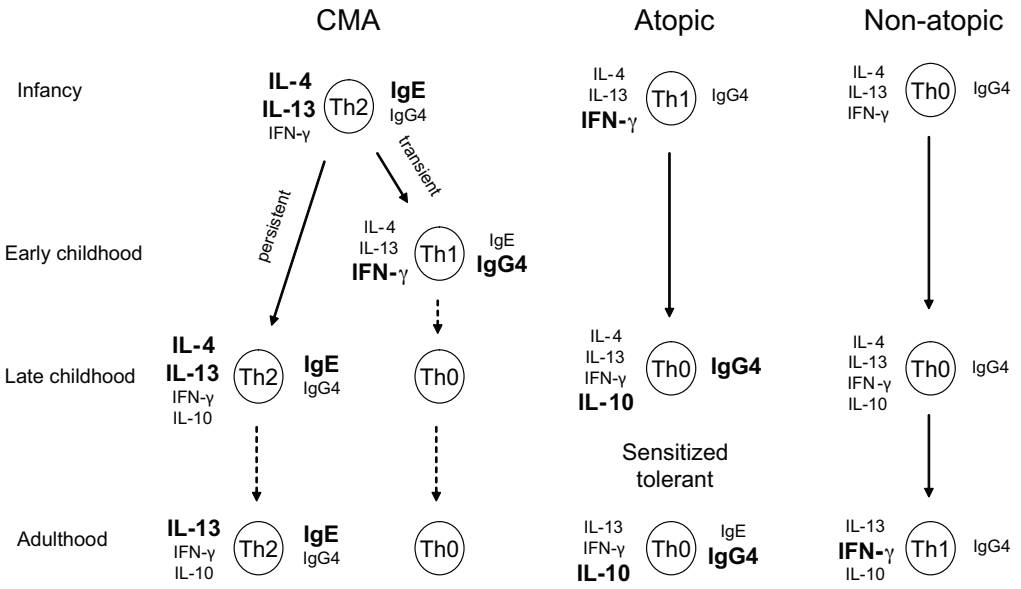

Figure 1.
Model that describes the adaptive immune response to CMP in three different subject groups in infancy, childhood and adulthood. The CMP-specific T cell and B cell response is shown in subjects with CMA, atopic individuals without CMA, and non-atopic subjects. The strength and polarization of the T cell cytokine response determines the effector B cell response, of which CMP-specific IgE and IgG4 are the most important parameters. The levels of cytokine and Ig responses are indicated by the size of the letters. Predominant responses for each subject group are depicted in bold.

volvement of sex hormones in CMA in adulthood. Comparatively, previous studies demonstrated that early age at menarche was associated with higher estrogen levels and an increased likelihood of allergic rhinitis, as compared to women with later onset of menarche.[42,43] In addition, pregnancy has been observed to enhance Th2 responses at the maternal-fetal interface, which may counterbalance proinflammatory Th1 responses that are harmful to the fetus.[27] This Th2 skewing may explain the observed development of allergy during or soon after pregnancy.[31] Interestingly, a previous study demonstrated that mothers of preterm infants less often had allergic rhinitis than mothers of infants with higher birth weight. It was suggested that a systemic Th2-skewed response is favorable for the maintenance of pregnancy, but increases the risk of atopic disease in the mother.[44] Nevertheless, the most important cofactor of development of CMA in adult subjects was the presence of previous atopic disease, which was reported by 53% of the patients.[31]

8.4.2 CMP-SPECIFIC IMMUNE RESPONSES IN ATOPIC CHILDREN AND SENSITIZED ADULTS WITHOUT CMA. The control group of atopic subjects without CMA is highly interesting, because these individuals share a Th2-skewed milieu with subjects with CMA, and yet tolerance to CM is maintained, here referred to as acquired tolerance. This may initially be realized by an "overshoot" in the CMP-specific T cell response towards a Th1 profile, as was observed in atopic infants without CMA, as well as in children with transient CMA upon development of tolerance.[25,37] At later age, these children develop a Th0 response, which is characterized by high levels of CMP-specific IL-10 production (Fig. 1).[39] Although the adult group of CM-sensitized subjects without CMA was different from the atopic children, who were not sensitized to CM, we observed a comparable Th0 response with high IL-10 release in CMP-stimulated PBMCs. The increased IL-10 response in atopic children and sensitized adults without CMA was accompanied by high levels of CMP-specific IgG4. Interestingly, a comparable allergen-specific immune response was observed in bee venom-allergic patients who developed tolerance after specific immunotherapy (SIT). The increase in allergen-specific IL-10 and IgG4 after SIT was accompanied by a decrease in specific IgE, and IL-10 was demonstrated in vitro to be responsible for the counterregulation of specific IgE and IgG4.[40,45] These data suggest that similar allergen-specific immune responses mediate active tolerance induction in allergic subjects by SIT and acquired tolerance to CM in atopic individuals.

A recent study by our group showed that children who were sensitized to CM but without CMA, developed severe allergic reactions upon accidental ingestion of CM, after a period of elimination of CM from the diet.[46] Hence, it is tempting to speculate that chronic exposure to CM in tolerant atopic subjects may play an important role in the maintenance of tolerance. More evidence supporting this hypothesis was provided by an animal model. It was demonstrated that exposure to allergen in presensitized rats induces pronounced regulatory T cell (Treg) activity and IL-10 responses. The maintenance of protective Treg activity was dependent on continuous allergen stimulation.[47] Even in non-atopic beekeepers, chronic exposure to bee venom increases allergen-specific production of IL-10 and IgG4, which suggests an active tolerance-inducing response.[40,48,49]

Although older atopic children and adults displayed an increased CMP-specific IL-10 and IgG4 response, atopic infants without CMA had a predominant Th1 response and low levels of specific IgG4. CMP-specific IL-10 responses may appear somewhat later in life than hallmark Th1 and Th2 cytokines, as was previously observed for cytokine responses in birch pollen-stimulated PBMCs of atopic and non-atopic children.[50] Comparatively, tolerance to CM in atopic subjects may initially be characterized by a Th1 response, whereas a balanced specific T cell response with high IL-10 release and subsequent induction of specific IgG4 is developed at later age (chapter 5, Fig. 1).

8.4.3 CMP-SPECIFIC IMMUNE RESPONSES IN NON-ATOPIC SUBJECTS. The non-atopic immune response to CMP is characterized by an attenuated T cell response and relatively low levels of specific IgG4 (chapter 5). CMP-specific T cells in non-atopic infants and children display a Th0 cytokine profile and have lower expression of activation markers than specific T cells from atopic subjects with and without CMA.[25,38,39] In contrast, the CMP-specific T cell response in non-atopic adults appears to be Th1-skewed, as high levels of IFN-γ and low IL-13 release were measured in CMP-stimulated PBMCs (chapter 4). The dichotomy between non-atopic children and adults may have been due to intrinsic differences in CMP-specific responses between TCCs and PBMCs, because IFN-γ was also higher than IL-13 in adults with CMA and sensitized subjects without CMA. However, CMP-specific Ig profiles were comparable in non-atopic children and adults, despite the differences in specific T cell responses. The lack of an atopic background in these subjects may reduce the need for upregulation of the immunomodulatory cytokine IL-10, as is observed in atopic subjects without CMA. In addition, and possibly as a result, CMP-specific IgG4 is lower in non-atopic than in atopic individuals (Fig. 1).

