Cow's milk allergy in infancy and childhood

Immunological and clinical aspects

Rogier Schade

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Koemelkallergie op de zuigelingen- en kinderleeftijd

Immunologische en klinische aspecten

(met een samenvatting in het nederlands)

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“A disease that can gause gastric upset and urticaria“

Hippocrates (460-370 BC)

Voor mijn ouders

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Chapter 1

General introduction

INTRODUCTION

Allergy is an important disease that affects a substantial proportion of the general population, and which has seen an increasing incidence during the past three decades. In the first years of life, the immune system is relatively immature and highly susceptible for the development of sensitization and allergy to environmental allergens such as foods and inhalants. This thesis focuses on allergy to cow's milk, one of the major food allergens during infancy and early childhood. Special attention will be paid to the role of antigen-specific T cell reactivity in the aetiology of cow's milk allergy (CMA).

ATOPY AND THE ALLERGIC MARCH

Atopy comes from the Greek 'atopos' which means 'out of place'. Persons with atopy have a hereditary predisposition to respond with allergic reactions against environmental antigens such as food- and inhalation-allergens. Immunologically, atopy expresses itself as sensitization; i.e. the development of allergen-specific IgE, which can be detected in the blood of atopic individuals[1]. Clinically, atopy is expressed through allergic conditions which tend to cluster in families, including atopic dermatitis (AD), allergic asthma and allergic rhinitis[2-4]. These three diseases together make up the 'atopic syndrome'.
The natural history of atopic diseases is characterized by a typical sequence of sensitization and manifestation of symptoms which appear during a certain age period. This is often called the 'allergic march'[5,6]. The symptoms can persist over years or decades, and often show a tendency for spontaneous remission with age. Usually, the 'allergic march' starts with the development of AD during the first 3-4 years of life[7]. This is commonly followed by the development of allergic asthma around the age of 4-5 years[8,9]. In the subsequent years, allergic rhinitis develops, which usually persists into adulthood[10]. The presentation of the different symptoms mostly overlap. Figure 1 shows a schematic representation of the incidence, and time of occurrence of the different symptoms[11].

ATOPIC DERMATITIS

Atopic dermatitis is a chronic eczematous skin disease which is characterized by a course of remissions and exacerbations[12]. Typical for AD are the pruritic, erythematous lesions with lichenification and thickening of the skin[13]. The diagnosis of AD is made on clinical grounds according to the criteria of Hanifin and & Rajka[12,14]. These criteria are based on the patient's history, family history and distinct clinical morphology and distribution of skin lesions.

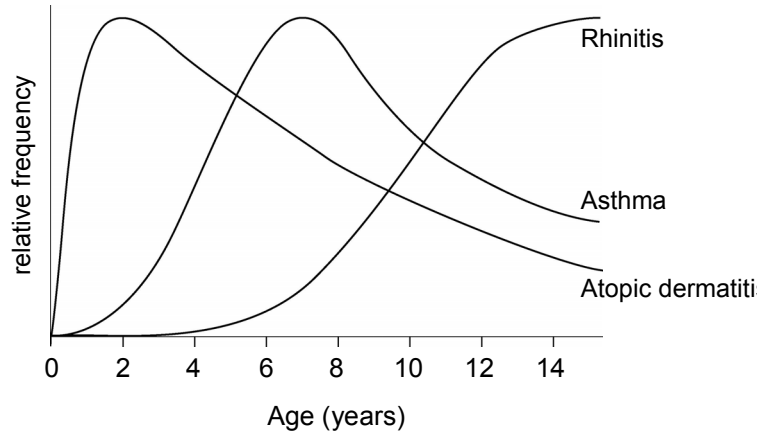

Figure 1. Schematic representation of the natural course of atopic disease in childhood ('allergic march'), adapted from reference 11.

AD is usually the first clinical presentation of the atopic state of an individual, and has an onset during infancy or early childhood[7]. It is estimated that 60% of the individuals who will develop AD, already have symptoms during the first year of life, and 85% during the first 5 years of life[15]. Overall, more than 10% of all children have AD at some point during childhood[16].
The relation between AD and allergic (atopic) disease is established through both clinical and immunological observations[17,18]. Clinically, patients with AD and allergy can experience a deterioration of their dermatitis after contact with allergens[7]. These contacts may also lead to acute cutaneous symptoms such as erythema and urticaria[19]. Immunologically, patients with AD generally have increased levels of IgE specific for common environmental allergens such as food and inhalation allergens[15]. While sensitization to food allergens is commonly found in infants with AD, inhalation allergens are more important in older children and adults with AD[20,21]. The allergen-specific IgE responses can be demonstrated in blood, but also in skin, by using allergen-specific test methods such as the skin-prick test[22,23].

COW'S MILK ALLERGY

Cow's milk is usually the first food antigen that is introduced into an infant's diet, and CMA is often the first presentation of the atopic state of an individual[24]. Ingestion of cow's milk leads to allergic reactions in approximately 2-3% of all infants, and individuals with an atopic predisposition have an increased risk of

developing CMA[25-28].
The most common symptom of CMA in childhood is AD[28,29]. It is estimated that CMA plays a pathogenic role in approximately 35-40% of infants with AD in the first year of life[30-32]. In these infants, ingestion of cow's milk leads to acute ('immediate-type') cutaneous symptoms in combination with aggravation of the eczema[29]. Besides cutaneous symptoms, CMA can also lead to immediate-type pulmonary and/or gastro-intestinal symptoms, or even systemic anaphylaxis[24]. It has been suggested that patients with CMA can also experience isolated 'late' or 'delayed-type' reactions. These are allergic reactions which are said to arise more than 24 hours after intake of cow's milk, and concern non-life-threatening symptoms such as infantile colic syndrome[33,34]. The existence of these isolated, delayed-type reactions however, has not yet been indisputably demonstrated in double-blinded test settings[35,36].
The diagnostic work-up of infants and children with suspicion of CMA is a multi-step process involving both *in vitro* and *in vivo* methods[35,37]. After a suspicion of CMA is established by careful examination of the medical history, and by physical examination, the first step is to investigate the presence of cow's milk specific sensitization, by using cow's milk-specific IgE-detection and/or SPT. The presence of cow's milk-specific sensitization indicates an immune reaction to milk, but does however not necessary mean clinical allergy. To prove clinical significant CMA, a provocation with milk is needed, preferably performed by using a double-blind, placebo-controlled food challenge (DBPCFC), as this is the most objective method to diagnose milk-induced symptoms[37,38].
In contrast to other food allergies, CMA in infancy is associated with the spontaneous development of clinical tolerance during early childhood. Prospective studies have shown that tolerance to cow's milk develops in more than 85% of the infants with CMA before the age of 3 years[25,26,39]. However, a small part of the patients retain their allergy until adolescence (persistent CMA)[40], and sometimes even into the second decade[41]. The mechanisms that determine the spontaneous development of clinical tolerance in patients with allergy to cow's milk are still under investigation.

THE IGE-RESPONSE TO COW'S MILK-PROTEINS

Allergen-specific IgE antibodies have been shown to be important in the aetiology of allergic reactions via their role in the degranulation of mast cells and basophilic granulocytes, but also through their accessory function in the presentation of allergens[42-44].
The protein-fraction of cow's milk is the major immunogenic component, and consists of at least 20 proteins that may provoke an antibody response in man[28,45-47]. The whey proteins, α-lactalbumin and β-lactoglobulin, together with the casein-

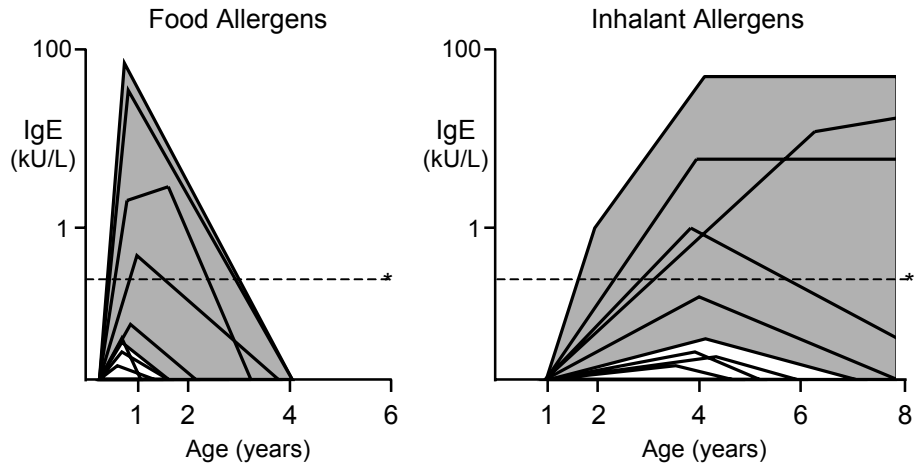

Figure 2. Postnatal IgE-responses to environmental allergens in normal and atopic children, derived from data in references 20 and 21. Curves are exemplary of the types of time-dependent changes seen in antibody levels of individual children; curves for atopic children all fall within the shaded area and those for normals are restricted to the clear areas. Asterisk represents cut-off level of commercially available assays.

proteins, are proposed to be the most important allergenic proteins, and the IgE-response to these proteins has been well investigated in infants and children[48-50].

A recent study, that investigated the natural course of sensitization to cow's milk allergens in a large population-based sample, estimated that the prevalence of IgE antibodies to cow's milk in an unselected population is approximately 6% at the age of 1 year[51]. It is clear however, that infants with an atopic predisposition have a greater risk of the development of IgE antibodies to cow's milk proteins (CMPs) than non-atopic infants[20,52,53]. In addition, the levels of CMP-specific IgE are generally higher in children with atopy compared with non-atopic children, who show mostly transient sensitization for cow's milk[20,21,54-56]. Interestingly, the kinetics of IgE responses to cow's milk, as well as other foods, differ significantly from the response to inhalant allergens such as house dust mite and pollen (Figure 2). While cow's milk-specific IgE can be detected in most atopic individuals shortly after birth, and rarely persists after early childhood, inhalant allergen-specific IgE only rarely appears before the age of 1 year, and usually persists into adulthood[20,21]. These differences most probably reflect the differences in timing and amount of exposure to these allergens[53,57].

The presence of CMP-specific IgE does not necessarily implicate clinically relevant CMA[37]. Studies have shown that only 30-40% of the individuals with CMP-specific IgE experience clinical symptoms after ingestion of cow's milk[58,59].

This means that, while IgE is important for the initiation of allergic responses, other presently unknown (but probably tissue-related) mechanisms prevent clinical relevant allergic disease in most individuals with CMP-specific IgE. Studies who investigated the levels of CMP-specific IgE in patients with clinically relevant CMA, showed that the spontaneous development of clinical tolerance for cow's milk is usually associated with a downregulation of the IgE-response[60-63]. However, in most tolerant patients, low-level IgE responses to cow's milk proteins continue to occur[37], which emphasizes that the presence of CMP-specific IgE alone does not determine allergy or tolerance to cow's milk.

T CELLS AND ATOPIC DISEASES

T cell responses to allergens have been shown to be important in the aetiology of atopic diseases[1,17,64,65]. CD4+ T helper (Th) cells are generally subdivided into 'Th1' and 'Th2' cells[66-68]. Th1 cells are defined as T cells that predominantly produce interferon (IFN)-γ, interleukin (IL)-2, and tumor necrosis factor-β, while Th2 cells are T cells that produce predominantly IL-4, IL-5, and IL-13[69,70]. Both cell-types have been associated with specific immunoregulatory functions[71]. Th1 cells evoke cell-mediated immunity and phagocyte-dependent inflammation[69], while Th2 cells mediate strong antibody responses, including those of the IgE-class[72], and eosinophil accumulation[73]. Although there seems to be a predominance of some T cell subsets in certain disease-states, numerous studies have shown that human T cells generally produce a heterogeneous cytokine profile[69,74]. These cells, which can produce equal amounts of both 'type-1' and 'type-2' cytokines are often called 'Th0' cells[69,70].

Investigations have shown a crucial role for allergen-specific CD4+ Th2 cells in the aetiology of atopic diseases such as AD[17,18,65,75]. Studies that investigated the systemic T cell response in patients with house-dust mite allergy and AD, showed that allergen-specific T cells in blood of allergic individuals generally display a Th2-pattern; i.e. high production of IL-4, IL-5 and IL-13, and little or no IFN-γ[76-78]. It was shown that these allergen-specific Th2 cells can infiltrate into the skin, and initiate an allergic skin-response by stimulation of IgE synthesis and eosinophil recruitment[18,64,79,80]. Subsequently, the T cell response in the skin switches to a response dominated by Th1-cytokines[64,81,82], which eventually results in the chronic skin lesions that are characteristic of AD[18].

The process by which circulating allergen-specific T cells extravasate and subsequently migrate to the skin is thought to be mediated by the expression of cutaneous lymphocyte antigen (CLA) [83-85]. It is proposed that CLA expression is the imperative signal for a T cell to selectively migrate to the skin, after which allergic skin inflammation is initiated by Th2 cytokine release[17,75,86,87]. Evidence for these mechanisms have so far primarily been investigated for inhalation allergens

such as house-dust mite and pollen. The role of food-specific T cell reactivity in the aetiology of food-induced skin symptoms is not yet clarified.

T CELL REACTIVITY AND CMA

There is evidence that cow's milk-specific T cell reactivity may play an important role in the aetiology of CMA, and cow's milk-induced symptoms. Cow's milk-reactive T cells in peripheral blood mononuclear cells (PBMCs) from cow's milk-allergic patients have been shown to proliferate in the presence of cow's milk proteins[88-100]. This demonstrates that T cells, specific for the proteins in cow's milk, are present in blood of patients with allergy to cow's milk. Studies that investigated the nature of these proliferative PBMC responses, suggested that CMP-specific T cells from allergic patients have an enhanced activated state, with high proliferative capacity[88-91,93-97,99].

Investigations that determined cytokine release by CMP-stimulated PBMCs showed high levels of IL-4 (Th2 cytokine), and lower levels of IFN-γ (Th1 cytokine)[92,98,100]. These results may suggest that the T cell response in cow's milk allergic patients is Th2-biased. Because of the relevance of allergen-specific Th2 cells in inhalation allergy[64,65], it could be speculated that CMP-specific T cells play a similar role in the aetiology of CMA. So far, however, studies into CMP-specific T cell reactivity and cytokine release have been mainly performed using bulk-cultures of T cells. A disadvantage of this is, that the observed reactivity and/or cytokine production is the sum of a polyclonal T cell response, with different antigen-specificities and different individual cytokine profiles. Results from previous studies in PBMCs therefore need to be verified by investigations at the clonal level.

More importantly, a well designed study comparing CMP-specific T cell reactivity in cow's milk allergic patients with age-matched atopic and non-atopic controls patients has not yet been performed. Although atopic individuals are prone to develop sensitization and allergy to cow's milk, not all atopic individuals do develop CMA. Investigations into the possible differences in T cell reactivity between atopic individuals with and without CMA should therefore be performed.

A remarkable feature of CMA is that, in contrast to other (food) allergies, it is associated with the spontaneous development of clinical tolerance during early childhood. This process has not yet been studied at the T cell level. It is presently unclear if clinical tolerance after CMA is associated with a downregulated T cell response to CMPs (immunological tolerance). If this is the case, this would imply that the CMP-specific T cells have been deleted from the T cell repertoire, or have been anergized. It could also be that T cell reactivity to CMPs is still present after the development of tolerance, but has been modulated, for instance with regard to production of cytokines.

AIM AND OUTLINE OF THIS THESIS

The aim of this thesis was to investigate the role of antigen-specific T cells in cow's milk allergic infants. Furthermore, the currently used methods to diagnose CMA, *in vivo* and *in vitro,* were evaluated.

The major research questions addressed in this thesis are:

- Do CMP-specific T cells in patients with allergy to cow's milk differ from CMP-specific T cells from individuals without CMA, with regard to specificity for the different proteins in cow's milk, production of cytokines, and expression of cell-surface markers?

- Is the CMP-specific T cell response from children that have spontaneously developed clinical tolerance to cow's milk, different from the CMP-specific T cell response during the allergic state?

- Are there differences between CMP-specific T cells in blood and skin of patients with CMA and allergic skin symptoms?

- Is *in vitro* IgE detection and/or skin prick testing sufficient to screen for cow's milk-specific sensitization?

- Can double-blinded cow's milk challenges be used in common clinical practice?

The role of cow's milk-specific T cells in the aetiology of CMA is described in Chapter 2-6. In Chapter 2, differences between the cow's milk-specific T cell response in infants with AD with and without CMA are examined, with emphasis on the production of Th1- and Th2-cytokines. Chapter 3 describes the presence and phenotype of CMP-specific T cells in blood of non-atopic infants without CMA. In Chapter 4, the changes that occur in the cow's milk-specific T cell response during the spontaneous development of tolerance for cow' milk in infants with CMA and AD are investigated. Chapter 5 provides a description of the differences between expression of cell-surface markers by cow's milk-specific T cells from infants with and without CMA, and the changes in expression of these markers during the spontaneous development of tolerance for cow's milk. In Chapter 6, cow's milk-specific T cells from blood and skin are investigated, and compared with regard to expression of the skin-specific cell-surface marker, cutaneous lymphocyte antigen. The currently used methods to diagnose CMA are studied in Chapter 7-8. Chapter 7 evaluates the screening for food-specific sensitization using *in vitro* IgE detection and skin prick testing, in infants and children referred for suspicion of food allergy. Chapter 8 provides a description of a new protocol for a double-blind, placebo-

controlled cow's milk challenge for infants and children with suspicion of CMA. Chapter 9 is a general discussion about the relevance of all presented data on CMP-specific T cells. In this chapter, a model is presented that describes the development of the CMP-specific T cell response during infancy and early childhood.

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Chapter 2

Differences in antigen-specific T cell responses between infants with atopic dermatitis with and without cow's milk allergy: relevance of Th2 cytokines

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ABSTRACT

Background Cow's milk is the most important food antigen in infancy, and may lead to acute cutaneous symptoms and atopic dermatitis (AD). The role of circulating allergen-specific T cells in the pathogenesis of food-allergic skin symptoms is still under investigation.

Objective This study was designed to analyze the cow's milk protein (CMP)-specific T cell response at the clonal level in infants with AD and cow's milk allergy (CMA), in comparison to infants with AD without CMA.

Methods We used an antigen-specific culturing system with autologous B cells as antigen-presenting cells to establish CMP-specific T cell clones, derived from peripheral blood mononuclear cells, in infants with AD. T cell reactivity, measured by lymphocyte stimulation test, and cytokine production, measured by ELISA, was compared between infants with AD, with and without CMA.

Results Both infants with, and without allergy to cow's milk had a CMP-specific T helper cell response directed against the major proteins in milk. Analysis of antigen-specific cytokine production showed that this response was Th2-skewed in infants with CMA, with production of high levels of interleukin (IL)-4, IL-5 and IL-13. In contrast, infants without CMA had a Th1-skewed response, with high levels of interferon-γ (IFN-γ) and low levels of IL-4, IL-5 and IL-13.

Conclusion These data confirm for the first time at the clonal level, that food allergy in infants with AD is associated with production of Th2 cytokines by circulating antigen-specific CD4+ T cells, while tolerance to food antigens is associated with low levels of these cytokines. This suggests a key role for the T helper cell-derived Th2 cytokines in food allergy-related skin symptoms.

INTRODUCTION

Atopic dermatitis (AD) is a chronic eczematous skin disease with early onset in infancy, characterized by a course of remissions and exacerbations[1]. At present, the pathogenesis of AD is not fully understood. In patients with AD and allergy towards inhalation-allergens such as house dust mite, circulating allergen-specific Th2 cells have shown to be important in the pathogenesis of allergic skin symptoms[2-4]. The infiltration into the skin of these T cells which produce high levels of interleukin (IL)-4, IL-5 and IL-13, but little or no interferon-γ (IFN-γ)[5,6], can mediate allergic skin inflammation through stimulation of IgE synthesis and eosinophil recruitment[7].

Evidence for these mechanisms in food-allergy are much less well established. Food-reactive T cells in peripheral blood mononuclear cells (PBMCs) from food-allergic patients with AD have been shown to proliferate in the presence of food antigens[8,9]. This capacity is however not restricted to the food-allergic patient, as PBMCs from food-tolerant individuals can also mount significant lymphoproliferative responses when stimulated *in vitro*[9]. Studies that determined cytokine production in food antigen-stimulated PBMCs, showed that food-allergic patients have a Th2-skewed response compared with non-allergic controls[10-14]. In these studies however, cytokines were measured in bulk cultures of T cells, making it difficult to extrapolate these results to the individual T cell. Food-reactive Th2 cells have been cloned from blood of patients with food allergy[15-17], but the food-specific T cell response in food-tolerant subjects has not been evaluated at the clonal level.
Cow's milk is among the first foods introduced into an infant's diet and thus one of the most common causes of food allergy in young children[18,19]. Cow's milk allergy (CMA) plays a pathogenetic role in approximately 35-40% of infants with AD[20-22]. In these patients, ingestion of cow's milk leads to acute cutaneous symptoms and/or aggravation of the eczema. The aim of this study was to analyze the CMP-specific T cell response in infants with AD and CMA. We used an antigen-specific culturing system to establish CMP-specific T cell clones (TCCs) from blood of infants with AD under the age of 1 year. A comparison of T cell reactivity and cytokine production was made between infants with CMA, and infants tolerant for cow's milk.

METHODS

Patients & control subjects

Twelve infants (7 males, 5 females; age 3.8-12.3 months, median 6.4) with AD according to the criteria of Hanifin & Rajka[23] were included in the study. The study was approved by the Medical Ethical Committee of the University Medical Center, Utrecht. After informed consent was obtained, a venous blood sample was taken. Cow's milk allergy was diagnosed by complete elimination of cow's milk from the infant's diet, followed by a cow's milk challenge. Infants that developed symptoms during challenge (Table 1) that disappeared during subsequent elimination were diagnosed with CMA (CMA patients). Infants without CMA (non-CMA patients) did not have any reactions, and no significant change in the severity of the eczema was noted throughout the entire elimination-provocation period. Age of CMA patients was not significantly different ($p = 0.94$) from non-CMA patients; 3.8 - 12.3 months (median 6.4) vs. 4.4 - 7.3 months (median 6.5).

Table 1. Characteristics of infants with AD, with and without CMA.

Patient	Symptoms*	IgE	SPT
1 †	Erythema, AD	-	-
2 ‡	-	-	-
3 †	Erythema, UR, AD	+	+
4 †	Erythema, AD	-	+
5 ‡	-	-	-
6 †	Erythema, AD	-	+
7 ‡	-	-	-
8 †	Erythema, UR	+	+
9 ‡	-	-	-
10 †	Erythema, UR, AD	+	+
11 ‡	-	-	+
13 ‡	-	-	-

† CMA patient
‡ non-CMA patient
* symptoms developed upon challenge
AD = worsening of atopic dermatitis
UR = urticaria
IgE = cow's milk-specific IgE detected by RAST
SPT = skin prick test for cow's milk

Cow's milk antigens

Purified cow's milk proteins (CMPs) (used at concentrations of 50 μg/ml): total casein, α-lactalbumin and β-lactoglobulin, and purified casein-subfractions (used at concentrations of 10 μg/ml): αS1-casein, αS2-casein, β-casein and κ-casein were kindly provided by Dr. E.C.H. van Beresteijn (Netherlands Institute for Dairy Research, Ede, The Netherlands). A mix of purified CMPs containing equal quantities of total casein, α-lactalbumin and β-lactoglobulin (each at a concentration of 50 μg/ml) is referred to as CMP-mix.

Culture media

Proliferation assays with PBMCs were performed with complete medium AIM V (Gibco, NY, USA). For culturing of T cell lines and TCCs, and in lymphocyte stimulation tests (LSTs), complete medium RPMI-1640 (Gibco) was used, supplemented with 10% pooled human AB serum. Epstein Barr virus (EBV)-transformed B cells were cultured in RPMI-1640 (Gibco), supplemented with 10% fetal calf serum (Gibco). Established TCCs were maintained in Iscove's Modified Dulbecco's Medium (Gibco) supplemented with 2% pooled human AB serum and

5% Yssel's medium[24]. All media were supplemented with penicillin (100 IU/ml), streptomycin (100 mg/ml) and glutamin (1mM) (Gibco).

Proliferation assays with PBMCs
PBMCs were isolated from heparinized venous blood by using Ficoll density gradient centrifugation. Recovered cells were cultured in quadruplicate (10^5 cells/well) at 37^0C under 5% CO_2 for 6 days in 96-well U-bottom culture plates (Greiner, Frickenhausen, Germany) in the presence or absence of antigen. After 6 days of culturing, proliferation was measured by using tritiated thymidine ([^{3}H]-TdR) incorporation; [^{3}H]-TdR (1μCi/well; Amersham, Aylesbury, UK) was added, and the cells were harvested after 18 hours. Thymidine incorporation was measured using a 1205 betaplate^{tm} counter (Wallac, Turku, Finland).

LST
LSTs were performed in triplicate in 96-well U-bottom plates (Greiner). Each well contained $4x10^4$ T cells, and $4x10^4$ irradiated (50 Gy) autologous EBV-transformed B cells as antigen-presenting cells. The EBV-transformed B cells were, before co-culture with T cells, incubated O/N with antigen. EBV-transformed B cells incubated without antigen were used as negative controls. After 24 hours of culturing, proliferation was measured by using [^{3}H]-TdR incorporation as described above.

