

ORIGINAL PAPER

Characterization of T cell epitopes in α s1-casein in cow's milk allergic, atopic and non-atopic children

B. Ruiter^{*1}, V. Trégoat^{†1}, L. M'Rabet[‡], J. Garssen^{†‡}, C. A. F. M. Bruijnzeel-Koomen^{*}, E. F. Knol^{*} and E. van Hoffen^{*}

^{*}Department of Dermatology/Allergology, University Medical Center, Utrecht, The Netherlands, [†]Biomedical Research Department, Numico Research B.V., Wageningen, The Netherlands and [‡]Utrecht Institute for Pharmaceutical Sciences, Utrecht, The Netherlands

Clinical and Experimental Allergy

Summary

Background One to two percent of infants suffer from IgE-mediated allergic reactions against cow's milk proteins. Most children develop clinical tolerance, but approximately 15% are still allergic by the age of 10 years. Little is known about the T cell epitopes in individual cow's milk protein in relation to allergy and tolerance.

Objective To identify T cell epitopes in α s1-casein, the most abundant milk protein, and to investigate T cell responses toward these epitopes in allergic, atopic and non-atopic children. **Methods** Allergen-specific T cell lines (TCLs) were derived from peripheral blood mononuclear cells of 11 cow's milk allergic, nine atopic and nine non-atopic children. T cell responses were measured to α s1-casein and to overlapping peptides (18-mers), spanning the α s1-casein molecule. Proliferation was determined by incorporation of ³H-thymidine, and cytokine production (IL-10, IL-13 and IFN- γ) was measured by ELISA.

Results Four main regions (amino acid (AA) residues 43–66, 73–96, 91–114 and 127–180) in the α s1-casein molecule were immunogenic to T cells, among which the AA residues 133–156 spanned the immunodominant part. Only subtle differences were found in peptide recognition between the subject groups. Some of the peptides induced slightly Th1- or Th2-skewed cytokine responses. The increased levels of IL-10 in response to α s1-casein observed in TCLs from atopic children appeared not to be linked to recognition of specific IL-10-inducing epitopes.

Conclusions The immunodominant sequence in α s1-casein is spanned by AA residues 133–156. Tolerance towards α s1-casein in atopic children may be mediated by an overall induction of IL-10 and not by recognition of certain T cell epitopes. The identified T cell epitopes in children with cow's milk allergy may be useful targets in developing peptide immunotherapy.

Keywords α s1-casein, atopy, cow's milk allergy, cytokine, IL-10, immunodominant, T cell epitope, tolerance

Submitted 16 June 2005; revised 4 November 2005; accepted 22 November 2005

Correspondence:

Els van Hoffen, Department of Dermatology/Allergology, G02.124, UMC Utrecht, PO Box 85500, 3508 GA, Utrecht, The Netherlands.
E-mail: E.vanHoffen@umcutrecht.nl

Introduction

Cow's milk allergy (CMA) is the most common food allergy in young children, affecting 1–2% of all infants. Most children develop clinical tolerance to cow's milk before the age of 3 years. However, approximately 15% of these infants retain their CMA in the second decade of life [1]. Casein is one of the major allergens in cow's milk. Cow's milk casein consists of four proteins: α s1-casein, α s2-casein, β -casein and κ -casein, representing 32%, 10%, 28% and

10% of the total milk protein, respectively [2]. α s1-Casein, a single-chain linear phosphoprotein of 199 AA residues, is the most abundant protein in cow's milk and is thought to be the most potent among all casein proteins in inducing a specific IgE response [3]. This protein has only a small amount of secondary structure (α -helices or β -sheets) and lacks disulfide bonds, resulting in a reduction of tertiary interactions [4]. IgE- and IgG-binding regions have been identified in α s1-casein. Interestingly, Chatchatee et al. [5] showed differences in epitopes recognized by IgE of children with persistent CMA compared with children with transient CMA. Inversely, IgG-binding epitopes revealed no

¹Both authors contributed equally to this manuscript.

discrimination between these groups. Differences in IgE-binding epitopes were also found for other casein proteins and were suggested to be a diagnostic tool to screen for persistent CMA [6].

The isotype switching of antigen-specific B cells to IgE is controlled by T cells that play a key role in the initiation of allergic symptoms, as well as in tolerance induction. The difference in IgE-binding epitope recognition between children with persistent and transient CMA, as well as immunological differences between CMA and tolerant subjects, may therefore be reflected in the T cell responses. To date, T cell epitopes in α s1-casein have only been described in allergic individuals. Nakajima-Adachi et al. [7] used synthetic peptides spanning the α s1-casein molecule to find T cell epitopes recognized by five T cell lines (TCLs) from two subjects with CMA. Elsayed et al. [8] used a similar approach, but found different epitopes. However, these data were limited to only one TCL.