8.5 ROLE OF IGE AND IGG IN ALLERGEN PRESENTATION IN CMA

An interesting characteristic of the immune response to CMP is the pronounced induction of specific IgG, both in allergic and tolerant subjects. Particularly the CMP-specific IgG4 response appears to be stronger than that for other allergens (chapter 5+6). In adults with CMA, the level of CMP-specific IgG4 is 1000-fold higher than IgE (chapter 5). Specific IgG has been suggested to block the binding of IgE to allergen, resulting in a reduction in IgE-facilitated antigen presentation (IgE-FAP).[51,52] We observed that in addition to specific IgE, both IgG4 and IgG1 are present in αs1-casein-Ig complexes. By contributing to complex formation, IgG appeared to reduce the binding of complexes to B cells via the low affinity IgE receptor CD23, especially at low allergen concentrations (chapter 6). The minimal allergen concentration needed for substantial complex binding to B cells, as the first step of IgE-FAP, was observed to be 100-fold higher for CMA than for birch pollen allergy (1-10 μg/ml for CMA versus 0.01-0.1 μg/ml for BPA). These data may indicate that IgE-FAP via B cells is approximately 100-fold less efficient in CMA than in birch pollen allergy. This suggests that IgE-FAP by B cells plays only a minor role in activation of CMP-specific T cells in CMA, because allergen presentation without IgE-FAP is also effective at these concentrations (Fig. 2).[53]

The presence of IgG in αs1-casein-Ig complexes may facilitate binding via the low affinity IgG receptor CD32. Although the role of this receptor in complex binding to B cells was observed to be minimal, a previous study showed that specific IgG enhanced antigen presentation by targeting antigen-Ig to CD32 on immature dendritic cells (DCs).[3] Simultaneous ligation of the activating receptor CD32a and the inhibitory receptor CD32b by IgG induces DC maturation, as well as release of proinflammatory cytokines and IL-10.[54,55] Importantly, CD32b has been shown in mice to be essential in creating the conditions of antigen presentation that lead to induction of Tregs that

mediate mucosal tolerance.[56] Thus, targeting of CMP-Ig complexes to CD32 on DCs may give rise to enhanced antigen presentation, as well as to induction of Tregs and production of cytokines that may attenuate the CMP-specific Th2 response (Fig. 2).
Although IgE-FAP by B cells appears not to play an important role in CMA, CMP-specific IgE may enhance the allergic response via other mechanisms. For example, it has been shown that IgE-FAP can also occur via the high affinity IgE receptor (FcεRI) on DCs,[57] monocytes in allergic patients,[58] and Langerhans cells in patients with atopic dermatitis.[59] It is apparent that CMP can bind to and cross-link FcεRI on mast cells and basophils in adults with CMA, despite high levels of potentially blocking CMP-specific IgG. Therefore, it can be expected that CMP also binds to FcεRI on APCs, resulting in internalization and subsequent presentation of allergen. Hence, IgE-FAP via FcεRI likely occurs in CMA.

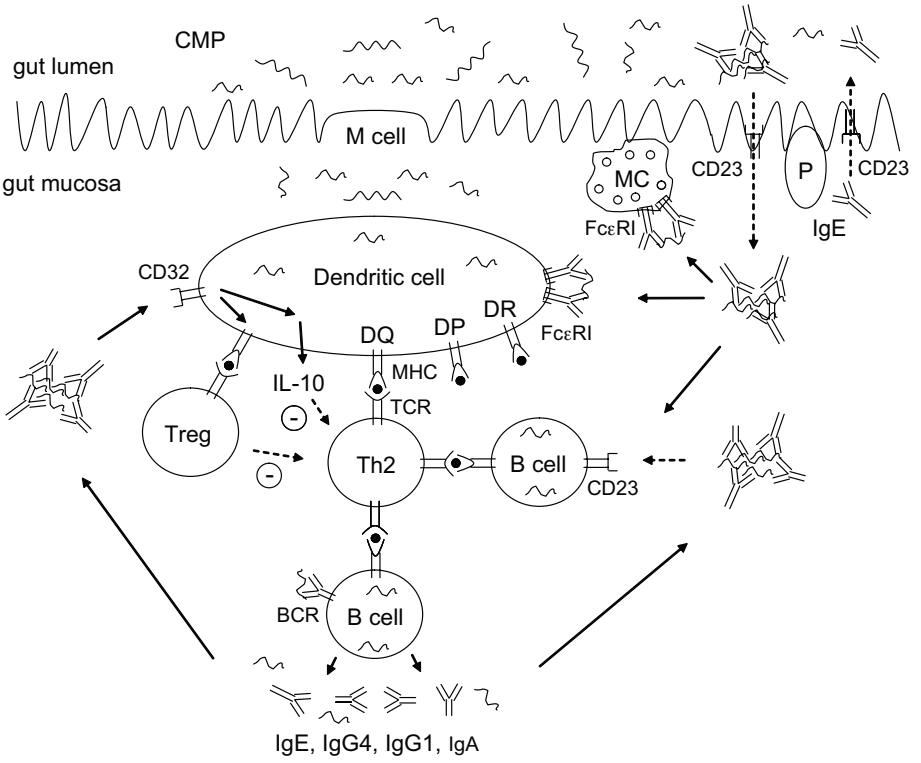

Figure 2.
The immune response to CMP in allergic patients. CMP is absorbed from the gut lumen mainly via microfold (M) cells and taken up by DCs. HLA-DQ may play an important role in presentation of CMP epitopes to T cells (chapter 3). Subsequently, a CMP-specific T cell response develops in CMA, dominated by Th2 cells. Specific T cells induce the production of IgE, as well as IgG and IgA, by CMP-specific B cells. Once CMA is established, CMP-specific IgE is produced by local plasma cells (P) and secreted into the gut lumen. As a result, traces of

CMP may be complexed with IgE in the gut lumen and taken up via CD23 on epithelial cells, leading to higher concentrations of IgE-CMP complexes in de mucosa. This may increase degranulation of local mast cells (MC), as well as uptake of CMP by DCs and other APCs via FcεRI or by B cells via CD23, resulting in increased CMP-specific T cell responses. Furthermore, specific IgE bound to FcεRI may directly mediate IgE-FAP of CMP-Ig complexes and uncomplexed CMP. Parallel to IgE, a pronounced CMP-specific IgG response is established (chapter 5), probably due to the relatively high amounts of ingested CMP before the onset of CMA, as well as the oral route of exposure. This may result in a decrease in IgE-FAP via CD23 and an increase in IgG-FAP via CD32, particularly at low allergen concentrations (chapter 6). Moreover, ligation of CD32 on DCs by complex-bound IgG may induce production of IL-10 and proinflammatory cytokines, as well as Treg activation. These effects may attenuate the CMP-specific Th2 response. Hence, specific IgG may break the vicious circle caused by IgE-FAP, by reducing the binding of IgE to allergen and by targeting allergen-Ig complexes to CD32. The marked induction of specific IgG upon exposure to CM could be part of a physiologic tolerogenic mechanism, observed in both atopic and non-atopic subjects. This mechanism may be particularly relevant in atopic individuals, in which the balance between specific IgE and IgG is probably decisive for the development of either CMA or tolerance.

An alternative way in which specific IgE may influence the CMP-specific T cell response is by enhancing the uptake of CMP from the gut lumen (Fig. 2). IgE has recently been described to facilitate transcytosis of IgE-allergen complexes in intestinal epithelial cells via CD23. In these cells, CD23 acts as a bidirectional IgE transporter, and facilitates apical to basal transcytosis of IgE-allergen complexes. Transcytosed allergen was still able to induce degranulation of mast cells, which indicated that the allergen was immunoreactive.[60] By this mechanism, CMP-specific IgE which is produced by local plasma cells and secreted into the gut lumen, appears to sample allergen and enhance its transport into the mucosa.[60,61] This may enhance local mast cell degranulation, as well as the CMP-specific T cell response.
In conclusion, CMP-specific IgE may be important in the maintenance of the allergic response in persistent CMA, despite the minor role of IgE-FAP via B cells. The efficient receptor-mediated allergen uptake via FcεRI on APCs and via CD23 in the intestine may cause traces of CMP to be sufficient for maintenance of the CMP-specific Th2 response. However, specific IgG may break this vicious circle caused by IgE-FAP, by reducing the binding of IgE to allergen and by targeting allergen-Ig complexes to CD32 (Fig. 2).

