

Role of Human Leucocyte Antigen DQ in the Presentation of T Cell Epitopes in the Major Cow's Milk Allergen α s1-Casein

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Key Words

Cow's milk allergy • Human leucocyte antigen DQ • T cell epitope • α s1-Casein

Abstract

Background: Little is known about the association between human leucocyte antigen (HLA) and cow's milk allergy (CMA). The aim of the present study was to determine the HLA restriction of T cell clones (TCCs) specific to α s1-casein, the most abundant milk protein, and to study possible HLA class II allele associations with CMA. **Methods:** α s1-Casein-specific TCCs were derived from 6 children with CMA, 9 atopic children without CMA and 5 non-atopic children. T cell epitope specificity was defined by stimulation with overlapping peptides, spanning the α s1-casein molecule. HLA restriction was determined in proliferation assays using antibodies blocking either HLA-DP, HLA-DQ or HLA-DR. HLA genotyping was performed in 32 subjects with CMA, 23 atopic and 22 non-atopic individuals. **Results:** Ten TCCs were restricted to HLA-DQ, 6 TCCs to HLA-DR and 4 TCCs to HLA-DP. The sequence in α s1-casein that was most immunogenic to T cells from children with CMA contained T cell epitopes restricted to DQB1*0201, DPB1*0401 and DRB1*1501. The DQB1*0501 allele frequency was lower in children with CMA

than in non-atopic children, but this difference could not be confirmed in an additional group of subjects with and without CMA. **Conclusions:** HLA-DQ plays a substantial role in the presentation of T cell epitopes in α s1-casein. However, HLA class II allele frequencies do not show major differences between cow's milk allergic, atopic and non-atopic subjects. T cell epitopes in the most immunogenic region are presented by various abundantly present HLA genotypes. Therefore, this sequence may be a suitable target for peptide immunotherapy.

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Introduction

Human leucocyte antigen (HLA) genotypes determine the repertoire of presented peptides to T cells. The HLA-DP, HLA-DQ and HLA-DR genes encode major histocompatibility complex II (MHC II) molecules which are present on the membrane of antigen-presenting cells (APCs). MHC II molecules present peptides from proteins that are internalized by APCs, such as allergens. HLA-DR molecules are generally known to be most abundantly present on the APC membrane, followed by HLA-DP, whereas expression of HLA-DQ is even lower [1]. In

the early 1980s, Marsh et al. [2] observed an association between an MHC-II-encoding HLA genotype and the immune response to the short ragweed pollen allergen Amb a 5 (Ra5). Since then, several HLA genotypes have been shown to be associated with respiratory and food allergies [3–6]. The correlation between an HLA genotype and sensitization to an allergen is strongest if the allergen contains a single immunodominant T cell epitope, as shown for the major mugwort pollen allergen Art v 1 [7]. To date, only one study reported an association of a particular HLA genotype with cow's milk allergy (CMA), being HLA-DQB1*0301/0304 (the HLA-DQ7 serotype) [8]. To estimate the functional relevance of an association between an HLA genotype and an allergic phenotype, it is necessary to determine the HLA restriction of the most prominent T cell epitopes in the allergen(s). Previously, a predominant HLA-DR restriction was found for T cell epitopes in the respiratory allergens cat dander [9] and grass pollen (Phl p 5) [10], as well as in the food allergens ovalbumin [11], peanut [12] and β -lactoglobulin, a cow's milk allergen [13].

The most abundant protein in cow's milk is α s1-casein, which represents 32% of total milk protein [14]. α s1-Casein is also the most allergenic casein protein [15]. Allergen-specific T cells play a key role in either an allergic or tolerant response towards cow's milk. Recently, we defined T cell epitopes in α s1-casein in polyclonal T cell lines from children with CMA, atopic children who were tolerant to cow's milk, and non-atopic children [16]. So far, it is not known which HLA genotypes determine the presentation of these epitopes. Therefore, the aim of the present study was to assess the individual contribution of HLA-DP, HLA-DQ and HLA-DR to the presentation of T cell epitopes in α s1-casein and to define CMA-associated HLA genotypes. α s1-Casein-specific T cell clones (TCCs) of children with CMA, atopic children without CMA and non-atopic children were tested for epitope specificity and HLA restriction. HLA genotyping was performed of these children, as well as adults with CMA, atopic adults sensitized to cow's milk but without allergic symptoms, and non-atopic adults.