Preparation of CMP-specific TCCs
Cow's milk protein-specific TCCs were established from heparinized venous blood as described previously[25,26]. In parallel to the proliferation assays, PBMCs were cultured in a 24-well flat bottom culture plate in the presence of CMP-mix. After 7 days, 50 IU/ml of both rIL-2 and rIL-4 (kind gift of Novartis Research Institute, Vienna, Austria) was added to the culture medium. To promote expansion of CMP-specific T cells, cultures were re-stimulated every 14 days with irradiated autologous EBV-transformed B cells that had been pre-incubated O/N with CMP-mix, as antigen-presenting cells. LST or determination of cytokine release was always done at the start of a re-stimulation cycle. After 2-3 weeks, when a polyclonal T cell culture was obtained, CMP-specificity was verified by LST. If these cultures indicated a high CMP-specific T cell proliferation, T cells were cloned by limiting dilution at 0.3, 1 or 3 cells/well in 96-well U-bottom culture plates in the presence of IL-2, IL-4 and irradiated autologous EBV-transformed B cells (10^4/well) that had been pre-incubated O/N with CMP-mix. Established clones were screened for CD4 or CD8 and TCR expression. Cells were stained with fluorescein isothiocyanate (FITC)-conjugated anti-CD4 (Leu-3a, Becton-Dickinson, San Jose, USA) and phycoerythrine-conjugated anti-CD8 (Leu-2a, Becton-Dickinson), or FITC-conjugated anti-α/β TCR (WT-31, Becton-Dickinson), fixed in paraformaldehyde, and analyzed with FACScan (Becton-

Dickinson). All TCCs were tested in LST to verify CMP-specificity, and to determine specificity for the different proteins in cow's milk.

CMP-specific cytokine release
To determine CMP-specific cytokine release, 10^6 cells of each TCC were incubated with 10^6 irradiated autologous EBV-transformed B cells that had been pre-incubated O/N with CMP-mix. Control cultures of TCCs and EBV-transformed B cells, pre-incubated without antigen were prepared in parallel. Stimulation was performed in a 24-well plate. After 24 hours of culture at 37^0C, supernatants were collected and stored at -20^0C. Cytokines were measured by ELISA according to the manufacturers recommendations; IL-4, IL-13, IFN-γ: Central Laboratory of the Blood Transfusion Service, Amsterdam, The Netherlands, IL-5: Endogen, Woburn, USA. The detection limit was 0.6 pg/ml for IL-4, 5 pg/ml for IL-5, 0.5 pg/ml for IL-13 and 2 pg/ml for IFN-γ. CMP-specific cytokine release by a TCC was calculated by subtracting control-stimulated cytokine production from cytokine production after stimulation with CMP-mix.

Statistical analysis
Non-parametric analysis (Wilcoxon signed-rank test and Mann-Whitney U test) was applied to determine significant differences between patient and control groups with regard to age, proliferative responses of PBMCs, and cytokine production by TCCs. Differences associated with *p* values of less than 0.05 were considered significant. Nonparametric Spearman's rank correlation was used to test for correlation between production of different cytokines.

RESULTS

CMP-specific proliferation in PBMCs
Antigen-specific proliferation of PBMCs was determined for the three major allergens in cow's milk: total casein, α-lactalbumin and β-lactoglobulin. Proliferative responses towards each of the three antigens were found in both CMA patients and non-CMA patients (Figure 1). In CMA patients, responses towards all three allergens were significantly higher than background responses ($p<0.05$). The range of these responses however was quite large and overlapped extensively between CMA and non-CMA patients. Mean T cell proliferation in CMA patients tended to be higher than in non-CMA patients, but these differences were not significant. Also, comparison of mean stimulation-index (ratio of counts per minute of antigen-stimulated cultures to unstimulated cultures) showed no significant differences in proliferative responses of PBMCs between CMA and non-CMA patients for each of the major cow's milk allergens (data not presented).

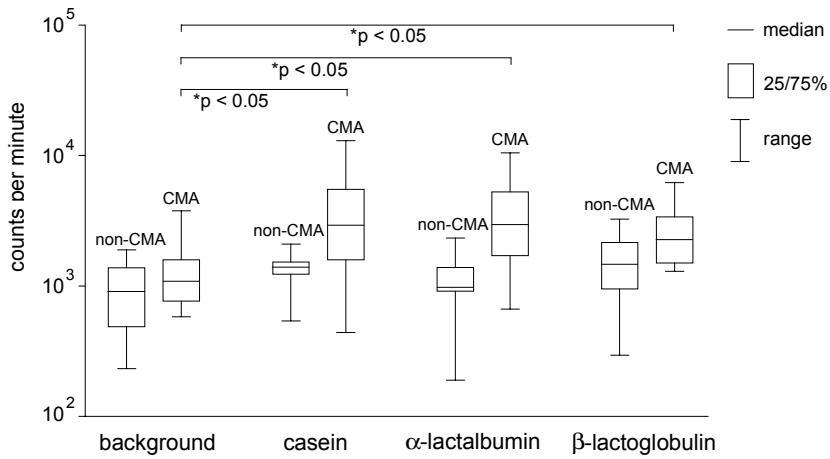

Figure 1. Proliferative responses of PBMCs from 12 infants with atopic dermatitis, 6 with cow's milk allergy (CMA) and 6 without cow's milk allergy (non-CMA), to the three major cow's milk allergens. Tritiated thymidine incorporation was measured 6 days after stimulation with the allergens.

Table 2. T cell clone reactivity towards the major cow's milk proteins and protein-subfractions in infants with AD, with and without CMA.

Pt	TCC reactivity	no*	Casein proteins					Whey proteins	
			αS1	αS2	β	κ	§	α-lac	β-lac
1 †	cas/whey	23		8		2	1	3	9
3 †	cas/whey	17	2	1		4	3	5	2
4 †	cas	3		3					
8 †	cas	7	2	3	1		1		
2 ‡	cas	4		1			3		
5 ‡	cas	3	1			1	1		
7 ‡	cas/whey	7				2			5
11 ‡	cas/whey	29					1		28

† CMA patient
‡ non-CMA patient
* number of TCCs obtained from each patient
§ TCCs specific for total casein, specificity for the casein-subfractions could not be determined
α-lac = α-lactalbumin
β-lac = β-lactoglobulin

CMP-specific TCCs: reactivity towards the various protein fractions in cow's milk

A total of 93 CMP-specific TCCs was obtained. Fifty TCCs were obtained from 4 CMA patients, 43 TCCs were obtained from 4 non-CMA patients. The cloning procedure did not yield TCCs in the other 4 patients. Flow-cytometric analysis showed that all clones were CD4+ and expressed the α/β TCR (data not presented). Specificity of the clones was determined for the casein and whey-proteins, and for the protein-subfractions (Table 2). TCC reactivity towards the casein and whey proteins was observed in both allergic and non-CMA patients and was not significantly different between the two groups.

Production of IL-4 and IFN-γ

Cow's milk protein-specific cytokine release was measured in 44 CMP-specific TCCs derived from CMA patients and 43 TCCs derived from non-CMA patients. Figure 2 shows production of IL-4 and IFN-γ. In CMA patients (Figure 2A), antigen-specific TCCs with a diverse cytokine pattern were found; TCCs showed isolated production of IL-4 or IFN-γ, or production of both cytokines. Clones from non-CMA infants (Figure 2B) showed a different pattern; none of the clones produced high levels of IL-4 (maximum 0.36 ng/ml), and 79% of these TCCs produced even less than 0.1 ng/ml of IL-4. A significant portion of the clones derived from non-CMA patients produced high amounts of IFN-γ, up to 30 ng/ml,

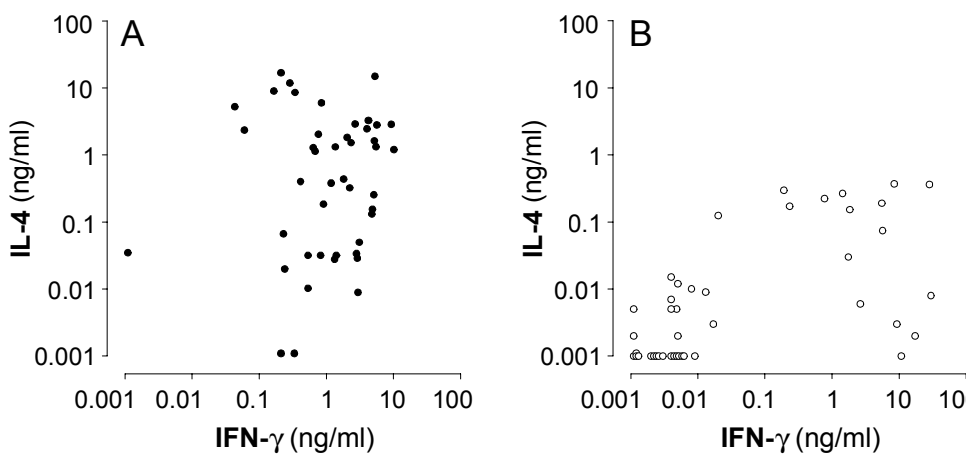

Figure 2. Antigen-specific production of IL-4 and IFN-γ by cow's milk-specific T cell clones established from infants with atopic dermatitis, with cow's milk allergy (A) and without cow's milk allergy (B). Cytokine production was measured in culture supernatants, 24 hr after stimulation with irradiated autologous EBV-transformed B cells, pre-incubated O/N with cow's milk proteins.

Table 3. Phenotype of cow's milk-specific T cell clones from infants with AD, with and without CMA.

Patient	no of TCCs	Th2*	Th0*	Th1*
1 †	20	2 (10%)	6 (30%)	12 (60%)
3 †	15	3 (20%)	9 (60%)	3 (20%)
4 †	3	2 (67%)	1 (33%)	0
8 †	6	4 (66%)	1 (17%)	1 (17%)
2 ‡	4	0	0	4 (100%)
5 ‡	3	0	0	3 (100%)
7 ‡	7	0	1 (14%)	6 (86%)
11 ‡	29	0	29 (100%)	0

† CMA patient
‡ non-CMA patient
* Th2 phenotype was defined by: IL-4 > 0.4 ng/ml and IFN-γ < 1 ng/ml
Th0 phenotype was defined by: IL-4 < 0.4 ng/ml and IFN-γ < 1 ng/ml, or IL-4 > 0.4 ng/ml and IFN-γ > 1 ng/ml
Th1 phenotype was defined by: IFN-γ > 1 ng/ml and IL-4 < 0.4 ng/ml

the rest (mostly TCCs from patient 11/TW) produced very small amounts of both IL-4 and IFN-γ. To phenotype the T cell clones according to their production of IL-4 and IFN-γ, an arbitrary classification was used that was adapted from a previous study with CMP-specific T cell clones[27] (Table 3).

Comparison of the CMP-specific cytokine response in infants with, and without CMA

To compare the overall Th1/Th2 skewing of the CMP-specific T cell response of infants with clinical reactions to cow's milk and infants without CMA, for each cytokine, mean production by all CMP-specific TCCs from each patient was calculated (Table 4). In infants with CMA, mean IL-4 production was markedly higher than in non-CMA infants, who showed a very low mean production of IL-4. This difference was statistically significant (Mann Whitney-U; $p<0.05$). Mean production of IL-5 and IL-13 was also markedly higher in CMA infants than in non-CMA infants. In contrast, mean production of IFN-γ was markedly higher in non-CMA infants, compared with the CMA infants. Although the differences in production of IL-5, IL-13 and IFN-γ did not reach statistical significance, when all clones from CMA patients (n=44) were pooled and compared with the mean cytokine production of all clones from non-CMA patients (n=43), these differences were all statistically significant (Mann Whitney-U; $p<0.001$) (data not presented).

Table 4. Mean cytokine production by cow's milk-specific T cell clones from infants with AD, with and without CMA.

Patient	No*	IL-4 (ng/ml)	IL-5 (ng/ml)	IL-13 (ng/ml)	IFN-γ (ng/ml)
1 †	20	0.76 ± 0.29	7.96 ± 1.23	7.85 ± 0.87	2.71± 0.64
3 †	15	1.86 ± 0.60	4.37 ± 1.20	3.97 ± 1.03	2.39 ± 0.53
4 †	3	14.53 ± 1.46	50.62 ± 18.78	14.28 ± 2.42	1.92 ± 1.67
8 †	6	3.08 ± 1.41	16.83 ± 7.13	11.09 ± 3.90	0.70 ± 0.15
2 ‡	4	0.1 ± 0.1	6.15 ± 6.07	5.23 ± 4.11	14.50 ± 5.09
5 ‡	3	0.04 ± 0.02	1.14 ± 0.90	1.11 ± 0.84	3.34 ± 1.18
7 ‡	7	0.2 ± 0.04	9.26 ± 4.33	6.12 ± 2.21	7.85 ± 4.04
11 ‡	29	0.02 ± 0.01	0.29 ± 0.06	0.19 ± 0.05	0.01 ± 0.01
CMA patients	n=4	5.06 ± 3.19*	19.94 ± 10.55	9.30 ± 2.21	1.93 ± 0.44
Non-CMA patients	n=4	0.09 ± 0.04*	4.21 ± 2.12	3.16 ± 1.47	6.43 ± 3.14

results are expressed as mean ± SEM
* number of TCCs obtained from each patient
† CMA patient
‡ non-CMA patient
§ differences between CMA and non-CMA patients are significantly different ($p<0.05$)

Correlation between IL-4 release, and release of IL-5 and IL-13

To determine the association between CMP-specific production of IL-4 and release of other Th2-type cytokines during stimulation with cow's milk, correlation analysis was performed (Figure 3). Both production of IL-5 (Figure 3A) and IL-13 (Figure 3B) was significantly correlated with IL-4 production.

DISCUSSION

In this study, we analyzed the CMP-specific T cell response in infants with AD and allergy for cow's milk, and in infants with AD tolerant for cow's milk. The results from the proliferation assays confirmed that lymphoproliferative responses to the major allergens in cow's milk exist in PBMCs of infants with AD[8,9,28].

To analyze the CMP-specific T cell response at the clonal level, CMP-specific T cell clones were established. We show that infants with AD have a CMP-specific T helper cell response, irrespective of their (cow's milk-) allergic state. This response may be directed against all the major proteins in cow's milk and is not restricted to a particular subfraction.

Determination of cytokine release showed a clear dichotomy between the

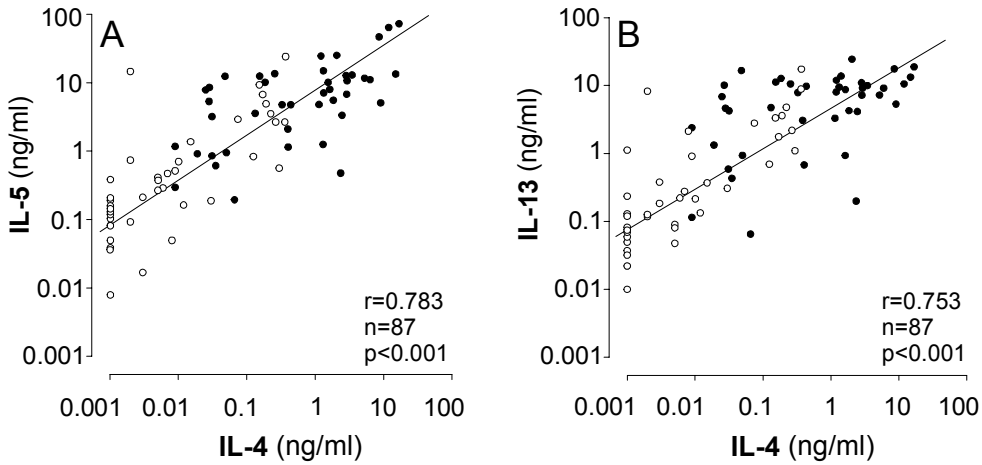

Figure 3. Correlation between antigen-specific production of IL-4 and IL-5 (A), and IL-4 and IL-13 (B) by cow's milk-specific T cell clones established from infants with atopic dermatitis, with and without cow's milk allergy. Closed circles represent clones from allergic infants, open circles represent clones from non-allergic infants. Cytokine production was measured in culture supernatants, 24 hr after stimulation with irradiated autologous EBV-transformed B cells, pre-incubated O/N with cow's milk proteins.

production of IL-4 in infants with, and without CMA. In TCC from infants with CMA, production of IL-4 was generally high, while none of the 43 clones derived from milk tolerant infants produced significant amounts of IL-4 (Figure 2).

The obtained TCCs were phenotyped according to their production of IL-4 and IFN-γ, based on criteria that were previously used to designate Th2, Th0 and Th1 subsets in CMP-specific TCCs[27]. According to this arbitrary classification, the CMP-specific T cells from infants with CMA were predominantly from the Th2-type and Th0-type, while the non-CMA infants did not have any CMP-specific Th2 cells, but had predominantly CMP-specific T cells from the Th1-type (Table 3).

Studies that evaluate human T cell clones generally designate distinct TCC-phenotypes similar to the Th1 and Th2 subsets that were originally found in murine models[29,30]. It has become clear however, that human T cells producing a clear-cut Th1 or Th2 cytokine profile as can be observed in mice, form two ends of a continuous spectrum in which most T cells produce a more heterogeneous cytokine profile[31-33]. Criteria for human Th2-Th0-Th1 subsets vary considerably between reports and each classification remains arbitrary[31,34]. For this reason, we analyzed differences between cytokine profiles of antigen-specific T cells from cow's milk-allergic and cow's milk-tolerant infants also by comparison of the absolute amounts of each individual cytokine (Table 4). This showed that in the infants with allergic reactions to milk, mean production of IL-4 was much higher than in the non-allergic infants. In contrast, mean production of IFN-γ was higher in the infants that

were tolerant for cow's milk. Three of these patients had a high IFN-γ production, while one patient had a cytokine response dominated by very low production of both IL-4 and IFN-γ.
In addition to IL-4, we determined the CMP-specific production of IL-5 and IL-13, two cytokines that are closely associated with the Th2 response, and which are potent inducers of allergic inflammation through recruitment of eosinophils and the induction of IgE synthesis[35,36]. In the CMA infants, production of IL-5 and IL-13 was high, while it was markedly lower in patients without CMA. Correlation analysis showed that release of both IL-5 and IL-13 was strongly correlated with IL-4 production, indicating that in CMP-specific T cells, release of Th2-cytokines is closely related.
Taken together, these results show that CMA in infants with AD is associated with a Th2-skewed CMP-specific T helper cell response in blood, while tolerance to cow's milk is associated with much lower levels of these cytokines and a Th1-skewed cytokine response.
The elaborate process of establishing antigen-specific T cell clones hampers the evaluation of large groups of patients. Due to the small sample size of 4 patients in each group, only a trend could be observed and statistical significance was not reached for all differences. However, when the TCCs from all allergic patients were pooled and mean cytokine production was compared with all clones from non-CMA patients, differences that were observed "at the patient level" between both groups were all statistically significant. This strongly suggests that these findings represent real differences that exist between cow's milk-allergic and cow's milk-tolerant infants with AD.
So far, only three previous studies have analyzed isolated T cell clones specific for CMPs[27,37,38]. In these studies, only the casein fraction of cow's milk was used for T cell culturing and, as a consequence, only casein-specific T cell clones were generated. Reekers and co-workers described 14 casein-specific T cell clones, established from 3 children with AD and allergic reactions to hen's egg and cow's milk[38]. These TCCs had been obtained using mitogenic, polyclonal stimulation, a method which may dramatically affect the primary responding T cell population[14], and may have resulted in only 43% of the obtained TCCs belonging to the CD4+ subset. Cytokine release by TCCs is also often determined after exposure to mitogenic stimuli, such as anti-CD3/anti-CD28 antibodies[17] or concavalin A[27,38]. However, *in vitro* cytokine release by TCCs activated through these non-specific stimuli can be markedly different from cytokine production following antigen-specific stimulation[15,17]. For these reasons, we propagated and activated the CMP-specific TCCs in our study using an antigen-specific culture system with autologous B cells as antigen-presenters, and did not use non-specific mitogens. We believe that in this antigen-specific *in vitro* system, the observed reactivity of T cells is a good representation of the *in vivo* reactivity.
In summary, reports that evaluate the T cell response against food antigens at the

clonal level are scarce[15-17,27,38]. The present study is the first to analyze food-specific T cell clones from food-tolerant individuals and to directly compare T cell reactivity and cytokine profiles from allergic and (age-matched) food-tolerant subjects. The results show that, while food allergy is associated with high production of Th2-cytokines, the food-specific T helper response in food-tolerant individuals is characterized by low levels of these cytokines. Especially the minute production of IL-4 in these infants, the hallmark cytokine of the allergic 'Th2' response, suggests a key role for the T helper-cell derived Th2 cytokines in food allergy-related skin symptoms. More studies on the mechanisms that determine Th1/Th2 skewing of the food antigen-specific T cell response[39] and the precise mechanisms through which circulating food-reactive Th2 cells mediate allergic inflammation in the skin[40] are needed to give future directions for the development of therapy in food allergy and AD.

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Chapter 3

Cow's milk-specific T cells in blood of non-allergic infants without atopy display a Th0-phenotype

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ABSTRACT

Cow's milk protein (CMP)-specific T cells are present in blood of infants with atopic dermatitis, irrespective of their CMP-allergic state. However, only T cells from patients with allergy to CMPs show a high production of Th2 cytokines. It is presently unclear if these results can be extrapolated to individuals without atopy. In this study, three non-atopic infants (mean age 8.0 months) were investigated, who did not have allergy to cow's milk or other foods, and in whom atopy was excluded by clinical and *in vitro* investigations. Using an antigen-specific culturing system, CD4+ CMP-specific T cell clones (TCCs) (n=75) specific for the major CMPs were established from blood. These TCCs showed a Th0-like phenotype, with a closely correlated production of IL-4 and IFN-γ. Release of IL-4 was lower compared to CMP-specific T cells from atopic patients with cow's milk allergy. These findings demonstrate for the first time at the clonal level, that a T helper cell response directed against the major proteins in milk is present in CMP-tolerant individuals without atopy. The 'Th0-like' cytokine release, and the low Th2 cytokine production, suggests that tolerance to milk in normal individuals is not caused by absence of circulating, CMP-specific T cells, but is probably determined by the cytokine profile of these cells.

INTRODUCTION

Food antigens are the most important allergens that cause allergic reactions during infancy and childhood. Antigen-specific T cells have been connected with the aetiology of food allergy and food-allergic reactions[1]. In a previous study we showed, at the clonal level, that cow's milk protein (CMP)-specific T cells are present in blood of infants with atopic dermatitis (AD), irrespective of their cow's milk-allergic state[2]. The CMP-specific T cells from patients that have allergic reactions to cow's milk however, show a significantly higher production of Th2 cytokines compared with patients that do not have cow's milk allergy (CMA)[2]. This finding suggests an important role for the Th2 cytokines in food allergy-related skin symptoms, similar to the role of Th2 cells in AD and inhalation allergy[3].

It is clear however, that atopic diseases are characterized by aberrant T cell responses[4,5]. Although studies have shown food-specific proliferation in PBMCs of normal, non-atopic controls[6-8], the presence of food-specific T cells has not yet been confirmed in non-allergic, non-atopic individuals at the clonal level so far. The previous observations on T cell cytokine release in atopic individuals can therefore not be extrapolated to normal individuals without an atopic predisposition.

The aim of the current study was to investigate the presence of T cells, specific for

CMPs, in blood of non-allergic individuals without atopic disease. An antigen-specific T cell culturing system with autologous B cells as antigen-presenting cells was used to establish CMP-specific T cell clones (TCCs) from blood of three non-atopic infants without allergy to cow's milk. Specificity of the TCCs for the various protein-fractions in milk, and cytokine release was investigated.

METHODS

We studied 3 non-atopic infants, age 6.4 - 9.9 months (mean 8.0), who were not allergic to cow's milk or other foods. Atopy was excluded by negative family history, absence of clinical atopic disorders (eczema, asthma or rhinitis), and by *in vitro* diagnostics: infants did not have increased total IgE levels, and were not sensitized for a panel of 12 common inhalation and food allergens as determined by CAP system FEIA (Pharmacia Diagnostics, Uppsala, Sweden). No systemic or topical immunomodulatory drugs were used. Blood samples were taken after informed consent was obtained.

CMP-specific TCCs were established from blood as described previously[2]. In short, PBMCs were isolated from heparinized venous blood, and subsequently cultured using an antigen-specific culturing system with irradiated autologous EBV-transformed B cells, pre-incubated O/N with CMPs, as antigen-presenting cells. Culturing was performed in the presence of IL-2 and IL-4 (kind gift of Novartis Research Institute, Vienna, Austria). If these cultures indicated a high CMP-specific T cell proliferation in the lymphocyte stimulation test (LST), T cells were cloned by limiting dilution. Established TCCs were tested in LST to determine specificity for the various different CMPs.

CMP-specific release of IL-4 and IFN-γ by the TCCs was determined as described previously[2]. In short, 10^6 cells of each TCC were incubated with 10^6 irradiated autologous EBV-transformed B cells, pre-incubated O/N with CMPs. Cytokine production was measured in cell-free supernatants obtained after 24 hours of culture, by ELISA. Student's t-test was used to determine differences between cytokine production. Spearman's rank correlation was used for correlation analysis. A p-value < 0.05 was considered significant.