8.6 THE IG RESPONSE IN FOOD ALLERGY AND INHALANT ALLERGY

The common immune response to non-pathogenic gut antigens is the induction of local and systemic immunological tolerance, known as oral tolerance. This mechanism is especially relevant for innocuous foreign antigens that enter the body at high doses, such as CM and other food antigens. Key players in the induction of oral tolerance are tolerogenic DCs present in the mucosa.[62,63] Furthermore, Tregs, TGF-β and perhaps IL-10 and are important in the induction of oral tolerance, as has been demonstrated in several animal studies.[64-66] More evidence for the involvement of TGF-β is provided by the observation that spontaneous production of this cytokine is decreased in lymphocytes and epithelial cells in the gut mucosa of children with food allergy.[67]

Despite the tolerogenic setting in which CMP is initially presented to T cells, an allergic response develops in subjects with CMA. The mechanism of oral tolerance is highly complex. Besides a decrease in local constitutive TGF-β production, various other factors may contribute to abrogation of tolerance in CMA (see paragraph 3.1 and 4.1).[29] Nevertheless, the oral route of allergen exposure, in combination with a relatively high dose of exposure to CMP before development of CMA, may provide an explanation for the differential allergen-specific Ig response in CMA as compared to other allergies. The possible influence of these two factors will be discussed below. CMP-specific IgG4 in children and adults with CMA was found to be higher than the specific IgG4 response for the causative allergens in subjects with hen's egg allergy, peanut allergy, house dust mite allergy and birch pollen allergy. Furthermore, specific IgG1 was comparable in CMA and peanut allergy, but lower in birch pollen allergy (chapter 5 + 6). The strong CMP-specific IgG4 response in CMA, as well as in subjects tolerant to CM, may partly be the result of high IL-10 production by DCs and Tregs in the intestine, which is an important site for activation of CMP-specific T and B cells (Fig. 2).[68,69] High amounts of this cytokine are present in the gut mucosa, and IL-10 has been shown to regulate inflammatory responses to intestinal bacteria.[70]
In addition, the dose of exposure to CMP before the onset of CMA may have influenced the specific Ig response. Chronic and high dose exposure to allergen has been reported to induce specific IgG4 responses.[48,71] Generally, CM is ingested in higher amounts than hen's egg and peanut, which may have induced a relatively strong IgG4 response. Particularly in infants, the amount of ingested CM in relation to body weight is very high, and induces a pronounced CMP-specific IgG1 response which develops into a balanced IgG1 and IgG4 response at later age (chapter 5).[72]
In conclusion, these factors may explain the high level of allergen-specific IgG in CMA, as compared to other allergies. As mentioned in paragraph 5, CMP-specific IgG in subjects with CMA may interfere with IgE-FAP via CD23 and mediate IgG-FAP via CD32. This may result in less feedback enhancement of the allergic response, as well as possible induction of CMP-specific Tregs and attenuation of the specific Th2 response (Fig. 2). Via these mechanisms, CMP-specific IgG may play a role in the spontaneous development of tolerance in most young children with CMA, which is enhanced by gastro-intestinal and immunological maturation as well (see paragraph 3.1).
In contrast to CMA, IgE-FAP via CD23 occurs at low allergen concentrations in allergies that are characterized by low specific IgG, such as birch pollen allergy. This may enhance the allergen-specific Th2 response, and subsequently increase the production of specific IgE, upon exposure to the relatively low allergen concentrations during the pollen season.[73,74] The possible differences in IgE- and IgG-FAP between CMA and birch pollen allergy may have implications for the clinical characteristics. For example, one could speculate that the difference in feedback mechanisms may influence the prevalence of these allergies, as the prevalence of CMA in older children and adults appears to be approximately 20-fold lower than the prevalence of birch pollen allergy.[17,22,75] The characteristics of the immune response to CM as compared to other allergens may positively influence the development and maintenance of tolerance. The pronounced induction

of specific IgG upon exposure to CM appears to be a normal tolerogenic mechanism, which may be particularly relevant in subjects with an atopic constitution (chapter 5). The balance between specific IgE and IgG is probably decisive for the development of either CMA or tolerance.

8.7 POSSIBLE THERAPIES FOR TREATMENT AND PREVENTION OF CMA

As was discussed previously, CMA is mostly transient in young children. However, CMA can persist in older children and develop at later age in adults. Although markedly lower than that of inhalant allergies, the prevalence of CMA in these age groups is still approximately 0.3%.[17,22,75] Moreover, older subjects with CMA are less likely to develop spontaneous tolerance to CM.[31] Hence, development of a tolerance-inducing therapy for CMA is desirable. The most effective therapy that has been applied to date in allergic subjects is SIT, which was observed to induce clinical tolerance in a.o. bee venom allergy, birch pollen allergy and house dust mite allergy.[45,76,77] The most frequently observed immunological effects of SIT include an increase in the ratio of allergen-induced Th1/Th2 cytokine production and IL-10 release, as well as increased levels of specific IgG.[78] Specific IgG, and IgG4 in particular, has been suggested to play a role in tolerance induction by blocking the binding of IgE to allergen, which leads to decreased histamine release by basophils and mast cells, as well as a reduction in IgE-FAP.[51,52,79] Besides its counter-regulatory role on specific IgE and IgG4, IL-10 may add to tolerance development by modulating the function of mast cells and T cells.[78]

A previous study from our group demonstrated that levels of birch pollen-specific IgG4 were markedly increased in patients with birch pollen allergy, who were treated for 1 year with SIT.[76] We observed that levels of birch pollen-specific IgG4 after SIT were equal to CMP-specific IgG4 in the same subjects, and these were comparable to CMP-specific IgG4 levels in subjects with CMA (unpublished data). Hence, increased levels of birch pollen-specific IgG4 appear to contribute to clinical tolerance, whereas comparable levels of CMP-specific IgG4 are not sufficient to prevent the allergic response in CMA. Although it may be possible to induce even higher CMP-specific IgG4 levels in subjects with CMA by means of SIT, the clinical efficacy may be questionable. Therefore, SIT or other tolerance-inducing therapies could probably be more effective when focused on other mechanisms. For example, therapy-induced release of IL-10 could inhibit the activation of mast cells, whereas downregulation of CMP-specific Th2 responses may eventually result in a decrease in specific IgE.[78]

8.7.1 USE OF IMMUNOGENIC CMP FRAGMENTS IN INDUCTION OF TOLERANCE. As was described in chapter 2, T cells from children with CMA were observed to bind at least 15 different epitopes in αs1-casein. Nevertheless, it was possible to define an immunodominant part, spanned by amino acid (AA) no. 133-168, which was bound by T cell lines (TCLs) from 7 out of 10 children with CMA. T cell epitopes in the immunodominant sequence were restricted to HLA

genotypes that are abundantly present in subjects with CMA (chapter 3). Therefore, peptides spanning this sequence may be suitable for use in SIT.
To date, SIT with peptides has been successfully applied in bee venom allergy and cat allergy.[80-82] A reduction of clinical symptoms upon allergen exposure was reported, as well as downregulation of systemic Th1 and Th2 responses, with concomitant induction of IL-10 production, and induction of Tregs.[78,81,83,84] An important advantage of SIT with T cell epitope-containing peptides as compared to conventional SIT with intact allergens is the inability of short peptides (< 20 AA) to cross-link IgE.[81,85,86] This results in a decrease of immediate allergic reactions as a side-effect of the therapy, although T cell-mediated late asthmatic reactions have been reported after SIT with peptides derived from Fel d 1, the major cat allergen.[87] These symptoms were observed in a minority of the treated subjects, and disappeared after repeated injections of peptides, although late-phase responses re-appeared upon exposure to peptides after a discontinuation of the treatment for at least one year. In CMA, peptide SIT may be appropriate for tolerance induction, as its clinical efficacy has been demonstrated in other allergies where side effects appeared to be tolerable. To induce a broad CMP-specific tolerizing T cell response in CMA, SIT should include a mix of peptides spanning immunodominant sequences in the most important CM allergens. To this aim, T cell epitopes should also be investigated in the other caseins and whey proteins, in a substantial number of patients.[1,88] A previous study investigating the efficacy of peptide SIT in bee venom allergy, showed that the use of three different peptides derived from the major bee venom allergen PLA2 was sufficient to induce tolerance to bee venom.[80] Comparatively, only few peptides spanning immunodominant sequences in each CMP may be enough to induce tolerance in CMA, yielding a limited number of different peptides to be included in a mix for peptide SIT. Whenever patient-specific T cell responses towards the CMP can be determined, this might reduce the necessity of all peptides, resulting in a patient-tailored immunotherapy.
An alternative way of inducing tolerance to CM in young children with CMA may be the use of extensively hydrolyzed CM formulas, which contain immunoreactive protein fragments that can induce T cell responses, but are too short to cross-link IgE. These formulas are generally used to supplement the diet of young children who have symptoms of CMA, or are at high risk of atopy.[89] Previously, a formula containing casein fragments of < 1200 Da (~ 11 AA) was observed to induce equally strong casein-specific T cell responses as a formula based on whole CMPs.[90] This indicates that a CM formula with epitope-containing peptides can induce T cell responses, whereas IgE binding is minimized.[91] In primary prevention studies, extensively hydrolyzed CM formulas have been shown to reduce the risk of development of CMA in infants at risk of atopy.[89] Whether activation of CMP-specific T cells by these formula can eventually lead to tolerance in established CMA remains to be investigated.