Besides T cell epitope recognition *per se*, also cytokine production in response to α s1-casein-derived epitopes may be different between CMA and non-CMA subjects. Our group has previously demonstrated that T cell clones isolated from infants with CMA produce higher amounts of IL-4, IL-5 and IL-13 upon stimulation with cow's milk proteins than T cells from atopic infants without CMA [9]. Tiemessen et al. [10] showed the same Th2-skewed phenotype in T cell clones from children with persistent CMA. Surprisingly, T cells from atopic children without CMA produced higher levels of IL-10 after cow's milk-specific stimulation than T cells from children with persistent CMA and non-atopic controls. This suggests that T cells from pre-disposed children need to up-regulate IL-10 production to create a tolerogenic microenvironment and thereby maintain tolerance to cow's milk proteins. It is not known whether this increased IL-10 production is induced by distinct T cell epitopes.

The aim of the present study was to define immunodominant T cell epitopes in α s1-casein and to evaluate whether specific epitope recognition and cytokine production are linked to allergy or tolerance. T cell responses were investigated in children with CMA, atopic children who were tolerant to cow's milk, and non-atopic children. Polyclonal casein-specific TCLs were used to determine T cell proliferation and cytokine production in response to whole α s1-casein, and to overlapping synthetic peptides spanning the entire α s1-casein molecule.

Methods

Allergic, atopic and non-atopic subjects

Eleven children with CMA (age: 0.3–14 years, mean 4.6), nine atopic children without CMA (age: 0.5–9.6 years, mean 3.8) and nine non-atopic controls (age: 0.5–10.1 years, mean 4.1) were included in this study. Most of these

children have been described previously [9, 10]. CMA was diagnosed by a positive skin prick test, a positive double-blind placebo-controlled food challenge with cow's milk and positive serum IgE levels specific for cow's milk, as determined by CAP System FEIA (Pharmacia Diagnostics, Uppsala, Sweden). Moreover, positive serum IgE levels specific for α s1-casein in children with CMA were detected by ELISA. Five children in the CMA group were allergic at the time of blood sampling, but later became tolerant to cow's milk. These children were, therefore, considered transiently cow's milk allergic. The other six children in this group were still allergic by the age of 6 years and were considered persistently allergic. In the atopic group, four children had a history of CMA but had become tolerant to cow's milk before the blood sample used in this study was taken. The remaining five children had a diagnosed food allergy, but no history of CMA. The non-atopic controls had no elevated serum IgE levels and no clinical or family history of atopy or allergy. Informed consent was obtained, and the study was approved by the Medical Ethical Committee of the University Medical Center, Utrecht.

Preparation and culture of cow's milk-specific T cell lines

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

Peptide synthesis

T cell epitope mapping was performed with 36 C-amidated peptides with a length of 18 AAs (Thermohybid, Ulm, Germany). The peptides were synthesized using the 9-fluorenyl-methoxycarbonyl technique and purified to > 95% by HPLC, according to the manufacturer's instruction. Of the 36 peptides, 32 peptides had a 12 AAs overlap and spanned the sequence of the most common variant B of the α s1-casein molecule (199 AAs). The post-translational phosphorylation on positions 46, 48, 64, 66, 67, 68, 75 and 115 was included. The remaining four peptides

were non-phosphorylated versions that covered the AA sequence 49–84 (Fig. 1).

T cell proliferation and cytokine release

To determine casein- or peptide-specific T cell proliferation and cytokine release, LSTs were performed in triplicate in 96-well U-bottom plates (Greiner, Frickenhausen, Germany) at 37 °C in a humidified 5% CO₂ atmosphere. These tests were carried out at the start of a re-stimulation cycle of the T cells in culture. Each well contained 3×10^4 T cells and 4×10^4 irradiated autologous EBV-B cells that had been pre-incubated overnight with either total casein, α s1-casein, α s2-casein, β -casein, κ -casein (all provided by Dr R. Floris, NIZO Food Research) or a synthetic peptide. All caseins and peptides were free of LPS-contamination and were tested at a final concentration of 1 μ M. Control cultures of TCLs and EBV-B cells without antigen were prepared in parallel to determine background levels of proliferation and cytokine production. After 24 h of culture, supernatants of TCL cultures were collected and stored at –20 °C. Tritiated thymidine ([³H]-TdR, 1 μ Ci/well; Amersham, Aylesbury, UK) was added to measure proliferation, and the cells were harvested after 18 h. [³H]-TdR incorporation was determined by a 1205 Betaplate counter (Wallac, Turku, Finland) and is expressed as counts per minute (cpm). Cytokine levels were measured in TCL culture supernatants by means of ELISA, according to the manufacturer's recommendations (IL-10, IL-13 and IFN- γ ; Sanquin, Amsterdam, the Netherlands). The detection limit was 2.3 pg/mL for IL-10, 1.2 pg/mL for IL-13 and 3.1 pg/mL for IFN- γ .