RESULTS

CMP-specific T cells: reactivity towards the various protein fractions in cow's milk

A total of 75 CMP-specific TCCs was obtained. Flow-cytometric analysis showed that all TCCs were CD4+ and expressed the α/β TCR (data not presented). For all TCCs, specificity towards the major CMPs, casein and whey, and their subfractions

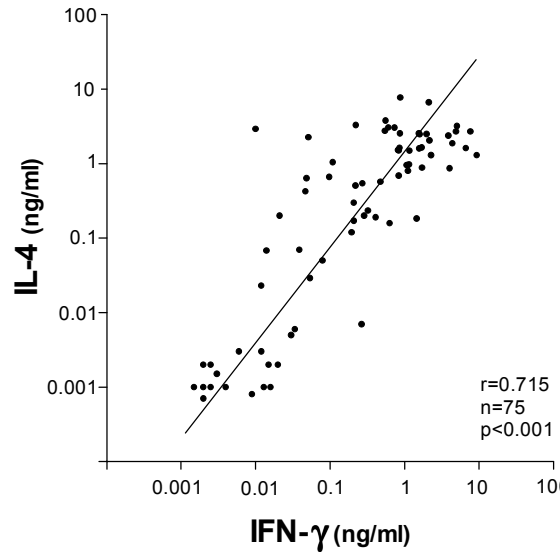

Figure 1. CMP-specific production of IL-4 and IFN-γ by CMP-specific TCCs derived from non-atopic infants without CMA. Most TCCs show a Th0-like phenotype, with comparable production of IL-4 and IFN-γ. Production of both cytokines is significantly correlated. ($p<0.001$). Cytokine production was measured in culture supernatants 24 hours after stimulation with irradiated, autologous, EBV-transformed B cells preincubated overnight with CMPs.

was determined. Sixty-six TCCs (88%) were specific for casein, 9 TCCs (12%) for whey. Specificities for casein subfractions were: αS1-casein (26 TCCs), αS2-casein (8 TCCs), β-casein (21 TCCs), κ-casein (8 TCCs), not determined (3 TCCs). Specificities for the whey subproteins: α-lactalbumin (4 TCCs), β-lactoglobulin (5 TCCs).

CMP-specific T cells display a Th0-phenotype

Figure 1 shows CMP-specific production of IL-4 and IFN-γ by individual TCCs. Most clones showed a Th0-like phenotype, with comparable production of IL-4 and IFN-γ. Correlation analysis showed that production of IL-4 and IFN-γ was significantly correlated in all TCCs: $r_s = 0.715$, $p<0.001$). Mean cytokine production for each patient is shown in Table 1.

Comparison of cytokine production with CMP-specific T cells from atopic infants

Cytokine production by the CMP-specific TCCs was compared with CMP-specific TCCs from age-matched atopic infants that have been described previously[2]. TCCs from infants with atopy and CMA show a Th2-like cytokine pattern, while atopic

Table 1. Mean cytokine production by cow's milk-specific TCCs from individual non-atopic infants without CMA.

Patient	IL-4 (ng/ml)	IFN-γ (ng/ml)
I	0.05 ± 0.02	0.05 ± 0.02
II	1.94 ± 0.56	2.19 ± 1.40
III	1.59 ± 0.22	1.62 ± 0.30

results are expressed as mean ± SEM

infants that do not have CMA display a Th1-skewed pattern[2] (Table 2). Mean cytokine production by the CMP-specific TCCs from the non-allergic, non-atopic infants shows a Th0-like cytokine profile, with a lower production of IL-4 than infants with CMA, and a lower production of IFN-γ than atopic infants without CMA (Table 2). No significant correlation was found between production of IL-4 and IFN-γ in TCCs from atopic infants (data not presented).

Table 2. Comparison of cytokine production by TCCs from non-atopic infants with atopic infants, with and without CMA, who have been described previously[2].

Patient group	IL-4 (ng/ml)	IFN-γ (ng/ml)
Non-atopic controls*	1.19 ± 0.58	1.29 ± 0.64
CMA patients†	5.06 ± 3.19	1.93 ± 0.44
Atopic controls*†	0.09 ± 0.04	6.43 ± 3.14

results are expressed as mean ± SEM of results from pooled clones from each individual patient
* infants without CMA
† infants with atopy

DISCUSSION

The findings in this report demonstrate for the first time at the clonal level, that a CMP-specific T helper cell response exists in non-atopic infants without CMA. This response is directed against all the major proteins in cow's milk and is not restricted to a particular subfraction.
The finding that, besides atopic individuals, also individuals without an atopic

predisposition have a CMP-specific T cells in blood, proves that clinical tolerance to milk in normal individuals is not caused by absence of CMP-specific T cell responses.
Analysis of cytokine release showed that the CMP-specific T cells display a balanced, relatively low production of IL-4 and IFN-γ, i.e. a Th0-like phenotype. This was clearly demonstrated by the significant correlation between IL-4 and IFN-γ in all (n=75) TCCs derived from these infants, which was absent in TCCs derived from atopic infants. Comparison of cytokine production with CMP-specific T cells from age-matched CMA patients showed that release of IL-4 by CMP-specific T cells from non-allergic, non-atopic infants is lower than in patients with allergy. Similar results were found for the production of IL-5 and IL-13 (data not presented). These findings add to the hypothesis that high release of Th2-cytokines by circulating antigen-specific T cells plays an important role in the aetiology of allergic reactions, whereas low release is associated with tolerance[3-5].
Allergen-specific T cell responses to foods are already present at birth in virtually all individuals, and are Th2-biased during the perinatal period[4,9]. From studies in PBMCs, it was suggested that T cell responses in individuals without an atopic predisposition postnatally deviate to a Th1-biased pattern, to prevent sensitization to environmental allergens[4,9]. The results described here, however, show that when investigated at the single cell level, the T cell response to milk in non-atopic individuals displays a balanced, Th0-like cytokine profile, with a comparable production of IL-4 and IFN-γ. This suggests that the fetal Th2-biased response to milk is indeed downregulated, but becomes a balanced Th0-response, instead of a Th1-biased response. It is clear that the strong inflammatory capacities of Th1-cytokines such as IFN-γ can be harmful. Therefore, as cow's milk is encountered in large quantities during infancy, its seems more logically that a balanced (i.e. Th0-like) cytokine reponse to milk is better than a Th1-response that is dominated by IFN-γ production.
In conclusion, these results demonstrate that non-atopic individuals have CD4+, CMP-specific T cells with a balanced, Th0-phenotype present in blood. This indicates that tolerance to milk in normal individuals is not caused by absence of circulating, CMP-specific T cells, but is probably determined by the low release of Th2-cytokines by these cells.

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Chapter 4

Spontaneous tolerance in cow's milk allergic infants is associated with a deviation towards a Th1-cytokine profile

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ABSTRACT

Background Food-specific T cell reactivity plays an important role in the aetiology of food allergy. Cow's milk allergy (CMA) is the major food allergy in infancy and is associated with the spontaneous development of tolerance within two years. This process is thought to be mediated by antigen-specific T cells, but has not yet been studied at the T cell level.

Objective This study was designed to analyze changes in the cow's milk protein (CMP)-specific T cell response during the spontaneous development of tolerance for cow's milk in infants with atopic dermatitis (AD) and CMA.

Methods We used an antigen-specific culturing system with autologous B cells as antigen-presenting cells to establish 65 CMP-specific T cell clones (TCCs) from blood of 4 infants with AD and CMA. CMP-specific TCC-reactivity, measured by lymphocyte stimulation test, and cytokine production, measured by ELISA, was compared before and after the spontaneous development of tolerance for each patient. To control for age-related changes we also studied 85 CMP-specific TCC from 4 age-matched infants with AD but without CMA.

Results CMP-specific production of the Th2 cytokines (IL-4, IL-5, and IL-13), which was abundant at the allergic state, was lower after the development of tolerance. In contrast, production of IFN-γ (Th1-cytokine) was increased. Age-matched control patients without CMA showed a Th1-skewed CMP-response during infancy, which changed to a Th0-like cytokine response after infancy.

Conclusions The spontaneous development of tolerance in cow's milk allergic infants is associated with a deviation from a Th2-biased to a Th1-biased CMP-specific T cell response. These results indicate that CMP-specific T cells play an important role in the development of allergy and tolerance to cow's milk in infants with CMA and AD.

INTRODUCTION

Food antigens are the main allergens that cause allergic reactions during infancy and childhood[1,2]. Antigen-specific T cells have been connected with the aetiology of food allergy and food allergic reactions. Food-specific T cell reactivity and cytokine production has been demonstrated in food-stimulated PBMCs[3-7] but as these results were observed in bulk cultures of T cells, they are difficult to extrapolate to the individual T cell. In a previous study, we have investigated differences in antigen-specific T cell reactivity between infants with and without

food allergy at the clonal level[8]. We found that food allergy in patients with atopic dermatitis (AD) is associated with high production of the Th2 cytokines by (circulating) food allergen-specific T cells. This suggests a key role for these cells in food allergy-related skin symptoms, similar to the role of Th2 cells in AD and inhalation allergy[9,10].

The most important food antigen in young children is cow's milk[11,12]. It is the aetiological factor of acute cutaneous symptoms and/or aggravation of the eczema in approximately 35-40% of infants with AD[13,14]. Cow's milk allergy (CMA) in infancy is associated with the spontaneous development of tolerance within 2-3 years. This feature results in more than 85% of the infants with CMA tolerating cow's milk without problems at the age of three years[2,15]. The immunological mechanisms that mediate this process are mostly unknown, and have only been investigated at the humoral level[16-18]. However, because of their key role in the aetiology of allergy to cow's milk, it is of importance to investigate the role of antigen-specific T cells in the development of tolerance to cow's milk.

In this study, we investigated changes in the cow's milk protein (CMP)-specific T cell response during the spontaneous development of tolerance for cow's milk in infants with CMA and AD. We used an antigen-specific T cell-culturing system with autologous B cells as antigen-presenting cells to establish CMP-specific T cell clones (TCCs) from blood of these infants, and from age-matched control infants with AD, but without CMA. We compared CMP-specific T cell reactivity before and after the spontaneous development of tolerance for cow's milk.

METHODS

Patients & control subjects

The study was approved by the Medical Ethical Committee of the University Medical Center, Utrecht. Four infants with AD and CMA were included in the study. Four infants with AD but without CMA were included as controls. Atopic dermatitis was diagnosed according to the criteria of Hanifin and Rajka[19]. CMA was diagnosed or excluded by complete elimination of cow's milk from the infant's diet, followed by a cow's milk challenge procedure[20]. All infants with CMA developed acute cutaneous symptoms. Table 1 shows characteristics of patients and controls. Age of CMA patients was not significantly different ($p = 0.79$) from non-CMA controls; 4.5 - 12.3 months (mean 7.0) vs. 6.1 - 7.4 months (mean 7.5). After informed consent was obtained, venous blood was taken from patients and controls.

One year after inclusion in the study, the allergic infants were re-evaluated for CMA via cow's milk challenge. At this timepoint, all CMA patients had become tolerant for CMPs and all the control patients still tolerated cow's milk without problems (Table 1). Venous blood samples were taken from patients and controls.

Table 1. Characteristics of infants with AD, with and without CMA.

Patient	Symptoms*	1st timepoint		2nd timepoint	
		IgE	SPT	IgE	SPT
Patient I	Erythema, AD	-	-	-	-
Patient II	Erythema, UR	+	+	-	-
Patient III	Erythema, UR, AD	+	+	+	+
Patient IV	Erythema, AD	-	+	-	+
Control I	-	-	-	-	-
Control II	-	-	-	-	nd
Control III	-	-	-	-	-
Control IV	-	-	-	-	-

* symptoms developed upon challenge during allergic state
AD = worsening of atopic dermatitis
UR = urticaria
IgE = cow's milk-specific IgE detected by Cap Pharmacia
SPT = positive skin prick test for cow's milk
nd = not done

Cow's milk antigens

Purified CMPs (used at concentrations of 50 μg/ml): total casein, α-lactalbumin and β-lactoglobulin, and purified casein-subfractions (used at concentrations of 10 μg/ml): αS1-casein, αS2-casein, β-casein and κ-casein, were kindly provided by Dr. E.C.H. van Beresteijn (Netherlands Institute for Dairy Research, Ede, The Netherlands). A mix of purified CMPs containing equal quantities of total casein, α-lactalbumin and β-lactoglobulin (each at a concentration of 50 μg/ml) is referred to as CMP-mix.

Culture media

For culturing of T cell lines and TCCs, and in lymphocyte stimulation tests (LSTs), complete medium RPMI-1640 (Gibco, New York, USA) was used, supplemented with 10% pooled human AB serum. Epstein Barr virus (EBV)-transformed B cells were cultured in RPMI-1640 (Gibco), supplemented with 10% fetal calf serum (Gibco). Established TCCs were maintained in Iscove's Modified Dulbecco's Medium (Gibco) supplemented with 2% pooled human AB serum and 5% Yssel's medium [21]. All media were supplemented with penicillin (100 IU/ml), streptomycin (100 mg/ml) and glutamin (1 mM) (Gibco).

LST

LSTs were performed in triplicate in 96-well U-bottom plates (Greiner, Frickenhausen, Germany). Each well contained $4x10^4$ T cells, and $4x10^4$ irradiated (50 Gy) autologous EBV-transformed B cells as antigen-presenting cells. The EBV-transformed B cells were, before co-culture with T cells, incubated O/N with antigen. EBV-transformed B cells incubated without antigen were used as negative controls. After 24 hours of culturing, proliferation was measured by using tritiated thymidine ([^{3}H]-TdR) incorporation; [^{3}H]-TdR (1μCi/well; Amersham, Aylesbury, UK) was added, and the cells were harvested after 18 hours. Thymidine incorporation was measured using a 1205 betaplate^{tm} counter (Wallac, Turku, Finland).

Preparation of CMP-specific TCCs

CMP-specific TCCs were established as described previously[8,22]. PBMCs were isolated from heparinized venous blood by using Ficoll density gradient centrifugation. Recovered cells were cultured in a 24-well flat bottom culture plate in the presence of CMP-mix. After 7 days, 50 IU/ml of both recombinant IL-2 and IL-4 (kind gift of Novartis Research Institute, Vienna, Austria) was added to the culture medium. To promote expansion of CMP-specific T cells, cultures were re-stimulated every 14 days with irradiated autologous EBV-transformed B cells that had been pre-incubated O/N with CMP-mix, as antigen-presenting cells. LST or determination of cytokine release was always done at the start of a re-stimulation cycle. After 2-3 weeks, when a polyclonal T cell culture was obtained, CMP-specificity was verified by LST. If these cultures indicated a high CMP-specific T cell proliferation, T cells were cloned by limiting dilution at 0.3, 1 or 3 cells/well in 96-well U-bottom culture plates in the presence of IL-2, IL-4 and irradiated autologous EBV-transformed B cells (10^4/well) that had been pre-incubated O/N with CMP-mix. Established TCCs were screened for CD4 or CD8 and T cell receptor (TCR) expression. Cells were stained with fluorescein isothiocyanate (FITC)-conjugated anti-CD4 (clone Leu-3a, BD Biosciences, San Jose, USA) and phycoerythrine-conjugated anti-CD8 (clone Leu-2a, BD Biosciences), or FITC-conjugated anti-α/β TCR (clone WT-31, BD Biosciences), fixed in paraformaldehyde, and analyzed with FACScan (BD Biosciences). All TCCs were tested in LST to verify CMP-specificity, and to determine specificity for the different proteins in cow's milk.

CMP-specific cytokine release

To determine CMP-specific cytokine release, 10^6 cells of each TCC were incubated with 10^6 irradiated autologous EBV-transformed B cells that had been pre-incubated O/N with CMP-mix. Control cultures of TCCs and EBV-transformed B cells, pre-incubated without antigen were prepared in parallel. Stimulation was performed in a 24-well plate, final volume 1 ml. After 24 hours of culture at 37^0C,

supernatants were collected and stored at -20^{0}C. Cytokines were measured by ELISA according to the manufacturers recommendations; IL-4, IL-10, IL-13, IFN-γ: Central Laboratory of the Blood Transfusion Service, Amsterdam, The Netherlands, IL-5: Endogen, Woburn, USA. The detection limit was 0.6 pg/ml for IL-4, 5 pg/ml for IL-5, 1.2 pg/ml for IL-10, 0.5 pg/ml for IL-13 and 2 pg/ml for IFN-γ. CMP-specific cytokine release by a TCC was calculated by subtracting control-stimulated cytokine production from cytokine production after stimulation with CMP-mix.

Statistical analysis

Parametric analysis (Student's t-test) was applied to determine significant differences between patient and control groups with regard to age, and differences in cytokine production between the two timepoints for each patient and control. Parametric analysis (Pearson's correlation) was used to test for correlation between production of different cytokines. Differences associated with *p* values of less than 0.05 were considered significant.

RESULTS

CMP-specific TCC: reactivity towards the various protein fractions in cow's milk

Forty-four TCCs were obtained from the 4 allergic patients at the first timepoint, 43 from the 4 age-matched control patients without CMA. At the second timepoint, 21 TCCs were isolated from 3 tolerant patients and 42 from 3 control patients. The cloning procedure did not yield TCCs in 1 tolerant patient and in 1 control patient at the second timepoint.

Flow-cytometric analysis showed that all clones were CD4+ and expressed the α/β TCR (data not shown). Specificity of the TCCs was determined for the casein and whey-proteins, and for the protein-subfractions (Table 2). TCC reactivity towards the casein and whey proteins was observed at the first timepoint in both allergic and controls and was not different between the two groups (Table 2A). At the second timepoint, few whey-specific TCCs were obtained; they were derived from the non-allergic controls (Table 2B). No major differences in casein-specific TCC reactivity were observed between patients and controls at the second timepoint.

Production of Th2 cytokines vs Th1 cytokines

For each individual patient with CMA, we studied changes in CMP-specific production of the hallmark Th2 cytokine (IL-4), and the hallmark Th1 cytokine (IFN-γ) during the development of tolerance. Figure 1A shows results of the three patients in which TCCs were obtained from both timepoints. In two patients with

Table 2A. First Timepoint: TCC reactivity towards the major CMPs in infants with AD and CMA, and age-matched controls.

Patient	no*	Casein proteins					Whey proteins	
		αS1	αS2	β	κ	§	α-lac	β-lac
Patient I	20		6		2	1	3	8
Patient II	6	2	3	1				
Patient III	15	2	1		3	3	4	2
Patient IV	3		3					
Control I	4		1			3		
Control II	3	1			1	1		
Control III	7				2			5
Control IV	29					1	28	

* number of TCCs obtained from each patient
§ TCC specific for total casein, specificity for the casein-subfractions could not be determined
α-lac = α-lactalbumin
β-lac = β-lactoglobulin

Table 2B. Second Timepoint: TCC reactivity towards the major CMPs and protein-subfractions in infants tolerant for CMPs, and age-matched controls.

Patient	no*	Casein proteins					Whey proteins	
		αS1	αS2	β	κ	§	α-lac	β-lac
Patient I	7	1	5			1		
Patient II	2	2						
Patient III	12	4		6	2			
Control I	11	10						1
Control II	18	14			1	2		1
Control III	13	6	3		3		1	

* number of TCCs obtained from each patient
§ TCC specific for total casein, specificity for the casein-subfractions could not be determined
α-lac = α-lactalbumin
β-lac = β-lactoglobulin

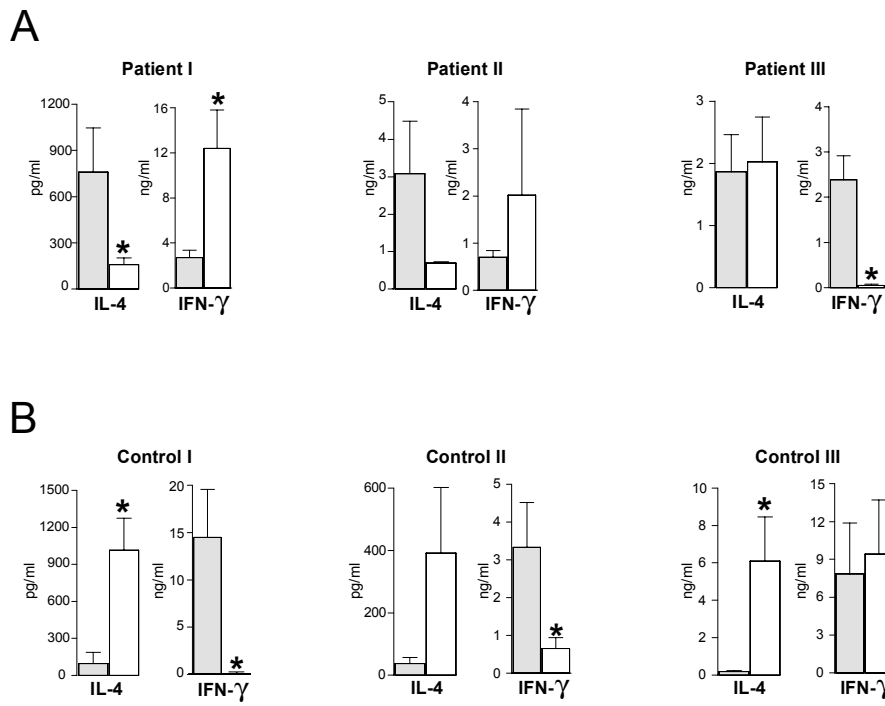

Figure 1. Mean production of IL-4 and IFN-γ by CMP-specific TCCs obtained from three infants with CMA and AD (Figure 1A) during the allergic state (closed bars) and after the development of tolerance for cow's milk (open bars), and from three age-matched controls with AD without CMA (Figure 1B) at both timepoints. Asterisk represents: $p<0.05$ (Student's t-test).

production of IL-4, and an increase in IFN-γ production. In the third patient, no change in IL-4 production was seen. Production of IFN-γ at the tolerant state was reduced to a very low level in this patient.

To control these results for age-related changes, we also investigated changes in the CMP-specific cytokine production between the two timepoints in each individual control patient. Figure 1B shows results of the three controls in which TCCs were obtained from both timepoints. In contrast to the patient group, IL-4 production in the controls showed a marked increase between the two timepoints, while IFN-γ production, which was high at the first timepoint, showed a decrease.

Results were tested for statistical significance using Student's t-test. In 1 patient and 1 control, statistical analyses were hampered by the fact that, at one of the timepoints, a low number (≤3) of clones was established. Despite clear differences (change in mean result of more than 75%) these results were only borderline significant due to the low number of clones.

Production of IL-5 and IL-13, and correlation with IL-4 release

In addition to IL-4, we also measured CMP-specific production of IL-5 and IL-13, two cytokines closely associated with the Th2 response. Table 3 shows results of mean production at both timepoints. Similar to IL-4, the development of tolerance in patients with CMA was accompanied by a marked reduction in production of IL-5 and IL-13 in 2 of these patients. The third CMA patient showed mainly a decrease in IL-13 at the development of tolerance. In contrast to the patient group, production of IL-5 and IL-13 was unchanged between the two timepoints in the control patients.

To study the association between the antigen-specific production of the Th2-cytokines during stimulation with cow's milk, correlations between production of IL-4, IL-5 and IL-13 were calculated. This showed that in all (n=87) TCCs established at the first timepoint, and in all (n=63) TCCs from the second timepoint, antigen-specific production of IL-4 and IL-5, production of IL-4 and IL-13, and production of IL-5 and IL-13 were significantly correlated ($p<0.001$, data not shown). There was no correlation between production of IFN-γ and any of the Th2-cytokines.

Table 3. Production of IL-5 and IL-13 by TCCs from infants with AD, with and without CMA, at two timepoints

Patient	IL-5 (ng/ml)		IL-13 (ng/ml)	
	1st timepoint	2nd timepoint	1st timepoint	2nd timepoint
Patient I	7.9 ± 1.2	0.5 ± 0.3	7.8 ± 0.8	3.4 ± 0.8
Patient II	16.8 ± 7.1	0.1 ± 0.0	11.0 ± 3.8	1.0 ± 1.0
Patient III	4.3 ± 1.2	4.4 ± 1.2	3.9 ± 1.0	1.0 ± 0.2
Patient IV	50.6 ± 18.7	*	11.1 ± 3.9	*
Control I	6.1 ± 6.0	3.0 ± 1.0	5.2 ± 4.1	3.5 ± 0.6
Control II	1.1 ± 0.9	2.2 ± 0.8	1.1 ± 0.8	0.9 ± 0.2
Control III	9.2 ± 4.3	7.6 ± 1.8	6.1 ± 2.2	2.6 ± 0.8
Control IV	0.2 ± 0.0	*	0.2 ± 0.0	*

* no TCCs obtained at this timepoint

Comparison of CMP-specific cytokine response in patients and controls, for both timepoints

To compare the overall Th1/Th2 skewing of the CMP-specific T cell response of infants with clinical reactions to cow's milk and infants without CMA, mean TCC cytokine production in each patient group was calculated. Table 4 shows results based on the three patients in which TCCs were obtained from both timepoints. Cow's milk allergic infants have a Th2-skewed cytokine pattern with higher levels

Table 4. Mean cytokine production in infants with AD, with and without CMA, at two timepoints.