8.7.2 PREVENTION OF CMA BY GOS/FOS. In chapter 7, a possible prophylactic treatment for CMA was described. In this study, a hypo-allergenic whey formula was supplemented with a prebiotic oligosaccharide mixture (GOS/FOS) and fed to infants at risk for development of atopic

disease. The GOS/FOS showed homologies with the neutral oligosaccharides present in human milk. Breast milk and formula supplemented with GOS/FOS were previously demonstrated to enrich the commensal bifidobacteria population to a higher extent than a formula without GOS/FOS.[92] In comparison with healthy infants, babies who developed allergy have been observed to be less often colonized with bifidobacteria during the first year of life, suggesting that these bacteria may have a protective function that prevents the development of allergy.[93] Treatment of infants with a combination of probiotic bacteria and galacto-oligosaccharides (GOS) has recently been observed to increase the commensal populations of bifidobacteria and lactobacilli, and resulted in a slight decrease in IgE-associated allergic disease.[94] Both types of bacteria have been demonstrated to induce high production of Th1 cytokines and IL-10 in vitro.[95] It is therefore tempting to speculate that these bacteria may balance the Th2-skewed immune response in infants, and hereby decrease the risk of atopic disease. Treatment with GOS/FOS was observed to decrease the levels of total IgG1-3, as well as IgE, which may be associated with the reduced incidence of atopic dermatitis in this group (chapter 7).[96] Reduction of the frequency of atopic dermatitis was previously observed after treament with *Lactobacillus rhamnosus* GG, but no changes in total or specific IgE levels were found.[97] This may suggest that GOS/FOS has a stronger immunomodulatory potential than supplementation with just one probiotic strain, which may be due to the broader range of commensal bacteria that are stimulated by a prebiotic mixture. GOS/FOS supplementation appeared to specifically modulate the immune response towards food antigens, since no decrease was observed in DTP-specific Igs. The decreased CMP-specific IgG1 response in treated infants suggests an overall suppression of the immune response to CMP, which may decrease the risk for development of CMA. However, this assumption needs confirmation in a follow-up study.

8.8 CONCLUDING REMARKS

Insight into the adaptive immune response to CM in allergic and tolerant individuals may provide starting points for treatment of CMA. In this thesis, it was demonstrated that T cell epitope recognition is not strikingly different between patients with CMA and tolerant subjects. The allergen-specific Ig response in CMA is marked by high levels of IgE, as well as IgG, which may influence its clinical characteristics. The pronounced induction of specific IgG upon exposure to CM appears to be a normal tolerogenic mechanism, which occurs in both atopic and non-atopic subjects. This mechanism may be particularly relevant in atopic individuals, as the balance between specific IgE and IgG appears to be decisive for the development of CMA or tolerance. Peptides spanning immunodominant sequences in the major CM allergens could be a good candidate for application in tolerance-inducing therapy in CMA. This may induce a tolerant immune response that is comparable to the acquired tolerance observed in atopic subjects without CMA, and is characterized by high IL-10 production by CMP-specific T cells, leading to reduced allergen-specific IgE and high IgG4.

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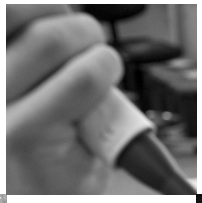
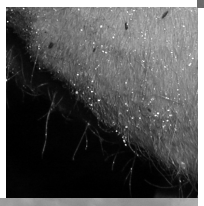


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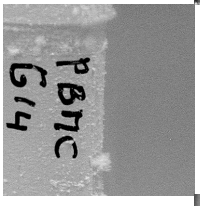
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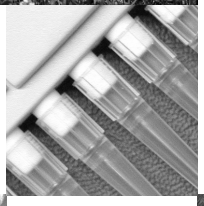
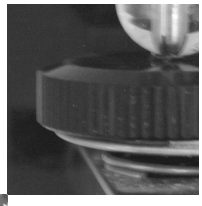
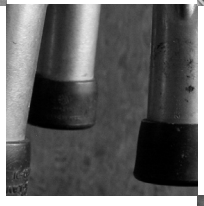
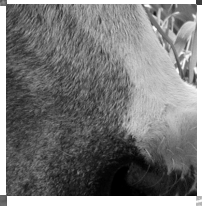
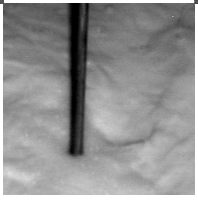


Summary/
Nederlandse samenvatting

SUMMARY

Allergy is best described as an inappropriate immune response against innocuous foreign antigens. Although the human body encounters a wide variety of foreign proteins each day, only a small proportion is known for their capacity to elicit an allergic response. The most allergenic food proteins are present in cow's milk (CM), hen's egg, peanut, tree nuts, fish and shellfish.
The key event in the elicitation of an allergic response is the production of allergen-specific immunoglobulin E (IgE), which binds to the allergenic protein. A unique feature of the IgE antibody is its capacity to bind with high affinity to mast cells and basophils. Upon cross-linking of bound IgE by the allergenic protein, these cells degranulate and release mediators such as histamine. It is these mediators that are responsible for the allergic reaction. In food allergy, symptoms can be diverse, ranging from cutaneous, oropharyngeal, gastro-intestinal, and respiratory organ involvement, to systemic anaphylaxis.
IgE-mediated cow's milk allergy (CMA) occurs in 1.5% of infants, as well as in 0.3% of older children and adults. CM and related products are an important source of protein and calcium in the diet, particularly at young age. Therefore, a CM-free diet can have serious implications for the development of young children with CMA, as well as for physiology at later age. In addition, CMA is inconvenient and potentially dangerous, and hereby affects the quality of life. Hence, insight into the immune response towards cow's milk protein (CMP) in CMA, as well as in tolerant individuals, is highly important and may provide potential targets for therapeutic strategies.
CMP-specific IgE and other immunoglobulins (Igs) are produced by plasma cells, which are B cells that have differentiated upon activation by T helper (Th) cells. The production of IgE requires Th2 cells that produce the cytokines interleukin-4 (IL-4) and IL-13. Th2 responses can be balanced by cytokines that are typical for Th1 and regulatory T cell responses, such as interferon-γ (IFN-γ) and IL-10, respectively. CMP-specific T cells recognize small fragments of CMPs, known as T cell epitopes. In chapter 2, these epitopes were studied in αs1-casein, the most abundant CMP. Polyclonal T cell lines (TCLs) were derived from children with CMA, atopic children who had other allergies but no CMA, and non-atopic subjects. T cell epitope mapping was performed with overlapping synthetic peptides, which spanned the entire sequence of αs1-casein. The immunodominant sequence in αs1-casein was observed to be spanned by AA residues 133-156. Only subtle differences were found in epitope recognition between T cells of children with CMA and tolerant subjects, which suggests that CMA is not caused by differential recognition of selective epitopes.
CMP-derived peptides are presented to T cells by major histocompatibility complex II (MHC II) molecules on antigen-presenting cells (APCs). MHC II molecules are encoded by the human leucocyte antigen (HLA) genes. These genes are divided into three groups, namely HLA-DP, -DQ, and -DR. HLA genes determine which CMP fragments are presented and influence the quality of epitope presentation, because peptides can bind strongly to one type of MHC II molecule and weakly to another. In chapter 3, the allele frequencies of HLA genes encoding MHC II were compared between cow's milk allergic, atopic and non-atopic children and adults. No major differences were

found between subjects with CMA and tolerant individuals. This suggests that the HLA genes do not contribute significantly to the genetic predisposition underlying CMA. The majority of T cell epitopes in αs1-casein were presented by HLA-DQ. This finding was unexpected, since expression of HLA-DQ is markedly lower than HLA-DR in APCs, and T cell epitopes in other allergens are mainly HLA-DR-restricted.