Statistical analysis

Background values were subtracted from antigen-specific proliferation and cytokine data, except for calculation of the stimulation indices (SIs). The SI of T cell proliferation to α s1-casein and the synthetic peptides was calculated as ratio between cpm measured in T cell cultures stimulated with α s1-casein or peptide (antigen-specific proliferation)

1 10 20 30 40
RPKHPKHQGLPQEVNENLLRFFVAPFPEVFGKEKVNEL
50 60 70 80
SKDIGSESTEDQAMEDIKQMEAESISSSEIIVPNSVEQKH
90 100 110 120
IQKEDVPSERYLGYLEQLRLKKYKVPQLEIVPNSAEERL
130 140 150 160
HSMKEGIHAQQKEPMIGVNQELAYFYPELFQFYQLDAYP
170 180 190 199
SGAWYYYVPLGTQYTDAPSFSDIPNPIGSENSEKTTMPLW

Fig. 1. Amino acid (AA) sequence of the most common variant B of α s1-casein. Underlined are the serine residues phosphorylated in the native protein. The serine residues at positions 64, 66, 67, 68 and 75 were present in the synthetic peptides in both phosphorylated and non-phosphorylated forms.

and cpm determined in T cell cultures without α s1-casein or peptide (background proliferation). A SI > 1.5 was considered positive, because antigen-specific proliferation measured in triplicate was in these cases significantly higher than background proliferation. Cytokine data were normalized by ¹⁰log-transformation. Differences in variance between groups were tested using the Fisher's test. A two-tailed Student's *t*-test was subsequently used to analyse differences in proliferation and cytokine levels. Differences associated with *P*-values < 0.05 were considered to be significant.

Results

Proliferation of T cell lines to the casein proteins

Casein-specific TCLs from 29 subjects were tested for proliferation in response to whole casein and the separate casein proteins, α s1-casein, α s2-casein, β -casein and κ -casein (Fig. 2a). α s1-Casein was shown to induce higher proliferation levels than β -casein (*P* < 0.01) and α s2-casein (*P* < 0.05). No differences in proliferation levels were found between children with CMA, atopic and non-atopic children (data not shown).

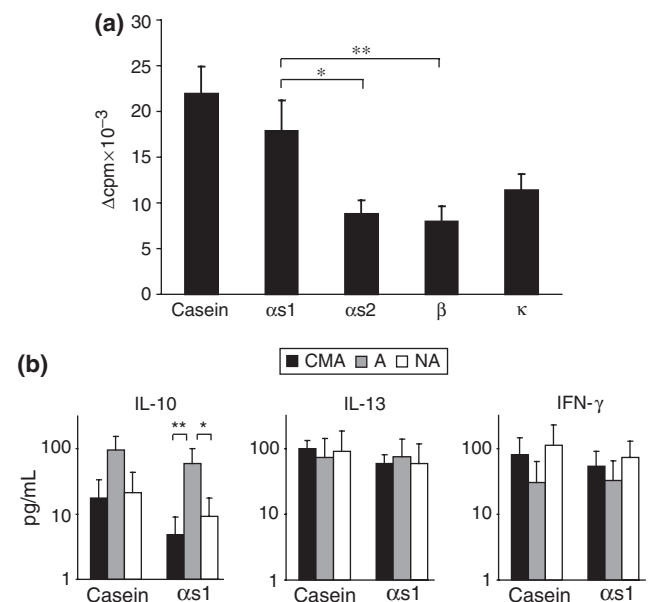


Fig. 2. Proliferation and cytokine responses of T cell lines (TCLs) to casein proteins. (a) Proliferation of TCLs (*n* = 29) in response to whole casein and the four casein proteins. Δ cpm = antigen-specific proliferation – background proliferation (shown as mean $\pm$ SEM). (b) Cytokine production of TCLs in response to whole casein and α s1-casein. The amounts of IL-10, IL-13 and IFN- γ were measured in culture supernatants of TCLs from 11 children with cow's milk allergy (CMA), nine atopic children (A) and nine non-atopic children (NA). Data were normalized using ¹⁰log transformation and are shown as mean $\pm$ SEM. Significant differences are indicated in the graphs (Student's *t*-test, **P* < 0.05, ***P* < 0.01).

Cytokine production induced by whole casein and α s1-casein

The levels of IL-10, IL-13 and IFN- γ secreted by TCLs in response to whole casein and α s1-casein are shown per subject group in Fig. 2b. Whereas levels of IL-13 and IFN- γ were comparable in the CMA, atopic and non-atopic group, a difference was observed in the levels of IL-10. TCLs from atopic children produced higher levels of IL-10 in response to α s1-casein than TCLs from the CMA ($P < 0.01$) and non-atopic ($P < 0.05$) groups.

Proliferation of T cell lines to synthetic peptides spanning α s1-casein

The TCLs were subsequently tested for α s1-casein epitope specificity using a panel of 32 overlapping peptides. Figure 3 shows the recognized peptides per TCL and per subject group. In total, 10 TCLs could be tested from the CMA group, eight TCLs from the atopic group and five TCLs from the non-atopic group. Of the transiently cow's milk allergic subjects no. 7 and 8, TCLs were propagated at both the allergic and tolerant time-points. These subjects were, therefore, included in both the CMA and atopic group.