	Patient group	IL-4 (ng/ml)	IL-5 (ng/ml)	IL-13 (ng/ml)	IFN-γ (ng/ml)
1st timepoint	CMA pts*	1.90 ± 0.66	9.72 ± 3.69	7.63 ± 2.05	1.93 ± 0.62
	non-CMA pts*	0.10 ± 0.04	5.52 ± 2.35	4.15 ± 1.54	8.56 ± 3.24
2nd timepoint	tolerant pts*	0.95 ± 0.55	1.58 ± 1.26	1.87 ± 0.80	4.82 ± 3.82
	non-CMA pts*	2.49 ± 1.80	4.32 ± 1.69	2.37 ± 0.78	3.39 ± 3.06

results are expressed as mean ± SEM
*results are based on 3 patients in each group

of IL-4, IL-5 and IL-13 than non-CMA infants. In contrast, non-allergic infants show a Th1-skewed cytokine profile with high production of IFN-γ. At the development of tolerance, the allergic group shows a Th1-skewed cytokine pattern, with low levels of IL-4 and high levels of IFN-γ. The age-matched control group shows a more Th0-like profile with comparable levels of IL-4, IL-5, IL-13 and IFN-γ.

CMP-specific production of IL-10

To investigate whether the spontaneous development of tolerance after CMA is accompanied by an increased production of IL-10 by antigen-specific T cells, we analyzed IL-10 production by the TCCs in this study (Table 5). IL-10 production was highly variable in individual TCCs. When compared as a group, the overall

Table 5. Production of IL-10 by TCCs from infants with AD, with and without CMA, at two timepoints

PATIENT	IL-10 (ng/ml) 1st timepoint	IL-10 (ng/ml) 2nd timepoint
Patient I	0.57	0.80
Patient II	1.70	0.01
Patient III	1.45	0.03
Patient IV	4.5	*
Mean	2.05 ± 0.85	0.27 ± 0.26
Control I	3.13	0.01
Control II	0.01	0.62
Control III	2.44	0.08
Control IV	0.12	*
Mean	1.67 ± 0.68	0.23 ± 0.19

* no TCCs obtained at this timepoint

trend did not show an increase, but a decrease in IL-10 production between the allergic and tolerant state. In the control group a similar trend was seen with a decrease in IL-10, which clearly suggests an age-related phenomenon. At both timepoints, mean TCC production of IL-10 was not different between patients and controls.

DISCUSSION

Food antigen-specific T cells have been shown to be important in the aetiology of allergic reactions to foods[3,5-8]. This has mainly been investigated by studying differences in T cell reactivity between food-allergic and food-tolerant individuals. The present study is the first to investigate the role of T cells during the spontaneous development of tolerance to foods. We compared, at the clonal level, the antigen-specific T cell response in cow's milk-allergic individuals during the allergic phase and after the spontaneous development of tolerance for cow's milk.
The analysis of cytokine production by the CMP-specific T cells showed marked differences in the CMP-specific release of the Th2 cytokines at both timepoints. While the production of IL-4, IL-5 and IL-13 was abundant in the allergic state, production of these cytokines was decreased after tolerance had developed. At the same time, an up-regulation in CMP-specific production of IFN-γ was seen. This resulted in a deviation of the cow's milk-specific T cell cytokine response towards a Th1-type profile. In contrast, age-matched control patients with AD but without CMA, showed an opposite phenomenon. During infancy, these patients had a Th1-skewed CMP-specific cytokine response. In the second year of life, production of IL-4 increased and IFN-γ decreased, causing a change in the CMP-specific T cell response towards a more Th0-like cytokine pattern.
The CMP-specific T cell response was investigated at the clonal level in this study. This makes it possible to investigate the reactivity of antigen-specific T cells without the disturbance of non-specific T cell reactivity. A disadvantage is that the elaborate process of establishing antigen-specific T cell clones hampers the evaluation of large groups of patients. The TCCs were propagated and activated using an antigen-specific culture system with autologous B cells as antigen-presenters, and without the use of non-specific mitogens. We believe that in this antigen-specific *in vitro* system, the observed reactivity of T cells is a good representation of the *in vivo* reactivity in these patients.
The release of Th2-cytokines by allergen-specific T cells has been shown to be important in the pathogenesis of allergic reactions[9,23,24]. They are potent inducers of allergic inflammation and act through recruitment of leukocytes and the induction of IgE synthesis[25,26]. The results from the present study show that the spontaneous development of tolerance in food allergy is associated with a down-regulation of these cytokines. This finding supplies further evidence for their importance in the

aetiology of food allergic symptoms. While a high production of IL-4, IL-5, IL-13 leads to allergic reactions, a low release of these cytokines by antigen-specific T cells is presumably the reason for the absence of allergic reactions in food-tolerant individuals.

It has been suggested that fetal and neonatal responses to environmental antigens such as foods, are initially Th2 in nature, and that these responses deviate to a Th1-pattern during the first year of life in normal individuals[27,28]. According to this view, allergic reactions (in atopic individuals) are caused by an aberrant persistence of the innate Th2 responses[29]. Our study shows that in allergy to cow's milk, the antigen-specific (Th2-) response deviates towards a Th1-response during the spontaneous development of tolerance. This suggests that if antigen-specific T cell responses are indeed initially Th2 in nature, then CMA in infancy could be viewed a result of a temporarily delay in the (normal) redirection of the Th2 response. The fact that CMA in infancy is remarkably transient, i.e. most patients become tolerant within 2 years, may support this idea. In this respect, the results from the cytokine analysis in the non-cow's milk allergic control group in our study are also of interest. In these patients, the (CMP-specific) Th1-skewed cytokine response that was present in the first year of life, changed into a Th0-like cytokine pattern. It is tempting to speculate that during infancy, when cow's milk is first encountered, persistence of the (fetal) Th2 response leads to allergy, while a vigorous CMP-specific Th1 response prevents sensitisation for milk antigens. After infancy, when the 'window of sensitisation'[30,31] has passed by, and allergy to milk has been successfully prevented, the CMP-specific cytokine response becomes more balanced (i.e. Th0-like). Further research into the mechanisms that determine T cell responses during the development of allergy and tolerance to foods, is needed to confirm this hypothesis.

In patients with allergy to for instance birch-pollen and bee venom, tolerance can be artificially induced via allergen-specific immunotherapy[32,33]. It has been shown that this induction of tolerance is accompanied by a switch from an allergen-specific Th2-skewed T cell response to a Th1-skewed response[34-36]. In addition, it was shown that allergen-specific immunotherapy induces an enhancement of IL-10 production by allergen-stimulated PBMCs[32,33]. This rise in production of IL-10 is thought to be caused by an increased IL-10 production by antigen-presenting cells such as B cells and monocytes[37]. In the present study, we found no evidence that spontaneous development of tolerance to food allergens is associated with an increased production of IL-10 by (food-)antigen-specific T cells. There was a tendency to a decreased antigen-specific IL-10 production by the TCC, but a similar decrease was seen in the control group, which suggests an age-related phenomenon. IL-10 production by antigen-presenting cells was not investigated in this study. We are therefore unable to determine whether spontaneous development of tolerance to cow's milk is associated with a rise in IL-10 production by antigen-presenting cells as occurs during immunotherapy[37].

Cow's milk consists of a number of different proteins. It has been clarified that allergy to cow's milk is not determined by an immunological response to a particular cow's milk protein subfraction[8,12,38]. The CMP-specific T cell clones in this study were established using a mix of casein- and whey-proteins, the two predominant protein-fractions in milk[38]. In the clones that we obtained from the tolerant patients in our study, we noted an absence of whey-specificity. This was probably caused by the lower number of clones obtained at this timepoint. In the age-matched controls, the percentage of established whey-specific TCCs was also lower, suggesting that T cell reactivity towards whey proteins is less predominant after infancy. To verify that the absence of whey-specific TCCs did not influence the conclusions from the cytokine analyses, we also performed all analyses while leaving out the whey-specific TCC, which showed the same overall result; i.e. skewing of the cytokine response from a Th2 to a Th1-biased response (data not shown). This emphasizes that deviation of T cell cytokine profiles and not T cell reactivity per se determines allergy and tolerance to cow's milk.
In summary, the release of Th2 cytokines by allergen-specific T cells has been shown to be important in the pathogenesis of food-allergic reactions. In this study we analyzed, at the clonal level, the cow's milk-specific T cell response in infants with AD and CMA during the spontaneous development of tolerance for cow's milk. We found that cow's milk-specific production of IL-4, IL-5 and IL-13, which was abundant during the allergic phase, had decreased after the development of tolerance, while at the same time IFN-γ production was up-regulated. This resulted in a deviation of the CMP-specific T cell cytokine response towards a Th1-type profile. These findings supply further evidence for the importance of T cell cytokine profiles in the aetiology of allergy and tolerance to food-antigens.

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Chapter 5

Cell-surface expression of CD25, CD26, and CD30 by allergen-specific T cells is intrinsically different in cow's milk allergic infants

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ABSTRACT

Background The release of Th2 cytokines by food-specific T cells is thought to be important in the etiology of food allergy. It has been suggested that the activation state of food-specific T cells also plays a significant role, but this has not yet been studied at the single cell level.

Objective To evaluate differences in the expression of cell-surface markers by cow's milk protein (CMP)-specific T cells between infants with and without cow's milk allergy (CMA) at the clonal level. In addition, changes during the spontaneous development of tolerance for cow's milk in infants with CMA were analyzed.

Methods We established CMP-specific T cell clones (TCCs) from blood of infants with CMA and atopic dermatitis (AD), from atopic controls with AD but without CMA, and from non-atopic controls. In addition, we established TCCs from the infants with CMA after they had spontaneously developed tolerance to CM. Expression levels of CD25, CD26, and CD30 by each TCC were analyzed by flow cytometry.

Results CMP-specific T cells from infants with CMA expressed much higher levels of CD25 and CD30 than CMP-specific T cells from infants without CMA. Expression of CD26 was much lower than in normal controls. After the development of tolerance for CM, expression of CD25 and CD30 was decreased, while the expression of CD26 was increased to normal levels.

Conclusion Antigen-specific T cells from patients with food allergy display an increased expression of cell-surface markers of activation, compared with patients without food allergy. This suggests an intrinsically stronger food-specific T cell response in food-allergic patients, and points to the key role of food-specific T cells in the pathogenesis of food allergy.

INTRODUCTION

Food antigens are the main allergens causing allergic reactions during infancy and childhood[1,2]. An important role in the pathogenesis of food allergy is attributed to food-specific T cell reactivity. Both in allergen-stimulated PBMCs[3-6], and at the clonal level[7] it has been shown, that the food-specific T cell response in patients with food allergy is Th2-skewed compared with food-tolerant individuals. As the Th2-cytokines (IL-4, IL-5 and IL-13) are important in the pathogenesis of allergic inflammation[8,9], these results have suggested a key role for allergen-specific T cells

in food allergy-related symptoms.
There are observations which suggest that not only production of Th2 cytokines, but also the activation state of food-specific T cells might play a role in the pathogenesis of food allergy. Food-specific T cell reactivity is present in food-allergic and food-tolerant individuals, but proliferative responses of T cells in patients with food allergy are higher than in food-tolerant patients[10-14]. In addition, patients with allergic disease have higher numbers of circulating, activated allergen-specific T cells bearing activation markers such as CD25 and CD30[15-18]. These studies however, investigated T cell reactivity in bulk cultures of T cells, which makes it difficult to extrapolate results to the individual T cell.
An important marker of T cell activation is CD25 (IL-2Rα), the high affinity receptor for IL-2[19]. CD30, a member of the tumor necrosis factor, is another marker for the activation state of a T cell[20]. The expression of CD30 by T cells has been associated with the preferential production of Th2 cytokines[21,22]. Similarly, the expression of CD26 (dipeptidyl-peptidase IV) by T cells has been associated with Th1 responses[23,24]. So far, no study has investigated the expression of these cell-surface markers by food-specific T cells at the single cell level.
Cow's milk is the most important food antigen in young children, and is the aetiological factor of cutaneous symptoms in approximately 35-40% of infants with atopic dermatitis (AD)[25,26]. Cow's milk allergy (CMA) in infancy is associated with the spontaneous development of tolerance within 2-3 years[2]. The aim of this study was to investigate the cow's milk protein (CMP)-specific T cell response in patients with CMA and AD at the clonal level, by analyzing levels of expression of cell-surface markers by the CMP-specific T cells in these patients. We used an antigen-specific T cell-culturing system with autologous B cells as antigen-presenting cells to establish CMP-specific T cell clones (TCCs) from blood. Expression of CD25, CD26, and CD30 by T cells derived from blood of infants with CMA was compared with CMP-specific T cells from age-matched control infants without CMA. In addition, we investigated changes during the spontaneous development of tolerance for cow's milk in infants with CMA.

METHODS

Patients & control subjects

The CMP-specific TCCs described in this study were derived from blood of 5 infants with CMA and AD, and from 4 atopic control infants with AD, but without CMA. These infants have been described elsewhere[7]. CMA was diagnosed by complete elimination of cow's milk from the infant's diet, followed by a cow's milk challenge. As non-atopic controls, 3 infants prior to cardiac surgery, in the absence of hemodynamic compromise were included. These infants did not have any atopic disorders (eczema, asthma or rhinitis), did not have increased total IgE levels, and

were not sensitized for a panel of 12 common inhalation and food allergens as determined by CAP system FEIA (Pharmacia Diagnostics, Uppsala, Sweden). They did not use systemic or topical immunomodulatory drugs. The three groups of infants were matched with regard to age, CMA patients: 4.5 – 10.4 months (mean 7.4); atopic controls: 6.1 - 7.4 months (mean 7.0); non-atopic controls: 6.4 - 9.9 months (mean 8.0).
In addition, CMP-specific TCCs were established from 3 of the infants with AD and CMA after they had spontaneously outgrown their CMA (age: 21.9-32.3 months, mean 26.5). To control for age-related changes, CMP-specific TCCs from 3 of the infants with AD but without CMA at the same age (age; 19.8-38.3 months, mean 26.0) were also established.
All blood samples were taken after informed consent was obtained, and the study was approved by the Medical Ethical Committee of the University Medical Center, Utrecht.

Reagents
A mix of purified CMPs (CMP-mix) containing equal quantities of total casein, α-lactalbumin and β-lactoglobulin (each at a concentration of 50 μg/ml) was used as antigen. Purified CMPs were kindly provided by Dr. E.C.H. van Beresteijn (Netherlands Institute for Dairy Research, Ede, The Netherlands). For culturing of T cell lines and TCCs, complete medium RPMI-1640 (Gibco, New York, USA) was used, supplemented with 10% pooled human AB serum. Epstein Barr virus (EBV)-transformed B cells were cultured in RPMI-1640 (Gibco), supplemented with 10% fetal calf serum (Gibco). Established TCCs were maintained in Iscove's Modified Dulbecco's Medium (Gibco) supplemented with 2% pooled human AB serum and 5% Yssel's medium. All media were supplemented with penicillin (100 IU/ml), streptomycin (100 mg/ml) and glutamin (1 mM) (Gibco).

Preparation of CMP-specific TCCs
CMP-specific TCCs were established as described previously[7,27]. PBMCs were isolated from heparinized venous blood, and subsequently cultured in the presence of CMP-mix. After 7 days, 50 IU/ml of both recombinant IL-2 and IL-4 (kind gift of Novartis Research Institute, Vienna, Austria) was added to the culture medium. Cultures were re-stimulated every 14 days with irradiated (50 Gy) autologous EBV-transformed B cells that had been pre-incubated O/N with CMP-mix, as antigen-presenting cells. If these cultures indicated a high CMP-specific T cell proliferation in lymphocyte stimulation test (LST), T cells were cloned by limiting dilution, in the presence of IL-2, IL-4 and irradiated autologous EBV-transformed B cells (10^4/well) that had been pre-incubated O/N with CMP-mix. All established TCCs were tested in LST to verify CMP-specificity.

Flow-cytometric analysis
Expression of CD4, CD8, TCR, CD25, CD26 and CD30 by the TCCs was analyzed by flow cytometry, 2 days after a re-stimulation with irradiated autologous EBV-transformed B cells that had been pre-incubated O/N with CMP-mix. Antibodies used were: fluorescein isothiocyanate (FITC)- conjugated mouse-anti-human CD4 (clone Leu-3a, BD Biosciences, San Jose, USA), phycoerythrine (PE)-conjugated mouse-anti-human CD8 (clone Leu-2a, BD Biosciences), FITC-conjugated mouse-anti-human α/β TCR (clone WT-31, BD Biosciences), FITC-conjugated mouse-anti-human CD25 (clone M-A251, BD Biosciences), PE-conjugated mouse-anti-human CD26 (clone M-A261, BD Biosciences), and FITC-conjugated mouse-anti-human CD30 (clone BerH8, BD Biosciences). Cells were stained, fixed in paraformaldehyde, and analyzed with FACScan (BD Biosciences). All clones were CD4+ and expressed the α/β TCR (data not presented). Expression levels of CD25, CD26, and CD30 by each TCC was measured as mean fluorescence intensity (MFI). Isotype-matched control antibodies were used to control for non-specific binding.

CMP-specific cytokine release
CMP-specific cytokine release by the TCCs was determined as described previously[7]. To determine CMP-specific cytokine release, 10^6 cells of each TCC were incubated with 10^6 irradiated autologous EBV-transformed B cells that had been pre-incubated O/N with CMP-mix. Control cultures of TCCs and EBV-transformed B cells, pre-incubated without antigen were prepared in parallel. Cytokines (IL-4, IFN-γ) were measured by ELISA according to the manufacturers recommendations (Central Laboratory of the Blood Transfusion Service, Amsterdam, The Netherlands).

Statistical analysis
Parametric analysis (Student's t-test) was applied to determine significant differences between patient and control groups with regard to age, mean MFI, and differences in cytokine production. Differences associated with p values of less than 0.05 were considered significant. Parametric analysis (Pearson's correlation) was used to test for correlation between expression of different cell-surface markers, and between surface marker expression and cytokine production.

RESULTS

Differences in expression of cell-surface markers by CMP-specific T cells from infants with CMA and controls
Differences in expression of CD25, CD26, and CD30 by TCCs derived from

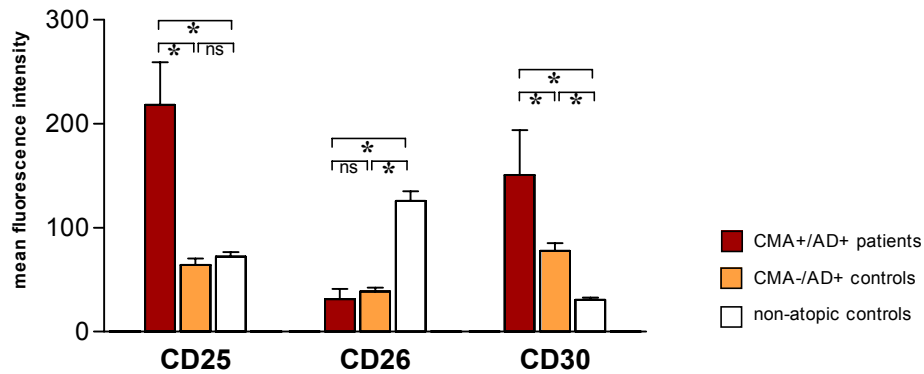

Figure 1. Mean fluorescence intensity of CD25-, CD26-, and CD30-expression by CMP-specific TCCs derived from infants with CMA and AD, age-matched atopic control patients with AD but without CMA, and age-matched non-atopic controls. Expression was measured by using flow cytometry, 2 days after stimulation with CMPs. *: $p<0.05$ (Student's t-test).

infants with CMA and AD (n=13), TCCs from atopic control infants without CMA (n=37), and TCCs from non-atopic control infants without CMA (n=74) were analyzed. Figure 1 shows MFI of CD25, CD26, and CD30 expression on CMP-specific TCCs derived from the three patient groups. Mean expression of CD25 was significantly higher on TCCs derived from infants with CMA compared with TCCs from infants without CMA ($p<0.05$). This accounted for both the atopic controls and the non-atopic controls. Expression of CD30 was also significantly higher on TCCs derived from infants with CMA, compared with TCCs from both control groups ($P<0.05$). In contrast, mean expression of CD26 was lower in infants with CMA, compared with non-atopic infants without CMA ($p<0.05$). Comparison of both control groups showed that atopic infants without CMA had comparable expression of CD25, but a higher expression of CD30 and a lower expression of CD26 than non-atopic controls.

Changes during the development of tolerance in patients with CMA

Expression of CD25, CD26 and CD30 on TCCs (n=18) derived from infants with CMA after they had spontaneously developed tolerance for CMP was analyzed, and compared with TCCs derived from these infants during the allergic state. In addition, TCCs (n=39) from age-matched atopic control infants were investigated. Figure 2 shows MFI of expression of CD25 (Figure 2A), CD26 (Figure 2B), and CD30 (Figure 2C) on CMP-specific TCCs derived from the infants with CMA before and after the development of tolerance, and from the age-matched atopic controls at both timepoints. The non-atopic infants at the first timepoint are shown as reference. There was a significant decrease in the mean expression of CD25 on

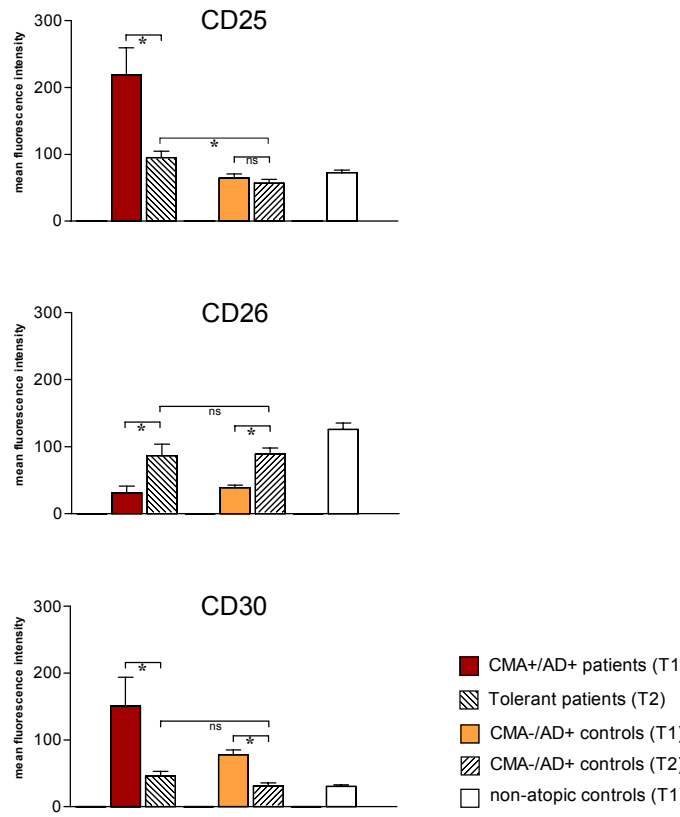

Figure 2. Mean fluorescence intensity of expression of CD25 (A), CD26 (B) and CD30 (C) by CMP-specific TCCs derived from infants with CMA and AD, before and after the development of tolerance for CMA, and age-matched atopic-control patients with AD without CMA at both timepoints. Non-atopic control infants from the first timepoint are shown as reference. T1 = first timepoint (before tolerance), T2 = second timepoint (after tolerance). Expression was measured by using flow cytometry, 2 days after stimulation with CMPs. *: $p<0.05$ (Student's t-test).

CMP-specific TCCs derived from the infants with CMA after they had developed tolerance to cow's milk, compared with TCCs derived from these infants during the allergic state. The same was true for the expression of CD30, which was high during the allergic state, and had significantly decreased after the development of tolerance. In contrast, mean expression of CD26, which was low during the allergic state, increased at the development of tolerance. At the tolerant state, the expression of CD25 by CMP-specific TCCs from infants with CMA was still higher than the non-atopic controls who had never had CMA. For CD26 and CD30, expression levels between both groups were comparable.

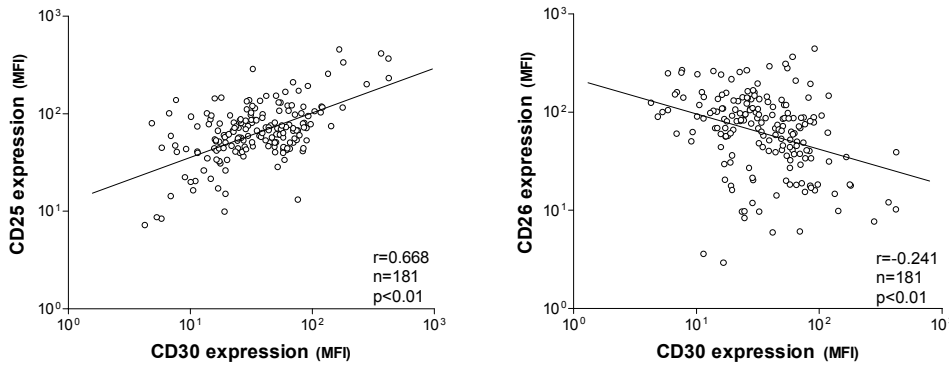

Figure 3. Correlation between expression of CD25 and CD30 (A), and between expression of CD26 and CD30 (B) by the CMP-specific TCCs investigated in this study. Expression was measured by using flow cytometry, 2 days after stimulation with CMPs.

Correlation between expression of different cell-surface markers

The relation between the antigen-specific expression of the different cell-surface markers on the CMP-specific TCCs described in this study was investigated. Results showed that the expression of CD25 and CD30 was significantly correlated ($r = 0.668$, $p<0.01$) in all TCCs investigated in this study (Figure 3A). There was a negative correlation ($r = -0.241$, $p<0.01$) between the expression of CD26 and CD30 in all TCCs (Figure 3B). No significant correlation was observed between the expression of CD25 and CD26 (data not shown).