Whereas CMA in infants and children is well documented, little information is available on CMA in adults. The immunological background of CMA may differ between affected children and adults, as adult subjects mainly develop CMA later in life. Chapter 4 dealt with the immune response to CMP in adults. In this study, peripheral blood mononuclear cells (PBMCs) were used to obtain an optimal polyclonal response. These cells were derived from adults with CMA, adults sensitized to CM but without CMA, and non-atopic adults. Proliferation and cytokine production of PBMCs was measured in response to CMP. Moreover, plasma levels of CMP-specific IgE and IgG4 were determined. Production of IL-13 in CMP-stimulated PBMCs and the ratio of IL-13/IFN-γ was higher in subjects with CMA than in non-atopic individuals. This underlines the role of specific Th2 responses in CMA, as was previously shown in infants and children with CMA. CMP-induced production of IL-10 by PBMCs, as well as plasma levels of CMP-specific IgG4, were higher in sensitized subjects without CMA than in non-atopic adults. The upregulation of IL-10 may be needed to balance the CMP-specific immune response in an atopic, Th2-skewed environment, and may play a role in tolerance to CM in sensitized adults without CMA by counterregulating specific IgE and IgG4.

In chapter 5, the CMP-specific antibody response was further investigated in infants, children and adults. Levels of specific IgE, IgG4, IgG1 and IgA for whole CMP and the six most abundant individual CMPs were measured in plasma of subjects with CMA, atopic individuals without CMA or sensitization to CM, and non-atopic subjects. In infants and children, αs1-casein and β-lactoglobulin induced the highest specific IgE response, whereas αs1-casein was the single most allergenic CMP in adults. Specific IgG4 and IgG1 responses were found to be highest to αs1-casein and β-lactoglobulin in all age groups, whereas κ-casein and α-lactalbumin induced the highest levels of IgA. CMP-specific IgG4 was higher in atopic children and adults without CMA, as compared to non-atopic individuals. This observation suggests that tolerance to CM in subjects with an atopic constitution is associated with elevated levels of specific IgG4, in combination with low specific IgE. A similar difference between atopic and non-atopic subjects was observed for IgG4 specific to ovomucoid, whereas house dust mite-specific IgG4 was not detectable in these subjects. Hence, the upregulation of specific IgG4 in tolerant atopic individuals may be related to the type of allergen and its regular dose of exposure.

Patients with CMA were observed to have high levels of CMP-specific IgG, in addition to high specific IgE. In chapter 6, it was shown that specific IgG for the causative allergen is higher in CMA than in peanut allergy (PA) and birch bollen allergy (BPA), whereas specific IgE levels are comparable. Allergen-specific IgG in CMA may reduce the efficiency of IgE-facilitated allergen presentation (IgE-FAP) to specific T cells, as is observed after birch pollen-specific immuno-

therapy in subjects with BPA. The first step of IgE-FAP by B cells is the binding of allergen-IgE complexes to the low affinity IgE receptor CD23. The optimal allergen concentration for complex binding to B cells in CMA was approximately 100-fold higher, and in PA 10-fold higher, than in BPA. This suggests that allergen-specific IgG in food-allergic patients competes with IgE for binding of allergen in allergen-Ig complex formation. Hereby it reduces CD23-mediated complex binding to B cells at low allergen concentrations. As a consequence, feedback enhancement of the specific Th2 response by IgE-FAP may be less prominent in CMA and PA than in allergies characterized by low specific IgG, such as BPA.

In chapter 7, a possible prophylactic treatment for atopic disease was described. A hypo-allergenic whey formula was supplemented with a prebiotic oligosaccharide mixture (GOS/FOS) and fed to infants at risk for development of atopic disease. Formula supplemented with GOS/FOS has previously been demonstrated to enrich the commensal bifidobacteria and lactobacilli populations. Both types of bacteria induce high production of Th1 cytokines and IL-10 in PBMCs *in vitro*, which suggests that these bacteria may balance the Th2-skewed immune response in infants, and hereby decrease the risk of atopic disease. Supplementation with GOS/FOS led to a significant reduction in the plasma level of total IgE, IgG1, IgG2 and IgG3, as well as CMP-specific IgG1. In contrast, levels of Igs specific for pathogen-derived proteins present in the DTP-vaccination were not affected. Hence, GOS/FOS supplementation appeared to specifically modulate the immune response towards food antigens, while leaving the response to pathogens intact. The decreased CMP-specific IgG1 response in treated infants suggests a suppression of the immune response to CMP, which may decrease the risk for development of CMA.

In conclusion, this thesis demonstrates that T cell epitope recognition and HLA genotype frequencies are not strikingly different between patients with CMA and tolerant subjects. The allergen-specific Ig response in CMA is marked by high levels of IgE, as well as IgG, which may influence its clinical characteristics. The pronounced induction of specific IgG upon exposure to CM appears to be a physiologic mechanism, which occurs in non-atopic and most prominently in atopic subjects. This mechanism may be particularly relevant in atopic individuals, as the balance between specific IgE and IgG appears to be decisive for the development of CMA or tolerance. Peptides spanning immunodominant sequences in αs1-casein and other major CM allergens could be a good candidate for application in tolerance-inducing therapy in CMA. This may induce an immune response that is comparable to the acquired tolerance observed in atopic subjects without CMA. This response is characterized by high IL-10 production by CMP-specific T cells, associated with reduced allergen-specific IgE and high IgG4.

NEDERLANDSE SAMENVATTING

Allergie is het best te omschrijven als een ongepaste immuunrespons tegen onschadelijke lichaamsvreemde stoffen. Hoewel het menselijk lichaam dagelijks wordt blootgesteld aan een scala aan lichaamsvreemde eiwitten, zijn er maar enkelen bekend vanwege de allergische symptomen die ze kunnen induceren. Voedseleiwitten die een allergische reactie kunnen veroorzaken zijn o.a. aanwezig in koemelk (KM), kippenei, pinda, noten, vissen en schaaldieren. Een allergische immuunrespons wordt gekenmerkt door de productie van allergeen-specifiek IgE. Een unieke eigenschap van IgE is, dat het met hoge affiniteit kan binden aan mestcellen en basofiele granulocyten. Als het allergeen vervolgens bindt aan het IgE op deze cellen, worden de cellen geactiveerd. Dit leidt tot degranulatie en uitscheiding van stoffen zoals histamine, welke verantwoordelijk zijn voor de allergische reactie. De symptomen van voedselallergie zijn divers en kunnen optreden in de huid, mond- en keelholte, maagdarmstelsel en luchtwegen. Ook kan een plotselinge sterke bloeddrukdaling optreden, wat bekend is als systemische anafylaxie.
IgE-gemedieerde koemelkallergie (KMA) komt voor in 1,5% van alle zuigelingen, evenals in 0,3% van de oudere kinderen en volwassenen. KM en hiervan afgeleide producten zijn een belangrijke bron van eiwit en calcium in de voeding, voornamelijk op jonge leeftijd. Daarom kan een noodgedwongen KM-vrij dieet de ontwikkeling van jonge kinderen met KMA en de fysiologie van oudere patiënten nadelig beïnvloeden. Daarnaast is KMA vervelend en potentieel gevaarlijk, wat de kwaliteit van leven vermindert. Vandaar dat inzicht in de immuunrespons tegen koemelkeiwit (KME) in zowel patiënten met KMA als personen die koemelk kunnen verdragen erg belangrijk is. Deze kennis zou bij kunnen dragen aan de ontwikkeling van therapieën voor KMA.
KME-specifiek IgE en andere specifieke antilichamen (immuunglobulines, Igs) worden geproduceerd door plasmacellen. Dit zijn B cellen die gedifferentieerd zijn na activatie door T helper (Th) cellen. B cellen gaan IgE produceren als ze geactiveerd worden door Th2 cellen die de cytokines interleukine-4 (IL-4) en IL-13 uitscheiden. Th2 responsen kunnen worden gedempt door cytokines die typerend zijn voor Th1 en regulatoire T cel responsen, zoals respectievelijk interferon-γ (IFN-γ) en IL-10. KMA-specifieke T cellen herkennen kleine fragmenten van koemelkeiwitten, die T cel epitopen worden genoemd. In hoofdstuk 2 zijn deze epitopen beschreven in αs1-caseïne, het eiwit dat in KM het meest voorkomt. KMA-specifieke T cellijnen werden opgekweekt uit witte bloedcellen, die waren geïsoleerd uit het bloed van kinderen met KMA, atopische kinderen die andere allergieën hadden maar geen KMA, en niet-atopische kinderen. Screening van T cel epitopen werd gedaan m.b.v. overlappende synthetische peptiden, die de gehele aminozuursequentie van αs1-caseïne omvatten. De sequentie die door de meeste T cellijnen werd herkend (de immunodominante sequentie) omvatte de aminozuren 133-156. Er waren slechts subtiele verschillen in T cel epitoopherkenning tussen kinderen met KMA en tolerante kinderen. Dit suggereert dat KMA niet veroorzaakt wordt door een verschil in herkenning van bepaalde T cel epitopen.
Peptiden van KME worden gepresenteerd aan T cellen door antigeen-presenterende cellen (APCs) via major histocompatibility complex II (MHC II) moleculen. De human leucocyte antigen