The magnitude of the SIs induced by α s1-casein and the peptides was comparable between the subject groups. On average, a TCL responded to three peptides, varying from one to six peptides.

Immunogenic peptides spanned AA residues 43–66, 73–96, 91–114 and 127–180. These peptides were recognized by at least three TCLs ($> 10\%$ of the total number of TCLs). The immunodominant region in α s1-casein was defined by AA residues 133–156; in total, 12 TCLs recognized epitopes in this region (52% of all tested TCLs). Three TCLs responded only to AA residues 133–150 and three TCLs to AA residues 139–156. Six TCLs were positive for both peptides and likely recognized epitopes in the overlapping part. Although T cell epitopes were found to be spread over the major part of α s1-casein, the AA sequence at the C-terminus (AA residues 175–199) was not recognized by any TCL.

A subtle difference in epitope recognition could be detected between children with and without CMA: the region spanned by AA residues 55–96 was only recognized by TCLs from children tolerant to cow's milk (atopic and non-atopic). No major differences in epitope recognition were found between persistently (subject no. 1–6) and transiently (no. 7A–10) allergic children in the CMA group. In the atopic group, TCLs from children who had previously become tolerant to cow's milk (no. 7B–11) and children who had a diagnosed food allergy other than CMA (no. 12–16) recognized corresponding epitopes.

A part of the α s1-casein sequence (AA residues 64–68) is highly phosphorylated in the native protein (see Fig. 1). Both phosphorylated and non-phosphorylated forms of

the peptides spanning AA residues 49–84 were tested. The state of phosphorylation did not have a consistent effect on T cell recognition. Therefore, only data obtained with phosphorylated peptides were included in Fig. 3.

Cytokine production induced by peptides spanning α s1-casein

To investigate whether higher levels of IL-10 secreted by TCLs from atopic children in response to whole α s1-casein were induced by certain parts of the α s1-casein protein, the levels of IL-10 were measured after stimulation with either α s1-casein or the peptides that induced proliferation in at least three TCLs. In addition, levels of IL-13 and IFN- γ were determined (Fig. 4a). To evaluate the variation in cytokine levels *per se* induced by the peptides, no differentiation was made between the subject groups. In general, the immunodominant region AA residues 133–156 induced the highest cytokine levels. Despite subtle differences, no particularly high levels of IL-10 could be detected for any peptide. The peptide with AA residues 43–60, which was recognized by TCLs of a relatively large proportion of atopic children (see Fig. 3), induced the highest ratios of IL-10/IL-13 and IL-10/IFN- γ . Regarding the levels of IL-13 and IFN- γ , most peptides evoked a Th0-like response (IL-13 $\approx$ IFN- γ). Although the data varied between the TCLs, peptides with AA residues 43–60, 49–66 and 163–180 seemed to generate a slightly Th2-skewed response (IL-13 $>$ IFN- γ). Two peptides (AA residues 91–108 and 127–144) elicited a Th1-skewed response (IFN- γ $>$ IL-13). IFN- γ levels induced by the peptides with AA residues 43–60 and 49–66 were lower than those evoked by AA residues 91–108 and 127–144 ($P < 0.01$).

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

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

Discussion

This is the first study that investigated T cell epitopes in α s1-casein, using substantial numbers of polyclonal TCLs