Materials and Methods

Subjects

A group of children and a group of adults were included in this study. The group of children consisted of 8 children with persistent CMA (age 6–14 years, mean 8.9), 9 children with transient CMA (age 0.4–1 year, mean 0.7), 13 atopic children without CMA (age 0.4–9.6 years, mean 2.9), and 10 non-atopic children (age

0.6–10.1 years, mean 4.3). Most of these children have been described previously [16–18]. The children in the transient CMA group became tolerant to cow's milk before the age of 4 years. The atopic children without CMA had a diagnosed allergy, but no history of CMA or sensitization to cow's milk. The group of adults was comprised of 15 subjects with CMA (age 26–68 years, mean 42), 10 atopic subjects sensitized to cow's milk but without CMA (age 23–54 years, mean 40), and 12 non-atopic adults (age 23–59 years, mean 31). CMA was diagnosed by a positive skin prick test with cow's milk and/or positive serum IgE levels specific for cow's milk, as determined by CAP System FEIA (Pharmacia Diagnostics, Uppsala, Sweden), and was confirmed by a positive double-blind placebo-controlled food challenge. All children and adults with CMA also had positive plasma IgE levels specific to α s1-casein, as determined by ELISA (unpublished observations). Adults sensitized to cow's milk but without CMA had a positive skin prick test and/or positive specific serum IgE levels, but a negative double-blind placebo-controlled food challenge. The non-atopic controls had no elevated serum IgE levels and no clinical or family history of allergy or atopy. Informed consent was obtained before venous blood samples were taken, and the study was approved by the Medical Ethical Committee of the University Medical Center, Utrecht.

Preparation and Culture of α s1-Casein-Specific TCCs

TCCs specific to α s1-casein were generated and cultured as described previously. Moreover, cytokine profiles and surface markers of the TCCs were determined in previous studies [17–19]. Most of the TCCs described in this study were derived from the cow's milk-specific polyclonal T cell lines that were used previously to define T cell epitopes in α s1-casein [16]. Multiple α s1-casein-specific TCCs could be isolated from all children of whom a TCC is mentioned here. However, TCCs from an individual subject mostly recognized the same synthetic peptides, indicating that the TCCs were likely derived from the same progenitor. Therefore, only one α s1-casein-specific TCC is described per child.

T Cell Proliferation to Synthetic Peptides and Blocking of Antigen Presentation

Overlapping synthetic peptides with a length of 18 amino acid (AA) residues (Thermohybid, Ulm, Germany), which spanned the sequence of the α s1-casein molecule, were used to screen the epitope specificity of the α s1-casein-specific TCCs. The peptides and this procedure have been described previously [16]. T cell proliferation tests were performed in triplicate in 96-well U-bottom plates (Greiner, Frickenhausen, Germany) at 37°C. Each well contained 4×10^4 irradiated autologous Epstein Barr virus transformed B cells (EBV-B cells) as antigen-presenting cells, which have been pre-incubated overnight with 1 μ M of α s1-casein (NIZO Food Research, Ede, The Netherlands) or peptide. To determine the type of MHC II molecules that presented the individual peptides, the EBV-B cells were subsequently incubated for 1 h with a 1:5 dilution of hybridoma culture supernatants containing murine monoclonal antibodies binding to either HLA-DP (B7/21), HLA-DQ (SPV L3) or HLA-DR (B8.11.2) (a gift from Dr. G. van Bleek, University Medical Center, Utrecht, The Netherlands), which have been shown to block antigen presentation [9]. Subsequently, T cells were added (2×10^4 /well). After 24 h of culture, tritiated thymidine (1 μ Ci/well; Amersham, Aylesbury, UK) was added to measure proliferation, and the cells were harvested

after 18 h. Tritiated thymidine incorporation was determined by a 1205 Betaplate counter (Wallac, Türkü, Finland) and is expressed as counts per minute. Control cultures of TCCs and EBV-B cells without antigen were prepared in parallel to determine background levels of proliferation, which ranged from 105 to 2,930 cpm (median 260).