Relation between expression of CD26/CD30 and production of Th1/Th2-cytokines

To investigate whether the relationship between the expression of CD26 and CD30 is associated with the production of Th1/Th2 cytokines in the TCCs described in this study, the correlation between the expression of CD26 and CD30 and the production of IL-4 and IFN-γ was calculated. Results showed no significant correlation between the expression of CD26 and IFN-γ (Figure 4A), or between the expression of CD30 and IL-4 (Figure 4B). In addition, we found no significant correlation between the expression of CD30 and the production of IFN-γ, or between the expression of CD26 and the production of IL-4 (data not shown).

DISCUSSION

Food antigen-specific T cells are important in the aetiology of allergic reactions to foods[1]. It has been shown that food-specific T cells from food-allergic and food-tolerant individuals differ greatly with regard to the production of Th1 and Th2

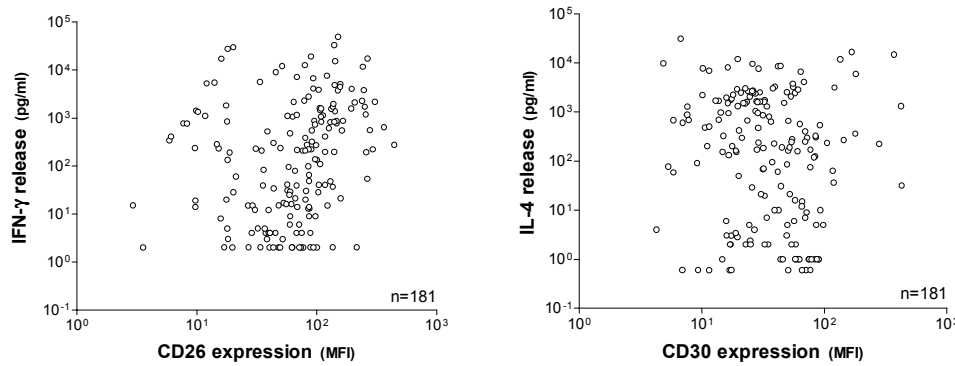

Figure 4. Correlation between expression of CD26 and production of IFN-γ (A), and expression of CD30 and production of IL-4 (B) by the CMP-specific TCCs investigated in this study. No significant correlations exist. Expression was measured by using flow cytometry, 2 days after stimulation with CMPs. Cytokine production was measured in culture supernatants 24 hours after stimulation with CMPs

cytokines[3-7]. In this study, we analyzed differences in food-specific T cell reactivity with regard to expression of cell-surface markers.

The results from our study show at the clonal level, that food-specific T cells from patients with food allergy express much higher levels of CD25 than food-specific T cells from patients without food allergy. CD25 (IL-2Rα) expression is regulated by IL-2, the major T cell growth factor, and is therefore an important marker for the activation state of a T cell[19]. The fact that the food-reactive T cells from food-allergic patients have a much higher expression of the receptor for IL-2, suggests that the antigen-specific T cell response in these patients is more pronounced than in patients without food allergy. From investigations in PBMCs it is known that food-specific T cells from patients with allergy to food have a higher proliferative capacity *in vitro* than cells from non-food allergic patients[10-14]. This suggests that the *in vivo* food-specific T cell response in patients with food allergy is intrinsically more vigorous. Our finding that allergen-specific T cells from infants with food allergy obtain a higher state of activation, might explain the food-allergic state of these patients. In this respect, the findings in the patients that had become spontaneously tolerant after cow's milk allergy are also of interest. We showed that in TCCs derived from these patients, the expression of CD25 is significantly lower than at the allergic state, whereas no change occurred in age-matched atopic controls, again suggesting that high CD25 expression by allergen-specific T cells and allergic state might be connected.

Determination of CD30 expression by the TCCs in our study confirmed the results of the expression of CD25. Analysis of expression of CD30 showed that antigen-specific T cells from cow's milk-allergic patients have a higher CD30 expression

than T cells from non-cow's milk allergic patients. The expression of CD30 by CMP-specific T cells from patients that had spontaneously developed tolerance was significantly lower than at the allergic state. These results from CD30 as marker for the activation state of T cells are comparable with the results of the CD25 expression, and indicate an enhanced activated state of T cells in patients with allergic symptoms. Indeed, analysis of the expression of CD25 and CD30 showed a significant positive correlation between expression of the two markers.

Our study shows that expression of CD26 by CMP-specific T cells from patients with CMA and AD is significantly lower than in normal controls. After the development of tolerance for cow's milk, CD26 expression increases, and returns to levels comparable with normal controls. Expression of CD26, the receptor for adenosine deaminase, by T cells is important for co-stimulatory signals, but its precise physiological role is unknown[28,29]. Several studies have shown that absence or low levels of CD26 on T cells mediates immunological diseases[28-31]. It is therefore tempting to speculate that low expression of CD26 by allergen-specific T cells from patients with food allergy contributes to the allergic state of these patients.

The expression of CD26 by T cells has been described to be preferentially associated with the release of Th1-cytokines[23,24]. Similarly, the expression of CD30 by T cells has been associated with Th2-mediated disease[21,22]. These associations have even led to the proposal for CD26 and CD30 as exclusive markers for Th1- and Th2-cells respectively[32]. Several subsequent studies however, have shown that expression of CD26 and CD30 is variable, and can be expressed by T cells on both ends of the Th1-Th2 spectrum, as well as Th0 cells[33-37]. We investigated the relation between the expression of CD30 and CD26, and the production of IL-4 and IFN-γ, the hallmark Th2-Th1 cytokines, in the TCCs described in our study. We found no correlation between the expression of CD30 and production of IL-4, nor between the expression of CD26 and IFN-γ production. In addition, we observed no association between CD30 and IFN-γ production, nor between CD26 expression and IL-4 release. These results with a large number (n=181) of TCCs confirm that on the level of the individual T cell, CD30 and CD26 can not be used as exclusive markers for Th2- and Th1-cells[33,35,36].

Interestingly, we noticed differences in the expression of CD26 and CD30 by T cells from the atopic and non-atopic control patients. Despite the fact that the atopic controls did not have CMA, the CMP-specific T cells showed a higher expression of CD30, and a lower expression of CD26 compared with non-atopic controls. This indicates that expression of these markers by T cells may reflect the atopic state of a patient. It has been shown that expression of CD30 is generally increased in atopic disease, both on the cell surface of T cells[38,39], as well as release of its soluble counterpart[40,41]. Studies that investigated CD30 expression on T cells however, mostly investigated differences in numbers of CD30-positive T cells. Our

study now shows that expression levels of CD30 on individual T cells are also increased in atopic disease. The exact immunological functions of CD26 and CD30 remain unknown, further studies therefore are needed to clarify the exact role of CD26 and CD30 in allergy and atopic disease.
In summary, it has been established that patients with food allergy have an antigen-specific Th2-skewed T cell response compared with food-tolerant patients. The results from the present study show that, following stimulation, antigen-specific T cells from patients with food allergy display an increased expression of cell-surface markers of activation, compared with patients without food allergy. This suggests an intrinsically stronger response of food-specific T cells in food-allergic patients, and points to the key role of food-specific T cells in the pathogenesis of food allergy.

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Chapter 6

The level of CLA expression, and not CLA per se, determines the localization of allergen-specific T cells

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ABSTRACT

Background Cutaneous lymphocyte antigen (CLA) is a cell surface protein that is proposed to play a role in the specific homing of circulating allergen-specific T cells to the skin. This hypothesis has mainly been addressed by assessing differences between skin and blood of patient and control groups with regard to numbers of CLA-positive T cells.

Objective To investigate the role of CLA in the context of CMA and AD, CLA expression was measured on T cells at the single cell level.

Methods Cow's milk protein (CMP)-specific T cell clones (TCCs) were established from blood of infants with cow's milk allergy (CMA) and atopic dermatitis (AD), and from age-matched controls without CMA. In addition, TCCs from skin and blood of older children with AD were compared. For each TCC, CLA expression after stimulation with CMP was analyzed by using flow cytometry.

Results CLA expression by CMP-specific TCCs in blood of infants with AD and CMA was not significantly different from infants without CMA; mean MFI resp. 29.7 ± 7.0 vs 26.1 ± 3.2. However, CLA expression on TCCs derived from skin of infants with AD was much higher than on TCCs from blood of these infants (mean MFI 280 ± 72 vs 56 ± 12, $p<0.0001$).

Conclusion CLA expression by allergen-specific T cells in blood of patients with allergen-induced skin symptoms is comparable to non-allergic infants when examined at the single cell level, which argues against an increased skin-homing potential. The finding that T cells in skin of patients with AD express much higher levels of CLA than T cells from blood, however, might indicate that CLA expression is important for the retention of allergen-specific T cells in the skin.

INTRODUCTION

Cutaneous lymphocyte antigen (CLA) is a cell surface protein that is expressed by T cells, and which is thought to be important for their selective migration to the skin[1-3]. Expression of CLA by a T cell aids in the migration to cutaneous sites through interactions with E-selectin, which facilitates the process of 'tethering and rolling' that is needed for extravasation[4-6]. It is proposed that CLA expression is the imperative signal for the T cell to selectively migrate to the skin, and CLA is therefore often referred to as the 'skin-specific homing marker'[3,7,8].
Several studies have shown an important role for CLA-expressing T cells in the

aetiology of allergic skin symptoms[9-15]. It was shown that in patients with atopic dermatitis and allergy to environmental allergens, allergen-specific T cells are predominantly CLA-positive, in contrast to T cells from non-allergic individuals[9-11]. Furthermore, it was shown that in skin of patients with allergic skin disease, the numbers of CLA-expressing T cells are higher than in blood, and are much higher than in skin of normal controls[12,13]. The finding that patients with allergic lung disease have virtually no antigen-specific T cells that express CLA, both in blood and at pulmonary sites, further established the role of CLA in the skin-specific homing of T cells[14,15].
However, the role of CLA expression by T cells has so far been mainly addressed by assessing differences between skin and blood of patient and control groups with regard to numbers of CLA-positive T cells. Few studies have investigated levels of CLA expression on individual T cells[16-18]. The aim of this study was to analyze the cell-surface expression of CLA at the single cell level. An antigen-specific T cell culturing system with autologous B cells as antigen-presenting cells was used to establish cow's milk protein (CMP)-specific T cell clones (TCCs). Differences in levels of CLA expression by CMP-specific TCCs derived from blood of infants with cow's milk allergy (CMA) and atopic dermatitis (AD), infants with AD without CMA, and non-atopic controls without AD were analyzed. In addition, differences in CLA expression by TCCs derived from skin and blood of children with AD were studied.

METHODS

Patients and control subjects

The CMP-specific TCCs described in this study were derived from blood of 5 infants with CMA and AD, and from 4 atopic control infants with AD, but without CMA. These infants have been described elsewhere[19]. CMA was diagnosed by complete elimination of cow's milk from the infant's diet, followed by a cow's milk challenge[20]. Infants that developed symptoms during challenge that disappeared during subsequent elimination were diagnosed with CMA. Infants without CMA did not have any reactions, and no significant change in the severity of the eczema was noted throughout the entire elimination-provocation period. As non-atopic controls, 3 infants prior to cardiac surgery, in the absence of hemodynamic compromise were included.
Atopy was excluded by negative family history, absence of clinical atopic disorders (eczema, asthma or rhinitis), and by *in vitro* diagnostics: infants did not have increased total IgE levels, and were not sensitized for a panel of 12 common inhalation and food allergens as determined by CAP system FEIA (Pharmacia Diagnostics, Uppsala, Sweden). No systemic or topical immunomodulatory drugs were used. The three groups of infants were matched with regard to age, CMA

patients: 4.5 – 10.5 months (mean 7.5); atopic controls: 6.0 - 7.5 months (mean 7.0); non-atopic controls: 6.5 - 10 months (mean 8.0).
Furthermore, to investigate differences between CMP-specific T cells in blood and skin, we established CMP-specific TCCs from blood and skin of 4 older children with AD (age: 22.0-38.5 months, mean 28.0). All 4 children were tolerant for cow's milk as proven by a cow's milk challenge.
All blood and skin samples were taken after informed consent was obtained, and the study was approved by the Medical Ethical Committee of the University Medical Center, Utrecht.

Reagents
A mix of purified CMPs (CMP-mix) containing equal quantities of total casein, α-lactalbumin and β-lactoglobulin (each at a concentration of 50 μg/ml) was used as antigen. Purified CMPs were kindly provided by Dr. E.C.H. van Beresteijn (Netherlands Institute for Dairy Research, Ede, The Netherlands). For culturing of T cell lines and TCCs, complete medium RPMI-1640 (Gibco, New York, USA) was used, supplemented with 10% pooled human AB serum. Epstein Barr virus (EBV)-transformed B cells were cultured in RPMI-1640 (Gibco), supplemented with 10% fetal calf serum (Gibco). Established TCCs were maintained in Iscove's Modified Dulbecco's Medium (Gibco) supplemented with 2% pooled human AB serum and 5% Yssel's medium. All media were supplemented with penicillin (100 IU/ml), streptomycin (100 mg/ml) and glutamin (1 mM) (Gibco).

Preparation of CMP-specific TCCs
CMP-specific TCCs were established from blood and skin as described previously[19,21]. To establish TCCs from blood, PBMCs were isolated from heparinized venous blood, and subsequently cultured in the presence of CMP-mix. After 7 days, 50 IU/ml of both recombinant IL-2 and IL-4 (kind gift of Novartis Research Institute, Vienna, Austria) was added to the culture medium. Cultures were re-stimulated every 14 days with irradiated (50 Gy) autologous EBV-transformed B cells that had been pre-incubated O/N with CMP-mix, as antigen-presenting cells. If these cultures indicated a high CMP-specific T cell proliferation in lymphocyte stimulation test (LST), T cells were cloned by limiting dilution, in the presence of IL-2, IL-4 and irradiated autologous EBV-transformed B cells (10^4/well) that had been pre-incubated O/N with CMP-mix[22.19].
To establish CMP-specific T cells from skin biopsies, a single-cell suspension was prepared from skin by enzymatic digestion according to the method described by Sager et al.[23]. Culture of recovered cells was started in a 48-well flat bottom culture plate in the presence of CMP-mix, IL-2, IL-4, and irradiated autologous PBMCs (10^6/well) as feeders. The cultures were maintained and tested as described for blood-derived TCCs. All established TCCs were tested in LST to verify CMP-specificity.

Flow-cytometric analysis
Expression of CLA by the TCCs was analyzed by flow cytometry, 2 days after a re-stimulation with irradiated autologous EBV-transformed B cells that had been pre-incubated O/N with CMP-mix. Antibodies used were: CLA (HECA 452, kind gift of Prof. Dr. R. Scheper, Free University of Amsterdam, Amsterdam, The Netherlands). Unlabeled CLA was detected by phycoerythrine-conjugated goat-anti-rat IgM (Immunotech, Marseille, France). Cells were stained, fixed in paraformaldehyde, and analyzed with FACScan (BD Biosciences). Results were expressed as mean fluorescence intensity (MFI). Expression levels of CLA by each TCC was measured as mean fluorescence intensity (MFI). Isotype-matched control antibodies were used to control for non-specific responses.

Statistical analysis
Parametric analysis (Student's t-test) was applied to determine significant differences between patient and control groups with regard to age, and differences in mean MFI. Differences associated with p values of less than 0.05 were considered significant.

RESULTS

CLA expression by CMP-specific T cells in blood of infants with and without CMA

The expression of CLA (MFI) was analyzed on TCCs derived from blood of infants with CMA and AD (n=13), TCCs from atopic control infants with AD, but without CMA (n=37), and TCCs from non-atopic control infants without CMA (n=74). CLA expression, measured as MFI, was generally low on CMP-specific T

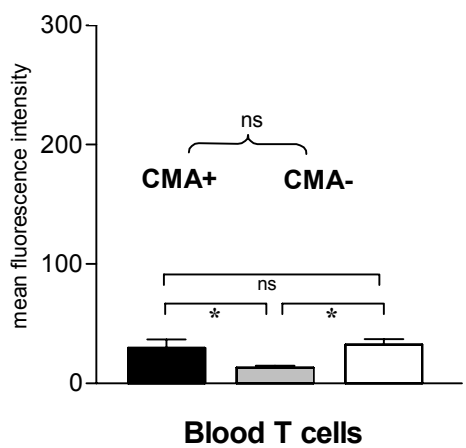

Figure 1. Mean expression of CLA on CMP-specific T cells in blood of infants with CMA and AD (closed bars), age-matched atopic controls without CMA (hatched bars), and non-atopic controls without CMA (open bars). *: $p<0.05$ (Student's t-test). NS = not significant.

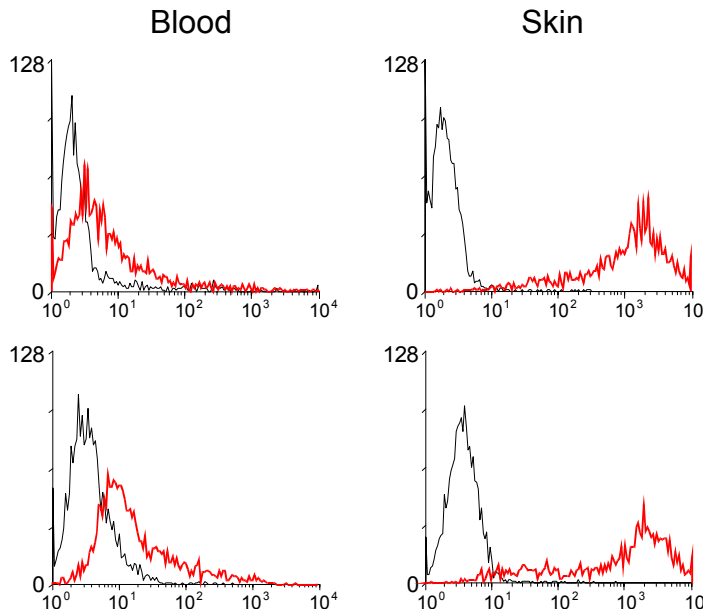

Figure 2. Representative results of flow-cytometric analysis of CLA expression on CMP-specific T cells in blood and skin of two children with AD.

cells derived from blood. Figure 1 shows mean MFI of CLA expression by CMP-specific TCCs derived from the three patient groups. When these subgroups were analyzed separately, results from infants with CMA and AD were comparable with normal controls whereas results from atopic controls were significantly lower than the other 2 groups. When expression of CLA by CMP-specific T cells from infants with CMA (mean MFI: 29.7 ± 7.0) was compared with CLA expression of all TCCs derived from infants without CMA (mean MFI: 26.1 ± 3.2), no significant differences were found.

Differences in CLA expression between CMP-specific T cells in blood and skin
To investigate differences in expression of CLA between T cells in blood and skin of patients with AD, CMP-specific TCCs derived from blood (n=57) and skin (n=38) of children with AD (mean age: 28.0 months) were compared. Results of flow-cytometric analysis showed significant differences in CLA expression between T cells from skin and blood. Representative examples of 2 patients are shown in Figure 2. Mean CLA expression on T cells derived from skin was more than 5-fold higher than expression on blood T cells (Figure 3).

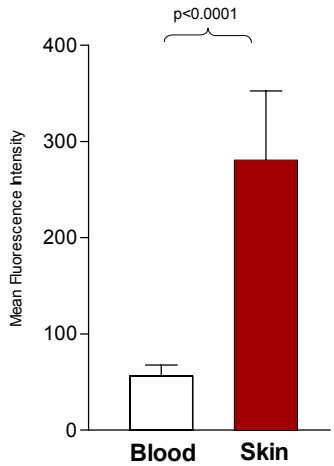

Figure 3. Mean expression of CLA on CMP-specific T cells in blood and skin of children with AD.

DISCUSSION

Allergen-specific T cells have been shown to be important in the aetiology of allergic reactions[24-27]. The expression of CLA by allergen-specific T cells is thought to be important for selective migration to the skin in patients with allergic skin disease[1-3]. This hypothesis has mainly been investigated by assessing differences between skin and blood of individuals with or without allergic skin disease with regard to numbers of CLA-expressing T cells[9-11,28]. In the present study, differences in the level of CLA expression by allergen-specific T cells from patients and controls were analyzed at the single cell level.

CMP-specific T cells from infants with CMA and AD were investigated. The results showed that CLA expression by allergen-specific T cells in blood of infants with CMA is not significantly different from CLA expression in non-atopic infants without CMA. A significant lower CLA expression was found in atopic control patients without CMA. However, as compared to the expression observed on skin TCCs, the level of CLA expression on blood TCCs is low in all 3 groups of infants. This, in addition to the fact that no differences exist between infants with CMA and non-atopic infants without CMA, questions the relevance of the significant lower expression in the atopic controls. Therefore, in our opinion, results show that CLA expression in blood is essentially comparable in all infants. This might indicate that the skin-homing potential of blood-derived T cells from allergic and non-allergic individuals is not different. Previous findings did suggest differences in skin-homing capacity between allergen-specific T cells from allergic and non-allergic individuals[2,9,10]. Abernathy-Carver and co-workers investigated children with CMA and allergic skin symptoms, and found that allergen-specific proliferative T cell

responses in these children are dominated by CLA-expressing T cells, in contrast to children who do not have cow's milk-induced skin symptoms[9]. Similar results were found when patients with allergy to environmental allergens were investigated[2,10]. However, these studies all evaluated differences in numbers of CLA-expressing T cells, rather than investigating the level of CLA expression by the disease-related T cells at the individual cell level. Until now, no study compared levels of CLA on isolated T cells from patients with and without allergy. Our study clearly shows that, at the single cell level, disease-related T cells in patients with allergic skin disease do not differ in CLA expression when compared to T cells from individuals without allergic skin disease. The previous suggested differences in skin-homing capacity of the allergen-specific T cells from (food) allergic and non-allergic individuals, must therefore be attributed to differences in numbers of CLA-expressing T cells, rather than to intrinsic differences in skin-homing capacity of the individual cells.

To investigate differences in expression of CLA between circulating T cells and T cells that reside in the skin, TCCs from blood and skin of older children with AD were established. Analysis of CLA expression by these TCCs showed that T cells from skin have a higher expression of CLA than T cells from blood. Expression of CLA on T cells from skin was on average approximately five times as high as CLA expression on blood T cells, and there was virtually no overlap between levels of CLA expression by skin and blood T cells (Figures 2 and 3). These findings indicate intrinsic differences in CLA expression by T cells in the blood and those that reside in the skin. Only 2 previous studies have evaluated levels of CLA expression on skin derived T cells[16,17]. Rossiter et al. investigated T cells from patients with allergy to house dust mite and showed that the allergen-specific T cells in skin of these patients expressed a much higher level of CLA compared with T cells from blood of these patients[17]. The same results were found when T cells from blood and skin of patients with delayed type hypersensitivity were investigated [16]. The results from our study now confirm these findings for food allergen-specific T cells in young children.

Taken together, findings from both this study and the literature show that there are intrinsic differences between levels of CLA expression by T cells in blood and those that reside in the skin. It is tempting to speculate that these differences in CLA expression determine the localization of allergen-specific T cells, either in the circulation or in the skin. The expression of CLA by T cells is thought to be helpful in the migration of T cells to cutaneous sites. It was shown that expression of CLA by T cells is not restricted to predefined T cell subsets, but is variable *in vivo*, and can be regulated by local tissue factors[6,18]. Previous studies, who showed that CLA is not an absolute prerequisite for blood T cells to extravasate into cutaneous sites[29,30] therefore suggested that CLA may be upregulated on T cells in skin after local activation[16,31]. It was suggested that expression of CLA by T cells is more important for the retention of specific T cells in the skin, rather than the process of

extravasation itself[29]. The observed differences in CLA-levels between blood- and skin-T cells suggest, that the level of CLA that is expressed by the T cell might be relevant in this issue. During allergen-specific immune surveillance, it is important that T cells stay in the skin, to mediate the tissue-specific immune response[3]. It is possible, that the (low) expression of CLA by allergen-specific T cells in blood is further upregulated after they have entered the skin, to facilitate the retention of these cells in the skin. The regulation of CLA by local tissue factors might play an important role in this process[6,18,32]. Future studies should therefore be focused on the regulation of levels of CLA expression, to elucidate the exact function of CLA expression by skin-homing T cells.
In summary, levels of CLA expression by allergen-specific T cells in blood of individuals with and without food allergy are comparable, suggesting that the skin-homing potential of circulating T cells from allergic and non-allergic individuals is not intrinsically different. However, T cells in skin of patients with AD express much higher levels of CLA than T cells in blood, suggesting intrinsic differences between CLA expression of T cells in the blood and those that reside in the skin. These results provide additional support for the critical role of CLA in the T cell localization in the skin, and suggest that differences in levels of CLA may be important for the retention of T cells in the skin.

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Chapter 7

Mismatch between screening for food-specific sensitization using in vitro IgE detection and skin prick testing

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ABSTRACT

Background Screening for food-specific sensitization using in vitro and/or skin tests has become an essential step in the diagnostic work-up of children with suspected food allergy.

Aim The aim of our study was twofold: to study patterns of sensitization for cow's milk, hen's egg and peanut in children referred to a tertiary hospital for suspected food allergy. In addition, we compared the screening for food-specific sensitization using in vitro IgE detection and skin prick testing in these children.

Methods We evaluated a cohort of 785 children referred to our pediatric allergy outpatient clinic for suspected food allergy. All patients were screened for food-specific sensitization by using in vitro IgE detection, and skin prick tests. Sensitization was established when at least one of both tests showed a positive result.

Results The most common allergen in the total group was hen's egg, with 51% of the referred children being sensitized. In children <4 years was hen's egg the most frequent allergen, in children >4 peanut. Comparison of screening-results in all sensitized children showed that the proportion of sensitized patients with a positive result in both in vitro- and skin test, was 56% at most. This means, that a large part of the sensitized children would have been missed if only one of both tests had been used for screening.