(HLA) genen coderen voor deze moleculen. HLA genen worden verdeeld in drie groepen, namelijk HLA-DP, -DQ en -DR. Deze genen bepalen welke KME fragmenten worden gepresenteerd. Tevens beïnvloeden de HLA genen de kwaliteit van de epitooppresentatie, aangezien peptides sterk kunnen binden aan het ene type MHC II en zwak aan het andere. In hoofdstuk 3 zijn de allelfrequenties van de MHC II-coderende HLA genen vergeleken tussen kinderen en volwassenen met KMA, atopische personen zonder KMA en niet-atopische personen. Er werden geen grote verschillen gevonden tussen patiënten met KMA en tolerante personen, wat suggereert dat de bijdrage van de HLA genen aan de erfelijke aanleg voor KMA gering is. Verder bleek het grootste deel van de T cel epitopen in αs1-caseïne gepresenteerd te worden door MHC II moleculen die gecodeerd worden door HLA-DQ. Dit was een onverwachte vondst, omdat de expressie van HLA-DQ in APCs veel lager is dan die van HLA-DR. T cel epitopen in andere allergenen worden dan ook voornamelijk gepresenteerd door HLA-DR-gecodeerd MHC II.

Over KMA in zuigelingen en kinderen is al vrij veel bekend, maar over KMA in volwassenen is nog maar weinig informatie beschikbaar. De immunologische achtergrond van KMA zou kunnen verschillen tussen aangedane kinderen en volwassenen, want volwassenen ontwikkelen meestal pas op latere leeftijd KMA. Hoofdstuk 4 beschrijft de immuunrespons tegen KME in volwassenen. In deze studie werden mononucleaire cellen uit perifeer bloed (PBMCs) gebruikt, die niet werden opgekweekt. Hiermee werd een zo polyclonaal mogelijke celpopulatie behouden. De cellen waren afkomstig van volwassenen met KMA, personen die gesensibiliseerd waren voor KM (d.w.z. specifiek IgE hadden voor KME) maar geen klinische symptomen van KMA, en niet-atopische volwassenen. De proliferatie en cytokineproductie van PBMCs werd gemeten na stimulatie met KME. Daarnaast werden de concentraties bepaald van KME-specifiek IgE en IgG4 in bloedplasma. De productie van IL-13 in KME-gestimuleerde PBMCs en de ratio van IL-13/IFN-γ waren hoger in personen met KMA dan in niet-atopische individuen. Dit benadrukt de rol van specifieke Th2 responsen in KMA, zoals eerder is aangetoond in zuigelingen en kinderen met KMA. De KME-geïnduceerde productie van IL-10 door PBMCs, evenals de plasmaconcentratie van KME-specifiek IgG4, waren hoger in gesensibiliseerde personen zonder KMA dan in niet-atopische volwassenen. De verhoogde IL-10 productie zou nodig kunnen zijn om de KME-specifieke immuunrespons, die neigt naar een Th2-gedomineerde respons in atopische personen, in balans te houden. IL-10 zou hiermee een rol kunnen spelen bij het behouden van tolerantie voor KM in gesensibiliseerde personen zonder KMA, door de productie van specifiek IgE te onderdrukken en die van IgG4 te versterken.

In hoofdstuk 5 werd de KME-specifieke antilichamenrespons verder onderzocht in zuigelingen, kinderen en volwassenen. De concentraties van specifiek IgE, IgG4, IgG1 en IgA voor totaal KME en de zes belangrijkste afzonderlijke koemelkeiwitten werden gemeten in plasma van patiënten met KMA, atopische personen zonder KMA en niet-atopische individuen. De specifieke IgE respons was het hoogst tegen αs1-caseïne en β-lactoglobuline in zuigelingen en kinderen met KMA, terwijl αs1-caseïne het meest allergeen was in volwassen patiënten. De specifieke IgG4 en IgG1 responsen waren het hoogst tegen αs1-caseïne en β-lactoglobuline in alle leeftijdsgroepen,

zowel in allergische als tolerante personen. Specifiek IgA was echter het hoogst voor κ-caseine en α-lactalbumine. KME-specifiek IgG4 was hoger in atopische kinderen en volwassenen zonder KMA, dan in niet-atopische personen. Deze bevinding suggereert dat tolerantie voor KM in personen met een atopische achtergrond geassocieerd is met verhoogde concentraties van specifiek IgG4, gecombineerd met laag specifiek IgE. Een vergelijkbaar verschil tussen atopische en niet-atopische personen werd gevonden voor IgG4 specifiek voor ovomucoid, een allergeen in kippeneiwit. Echter, huisstofmijt-specifiek IgG4 was niet detecteerbaar in deze personen, maar enkel in individuen allergisch voor huisstofmijt. Dit wijst erop dat de versterkte IgG4 respons in tolerante atopische personen gerelateerd is aan het type allergeen en de bijbehorende dosis van blootstelling.

Patiënten met KMA hebben niet alleen hoge concentraties KME-specifiek IgE, maar ook hoog specifiek IgG. In hoofdstuk 6 is beschreven dat specifiek IgG voor het veroorzakende allergeen hoger is in KMA dan in pinda-allergie (PA) en in berkenpollen-allergie (BPA), terwijl de concentraties van specifiek IgE vergelijkbaar zijn. Allergeen-specifiek IgG in KMA zou de efficiëntie van IgE-gefaciliteerde allergeen presentatie (IgE-FAP) aan specifieke T cellen kunnen verminderen, zoals eerder beschreven is na berkenpollen-specifieke immuuntherapie in personen met BPA. De eerste stap van IgE-FAP door B cellen is de binding van allergeen-IgE complexen aan de lage-affiniteits IgE receptor CD23. De optimale allergeenconcentratie voor complexbinding aan B cellen was in KMA ongeveer 100 keer hoger, en in PA 10 keer hoger, dan in BPA. Deze data suggereren dat allergeen-specifiek IgG in voedselallergische patiënten concurreert met IgE om binding aan allergeen bij de vorming van allergeen-Ig complexen. Hiermee verlaagt IgG de CD23-gemedieerde complexbinding aan B cellen bij lage allergeenconcentraties. Als gevolg hiervan zou de versterking van de specifieke Th2 respons door IgE-FAP minder prominent kunnen zijn in KMA en PA dan in allergieën die gekenmerkt worden door lage concentraties specifiek IgG, zoals BPA.