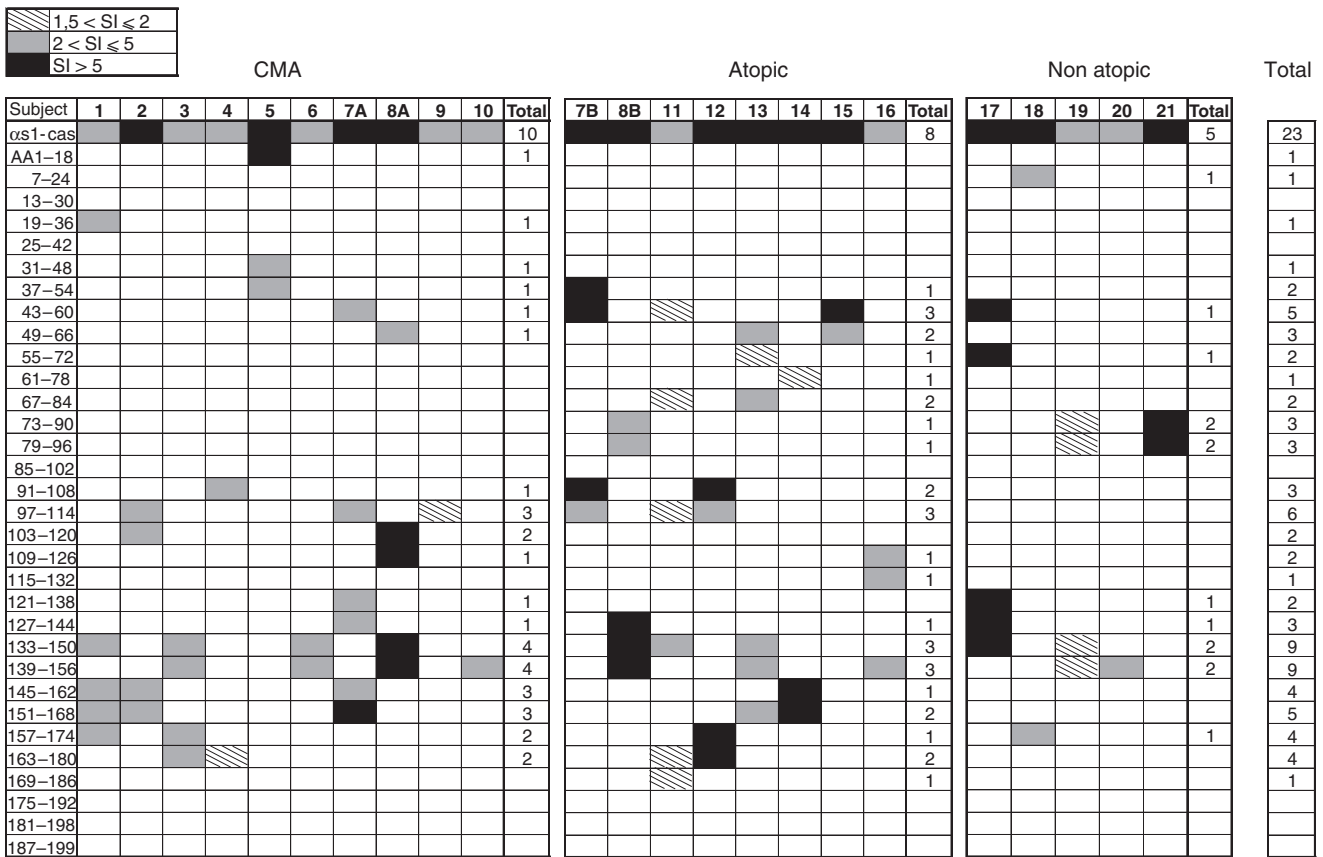


Fig. 3. Proliferation of T cell lines (TCLs) to overlapping synthetic peptides, spanning the α s1-casein molecule. TCLs from 10 children with cow's milk allergy (CMA), eight atopic and five non-atopic children were assessed for their proliferation to α s1-casein and the peptides (18-mers). Peptides inducing a SI > 1.5 are indicated.

from cow's milk allergic, atopic and non-atopic children. Four main regions (AA residues 43–66, 73–96, 91–114 and 127–180) in the α s1-casein molecule were immunogenic to T cells. The immunodominant part (AA residues 133–156) was recognized by 12 out of 23 TCLs (52 %). The immunogenic AA sequences reported in the studies of Nakajima-Adachi et al. [7] (AA residues 31–50, 76–125, 136–170) and Elsayed et al. [8] (AA residues 1–18, 136–155) evoked responses in at least one TCL in the present study, and included the immunodominant sequence. In contrast to these reports, the large number of TCLs enabled us to define more and less immunogenic regions in α s1-casein. No striking differences were found in epitope recognition between the three subject groups. This is in accordance with other studies defining T cell epitopes in allergens in allergic and non-allergic individuals [11–13]. However, T cell epitopes in Ves v 5, a major allergen from wasp venom, differed between vespid-allergic and non-allergic subjects [14]. In the present study, the region covered by AA residues 55–96 was only recognized by T cells from children tolerant to cow's milk (atopic and non-atopic subjects). Although larger groups are needed to confirm these data, this sequence might play a tolerogenic role.

Interestingly, some of the peptides that were not or only slightly immunogenic to TCLs, spanned regions in α s1-casein that contain highly immunogenic IgE-binding epitopes. IgE- and IgG-binding epitopes in α s1-casein were previously identified by Chatchatee et al. [5] using sera from 24 cow's milk allergic children. Although these subjects were different from the children included in the present study, it is interesting to compare the data obtained in these groups of children. Whereas T cell epitopes in subjects with CMA in our study are mainly located in the sequence spanned by AA residues 133–168, IgE-binding regions seem to be more diverse. The AA residues 17–36, 69–78, 109–120, and 173–194 contain hardly any T cell epitopes, but strong IgE-binding epitopes. Two of these regions, namely AA residues 69–78 and 173–194, were only bound by IgE from children with persistent CMA and not by IgE from younger children who were likely to outgrow their allergy [5]. In contrast to these findings, it was not possible to define AA sequences that were differentially recognized by T cells from children with persistent CMA as compared with those with transient CMA in our study.