Conclusions The results from this study show a substantial mismatch between the outcome of in vitro IgE detection and skin prick testing when screening for food-specific sensitization in children with suspected food allergy. Screening for food-specific sensitization in the pediatric age group should therefore be performed using both in vitro IgE detection as well as skin prick testing, to prevent false-negative results.

INTRODUCTION

Food allergy is the most common allergy during infancy and childhood[1,2]. It is estimated that 2-8% of all infants and young children have adverse reactions to foods[3-7]. The predominant food allergens are cow's milk, hen's egg, peanut and to a lesser extent soy. These allergens may induce a repertoire of symptoms, such as acute effects in the lung or skin, but may are also associated with more chronic disease such as atopic dermatitis[2].
The diagnostic work-up of food allergy in children has gained more attention in the

recent years[8-10]. After the suspected food has been identified by examining the patients medical history, the golden standard to diagnose clinical relevant food allergy is a provocation with the food in question. This challenge is preferably performed using a double-blind, placebo-controlled protocol (DBPCFC), as this is the most objective method to diagnose food-induced symptoms[11]. However, because of the time, expense, and discomfort to the patient, it is often not possible to examine large numbers of foods using a challenge procedure. It is therefore common practice to screen for food-specific sensitization using in vitro and/or skin prick testing[8,10,12]. These methods, which are not time-consuming to the patient, establish the presence of IgE-antibodies to the food, indicating a food-specific immune reaction. This establishes the need for a provocation with the food, to determine clinically relevant food allergy. Screening for food-specific sensitization using in vitro and/or skin tests thus provides guidance for selecting food for DBPCFCs, and has therefore become an essential step in the diagnostic work-up of children with suspected food allergy[1,10].
In this study we evaluated a cohort of 785 children referred to our (tertiary) hospital for suspected food allergy between 1995 and 2001. All children were screened for food-specific sensitization by using in vitro IgE detection, and skin prick tests. We analyzed patterns of sensitization for the three major food allergens; cow's milk, hen's egg and peanut in these children. To compare the screening for food-specific sensitization using in vitro IgE detection or skin prick testing, we evaluated all sensitized patients for differences between outcome of both tests.

PATIENTS AND METHODS

Patients

We evaluated a cohort of 785 children referred to our pediatric allergy outpatient clinic between September 1, 1995 and March 1, 2001. All children had been referred for suspected food allergy based on medical history and physical examination. Table 1 shows the indications for screening for food-specific sensitization in children referred to our hospital. Median age was 1.6 years (range: 0-15.7). All children were screened for food-specific sensitization by using in vitro IgE detection, and skin prick tests (SPTs). Sensitization was defined as a positive result in either the in vitro IgE detection test, and/or the SPT.

In vitro IgE detection

Serum samples from all patients were analyzed for specific antibodies to cow's milk, hen's egg, and peanut. Analyses were done with the CAP system FEIA (Pharmacia Diagnostics, Uppsala, Sweden) in accordance with manufacturer's instructions. An antigen-specific IgE-value of more than 0.35kU/L was considered as positive.

Table 1. Indications for screening for food-specific sensitisation in children with suspicion of food allergic symptoms.

Skin	Urticaria/angioedema
	Erythematous pruritic rash
	Atopic dermatitis
Respiratory tract	Wheezing/repetitive cough
	Nasal congestion
	Pruritus/sneezing
	Laryngeal oedema
	Rhinorrhea
Gastrointestinal tract	Abdominal cramping/colic
	Vomiting or reflux
	Diarrhea
	Pruritus and swelling of the lips tongue or oral mucosa
	Nausea
Cardiovascular	Hypotension/shock

Skin prick test

SPTs were performed using European and American recommendations[12,13]. Tests were performed on the backs of infants and on the forearm of older children. Tests were performed using a lancet technique using extracts of the relevant foods: cow's milk, hen's egg, and peanut (ALK-Abelló, Nieuwegein, The Netherlands). Results were evaluated after 15 minutes. A positive skin test was classified as a wheal of 3mm or larger. Histamine (10 mg/ml) and a diluent solution were used as positive and negative controls respectively.

Statistical analysis

Non-Parametric analysis (Chi-square test) was applied to determine significant differences in outcome of in vitro- and skin test results between the different age groups. Differences associated with p values of less than 0.05 were considered significant.

RESULTS

Patterns of sensitization for the three allergens

We investigated patterns of sensitization for cow's milk, hen's egg and peanut in the 785 referred children. Sensitization was defined as a positive in vitro IgE detection test and/or a positive SPT. Figure 1 shows percentages of sensitization in the total study group. The most common allergen in the total group was hen's egg, with 51% of the referred children being sensitized. Second most common allergen was peanut (38%) followed by cow's milk (35%).

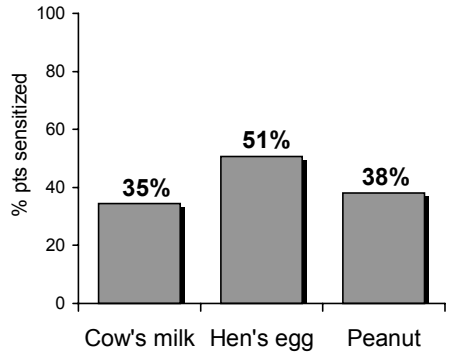

Figure 1. Percentages of sensitization for cow's milk, hen's egg, and peanut in the total study group (n=785). Sensitization was defined as a positive result in either the in vitro IgE detection test, and/or the SPT.

To evaluate possible age-related differences in food-specific sensitization between infants, young children and older children, three age groups were defined; 0-1 years, 1-4 years, and children older than 4 years. Figure 2 shows percentages of sensitization in these age groups. In infants, hen's egg was the most common allergen (48%), followed by cow's milk, and peanut. Hen's egg was also the most common allergen in young children, with a high percentage of children being sensitized (58%). In this age group, peanut sensitization was more common than in infancy, and was similar to cow's milk sensitization. In the group of children who were older than 4 years, peanut was the most important allergen, with 48% of the referred children being sensitized. Both hen's egg and cow's milk sensitization was less common than in the group of children from 1-4 years.

Mismatch between in vitro IgE detection and skin prick testing

We compared the screening for food-specific sensitization using in vitro IgE detection or skin prick testing. Therefore, we evaluated all sensitized patients for differences between outcome of both tests. Figure 3 shows the outcome of in vitro testing, and skin testing, in all patients sensitized for cow's milk (Figure 3A), hen's egg (Figure 3B), and peanut (Figure 3C). There was a considerable mismatch between the outcome of both tests. For all three allergens applied that a large proportion of the sensitized patients had a positive result for only 1 of both tests. For cow's milk, the proportion of sensitized patients that had only 1 positive test was 54%. For hen's egg this was 44%, and for peanut 45%.

To analyze the observed mismatch between in vitro testing and skin testing, we investigated if the group with a positive result for only the in vitro test (and with negative skin test), did not solely consist of patients with marginally elevated allergen-specific IgE levels. This analysis showed that, for cow's milk, 47% of the patients in this group allergen-specific IgE levels >0.7kU/L. For hen's egg, this proportion was 54%, for peanut it was 65%.

Furthermore, we evaluated if the observed mismatch between in vitro and skin-tests was different in infants compared with children older than 1 year (Figure 4).

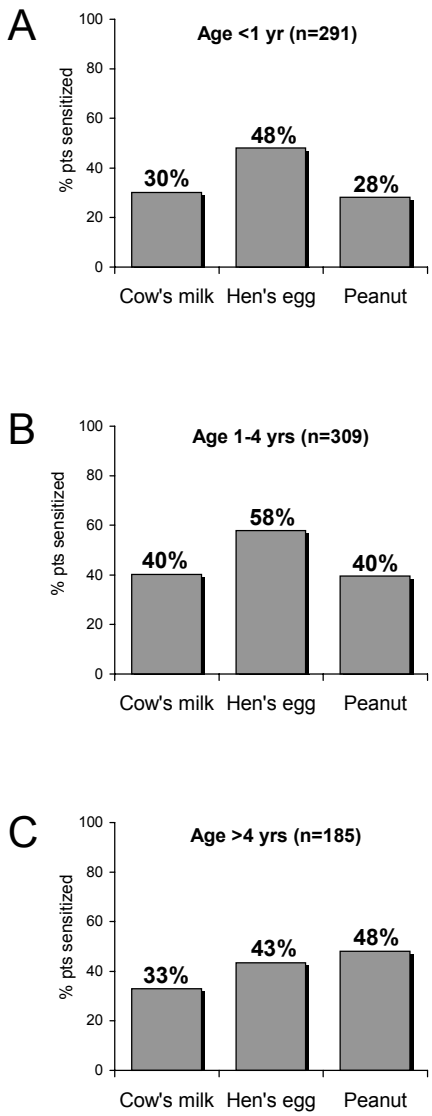

Figure 2. Percentages of sensitization for the three allergens in the age groups: less than 1 yr (A), 1-4 years (B), and older than 4 years (C). Sensitization was defined as a positive result in either the in vitro IgE detection test, and/or the SPT.

Results showed, for both cow's milk (Figure 4A) and hen's egg (Figure 4B), that the proportion of sensitized patients with only 1 positive test was slightly larger in the group of children under 1 year of age, but these differences were not significant (cow's milk p=0.17, hen's egg p=0.35). For peanut (Figure 4C) the proportion of sensitized patients that had only 1 positive test was also larger in the group of children under 1 year of age, and this difference was significant (p=0.02).

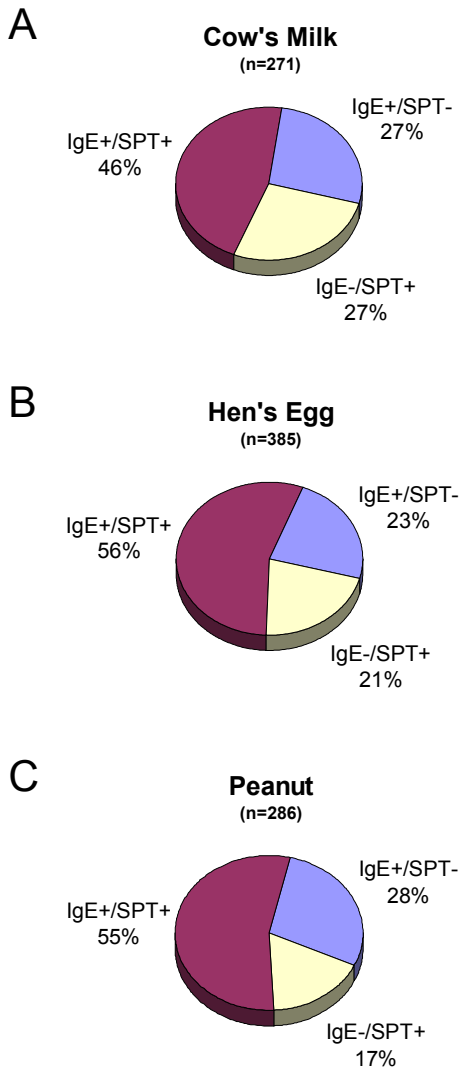

Figure 3. Differences between outcome of in vitro IgE test and SPT, in all patients sensitized for cow's milk (A), hen's egg (B), and peanut (C). IgE+ = positive in vitro IgE-test. SPT+ = positive SPT.

DISCUSSION

In this study we evaluated a cohort of 785 children, referred for suspected food allergy to a tertiary hospital. We analyzed patterns of sensitization for the major food allergens: cow's milk, hen's egg and peanut in these children. The incidence of sensitization in the total study population was 35% for cow's milk, and 38% for peanut. The most common allergen was hen's egg, with 51% of the referred

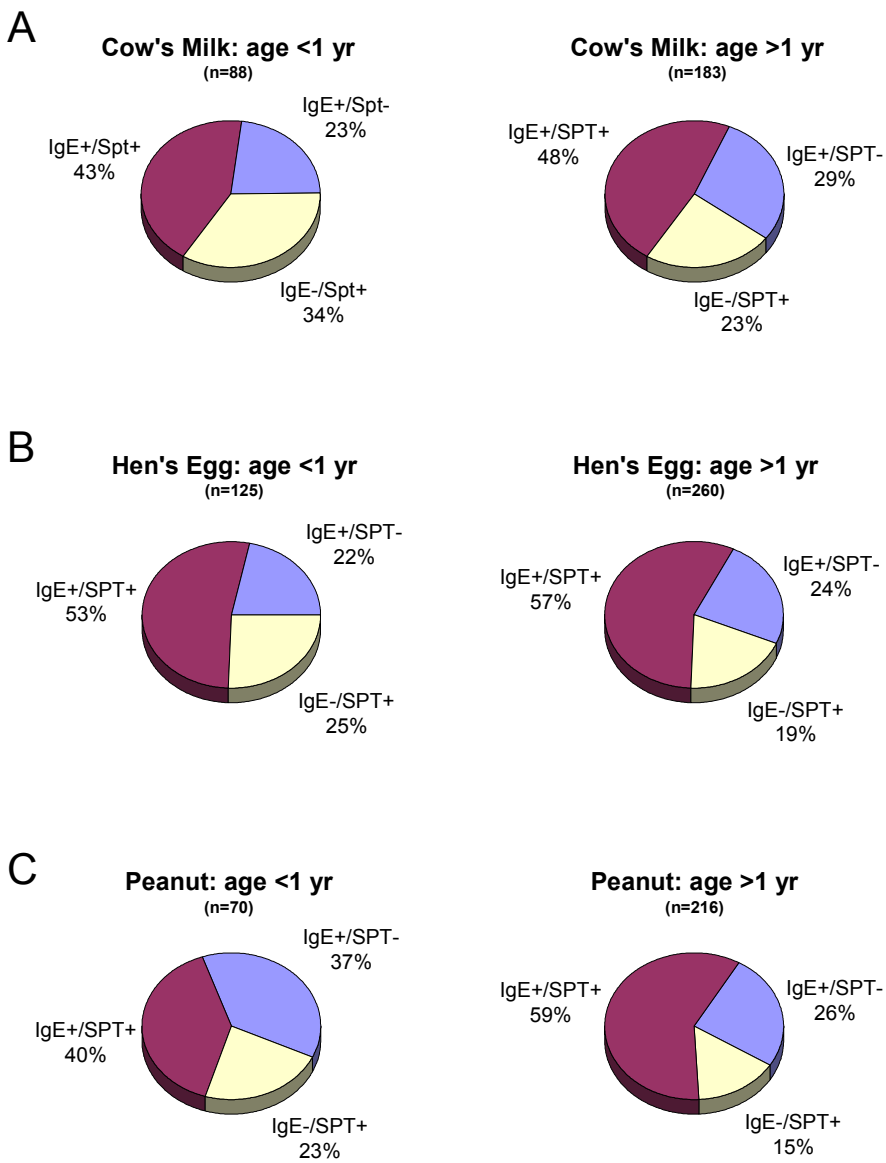

Figure 4. Differences between outcome of in vitro IgE test and SPT, in patients sensitized for the cow's milk (A), hen's egg (B), and peanut (C) in two agegroups: less than 1 yr, and older than 1 year. IgE+ = positive in vitro IgE-test. SPT+ = positive SPT. Differences between the two agegroups are not significant for cow's milk and hen's egg (Chi-square test; p=0.17 and p=0.35), and are significant for peanut (Chi-square test; p=0.02).

children being sensitized. This percentage varied between the different age groups, but in all children younger than 4 years of age hen's egg was the most frequent allergen. In children older than 4 year was peanut the most frequent allergen, with 48% of the referred children being sensitized. Still, 43% of the referred children in this age group were sensitized for hen's egg.

The results from this cohort confirm that hen's egg is the most important food allergen in infants and young children. In a recent study, Kulig and co-workers studied the natural course of sensitization to food allergens in a large population-based sample, and found that hen's egg is the most frequent food antigen that leads to sensitization during infancy and childhood[14]. Based on their cohort sample they estimated that the prevalence of hen's egg sensitization in an unselected population is approximately 6% at the age of 1 year. In addition, they concluded that sensitization to hen's egg at the age of 12 months is the most predictive marker for occurrence of sensitization to inhalant allergens at a later age[6,14], which has been confirmed by others[15]. This emphasizes that already during the first year of life, hen's egg is a very important allergen. Cow's milk, being usually the first food antigen that is introduced into an infants' diet, is often considered as the major food antigen in infancy. The results from our study, show however that hen's egg allergens should also be taken into account when an infant is evaluated for suspicion of food allergic symptoms. Striking in this respect is, that nearly 50% of the infants referred to our hospital had already been sensitized for hen's egg.

To compare the screening for food-specific sensitization using in vitro IgE detection or skin prick testing, we evaluated all sensitized patients in our study for differences between the outcome of both tests. The results showed that, for all three allergens, a large part (44-54%) of the sensitized patients had a positive result in only one of both tests. The proportion of sensitized patients who had a positive result in both the in vitro test and the skin test varied slightly between the three allergens, but was 56% at most. This is low, and shows a substantial mismatch between the detection of food-specific sensitization using in vitro IgE detection and skin prick testing. IgE-mediated immune responses in infants generally are less developed than in older children and adults[10,16,17]. As a large part (33%) of the patients referred to our hospital consisted of infants, we evaluated if the observed mismatch between in vitro and skin-testing was different in infants compared with children older than 1 year. This analysis showed, for cow's milk and hen's egg, no differences between infants and older children. For peanut, an even larger proportion of infants had a positive result in only one of both tests compared with older children. This indicates an even greater mismatch between both tests in this age group.

Screening for food-specific sensitization using in vitro and/or skin tests has become an essential step in the diagnostic work-up of children with suspected food allergy as it provides guidance for selecting foods for DBPCFCs[1,10]. In several clinics, screening for sensitization is performed using only one of both test-methods

because of time, expense or discomfort for the patient. The results from our study clearly show that screening using only one of both tests is insufficient. If only the in vitro-test had been used for screening in our study group, depending on the allergen, 17-27% of the sensitized patients would have been missed. In case the SPT had been solely used, 23-28% of the patients would have been missed. This means that it is imperative for pediatric clinicians to use both test-methods when evaluating a child with suspected food allergy. Screening for food-specific sensitization should be performed using both in vitro-IgE detection and skin testing to prevent false-negative screening results. This applies for the total pediatric age group, and certainly for infants.

Results of in vitro and skin-tests in this study were expressed as positive or negative, based on standardized and generally accepted cut-off values that are widely used[12,13,18]. These values determine the presence of an IgE-mediated immune response to the food, which is not present in non-atopic individuals, and which is therefore generally accepted to be the best reference point in the screening for food-specific sensitisation[8,10,11]. Recently, it has been suggested that not just the presence or absence of food-specific IgE antibodies, but also the level of the specific IgE titer is associated with the risk of clinical reactivity[19-21]. These studies have tried to define cut-off levels which make it possible to diagnose or exclude clinical reactivity with greater than 95% certainty, without performing food challenges. This same principle has also been suggested for the size of the wheal that appears during the SPT[22]. The results of these studies are promising, however further studies with large cohorts of patients are needed to standardize these cut-off values, before they can be implemented into clinical practice.

In summary, detection of allergen-specific IgE by in vitro methods and skin prick testing are important methods to detect sensitization for food allergens. In this study we evaluated the screening for sensitization for the three major food allergens; cow's milk, hen's egg and peanut in a large cohort of children with suspected food allergy. The results from this study show that there is a considerable mismatch between the outcome of in vitro IgE detection and skin prick testing when screening for food-specific sensitization in the population of children referred for suspected food allergy. This means that screening in the pediatric age group should be performed using both in vitro IgE detection as well as skin prick testing, to prevent false-negative results.

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Chapter 8

Protocol for a double-blind, placebo-controlled cow's milk challenge to diagnose immediate-type hypersensitivity reactions in infants and children

RP Schade

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ABSTRACT

The golden standard to diagnose clinical significant cow's milk allergy (CMA) is by means of a milk-provocation using a placebo-controlled protocol with blinded patients and observers. In most pediatric settings however, provocations are usually performed using open challenges, because this is less time-demanding and labor-intensive. We have therefore designed a protocol for a double-blind, placebo-controlled cow's milk challenge (DBPCCMC) that can be used to diagnose immediate-type allergic reactions to milk in a normal pediatric clinical setting. Our experience with this protocol in 154 children referred to our pediatric outpatient clinic over a two-year period, showed that it is possible to perform blinded-challenges routinely in the diagnostic work-up of children with suspicion of CMA. The protocol that we describe, provides a safe and efficient method to accurately diagnose immediate-type allergic reactions in infants and children. As it is generally accepted that DBPCCMCs are superior to open challenges because of the significant improved value, and parental acceptance of the diagnosis, we propose that DBPCCMCs should be implemented into routine pediatric practice, to prevent inaccurate diagnosis and unnecessary elimination diets.

INTRODUCTION

CMA is one of the major food allergies during infancy and childhood[1,2]. It is estimated that cow's milk leads to allergic reactions in 2-3% of all infants and children[3-5]. The immediate-type allergic reactions that can occur after ingestion of milk vary from cutaneous symptoms such as urticaria and erythematous rash, to vomiting and respiratory wheezing[2].

Despite advances in the understanding of the mechanisms of CMA[6], the existing in-vitro methods for the detection of clinically significant CMA are of poor diagnostic accuracy[7,8]. The only way to diagnose clinically relevant CMA is therefore by means of a clinical provocation with cow's milk. In most pediatric settings, this is performed by an open feeding with milk (open challenge). It has been shown however, that allergic reactions diagnosed during open challenges are often difficult to reproduce with a provocation during which both patients and observers are unaware of the challenge-content (blinded challenge)[9]. It is therefore clear, that the golden standard to diagnose CMA is by means of a double-blind, placebo-controlled milk challenge, to prevent false-positive results[7,8,10].

The principles on which blinded milk challenges should be based are widely accepted[10,11], and several protocols have been described[9,12-14]. Performing double-blind challenges, however, can be very time-consuming and labor-intensive, and most described protocols are therefore limited to use in research-settings. To prevent inaccurate diagnosis and the costs and burden of unnecessary elimination

diets, it is important that the diagnosis of CMA in routine practice should also be established using double-blind challenges[15].
For these reasons, we designed a protocol for a double-blind, placebo-controlled cow's milk challenge (DBPCCMC) that can be used to diagnose immediate-type allergic reactions to milk routinely in an outpatient clinical setting. In this report, we describe the protocol, and our experience with it during the diagnostic work-up of 154 infants and children suspected of CMA in our hospital, during the period 1999-2001.

DESCRIPTION OF THE CHALLENGE-PROTOCOL

Challenge formulas

For each DBPCCMC, 2 challenge formulas were prepared. The placebo-formula contained no cow's milk proteins, and was based on Nutramigen (Mead Johnson, Woerden, The Netherlands); a commercially available extensively hydrolyzed infant formula. The content of the active-formula was constructed in a manner so that the cow's milk protein-content was similar to that of regular infants formula (1,8 gr/100 ml). This was done by a mixture (ratio 11:3) of Nutramigen and Protifar (Nutricia, Zoetermeer, The Netherlands); a cow's milk protein-enriched formula. Both placebo-, and active-formula were comparable with regard to aspect, odor and taste. Food technicians prepared both formula's, which were put in similar bottles to assure blinding, and were subsequently randomly recoded into 'formula A' and 'formula B'.

Challenge routine

Children that underwent the DBPCCMC were admitted to the day-care ward of our hospital. After a physical examination, the DBPCCMC was started, always with 'formula A'. First step was application of a droplet of formula to the lips of the child. If no reaction had occurred after 15 minutes, the challenge was continued following a scheme with increasing amounts of formula (Table 1). If 1 hour after the last dose no allergic reactions had occurred, the second part of the challenge

Table 1. Challenge protocol for each part of the DBPCCMC.

Step	Time (minutes)	Amount
1	0	droplet
2	15	10ml
3	40	20ml
4	60	30ml
5	80	40ml
6	110	60ml
7	150	90ml

was started. This was performed with 'formula B', and was done using the same scheme as with 'formula A'. If no allergic reactions had occurred during the challenge with both formula's, the child was sent home at the end of the challenge day. An open feeding with the child's regular (cow's milk proteins containing-) food was started the next day.

Diagnosis of CMA
Challenges were performed while both patients and parents, as well as attending nurses and physicians were unaware of the contents of the formula (double-blinded). Before each part of the DBPCCMC, a routine physical examination was done by an attending pediatrician, and results were recorded on a special challenge-form. When symptoms of an allergic reaction occurred during the challenge, these were investigated by the same pediatrician, and recorded at the form. If reactions were unclear or only subjective, the last dose was repeated before the challenge proceeded with the next step. If objective allergic symptoms occurred, and persisted and/or deteriorated during the next step, the challenge was stopped, and medication was given if necessary. Subsequently the code was opened, and the diagnosis of CMA was confirmed when the symptoms had occurred during feeding with the active-formula.