In hoofdstuk 7 is een behandeling beschreven die de kans op ontwikkeling van atopische aandoeningen zou kunnen verkleinen. Een mix van oligosacchariden (GOS/FOS) met prebiotische activiteit werd toegevoegd aan hypoallergene babymelk op basis van wei-eiwitten uit KM. Deze voeding werd gegeven aan zuigelingen met een verhoogd risico op de ontwikkeling van atopische ziekten. Eerder is aangetoond dat babymelk met GOS/FOS de groei bevordert van commensale darmbacteriën, zoals bifidobacteriën en lactobacillen. Beide soorten bacteriën induceren een hoge productie van Th1 cytokines en IL-10 in PBMCs *in vitro*. Deze bacteriën zouden daarom kunnen helpen bij het in balans brengen van de Th2 respons in zuigelingen, wat het risico op atopische aandoeningen zou verminderen. Het gebruik van babymelk met GOS/FOS leidde tot een verlaging van de plasmaconcentraties van totaal IgE, IgG1, IgG2 en IgG3, evenals van KME-specifiek IgG1. Echter, de concentraties van Igs specifiek voor eiwitten van pathogenen, die gebruikt worden in de DTP-vaccinatie, bleven onveranderd. Toevoeging van GOS/FOS aan babymelk lijkt dus specifiek de immuunrespons tegen voedseleiwitten te dempen, terwijl de respons tegen pathogenen intact blijft. De verlaagde KME-specifieke IgG1 respons in behandelde zuigelingen wijst op een onderdrukking van de immuunrespons tegen KME, wat het risico op ontwikkeling

van KMA mogelijk zou kunnen beperken. Samenvattend wordt in dit proefschrift aangetoond dat T cel epitoopherkenning en frequenties van HLA genotypen niet duidelijk verschillen tussen patiënten met KMA en tolerante personen. De allergeen-specifieke Ig respons in KMA wordt gekenmerkt door een hoge concentratie van zowel IgE als IgG, wat de klinische karakteristieken van KMA zou kunnen beïnvloeden. De sterke inductie van specifiek IgG na blootstelling aan KM lijkt een fysiologisch mechanisme, wat plaatsvindt in niet-atopische en met name in atopische personen. Dit mechanisme zou belangrijk kunnen zijn in atopische individuen, omdat de balans tussen specifiek IgE en IgG beslissend lijkt voor het ontwikkelen van KMA of tolerantie. Peptiden die de immunodominante aminozuursequenties omvatten van αs1-caseïne en andere belangrijke KM allergenen zouden een goede kandidaat kunnen zijn voor toepassing in tolerantie-induceren-de therapie in KMA. Deze therapie zou een immuunrespons kunnen induceren die vergelijkbaar is met de tolerante respons in atopische personen zonder KMA. Deze respons wordt gekenmerkt door hoge IL-10 productie door KME-specifieke T cellen, gepaard gaand met gereduceerd aller-geen-specifiek IgE en verhoogd IgG4.

Mel

PBMC
6/14

Dankwoord

DANKWOORD

Zo, tiskeburt...wat fijn dat het af is. Het resultaat van 4,5 jaar pipetteren en schrijven, samengevat in een stemmig zwart-wit boekje. Nu is een mooi moment om alle mensen te bedanken die hieraan hebben bijgedragen, in het traditioneel best gelezen stukje proefschrift.
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Waar Els en Edward mij direct begeleidden, waakte Carla als promotor over de grote lijn. Carla, jij hebt die typische professorenkwaliteit om zonder volledig ingewijd te zijn toch de goede vragen te stellen. Hiermee heb je me scherp gehouden in de laatste anderhalf jaar van mijn promotie. Dat een professor ook toegankelijk kan zijn, bewees je onlangs nog op het feestje van Kim, waar we samen hebben geswingd op de fijnbesnaarde tonen van de gevierde volkszanger van dienst.

Dan de medebewoners van mijn natuurlijke habitat op het AZU, de voedselkamer. In chronologische volgorde: Machteld, met wie ik menig gezellig moment heb gedeeld, met een ijsje in de binnentuin of met een biertje in de Basket of Primus. Jou en Rogier moet ik ook bedanken voor het verzamelen van het patiëntenmateriaal wat ik voor een groot deel van mijn onderzoek heb gebruikt. Je bent alweer bijna klaar met je postdoc onderzoek in Londen, succes met je nieuwe job in Rotterdam. Suzanne, mijn kennis van het levenslied en Willeke Alberti in het bijzonder heb ik voornamelijk te danken aan jou en aan Kim, die vol overgave meezong in de muis van haar computer op de vrijdagmiddag-smartlapmomenten. Kimmie, we begonnen tegelijk aan ons onderzoek en deelden zowel promovendus-stressmomenten als licht beschonken ritjes huiswaarts na feestelijkheden. Succes met het restant van je dermat-opleiding. Adrie, jouw ruime ervaring

met labtechnieken kwam goed van pas in onze groep met allemaal jonkies. Ik vond het heel gezellig om met je samen te werken, dank je voor je werk aan hoofdstuk 4 van dit boekje en hoop dat je lekker gaat genieten van je pensioen. Annebeth, we hebben vaak gezellig gekletst, gevoetbald in het park en samen met Kim romantisch in het reuzenrad gezeten in Wenen. Veel succes met de laatste onderzoeksloodjes. Stansie, jij gaf het labwerk een flinke gezelligheids-impuls. Het is toch bijzonder als een vrouw je bij kan praten over zowel Champions League als Grey's Anatomy. Toen kwam er een paleis te koop en dachten jullie: Gewoon doen. Veel succes met klussen en verhuizen. La Titia, nieuwste bewoner van onze kamer maar meteen al belangrijk voor mijn gezondheid. Bedankt voor je gezelligheid en goede zorgen. Jaap, deeltijd-kamergenoot, dank voor je werk voor hoofdstuk 5 en je droge opmerkingen.

De voedselkamer mag dan wel de leukste zijn van Dermatologie, ook daarbuiten waren er veel mensen belangrijk voor mijn onderzoek en de leuke tijd die ik bij dermat heb gehad. Bijvoorbeeld Inge, de superprikker die me geholpen heeft met bloed verzamelen van niet-atopische controles (lees: labgenoten). Dirk Jan, die immer paraat stond voor compu-gelinkte vragen en nu ongetwijfeld aan de weg timmert in Boston. Evert, eveneens digitaal deskundige en altijd in voor wat wetenschappelijke reflectie. Bennie (tevens bekend als Onno), die op het lab een komisch duo vormt met Peter, overigens ook een compu-handyman. Jullie melige grappen vormden een mooi tegenwicht voor de wetenschappelijke serieuzigheid. Sara, fijn dat we de jive even hebben opgefrist op het feestje van Kim. Maurice, droom lekker verder van dat gigantische zeiljacht wat er vast ooit gaat komen. Mayke, veel succes met de laatste worstelingen met die gigantische databases. Marloes en Annemieke, bedankt voor de gezelligheid tijdens het labwerk. Ans, dank je wel voor je hulp bij het uitzoeken van patiëntenmateriaal. De secretaresses Jantine en Miranda, bedankt voor jullie hulp en de gezellige praatjes tussendoor. André, Suzanne en de andere dermat-stafleden, evenals Marja, Ines en de andere assistenten, bedankt voor jullie hulp en/of gezelligheid. En natuurlijk Kees, jouw kennis van statistiek, antieke computers en bijbehorende programma's is onmisbaar.

Ook mensen van buiten dermat hebben bijgedragen aan dit proefschrift. Joost, je was dermat-parttimer maar je expertise was heel belangrijk voor hoofdstuk 5 en 6 van dit boekje. Veel succes met je nieuwe functie in Deventer. Johan, Laura, Joyce en Virginie, bedankt voor jullie input namens Numico Research en voor het feit dat jullie je sterk hebben gemaakt voor de financiering van het onderzoek. Marcel, Erik, Anette en de diagnostiek-analisten van Pathologie, dank voor het mede mogelijk maken van de HLA-paper en het beantwoorden van mijn vele beginnersvragen over DNA-sequencing.
Drie studenten hebben mij geholpen met het onderzoek. Hoo Yin en Karin, mijn dank aan jullie voor het includeren van de volwassen patiënten en controles en de proeven die hebben geleid tot een deel van hoofdstuk 5. Hopman Willem, de grote vuurmaker, bedankt voor je voorbereidende werk voor hoofdstuk 6, hopelijk leidt het nog tot een mooie paper. Veel succes tijdens je

stage bij Novartis. Gezelligheid op en om het lab is zeer belangrijk voor het functioneren van de promovendus. Hiervoor wil ik de labgenoten van Longziekten dan ook hartelijk danken en de onlangs gespeelde memorabele voetbalpot niet onvermeld laten. Mooi was dat, ook al gaat het gerucht dat we van jullie verloren hebben. Ik heb gemerkt dat studenten een zeer positieve invloed kunnen hebben op de sfeer en gezelligheid op het lab en binnen de groep. Hiervoor wil ik alle studenten bedanken die de afgelopen 4,5 jaar bij dermat een stage danwel co-schap hebben gelopen, you know who you are.
Om nog even terug te komen op de Keystone-meeting: Berber, mede door jou was het in Breckenridge heel gezellig. Veel succes met de kinderarts-opleiding in Tilburg.
Wayne, a year ago we met at the Keystone meeting, had a nice talk and played a few games of fussball. You suggested to get in touch again by the end of my PhD, and the result is that I will start working in your group in August. Thank you for giving me this opportunity.