Although no clear differences were found in epitope recognition between the subject groups, cytokine responses

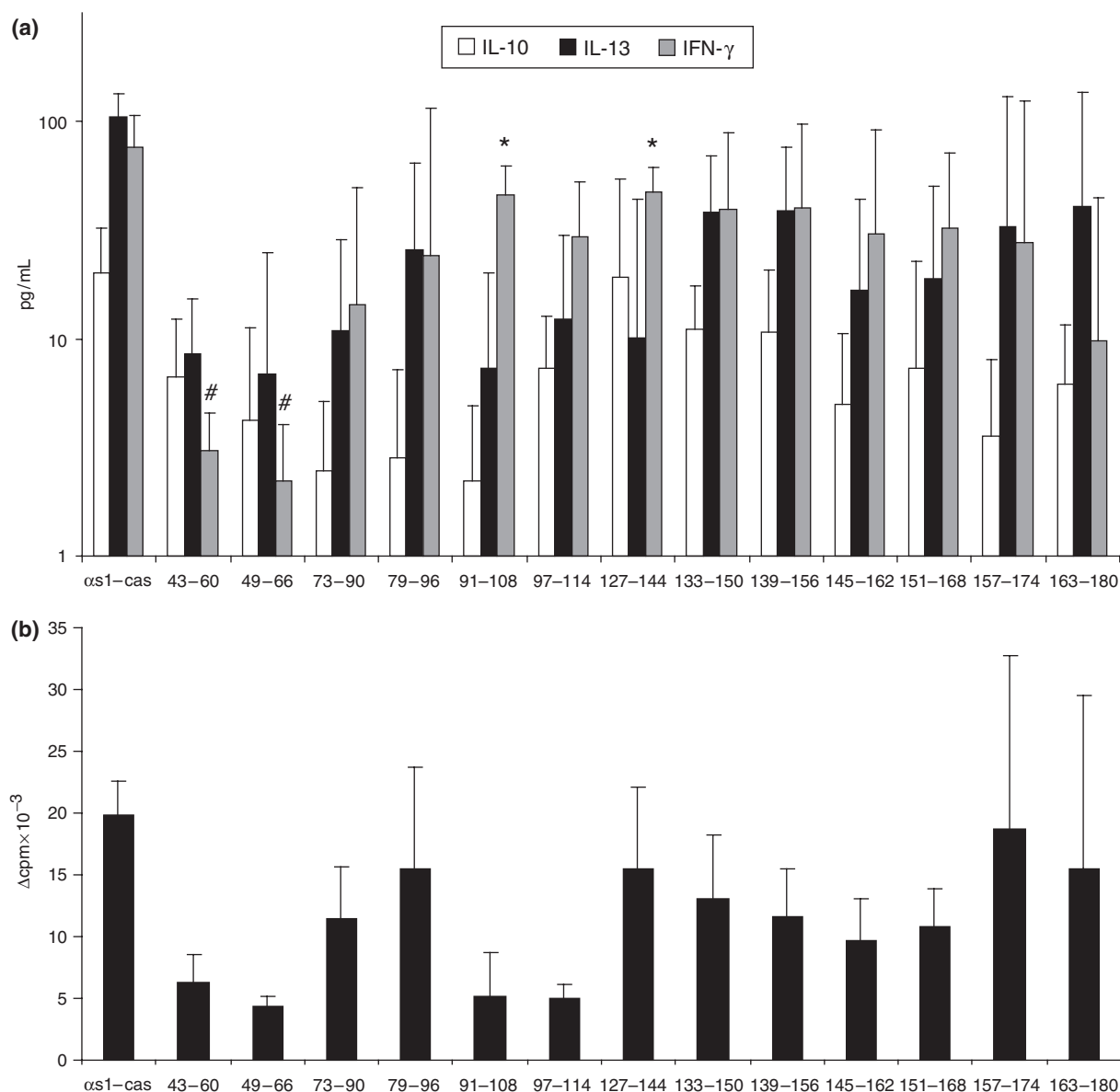


Fig. 4. Cytokine responses and proliferation of T cell lines (TCLs) to the most immunogenic synthetic peptides. (a) Cytokine production in response to synthetic peptides that were recognized by at least three TCLs, irrespective of the subject group. The levels of IL-10, IL-13 and IFN- γ , induced by α s1-casein and by the peptides, were determined in culture supernatants. Data were normalized using $^{10}\log$ transformation and are expressed as mean + SEM. Significant differences are indicated in the graph (Student's *t*-test, * vs. # = *P* < 0.01). (b) Proliferation of these TCLs in response to α s1-casein and to the most immunogenic peptides. Δ cpm = antigen-specific proliferation – background proliferation (shown as mean + SEM).

could well be discriminative. Therefore, levels of IL-10, IL-13 and IFN- γ were measured in TCLs in response to the different casein proteins and to the peptides spanning α s1-casein. IL-13 was preferred to IL-4 as a representative Th2-cytokine, because it acts not only synergistically with IL-4, but also independently in regulation of IgE synthesis [15]. The cytokine data reported in this study confirm and extend the findings of Tiemessen et al. [10]. In the present polyclonal system, α s1-casein induced higher levels of IL-10 in the atopic group compared with the CMA and non-atopic group. Nevertheless, the higher

levels of IL-10 in the atopic group could not be related to recognition of certain IL-10-inducing peptides. Cytokine responses to the region with AA residues 73–96, which was only recognized by T cells from children tolerant to cow's milk, were neither Th1-skewed nor demonstrated high IL-10 levels. Overall, most peptides seemed to elicit a Th0-like cytokine pattern (IFN- γ $\approx$ IL-13), comparable with the responses evoked by whole casein and α s1-casein. Two peptides induced a Th1-skewed (IFN- γ > IL-13) response and three peptides generated a slightly Th2-skewed pattern (IL-13 > IFN- γ). Despite