RESULTS IN 154 INFANTS AND CHILDREN WITH SUSPICION OF CMA

Description of the study-cohort
Between June 1, 1999 and March 1, 2001, 154 children underwent a DBPCCMC-challenge to diagnose or exclude CMA. Eighty-five (55%) of these children were boys, 69 (45%) were girls. Age varied between 0.25-14 years (median 1.5 years). Fifty-eight (38%) were younger than 1 year of age (infants), 41 (27%) were 3 years or older.
All children had been referred to our pediatric allergy outpatient clinic because of symptoms suspicious for CMA, and had been on a cow's milk-free diet (elimination) for at least 1 month prior to challenge. Table 2 shows symptoms and/or indications for DBPCCMC in these children. Symptoms were mostly limited to the three following organ systems: skin, gastro-intestinal tract and the respiratory tract. The most common complaint was atopic dermatitis, with 113 (73%) of the referred children having this symptom. Abdominal cramping and/or colic was also very common, with 42 (27%) of the children having this symptom. Overall, 120 children (78%) had cutaneous symptoms, 64 (42%) had symptoms in the gastro-intestinal tract, and 30 children (19%) had respiratory symptoms. Symptoms in multiple organ systems were reported very commonly; 61 (40%) children had symptoms in more than 1 organ system, 3 children (2%) had symptoms in all three tracts. The combination of skin and gastro-intestinal

Table 2. Referral-indication for DBPCCMC in the total study group.

Organ	Symptom	No*
Skin	Urticaria/angioedema	13
	Erythematous pruritic rash	7
	Atopic dermatitis	113
Respiratory tract	Wheezing/repetitive cough	29
	Nasal congestion	2
	Pruritus/sneezing	1
	Laryngeal oedema	1
Gastrointestinal tract	Abdominal cramping/colic	42
	Vomiting or reflux	22
	Diarrhea	7
Other	Failure to thrive	1
	Positive family history for CMA	3

* number of children with this symptom

symptoms was the most common combination. Sixty (99%) of the patients that had symptoms in more than 1 organ-system did have skin symptoms. Three children that were referred for DBPCCMC had been on a cow's milk-free diet (elimination) since birth, because of a positive family history of CMA. These patients had never encountered cow's milk prior to the challenge.

Outcome of DBPCCMCs

In 133 of the 154 children that were tested, the diagnosis of CMA was rejected because no objective symptoms of allergy occurred during DBPCCMC. Although subjective symptoms, such as mild gastro-intestinal distress or irritability, were reported by some patients during the test, these symptoms invariably disappeared during the next steps in the challenge, with increasing amounts of formula. All 133 children completed the complete challenge protocol without objective allergic symptoms, and cow's milk could be re-introduced into the diet at home in all 133 children, without the occurrence of immediate-type allergic reactions. No reactions to placebo were seen in this group.

Symptoms during DBPCCMC in the CMA-patients

The DBPCCMC showed a positive result in 21 (14%) of the referred children. These children were diagnosed with CMA. Age of CMA-patients varied between 0.25-14 years (median 1.3 years). Eight (38%) were less than 1 year of age, 7 (33%) were 3 years or older. The symptoms that occurred during the challenge in the patients that were diagnosed with CMA varied, but mostly showed a similar pattern. In 5 of the 21 CMA-patients (24%), application of a droplet of cow's milk at the lip already resulted in urticarial reactions and/or erythema around the mouth

and in the face. In 1 of these infants, this was accompanied by symptoms of angioedema and inspiratory stridor, at which point the challenge was stopped. In the other 4, the symptoms of erythema returned and progressed during the next 3 steps in the challenge procedure (10-30ml). Eight of the 16 CMA-patients who did not react to the application of a droplet of cow's milk to the lips, developed the first symptoms of an allergic reaction during the next 3 steps in the challenge procedure (10-30ml). The symptoms in these patients were urticaria and/or erythema of the skin. This was mostly present around the mouth and in the face. One of these patients reported severe abdominal cramping. The symptoms in these patients aggravated during the increase of the milk-intake, upon which the test was discontinued. Of the 8 patients who developed the first allergic symptoms during the later stage of the DBPCCMC (40-90ml), 6 had skin symptoms. This concerned urticaria and/or erythema of the skin, mostly in the face. One of these patients had accompanying pulmonary problems, i.e. wheezing and inspiratory stridor. The two patients who did not have skin symptoms, reacted to cow's milk ingestion with vomiting. One of these also experienced severe abdominal cramping.
Taken together, 19 (90%) of the CMA-patients had cutaneous symptoms at one or more points during the DBPCCMC. In 2 of these patients this was accompanied by respiratory symptoms, in 1 by gastro-intestinal problems. Two CMA-patients (10%) did have isolated gastro-intestinal symptoms (vomiting). No reactions to placebo were seen in the CMA-patients.

Results of screening for cow's milk-specific sensitization in CMA-patients
Most of the children that were referred for DBPCCMC had been tested for cow's milk-specific sensitization prior to the DBPCCMC. This was done by using IgE-detection with the CAP system FEIA (Pharmacia Diagnostics, Uppsala, Sweden), and/or skin testing using the skin prick test (SPT)[16,17]. Results were available for 20 of the 21 CMA-patients. Of these, 18 (90%) were sensitized for cow's milk, as determined by a positive result in IgE-detection and/or SPT. In 2 CMA-patients, sensitization had not been proven, but screening had only been performed using SPT. These patients were both less than 6 months of age at the time of challenge. The patient in which no screening had been performed prior to DBPCCMC was 6 months old at the time of challenge.

DISCUSSION

The golden standard to diagnose clinical significant allergic reactions to cow's milk is by means of a provocation using a placebo-controlled protocol with blinded patients and observers[8,10]. However, in routine pediatric settings, milk-provocation is usually performed by open challenge, because this is less time-demanding and labor-intensive. We designed a protocol for a DBPCCMC that can be used to

diagnose immediate-type allergic reactions to milk in a routine pediatric clinical setting. The protocol was tested in a cohort of children (n=154) that were referred for milk-challenge over a two-year period in our hospital. A diagnosis of immediate-type CMA was established in 21 children (14%), and could be excluded in 133 (86%). There was a good correlation between the symptoms that were reported during previous ingestion of milk and outcome of DBPCCMC in the CMA-patients. The challenge-formula's that were used, were acceptable for children of all ages, including infants and pre-adolescent children, showing that this protocol can be used to diagnose immediate type reactions to cow's milk in virtually every child with suspicion of CMA.

The described protocol provides for a safe way of challenging children at risk for allergic reactions, by starting with small dosages of challenge-formula. In children that were considered to be at high-risk for allergic reactions, we usually prepared an IV-line for precautionary measures. None of the tested children experienced life-threatening allergic reactions that required additional hospitalization. Furthermore, we wanted to prevent false-negative challenges that could lead to anaphylactic reactions during the re-introduction of milk at home. Studies from literature have suggested that immediate-type allergic reactions can be ruled out when an individual has ingested up to 10 gr of dehydrated food without problems[8,18]. As dehydrated milk consists for approximately a third of proteins[19], this would suggest a 'secure dose' of 3.3 gr milk proteins. The challenge-formulas were therefore constructed so that children who completed the entire protocol had ingested at least 4.5 gr of pure milk proteins. As a result, all immediate-type reactions that occurred in our cohort, occurred during the hospital-admission, and none of the 133 children with a negative challenge-result experienced immediate-type reactions at home during re-introduction of milk. This shows that the described protocol provides a safe procedure to reproduce allergic symptoms, and prevents unwanted false-negative results.

It is generally accepted that double-blind, placebo-controlled challenges are superior to open challenges because of the significant improved diagnostic value[8,10,11]. Moreover, studies have shown that the parental acceptance of a diagnosis of CMA based on DBPCCMC, is much higher than a diagnosis that has been established during open challenge[9]. This is important, as it may drastically improve adherence to the assigned elimination diet. For these reasons, we propose that double-blind, placebo-controlled milk challenges should be implemented into routine pediatric practice, to improve accurate diagnosis in infants and children with suspicion of CMA. Results from our two-year experience show that the hereby-presented protocol is a safe and efficient method to diagnose immediate-type reactions in infants and children, and can be used routinely in a pediatric outpatient clinic. Usage of this protocol instead of open challenges may reduce false-positive diagnosis and unnecessary elimination diets.

Most of the children that were referred for DBPCCMC in our study-cohort had

been screened for cow's milk-specific sensitization by using in vitro IgE-detection and/or SPT. Results showed that all but three patients that were diagnosed with CMA during DBPCCMC were sensitized for cow's milk. This supports the current hypothesis that cow's milk-specific sensitization, i.e. the presence of an IgE-mediated immune reaction to milk, is important in the aetiology of immediate-type reactions[2,6]. In the three patients in whom cow's milk-specific sensitization was not established, screening had only been performed using the SPT. We recently evaluated the screening for food-specific sensitization in a large group of patients with suspicion of food allergy (n=785), and noted a significant mismatch between results of in vitro IgE detection and skin prick testing, especially in infants[20]. This means that we cannot exclude sensitization to cow's milk in these 3 patients, who were all younger than 7 months of age. Overall, in our study, none of the children that were older than 1 year of age and were not sensitized for cow's milk, showed immediate-type reactions during DBPCCMC. It is therefore tempting to speculate that patients who are not sensitized, and who are older than 1 year of age, do never experience immediate-type reactions to cow's milk. This means that these patients do not have to be challenged, which would drastically reduce the need for challenges in a routine pediatric setting. This principle has also been suggested by Sampson and co-workers, who suggested from a statistical model that patients with cow's milk-specific IgE levels of less than 0.8 kU/L, have a predictive value for a negative outcome of the DBPCCMC of more than 95%[21,22]. These observations have been done in a relatively small number of patients, and have therefore to be extended to larger groups before they can be applied in routine pediatric practice.

The protocol that we present in this report is designed to diagnose 'classical', immediate-type allergic reactions to cow's milk. It has been suggested that patients with CMA can also have isolated 'late' or 'delayed-type' reactions[2]. These are allergic reactions who are said to arise only after more than 24 hours intake of cow's milk[13,14]. This does not concern acute, life-threatening symptoms, but comprises a negative influence of milk-ingestion on chronic conditions such as infantile colic or atopic dermatitis, in the absence of acute reactions. To prove these reactions using DBPCCMC is difficult, as it requires a prolonged double-blind, controlled administration of cow's milk proteins or placebo in high doses, corresponding to normal daily intake[23]. We have recently performed a pilot-study with a protocol for a prolonged-DBPCCMC, in which infants with suspicion of delayed-type reactions to cow's milk were given blinded test-formulas for 2x1 weeks (unpublished data). Both test-formulas contained normal amounts of proteins, but only the active-formula contained intact proteins from cow's milk. Results showed that it is possible to perform these kind of prolonged challenges in a double-blind manner at home. However, as these challenges are very labor-intensive, they should be reserved for research-settings, or for special cases, in which routine diagnostics fails to establish an unequivocal diagnosis of delayed-type CMA.

In summary, we show that it is possible to perform double-blind, placebo-controlled milk challenges routinely in infants and children that are referred to a pediatric outpatient clinic. The protocol that is presented here provides a safe and efficient method to diagnose immediate-type allergic reactions to cow's milk in a double-blind manner. Implementation of this protocol into routine pediatric practice can reduce inaccurate diagnosis and the costs and burden of unnecessary elimination diets.

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Chapter 9

General discussion

INTRODUCTION

Cow's milk allergy (CMA) is one of the major allergies during early childhood, and is usually the first presentation of the atopic state of an individual. Unlike other (food) allergies, CMA in infancy is associated with rapid and spontaneous development of clinical tolerance. Elucidation of the mechanisms that determine the development of allergy and tolerance to cow's milk, may not only provide insights for therapeutic strategies for CMA, but also other (food) allergies. The studies presented in this thesis investigate the role of cow's milk protein (CMP)-specific T cells. In this chapter, findings on the relevance of circulating CMP-specific T cells in the aetiology of CMA are summarized and discussed. A model is presented (Figure 1) which describes the development of the CMP-specific T cell response in blood of atopic and non-atopic individuals during infancy and early childhood.

THE RELEVANCE OF CMP-SPECIFIC T CELL REACTIVITY IN THE AETIOLOGY OF CMA

In this thesis, it was shown that CMP-specific T cell reactivity can be demonstrated in blood of infants with CMA and AD, but also in infants with AD without CMA (Chapter 2 to 5). This clearly shows that the presence of a CMP-specific T cell response per se, does not determine CMA. T cells, specific for the proteins in milk, were also demonstrated in blood of non-atopic infants (Chapter 3, 5), which shows that this conclusion can be extrapolated to individuals without atopy. The presence of CMP-specific T cell reactivity in blood of young children that had outgrown their CMA (Chapter 4, 5), further emphasizes that CMA is not caused by a CMP-specific T cell response per se.
Cow's milk consists of several different protein-fractions that may provoke an immune reaction in man, the most important being the whey and casein proteins[1-3]. From investigations at the humoral level (IgE), it is known that no particular protein-fraction in milk accounts for the allergenicity of cow's milk[4-7]. In this thesis, it was shown that T cells specific for both protein-fractions are present in blood of infants with and without CMA, irrespective of their atopic state (Chapter 2 to 4). This demonstrates that, both at the humoral and at the T cell level, there is no immuno-dominant protein-fraction in cow's milk. Intact milk-proteins consist of multiple joined aminoacids[8], sometimes up to 200, while T cells, as well as IgE-antibodies, recognize only small parts of the proteins (epitopes), consisting of several aminoacids[9-11]. Investigations into IgE-binding epitopes have shown that patients with and without CMA do differ in their epitope-recognition of the various CMPs[12,13]. This might suggest that also for T cell epitopes, differences exist between patients and controls, which would lead to different T cell responses

towards the various CMPs. In this thesis, no epitope-analysis was performed, therefore these findings cannot be verified for T cell-binding epitopes.
From investigations[14-16] in patients with allergy to inhalation-allergens it is known that circulating Th2 cells are important in the initiation of allergic tissue-inflammation, through stimulation of IgE synthesis and eosinophil recruitment[17-20]. The investigations in this thesis demonstrate that also in infants with allergy to cow's milk, cytokine release by the circulating CMP-specific, i.e. disease-relevant, T cells is Th2-biased, in contrast to infants without CMA (Chapter 2, 3). This had already been suggested by previous studies with PBMCs[21-23], but is confirmed at the clonal level in this thesis. The Th2-biased nature is demonstrated by the production of IL-4, the hallmark cytokine of the Th2-response, which is higher in infants with CMA compared to (age-matched) atopic, and non-atopic infants without CMA (Chapter 2, 3). This Th2-skewing of the CMP-specific T cell response is further reflected by high release of IL-5 and IL-13, two cytokines that are also closely associated with release of IL-4 (Chapter 2, 3).
In combination with this Th2-biased cytokine pattern, the activation state of antigen-specific T cells is probably also of importance in the aetiology of allergic reactions to CMPs. Analysis of the cell surface expression of CD25 (IL-2Rα), the high affinity receptor for IL-2[24] showed that the expression of this activation-marker by CMP-specific T cells of infants with CMA is significantly higher than in atopic and non-atopic infants without CMA (Chapter 5). Similar results were found for cell surface expression of CD30, another marker of T cell activation[25], which is closely associated with CD25 expression (Chapter 5). Investigations in PBMCs showed that CMP-specific T cells from patients with CMA *in vitro* generally have a higher proliferative capacity than T cells from individuals without CMA[26-35]. The studies in this thesis suggest at the clonal level that CMP-specific T cells from patients with CMA have an enhanced activated phenotype. These findings may indicate that the *in vivo* CMP-specific T cell response in patients with CMA is by itself more vigorous. In line with this hypothesis, it was demonstrated in this thesis that the development of clinical tolerance for CMA is associated with a downregulation of the CMP-specific (Th2) response. Both Th2-cytokine production (Chapter 4), and activation level (Chapter 5) of the circulating CMP-specific T cells is lower after the development of tolerance for CMPs. This indicates that certain, currently unknown, mechanisms cause a change in the CMP-specific T cell response, resulting in a clinical CMP-tolerant state of a patient with CMA.
Interestingly, in this thesis, differences between patients with and without atopy were found with respect to T cell expression of CD26 (the receptor for adenosine deaminase) and CD30. It was shown that the expression of CD26 by CMP-specific T cells from atopic individuals is higher, and the expression of CD30 is lower than in individuals without atopy (Chapter 5). The exact functions of CD26 and CD30 remain unknown[36-39], but these findings suggest that the expression of these

markers by T cells is a reflection of the atopic state of a patient. The previously suggested[40-42] association between the cell-surface expression of both markers and the production of Th1 and Th2 cytokines by CMP-specific T cells was also investigated (Chapter 5). This confirmed earlier studies which demonstrated that on the level of the individual T cell, CD26 and CD30 cannot be used as exclusive markers for Th2- and Th1-cells[43-45].
This thesis focused on infants with CMP-induced allergic inflammation in the skin. The expression of cutaneous lymphocyte antigen (CLA), a cell surface protein, is proposed to play a key role in the skin-infiltrating capacity of circulating antigen-specific T cells[46-48]. Analysis of the expression of CLA by the CMP-specific T cells in blood of patients with CM-induced skin symptoms showed that CLA-expression is comparable to T cells from individuals without allergic skin disease (Chapter 6). This suggests that the skin-homing potential of these T cells is not intrinsically different, but that other factors[49], perhaps in combination with the cytokine pattern and activation state of the T cell, determines migration to the skin, and the development of an allergic skin reaction.

THE DEVELOPMENT OF THE CMP-SPECIFIC T CELL RESPONSE DURING INFANCY AND EARLY CHILDHOOD

The T cell response in individuals without an atopic predisposition

Proliferative responses to the proteins in cow's milk have been shown in cord blood of atopic and non-atopic individuals[50-60]. This means that already at birth CMP-specific T cells are present in blood of atopic and non-atopic individuals. Priming of T cells against CMP-proteins thus may occur *in utero*, through mechanisms which are currently unknown[61-63]. Recent studies by Prescott and co-workers showed that the allergen-specific T cell response to foods in both atopic and non-atopic individuals is Th2-skewed at birth[58,59,64]. This is probably secondary to the fact that T cell responses in all individuals are Th2-skewed *in utero*, to prevent the toxic effects of the Th1-cytokines on the placenta and the 'allogenic' fetus[65,66]. Postnatally however, the development of T cell responses to environmental allergens in individuals with or without an atopic predisposition is essentially different[59,62,64]. It was suggested that in non-atopic individuals these Th2-responses are gradually suppressed, to prevent sensitization to environmental allergens[64]. Results presented in this thesis demonstrated that the T cell response to cow's milk in non-atopic individuals is indeed associated with a low production of the Th2-cytokines (Chapter 3). This indicates that allergy to CMPs in these individuals is successfully prevented by a downregulation of the fetal Th2-response.
Investigations in PBMCs have suggested that antigen-specific T cell responses generally deviate to a Th1-biased pattern in individuals without an atopic

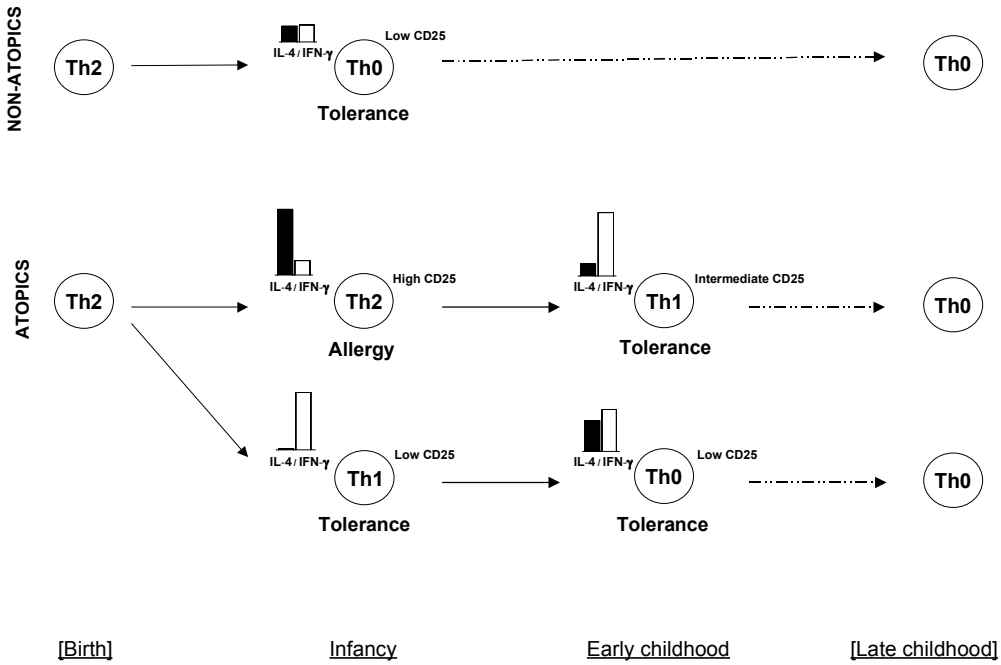

Figure 1. Model that describes the development of the CMP-specific T cell response in non-atopic and atopic individuals. Results from birth are extrapolated from data in references 58 and 64. Results from infancy and early childhood are described in this thesis (Chapter 2-5). Results from late childhood are extrapolations of results described in this thesis (Chapter 2-5).

predisposition[62,64]. However, the studies in this thesis show that, when investigated at the single cell level, the T cell response to milk in non-atopic infants displays a balanced, Th0-like cytokine profile, with equal production of IL-4 and IFN-γ (Chapter 3). As the strong inflammatory capacities of Th1-cytokines, such as IFN-γ, can be harmful, it seems logical that a balanced (i.e. Th0-like) cytokine response to environmental allergens is better than a full-blown Th1-response that is dominated by IFN-γ release. Therefore, it is likely that the T cell response to milk in non-atopics remains Th0-like throughout life, although this has yet to await formal prove. Studies have shown proliferative responses to cow's milk in PBMCs of non-atopic adults[34,67] which demonstrates the persistence of a CMP-specific T cell response in blood of non-atopics into adulthood. Cytokine release by these CMP-specific T cells however has not been investigated at the single cell level so far.

The T cell response in atopic patients with CMA

Studies that investigated the postnatal development of allergen-specific T cell responses concluded that, in contrast to individuals without atopy, the fetal allergen-specific (Th2) responses in individuals with an atopic predisposition remain Th2-skewed during infancy and childhood[59,60,64]. However, these studies made no distinction between the allergen-specific T cell response in atopic patients with, and without allergic reactions to that specific allergen. In this thesis, it was demonstrated at the clonal level, that atopic infants with allergy to CMPs have a Th2-biased, CMP-specific T cell cytokine response in blood (Chapter 2). This T cell response, with high levels of IL-4, IL-5 and IL-13, most probably mediates allergic tissue inflammation similar to the mechanisms that have been shown for allergic reactions to inhalants[17,19,20]. The finding that CMA in infancy is associated with a Th2-biased T cell response, may indeed indicate that CMA is the result of a temporary delay in the (normal) redirection of the fetal Th2 response. The fact that CMA in infancy is remarkably transient, i.e. most patients become spontaneously tolerant to milk within 2 years[68,69], supports this hypothesis.

Investigations in this thesis showed that this spontaneous development of clinical tolerance to milk in infants with CMA is associated with a downregulation of Th2-cytokine release, while at the same time IFN-γ production is up-regulated (Chapter 4). This results in a deviation of the CMP-specific T cell cytokine response towards a Th1-type profile. It is tempting to speculate that in infants that have developed allergy to milk, an 'overshoot' deviation of the (Th2-biased) CMP-specific T cell response to a Th1-profile, is needed to induce the CMP-tolerant state in these patients. The CMP-specific T cells still show an enhanced activated state at this timepoint, as reflected by the higher cell surface expression of CD25 than control patients (Chapter 5). These findings suggest that the increased Th1-cytokine release, in combination with the enhanced activated phenotype is needed for the maintenance of the CMP-tolerant state in these patients. It is likely that, in due time, this CMP-specific Th1-biased T cell response will be downregulated to a balanced, Th0-like response. As argued before, a full-blown Th1-response with high levels of IFN-γ may have harmful effects. Therefore, it is plausible that with increasing age, patients that have become clinically tolerant to milk after 'transient' CMA will obtain a T cell response that is comparable with atopic and non-atopic individuals who never had CMA.

The T cell response in atopic individuals without CMA

To investigate the CMP-specific T cell response in individuals with an atopic predisposition, but who do not have allergy to cow's milk, infants with AD without CMA were investigated in this thesis. These patients had existing atopic disease, both immunologically and clinically, but they were not allergic to cow's milk as proven by formal challenge. In accordance with the hypothesis that production of high levels of Th2 cytokines by allergen-specific T cells is important in the

aetiology of allergy, it was found that CMP-specific T cells from these infants show a low production of the Th2 cytokines (Chapter 2). Of interest however, is the finding that the majority of these infants have CMP-specific T cells that display a Th1-phenotype. This is in contrast with the findings from previous studies who suggested that atopic individuals have a prolonged period of fetal Th2-skewed allergen-specific T cell responses[59,60,64]. However, these studies did not discriminate between T cell responses in atopic patients with, and without allergic reactions to the studied allergen.
The finding that, when investigated at the single cell level, CMP-specific T cells from atopic individuals who do not have CMA display a Th1-phenotype, raises important questions. The hallmark characteristic of individuals with an atopic predisposition is their tendency to develop sensitization and allergy to common allergens. The fact that these patients had not developed CMA means that, despite their atopic state, allergy to cow's milk has somehow been successfully prevented. It is tempting to speculate that the Th1–biased nature of the cytokine response is of importance in this respect. Atopic individuals have an increased risk of developing allergic sensitization during the first year of life, the so-called 'window of sensitization'[64,70,71]. Usually CMPs are often encountered in large quantities during infancy. It can be argued that during this period, with its lingering fetal T cell responses which cause a Th2-biased milieu in blood, the T cell response to milk is more Th1-skewed in these patients to prevent sensitization. The finding that the Th1-skewed cytokine response that is present in the first year of life, changes into a Th0-like cytokine pattern after infancy (Chapter 4), is in accordance with this hypothesis. Most probably, after infancy, when the 'window of sensitization' has passed and allergy to milk has been successfully prevented, there is no longer need for a 'compensatory', Th1-biased T cell response. To prevent the potential harmful effects of a full blown Th1-response upon exposure to cow's milk, the response is downregulated to a balanced, Th0-like response.