Doug and Bonnie, I would like to thank you for the nice days that I've spent with you last year before travelling to Breckenridge, as well as for reading my resume and supporting me in the last months of writing of my thesis. Sandra, thank you for correcting my application letter and giving it a more American style. I hope you will do great in Denver and wish you success in finishing your PhD.
Mijn paranimfen, Rinze en Marcel. Rinze, komende zomer is het alweer twaalf jaar geleden dat we samen in het A-novitiaat zaten bij Unitas S.R. Niet lang daarna kwamen we in dezelfde jaarclub terecht en onze grote overlap aan interesses zorgde voor een mooie vriendschap. Marcel, enkele maanden geleden had je nog nooit van het begrip "paranimf" gehoord. Eigenlijk is het ook maar een bizar woord. Ik ken je al vanaf de vierde klas van de middelbare school, mooi was die tijd. Gezellig dat we elkaar de laatste tijd weer vaak spreken en dat je me routineus inmaakt op de squashbaan. Heren, bedankt dat jullie mijn paranimfen willen zijn.
Ontspanning naast het werk is onmisbaar, want voor je het weet heb je een muisarm of een pipetteerduim. Een aantal mensen heeft hieraan significant bijgedragen, zoals de heren van dé JC. Ik zal vanaf augustus een tijdje verstek moeten laten gaan op clubeten, maar ik heb nu al zin in het clubweekend, dat wordt natuurlijk beremooi. Studiegenoten, huyschgenooten, bestuursgenoten, middelbare schoolgenoten, vrienden, dank voor het foebele, foebel kijke, de weekendjes, de spelletjes, de filmpjes, het schaatsen, het dansen, het feesten, de biertjes hier, de biertjes daar, kletsen over wetenschap en het edele promovendusschap, kletsen over alles daar omheen, en de gezelligheid in het algemeen.

Gezien mijn agrarische achtergrond zou je bijna denken dat mijn keuze voor onderzoek aan koemelkallergie niet toevallig was. Opgroeien op een boerderij wordt steeds specialer in Nederland. Mij is het goed bevallen, wat te danken is aan mijn ouders. Pap, je hebt me leren koeien melken, trekker rijden en motor rijden (nu alleen dat rijbewijs nog..), en was altijd nieuwsgierig naar mijn vorderingen op de universiteit, in de studie en later als OIO. Mam, met jou bezocht ik de open

dag van Medische Biologie in Utrecht, waar het allemaal begon. Je hebt me geholpen bewust te worden van mijn interesses, wat heel belangrijk is geweest voor het tot stand komen van dit boekje. De broeders, Jan Pieter en Jeroen, en de vriendinnen, Jeanine en Madelon, bijzonder dat we bijna allemaal in de afrondende fase zitten en binnenkort de grote mensenwereld betreden, waar JP al een jaartje kiezen trekt. Veel succes daarmee en met de nieuwe projecten.
Aan veel mensen heb ik steun gehad de afgelopen jaren, maar er is er eigenlijk maar één die echt weet hoe een Bert is in tijden van promotiedrukte. Yvette, dank je wel voor je aandacht en je knuffels, evenals je hulp bij het illustreren van dit boekje met de mooie foto's die je hebt gemaakt op de boerderij en het lab. Nu gaan we een nieuw avontuur tegemoet in New York, New York, en het wordt vast mooi.

Mel

PBMC
6/14

Curriculum Vitae/ List of publications/ Abbreviations

CURRICULUM VITAE

Bert Ruiter werd geboren op 7 mei 1977 te Ermelo. Na het behalen van het VWO diploma aan het Christelijk College Groevenbeek te Ermelo, begon hij in 1995 aan de studie Medische Biologie aan de Universiteit Utrecht. In het kader van deze studie werden twee stages voltooid. Als eerste werd op het Rudolph Magnus Instituut voor Neurowetenschappen te Utrecht onderzoek gedaan naar de fosforylering van pre- en postsynaptische signaaleiwitten in de hippocampus van de rat. Deze eiwitten zouden een rol kunnen spelen bij ruimtelijke verkenning en geheugenprocessen. Het onderzoek werd begeleid door dr. Els van Dam en dr. Pierre de Graan. Tijdens de tweede stage verrichtte hij bij de vakgroep Pedriatische Immunologie in het Wilhelmina Kinderziekenhuis te Utrecht onderzoek naar de verlaging van G-eiwit receptor kinase 6 in humane T cellen na milde oxidatieve stress. De resultaten suggereerden dat zuurstofradicalen, die geproduceerd worden gedurende een chronische ontsteking zoals bij reuma, de signaaltransductie kunnen beïnvloeden. De begeleiders van dit onderzoek waren dr. Annemieke Kavelaars en prof. dr. Cobi Heijnen. Naast de studie was hij actief in diverse commissies van de Medisch Biologen Vereniging Mebiose en was hij een jaar secretaris in het bestuur van deze vereniging.
Na het behalen van het doctoraal examen Medische Biologie in 2002 is hij begonnen als OIO bij de afdeling Dermatologie/Allergologie in het UMC Utrecht, onder leiding van dr. Els van Hoffen, dr. Edward Knol en prof. dr. Carla Bruijnzeel-Koomen. De resultaten van het hier verrichte onderzoek zijn beschreven in dit proefschrift. Vanaf augustus 2007 zal hij werkzaam zijn als postdoctoraal onderzoeker in de groep van dr. Wayne Shreffler en prof. dr. Hugh Sampson, binnen de afdeling Pediatric Allergy and Immunology van de Mount Sinai School of Medicine in New York.

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A specific mixture of short chain Galacto-oligosaccharides and long chain Fructo-oligosaccharides induces a beneficial immunoglobulin profile in infants at high risk for allergy.
Submitted

ABBREVIATIONS

A	atopic
AA	amino acid
AD	atopic dermatitis
APC	antigen-presenting cell
AU	arbitrary units
BCR	B cell receptor
BPA	birch pollen allergy
CD	cluster of differentiation
CD23/FcεRII	low-affinity IgE receptor
CD32/FcγRII	low-affinity IgG receptor
CMA	cow's milk allergy
CMP	cow's milk protein
CPM	counts per minute
DBPCFC	double-blind placebo-controlled food challenge
DC	dendritic cell
DTP	diphtheria, tetanus and polio
EBV-B cell	Epstein Barr-virus-transformed B cell
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence-activated cell sorting
FcεRI	high-affinity IgE receptor
Fmoc	9-fluorenyl-methoxycarbonyl
GALT	gut-associated lymphoid tissue
GOS/FOS	galacto-oligosaccharides/fructo-oligosaccharides
HLA	human leucocyte antigen
HPLC	high performance liquid chromatography
HSA	human serum albumin
IFN-γ	interferon-γ
Ig	immunoglobulin
IgE-FAP	IgE-facilitated allergen presentation
IL	interleukin
IQR	interquartile range
IT	immunotherapy
LPS	lipopolysaccharide
LST	lymphocyte stimulation test
mAb	monoclonal antibody
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
NA	non-atopic

OD	optical density
p-value	probability value
PA	peanut allergy
PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
RAST	radioallergosorbent test
RP	reference population
SCORAD	scoring atopic dermatitis
SEM	standard error of the mean
SI	stimulation index
SPT	skin prick test
TCC	T cell clone
TCL	T cell line
TCR	T cell receptor
TGF-β	transforming growth factor-β
Th cell	T helper cell
TNF-α	tumor necrosis factor-α
Treg	regulatory T cell
[^{3}H]-TdR	tritiated thymidine