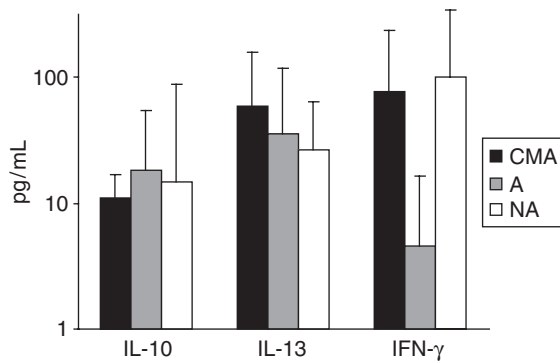


Fig. 5. Cytokine production of T cells in response to the peptides spanning AA residues 133–156. Five T cells from children with CMA, four T cells from atopic children and three T cells from non-atopic children were assessed for their production of IL-10, IL-13 and IFN- γ in response to the peptides spanning AA residues 133–150 and 139–156. Data were normalized using $^{10}\log$ transformation and are shown as mean + SEM.

these subtle differences between cytokines relative to each other, no particularly high levels of IL-10, IL-13 or IFN- γ were found for any peptide. Reefer et al. [16] observed that of two peptides spanning the most immunogenic region in the major cat allergen (Fel d 1), one peptide induced strikingly high levels of IL-10 and the other evoked high IFN- γ levels. These responses were seen in PBMCs from both cat-allergic and non-allergic subjects. This discrepancy with our data might be because of intrinsic differences between the inhalation allergen Fel d 1 and the food allergen α s1-casein. However, as the cytokine levels in the present study showed large variation between the T cells and because the numbers of T cells that responded per peptide were limited, unequivocal conclusions cannot be drawn. Future studies testing proliferative and cytokine responses to the peptides in PBMCs of larger subject groups may reveal differences in cytokine levels induced by distinct peptides. The data discussed above suggest that tolerance to α s1-casein in atopic children is mediated by a general induction of IL-10 and not by recognition of distinct IL-10-inducing T cell epitopes. In concordance with these findings, PBMCs from bee venom-allergic subjects receiving immunotherapy (IT) and from non-allergic beekeepers secreted more IL-10 in response to the major allergen (phospholipase A) than PBMCs from allergic patients [17]. Despite differential IL-10 levels, T cells from these three subject groups recognized the same epitopes in the bee venom allergen [11].

With respect to the induction of tolerance in CMA, immunotherapy with short peptides (12–18 AA residues in length) could be considered. Peptide immunotherapy has the advantage that it is not limited to the use of parts of the allergen that do not contain IgE-binding epitopes, as short peptides are not able to cross-link IgE-receptors on mast cells [18]. In humans, immunotherapy with peptides has been reported in bee venom allergy [19] and in cat

allergy [20]. The latter study showed that peptide immunotherapy is associated with increased IL-10 secretion in PBMCs upon antigen-specific stimulation. The possibility of inducing tolerance to α s1-casein with an immunodominant peptide has already been shown in mice [21]. Therefore, multiple dosing with the peptides that were found in this study to be most immunogenic to T cells from CMA subjects, may induce tolerance by an overall enhancement of antigen-specific secretion of IL-10.

In conclusion, T cell epitopes were identified in α s1-casein, the most immunogenic casein protein. The sequence spanned by AA residues 133–156 was found to be immunodominant. No major differences were observed in epitope recognition between allergic and tolerant subjects. T cells from atopic children who are tolerant to cow's milk produce more IL-10 in response to α s1-casein than T cells from allergic or non-atopic children. However, this could not be linked to specific T cell epitopes. The information obtained in this study may be useful for the development of peptide immunotherapy to induce tolerance towards α s1-casein, a major cow's milk allergen.

Acknowledgements

This study was funded in part by Numico Research B.V., Wageningen, the Netherlands. We thank Rogier P. Schade, MD, PhD, Machteld M. Tiemessen, PhD, and Adrie G. van Ieperen-van Dijk, BSc, for inclusion of the subjects and generation of T cells. R. Floris, PhD (NIZO Food Research, Ede, the Netherlands) is acknowledged for the purified cow's milk proteins and the Bloodbank Utrecht for providing us with the human AB plasma. Furthermore, we thank Grada M. van Bleek, PhD, for helpful practical advice, and Henk-Jan Schuurman, PhD, for critically reading the manuscript.