REGULATION OF THE CMP-SPECIFIC T CELL RESPONSE

The results presented in this thesis show differences in the postnatal development of the T cell response to cow's milk, between individuals with and without an atopic predisposition. More importantly, differences in the development of the T cell response between atopic individuals who did or did not develop CMA were demonstrated. This finding, in combination with the fact that the spontaneous development of clinical tolerance is apparently associated with a modulation of the CMP-specific T cell response, shows that understanding of the regulatory mechanisms that define T cell responses is of importance for the development of therapy for CMA.
Several observations indicate that the antigen-presenting cell (APC)-apparatus is

probably crucial in the orchestration of T cell responses. It has been shown that the signals that APCs in the central lymphoid organs provide during the priming of T cell responses in the perinatal period, can determine the outcome of the Th1- or Th2-biased nature of the T cell response[72,73]. Work by Kalinski and co-workers showed that distinct subsets of dendritic cells (DCs) exist, that are responsible for primary polarization of naive T cells in the lymph nodes[73,74]. These DC-subsets provide stimulatory signals during antigen-presentation that determine if the T cell cytokine profile becomes Th1- or Th2-biased[74,75]. Although the exact signals remain to be elucidated, both soluble factors such as IL-12, as well as cell-surface interactions are probably of importance in this process[73,75].

During the postnatal development of allergen-specific T cell responses, the peripheral APC-network is proposed to play a crucial role[64,71,76]. It has been demonstrated that the DC-network in the lung can orchestrate the postnatal T cell response towards inhalation allergens[77-80]. The mechanisms that regulate the postnatal immune-response to foods are less clarified, but are under investigation[81]. It is clear that antigen-presenting cells in the gut-associated lymphoid tissue are of vital importance in the induction of oral tolerance[82-84]. However, both the nature of the relevant APCs, and the exact processing events are currently unclear[83,84].

Furthermore, the possible role of IL-10 in the regulation of CMP-specific T cell responses should be discussed. In adults with established allergy to birch-pollen and bee venom, tolerance can be artificially established via allergen-specific immunotherapy. This induces a deviation of the allergen-specific T cell response from a Th2-response to a Th1-skewed response[85-88]. In this thesis, it was shown that the spontaneous development of tolerance for CMPs is associated with a similar Th2-Th1 switch (Chapter 4). In immunotherapy, an increase in production of IL-10 by allergen-stimulated PBMCs can be found[87,89]. This cytokine is predominantly being produced by antigen-presenting cells such as B cells and monocytes[90,91]. It is thought that IL-10 is important for the modulation of the T cell response through inhibition of proliferative responses and cytokine production[91-93]. In this thesis, no evidence was found that the spontaneous development of tolerance to CMPs is associated with an increased production of IL-10 by the CMP-specific T cells in blood (Chapter 4). The antigen-presenting cell apparatus however, was not investigated in this thesis. Therefore, the exact role of IL-10 in the regulation of the CMP-specific T cell response remains to be clarified. It might be that, similar to (artificially) induced tolerance, IL-10 production by antigen-presenting cells plays an important role in the development of spontaneous tolerance to cow's milk. Future studies should therefore focus on the role of the antigen-presenting cell apparatus, and its IL-10 production, in the regulation of the CMP-specific T cell response.

DIRECTIONS FOR FUTURE STUDIES

Extension of current observations to adults and patients with persistent CMA

The studies in this thesis have elucidated the nature of the CMP-specific T cell response in atopic and non-atopic individuals in infancy and childhood. Future studies should be focused on the extension of the observations that were made in this thesis. Long-term follow-up of T cell responses in the patients that have become tolerant after CMA is needed, to see if the spontaneous acquisition of clinical tolerance is associated with a life-long, Th1-biased T cell response to cow's milk, or indeed develops into a downregulated, Th0-response. In addition, the CMP-specific T cell response in atopic and non-atopic adults who never had CMA, should be analyzed for cytokine production, to verify the hypotheses that were made in this thesis.

CMA in infancy is a transient disease that usually disappears during early childhood[69,94]. However, in a small proportion of patients, clinical tolerance does not develop during childhood, causing 'persistent CMA'. It is important to investigate if the CMP-specific T cell response in these patients is intrinsically different from the T cell response in 'common' (i.e. transient) CMA. The studies in this thesis suggested that 'common' CMA is a temporary error in the normal redirection of the fetal Th2-response. For unknown reasons, this redirection fails completely in certain patients, leading tot persistent CMA. The mechanisms that determine the development of clinical tolerance to milk in patients with transient CMA, and the reasons why this fails, or only develops after several years in patients with persistent CMA, need to be clarified.

The fate of T cells after the development of clinical tolerance

This thesis showed that the development of clinical tolerance to cow's milk in patients with CMA is associated with significant changes in the CMP-specific T cell response. This suggests that certain, currently unknown, mechanisms modulate the reactivity and cytokine release of the CMP-specific T cells. It is unclear however, whether the CMP-specific T cells that were demonstrated at both timepoints, are essentially the same T cells. One could also speculate that, in order to acquire clinical tolerance to milk, the highly-activated CMP-specific Th2-cells are deleted from the T cell repertoire, or become anergized. This implies that newly-formed CMP-specific T cells, exhibiting a Th1-phenotype, shift the balance towards a clinical tolerant response. At the moment it is unknown, which mechanism is responsible for the observed changes in the CMP-specific T cell response. Future research should set out to solve this question. Studies by Van Reijsen and co-workers showed that it is possible to use the T cell receptor as a 'fingerprint' to trace identify identical T cell clones[95,96]. The exact DNA-sequence of the variable part of the β-chain of the T cell receptor, which is unique for each T cell, was used as a identification marker for that specific TCC. Using probes with a

similar sequence they showed that it is possible to trace T cells in polyclonal T cell cultures obtained from the same patient at different timepoints over a period of several years[95,96]. This approach may provide insight into the mechanisms that determine the fate of T cells after the development of clinical tolerance to cow's milk.

The role of circulating T cells in the aetiology of CMP-induced skin inflammation

The findings in this thesis show significant differences in the CMP-specific T cell response in blood of patients with CMA and AD compared to infants without CMA, especially with regard to the production of Th2 cytokines. From investigations in patients with inhalation-allergy, it was suggested that skin-infiltrating, allergen-specific Th2-cells in blood are crucial in the initiation of the allergic skin inflammation[17,97,98]. The skin compartment in patients with CMP-induced skin symptoms however has not yet been investigated. Extrapolation of reactivity and cytokine release of circulating CMP-specific T cells to mechanisms in the skin remains therefore hypothetical. Future studies should investigate the presence and phenotype of CMP-specific T cells in skin of CMP-allergic individuals to elucidate the mechanisms of CMP-induced skin inflammation.

The expression of CLA is proposed to play a key role in the skin-infiltrating capacity of a T cell[46,48,99]. Results from this thesis suggested that the expression of CLA by CMP-specific T cells in blood of patients with CM-induced skin inflammation is not essentially different from individuals without allergic skin disease. Interestingly however, it was found that the level of CLA-expression by T cells in blood is significantly lower than CLA-expression by skin T cells (Chapter 6). These findings imply that the level of CLA-expression, rather than the expression per se, determines the (skin-) localization of allergen-specific T cells. Future research into the mechanisms by which CMP-specific T cells in blood are connected to allergic skin inflammation, should therefore include the regulation of the level of CLA-expression by allergen-specific T cells.

Clinical manifestations of CMA

Besides the efforts to unravel the aetiology of CMA and CMP-induced skin symptoms, future studies should focus on the improvement of the clinical characterization of patients with CMA. The studies in this thesis have focused on patients with immediate-type reactions to cow's milk. It has been shown that there are also patients with CMA who experience isolated 'late' or 'delayed-type' reactions[100,101]. These are allergic reactions who arise only after prolonged intake of cow's milk, and are not life-threatening. It has even been suggested that patients with CMA can be subdivided into 3 different 'disease-profiles'. Hill and co-workers showed that besides the immediate-type reactors, there are CMA-patients who experience isolated gastro-intestinal symptoms, 1-20 hours after milk

ingestion (intermediate reactors)[101,102]. Furthermore, they suggested that there is a (late-reacting) subgroup of patients with AD, who show deterioration of their AD, 24 hours after milk-ingestion, in the absence of immediate-type reactions[101,102]. The existence of these subgroups of CMA patients, however, has not yet been indisputably demonstrated in double-blind test-settings that controlled for other, disturbing factors. To prove delayed-type reactions using double-blinded challenges, a prolonged, controlled administration of CMPs or placebo in high doses, corresponding to normal daily intake, is required. Results from a pilot-study that was mentioned in Chapter 8, showed that it is possible to perform these kind of prolonged challenges in a double-blinded manner at home. Exact knowledge of the heterogeneity of symptoms that patients with CMA may experience, and the changes in 'disease-patterns' that may occur with increasing age[103], is essential for future research into CMA.

CONCLUSION

In this thesis, the role of antigen-specific T cells in the aetiology of CMA and AD was investigated. Distinct differences between T cells in blood of infants with and without CMA were found, especially with regard to reactivity and cytokine-release. These differences were reflected by the change in the CMP-specific T cell response that was observed during the spontaneous remission of CMA in patients with CMP-induced skin disease. The presented findings strongly suggest that the nature of the CMP-specific T cell response in blood determines the outcome of allergy and tolerance to cow's milk.
Furthermore, the investigations confirmed that differences exist between the CMP-specific T cell response in atopic and non-atopic individuals during infancy and early childhood. This suggests that the mechanisms that regulate the T cell response to milk are of vital importance in the development of clinical tolerance to CMPs. Future studies should therefore be focused on the elucidation of these mechanisms, to give directions for the development of therapeutic and preventive strategies in allergy to cow's milk.

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SUMMARY

Allergy is an important disease that affects a substantial proportion of the general population, and which has seen an increasing incidence during the past three decades. Cow's milk allergy (CMA) is one of the major food-allergies during infancy and early childhood. This thesis focuses on patients with CMA and atopic dermatitis (AD), which is the major presentation of CMA in the majority of patients during childhood. The aim of this thesis is to investigate the role of antigen-specific T cells in the etiology of CMA and AD. In addition, the currently used methods to diagnose CMA, *in vivo* and *in vitro,* are evaluated.

In **Chapter 1**, a general introduction to CMA and AD in infancy and childhood is given. CMA plays a pathogenic role in approximately a third of the infants with AD in the first year of life, and is associated with the spontaneous development of clinical tolerance within a relatively short period of two to three years. Similar to inhalant-allergy, in which Th2 cells that secrete high levels of interleukin (IL)-4, IL-5 and IL-13 are important, T cell reactivity towards the proteins in cow's milk has been implicated in the aetiology of allergy to cow's milk. This has however not been thoroughly investigated so far.

Chapter 2 describes differences in the cow's milk protein (CMP)-specific T cell response between blood of infants with AD with and without CMA. Using an antigen-specific T cell culturing system with autologous B cells as antigen-presenting cells, CMP-specific T cell clones were established from blood. Results show that T cells specific for the various proteins in milk are present in blood of infants with AD, irrespective of their atopic state. Analysis of cytokine release shows that the CMP-specific T cell response in infants with CMA is Th2-skewed, with production of high levels of IL-4, IL-5 and IL-13. In contrast, infants without CMA have a Th1-skewed response, with high levels of interferon (IFN)-γ and low levels of the Th2-cytokines.

In **Chapter 3**, infants without an atopic predisposition and without allergic disease are investigated. Results show that in these individuals, a T helper cell response directed against the major proteins in milk is evident. These T cells show a balanced, Th0-like cytokine production with an equal production of IL-4 and IFN-γ. Comparison of these T cells with those described in **Chapter 2** emphasizes the Th0-like phenotype of cells, and the low release of Th2 cytokines. This suggests that similarly to individuals with an atopic predisposition, tolerance to milk in normal individuals is not caused by absence of circulating, CMP-specific T cells, but is probably determined by low release of Th2-cytokines by these cells.

Chapter 4 investigates the changes that occur in the CMP-specific T cell response during the spontaneous development of clinical tolerance for cow's milk in infants with CMA and AD. Results show that CMP-specific T cells are still present in blood of CMA patients that have become clinically tolerant for CMPs. However, production of the Th2 cytokines by these T cells is lower than during the allergic

state. In contrast, production of IFN-γ is increased, which results in a Th1-skewed cytokine pattern. These results emphasize the important role of antigen-specific Th2 cytokine-release in the development of allergic reactions to CMPs.
In **Chapter 5**, the expression of several cell-surface markers by CMP-specific T cells is investigated. Results show that the expression of the activation markers CD25 and CD30 by CMP-specific T cells is significantly higher in infants with CMA, which suggest a higher activation state of these T cells. After the development of clinical tolerance for CMPs, cell-surface expression of activation markers decreases, suggesting a downregulation of CMP-specific T cell reactivity. Comparison of the expression of CD26 and CD30 with the release of IL-4 and IFN-γ confirms earlier studies which demonstrated that on the level of the individual T cell, CD26 and CD30 cannot be used as exclusive markers for Th2- and Th1-cells.
In **Chapter 6**, CMP-specific T cells from blood and skin are investigated, and compared with regard to cell-surface expression of the skin-specific homing marker; cutaneous lymphocyte antigen (CLA). Results show that expression of CLA by CMP-specific T cells in patients with CMA and AD is comparable with patients without CMA. This suggests that the skin-homing potential of T cells from allergic and non-allergic individuals is not essentially different. Analysis of CLA-expression by T cells in skin of patients with AD however, shows significant higher expression levels of CLA on skin T cells compared to T cells from blood. This finding suggest that the level of CLA-expression might be important for the retention of allergen-specific T cells in the skin.
Chapter 7 evaluates the screening for food-specific sensitization using *in vitro* IgE detection and skin prick-testing in infants and children. Results show a substantial mismatch between the outcome of both tests when screening for food-specific sensitization in infants and children with suspected food allergy. This indicates that the screening for food-specific sensitization in the pediatric age group should be performed using both in vitro IgE detection as well as skin prick testing, to prevent false-negative results.
Chapter 8 provides a description of a new protocol for a double-blind, placebo-controlled cow's milk challenge (DBPCCMC) to diagnose immediate-type hypersensitivity reactions in infants and children, in a normal pediatric clinical setting. Results of the use of this protocol in a cohort of 154 children referred to the pediatric outpatient clinic of the University Hospital in Utrecht showed that it is possible to perform blinded-challenges routinely in the diagnostic work-up of children with suspicion of CMA. This indicates that DBPCCMCs should be implemented into routine pediatric practice, which may prevent inaccurate diagnosis and unnecessary elimination diets.
Finally, **Chapter 9** is a general discussion which summarizes and discusses the presented data on CMP-specific T cells in this thesis. In this chapter, a model is presented that describes the postnatal development of the T cell response to cow's

milk in atopic and non-atopic individuals. Especially during infancy, clear differences exist between the CMP-specific T cell response in atopic and non-atopic individuals with regard to cytokine release. This suggests that the mechanisms that regulate the T cell response to milk are of vital importance in the development of clinical tolerance to CMPs. Future studies should therefore be focused on the elucidation of these mechanisms, to give directions for the development of therapeutic and preventive strategies in allergy to cow's milk.

SAMENVATTING

Allergie is een ziekte die een substantieel deel van de bevolking treft, en waarvan die incidentie de afgelopen decennia fors is gestegen. Koemelkallergie (KMA) is een van de belangrijkste voedselallergieen tijdens de zuigelingen- en peuterleeftijd. Dit proefschrift gaat over patiënten met KMA en atopische dermatitis (AD), de belangrijkste uitingsvorm van KMA tijdens de kinderleeftijd. Het doel van dit proefschrift is om de rol van antigeenspecifieke T cellen bij het ontstaan van KMA en AD te bestuderen. Verder worden enkele *in-vivo* en *in-vitro* methoden om KMA te diagnosticeren geëvalueerd.

In **Hoofdstuk 1** wordt een algemene inleiding op KMA en AD op de kinderleeftijd gegeven. KMA speelt een pathogenetische rol in ongeveer eenderde van de zuigelingen met AD tijdens het eerste levensjaar. Zuigelingen KMA ontwikkelen spontaan klinische tolerantie voor koemelk (KM) binnen een relatief korte periode van 1-3 jaar. Bij allergie voor inhalatieallergenen worden Th2 cellen, die grote hoeveelheden van de cytokines: interleukine (IL)-4, IL-5 en IL-13 produceren, zeer belangrijk geacht in het ontstaan van de allergische reacties. Op dezelfde manier wordt T cel reactiviteit tegen de eiwitten in koemelk belangrijk geacht bij het ontstaan van KMA, echter dit is tot nu toe nog niet goed onderzocht.

Hoofdstuk 2 beschrijft de verschillen tussen de KM-specifieke T cel respons in bloed van zuigelingen met AD, met en zonder KMA. Met behulp van een antigeenspecifiek kweeksysteem dat gebruik maakt van autologe B cellen, werden KM-specifieke T cel klonen gekweekt uit bloed. De resultaten laten zien dat T cellen, specifiek voor de eiwitten in koemelk, aanwezig zijn in het bloed van zuigelingen met AD, onafhankelijk van het feit of ze KMA hebben. Analyse van de cytokine productie laat zien dat deze cellen een 'Th2-achtige' cytokine productie hebben; dwz een hoge productie van IL-4, IL-5 en IL-13. Zuigelingen zonder KMA hebben daarentegen een respons die Th1-achtig is, dwz een hoge productie van interferon (IFN)-γ en een lage productie van de Th2-cytokines.

In **Hoofdstuk 3** worden zuigelingen onderzocht, die geen atopische predispositie, en geen allergische ziekten hebben. De resultaten laten zien dat deze kinderen een T cel respons hebben, gericht tegen de belangrijkste eiwitten in KM. Deze cellen produceren een gebalanceerde, Th0-achtig cytokine patroon, dwz een gelijke productie 'Th1-' en 'Th2-cytokines'. De vergelijking met de T cellen die beschreven zijn in **Hoofdstuk 2** benadrukt dit Th0-achtige cytokine patroon, en de lage productie van de Th2-cytokines. Dit suggereert dat tolerantie voor melk in normale personen niet veroorzaakt wordt door een afwezigheid van circulerende, KM-specifieke T cellen,
maar, net als bij mensen met een atopische predispositie, waarschijnlijk veroorzaakt wordt door de lage productie van Th2-cytokines door deze cellen.

Hoofdstuk 4 onderzoekt de veranderingen die optreden in de KM-specifieke T cel respons tijdens de spontane ontwikkeling van tolerantie voor KM bij zuigelingen

met KMA en AD. De resultaten laten zien dat KM-specifieke T cellen nog steeds aanwezig zijn in het bloed van patiënten met KMA die klinisch tolerant geworden zijn voor KM-eiwitten. De productie van de Th2-cytokines door deze cellen is echter lager dan tijdens de (KM)allergische fase, terwijl de productie van IFN-γ is gestegen. Dit resulteert in een Th1-achtig patroon. Deze resultaten benadrukken de belangrijke rol van antigeenspecifieke (Th2) cytokine productie bij het ontstaan van allergische reacties op KM-eiwitten.
In **Hoofdstuk 5** wordt de expressie van verschillende celmembraaneiwitten op T cellen onderzocht. De resultaten laten zien dat de expressie van de activatie-'markers' CD25 en CD30 door KM-specifieke T cellen significant hoger is bij zuigelingen met KMA, wat suggereert dat de activatiestatus van deze cellen hoger is. Na het ontstaan van klinische tolerantie voor KM-eiwitten daalt de expressie van deze activatie-'markers', wat suggereert dat de KM-specifieke T cel reactiviteit afneemt. Vergelijking van de expressie van de membraan-eiwitten CD26 en CD30 met de productie van IL-4 en IFN-γ bevestigt eerdere studies die lieten zien dat, op het niveau van de individuele T cel, de expressie van CD26 en CD30 niet gebruikt kan worden om Th1- en Th2-cellen te onderscheiden.
In **Hoofdstuk 6** worden KM-specifieke T cellen in bloed en huid onderzocht, en vergeleken mbt de expressie van 'cutaneous lymphocyte antigen' (CLA), de huidspecifieke 'homing-marker'. De resultaten laten zien dat de expressie van CLA door KM-specifieke T cellen in bloed van patiënten met KMA en AD vergelijkbaar is met T cellen van patiënten zonder KMA. Dit suggereert dat het vermogen van T cellen in bloed van allergische en niet-allergische patiënten om specifiek naar de huid te 'homen' niet essentieel verschillend is. De analyse van T cellen in de huid van patiënten met AD laat echter zien dat expressie van CLA door deze cellen significant hoger is dan T cellen in bloed van deze patiënten. Dit suggereert dat het niveau van expressie van CLA belangrijk zou kunnen zijn voor het achterblijven van allergeenspecifieke T cellen in de huid.
Hoofdstuk 7 evalueert de screening op voedsel-specifieke sensibilisatie mbv *in vitro* IgE-detectie en huidprik testen bij zuigelingen en kinderen. De resultaten laten een substantiële 'mismatch' zien tussen de uitkomst van beide testen bij het screenen op voedsel-specifieke sensibilisatie bij zuigelingen en kinderen met de verdenking op voedselallergie. Dit geeft aan dat de screening op voedsel-specifieke sensibilisatie op de kinderleeftijd gedaan zou moeten worden gebruik makend van beide testen, om foutnegatieve uitkomsten te voorkomen.
Hoofdstuk 8 beschrijft een nieuw protocol voor een dubbelblinde, placebogecontroleerde koemelkprovocatie dat gebruikt kan worden om, in een alledaagse pediatrische kliniek, 'immediate-type' allergische reacties op koemelk te diagnosticeren bij zuigelingen en kinderen. De ervaringen met dit protocol, in een cohort van 154 kinderen die verwezen werden naar de polikliniek voedselallergie van het Universitair Medisch Centrum in Utrecht, laten zien dat het mogelijk is om routinematig geblindeerde provocaties te verrichten bij kinderen met de verdenking

op KMA. Dit suggereert dat dubbelblinde provocaties geïmplementeerd moeten worden in de alledaagse pediatrische praktijk, wat inaccurate diagnostiek en onnodige eliminatiediëten zou kunnen voorkomen.
Tot slot, **Hoofdstuk 9** is een algemene discussie die de onderzoeksgegevens over de KM-specifieke T cellen samenvat, en bediscussieert. In dit hoofdstuk wordt een model gepresenteerd dat de postnatale ontwikkeling van de T cel respons tegen koemelk in atopische en niet-atopische personen beschrijft. Met name op de zuigelingenleeftijd zijn er duidelijke verschillen tussen de KM-specifieke T cel respons van atopische en niet-atopische personen, vooral met betrekking tot cytokine productie. Dit suggereert dat de mechanismen die de T cel respons tegen melk reguleren zeer belangrijk zijn voor het ontstaan van klinische tolerantie voor koemelkeiwitten. Toekomstige studies moeten daarom gericht worden op het ophelderen van deze mechanismen. Dit kan richting geven aan de ontwikkeling van therapeutische en preventieve strategieën voor allergie tegen koemelkeiwitten.

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CURRICULUM VITAE

Rogier Schade werd geboren op 7 oktober 1971 in Nijmegen. Hij behaalde het VWO diploma in 1989 aan het Titus Brandsma Lyceum in Oss. Hierna studeerde hij gedurende 1 jaar elektrotechniek aan de Technische Universiteit in Eindhoven. In 1990 begon hij met de studie Geneeskunde aan de Universiteit in Utrecht. Het propedeutisch examen werd behaald in 1991 en het doctoraal examen in 1995. Gedurende de studie geneeskunde verrichte hij onderzoek naar de prenatale diagnostiek van aangeboren hartafwijkingen en ritmestoornissen bij de afdeling Cardiologie in het Wilhelmina Kinderziekenhuis in Utrecht onder begeleiding van Dr. E.J. Meijboom. Ook was hij student-assistent bij de afdeling Anatomie in het Academisch Ziekenhuis in Utrecht. In 1996 werd begonnen met de co-assistentschappen en het artsexamen werd behaald in 1998. In de laatste fase van zijn co-schappen werkte hij gedurende 3 maanden als zaal-assistent op de afdeling Pediatrie en Kindergesondheit in het Tygerberg Hospitaal in Kaapstad, Zuid-Afrika (hoofd: Prof.dr. P. Hesseling).
Met het onderzoek dat beschreven is in dit proefschrift werd aangevangen in juni 1998. Het onderzoek werd uitgevoerd bij de afdeling Dermatologie/Allergologie (hoofd: Prof.dr. C.A.F.M. Bruijnzeel-Koomen), en de afdeling Algemene Kindergeneeskunde (hoofd: Prof.dr. J.L.L. Kimpen) van het Universitair Medisch Centrum in Utrecht, onder begeleiding van Dr. E. Knol en Dr. E. van Hoffen.
Per 1 januari 2002 zal hij beginnen met de opleiding tot kinderarts aan de afdeling Kindergeneeskunde van het Leids Universitair Medisch Centrum (hoofd: Prof.dr. J.M. Wit).
Rogier Schade is getrouwd met Anke Busch.