References

- 1 Høst A, Halken S, Jacobsen HP, Estmann A, Mortensen S, Mygil S. The natural course of cow's milk protein allergy/intolerance [abstract]. *J Allergy Clin Immunol* 1997; **99**:S490.
- 2 Wal J-M. Bovine milk allergenicity. *Ann Allergy Asthma Immunol* 2004; **93**:S2–11.
- 3 Bernard H, Créminon C, Yvon M, Wal J-M. Specificity of the human IgE response to the different purified caseins in allergy to cow's milk proteins. *Int Arch Allergy Immunol* 1998; **115**:235–44.
- 4 Kumosinski TF, Brown EM, Farrell Jr HM. Three-dimensional molecular modeling of bovine caseins: α s1-casein. *J Dairy Sci* 1991; **74**:2889–95.
- 5 Chatchatee P, Järvinen K-M, Bardina L, Beyer K, Sampson HA. Identification of IgE- and IgG-binding epitopes on α s1-casein: differences in patients with persistent and transient cow's milk allergy. *J Allergy Clin Immunol* 2001; **107**:379–83.

- 6 Järvinen K-M, Beyer K, Vila L, Chatchatee P, Busse PJ, Sampson HA. B-cell epitopes as a screening instrument for persistent cow's milk allergy. *J Allergy Clin Immunol* 2002; 110:293-97.
- 7 Nakajima-Adachi H, Hachimura S, Ise W *et al*. Determinant analysis of IgE and IgG4 antibodies and T cells specific for bovine α s1-casein from the same patients allergic to cow's milk: existence of α s1-casein-specific B cells and T cells characteristic in cow's milk allergy. *J Allergy Clin Immunol* 1998; 101:660-71.
- 8 Elsayed S, Eriksen J, Oysaed LK, Idsoe R, Hill DJ. T cell recognition pattern of bovine milk α s1-casein and its peptides. *Mol Immunol* 2004; 41:1225-34.
- 9 Schade RP, Van Ieperen-Van Dijk AG, Van Reijssen FC *et al*. Differences in antigen-specific T cell responses between infants with atopic dermatitis with and without cow's milk allergy: relevance of Th2 cytokines. *J Allergy Clin Immunol* 2000; 106:1155-62.
- 10 Tiemessen MM, Van Ieperen-Van Dijk AG, Bruijnzeel-Koomen CAFM, Garssen J, Knol EF, Van Hoffen E. Cow's milk-specific T cell reactivity of children with and without persistent cow's milk allergy: key role for IL-10. *J Allergy Clin Immunol* 2004; 113:932-9.
- 11 Carballido JM, Carballido-Perrig N, Kagi MK *et al*. T cell epitope specificity in human allergic and non-allergic subjects to bee venom phospholipase A2. *J Immunol* 1993; 150:3582-91.
- 12 Ebner C, Schenk S, Najafian N *et al*. Non-allergic individuals recognize the same T cell epitopes of Bet v 1, the major birch pollen allergen, as atopic patients. *J Immunol* 1995; 154:1932-40.
- 13 Haselden BM, Syrigou E, Jones M *et al*. Proliferation and release of IL-5 and IFN γ by peripheral blood mononuclear cells from cat-allergic asthmatics and rhinitics, non-cat-allergic asthmatics, and normal controls to peptides derived from Fel d 1 chain 1. *J Allergy Clin Immunol* 2001; 108:349-56.
- 14 Bohle B, Zwölfer B, Fischer GF *et al*. Characterization of the human T cell response to antigen 5 from *Vespula vulgaris* (Ves v 5). *Clin Exp Allergy* 2005; 35:367-73.
- 15 Punnonen J, Aversa G, Cocks BG *et al*. Interleukin 13 induces interleukin 4-independent IgG4 and IgE synthesis and CD23 expression by human B cells. *Proc Natl Acad Sci USA* 1993; 90:3730-4.
- 16 Reefer AJ, Carneiro RM, Custis NJ *et al*. A role for IL-10-mediated HLA-DR7-restricted T cell-dependent events in development of the modified Th2 response to cat allergen. *J Immunol* 2004; 172:2763-72.
- 17 Akdis CA, Blesken T, Akdis M, Wuthrich B, Blaser K. Role of interleukin 10 in specific immunotherapy. *J Clin Invest* 1998; 102:98-106.
- 18 Kane P, Erickson J, Fewtrell C, Baird B, Holowka D. Cross-linking of IgE-receptor complexes at the cell surface: synthesis and characterization of a long bivalent hapten that is capable of triggering mast cells and rat basophilic leukemia cells. *Mol Immunol* 1986; 23:783-90.
- 19 Müller U, Akdis CA, Fricker M *et al*. Successful immunotherapy with T cell epitope peptides of bee venom phospholipase A2 induces specific T cell allergy in patients allergic to bee venom. *J Allergy Clin Immunol* 1998; 101:747-54.
- 20 Oldfield WL, Larché M, Kay AB. Effect of T cell peptides derived from Fel d 1 on allergic reactions and cytokine production in patients sensitive to cats: a randomised controlled trial. *Lancet* 2002; 360:47-53.
- 21 Hirahara K, Hisatsune T, Choi CY, Kaminogawa S. Profound immunological tolerance in the antibody response against bovine α s1-casein induced by intradermal administration of a dominant T cell determinant. *Clin Immunol Immunopathol* 1995; 76:12-8.