Sequencing-Based Typing of HLA-DPB1, HLA-DQB1 and HLA-DRB1

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

Data Analysis

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

Results

HLA Restriction and Epitope Specificity of α s1-Casein-Specific TCCs

A panel of 32 overlapping synthetic peptides, which spanned the AA sequence of the α s1-casein molecule, was used to determine the epitope specificity and HLA restriction of 20 α s1-casein-specific TCCs. TCCs specific to this protein were generated previously from 6 children with CMA, 9 atopic children without CMA and 5 non-atopic children [17, 18]. In the CMA group, subjects 1–4 were persistently allergic, whereas subjects 5 and 6 had transient CMA. For every TCC, the stimulation index (antigen-specific proliferation/background proliferation) induced by the recognized peptide(s) was at least 7. HLA-blocking antibodies reduced proliferation of the responding TCC by at least 50%. Data from a representative experiment are shown in figure 1a. Antibodies against HLA-DQ, but not HLA-DP or HLA-DR, inhibited proliferation of this TCC by 75%, indicating that the TCC was restricted to HLA-DQ. Figure 1b summarizes the recognized peptides and HLA restriction per TCC and per subject group. Ten TCCs responded to a peptide presented by HLA-DQ, 6 TCCs recognized an HLA-DR- and 4 TCCs an HLA-DP-bound peptide. No major differences in HLA restriction and peptide recognition were observed

between the subject groups. The sequence spanned by AA residues 133–156 was most frequently recognized by the TCCs. In general, the peptide recognition pattern of the TCCs was comparable with that of the T cell lines tested previously [16].

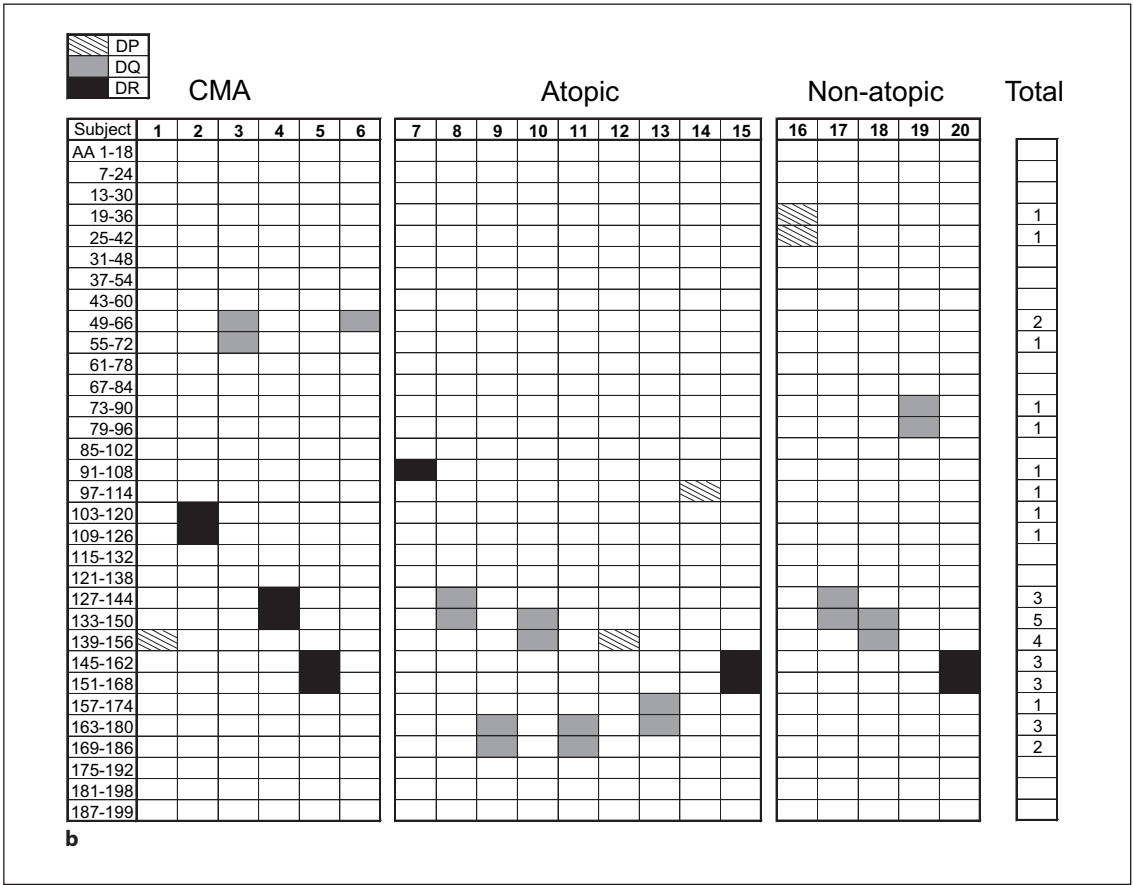
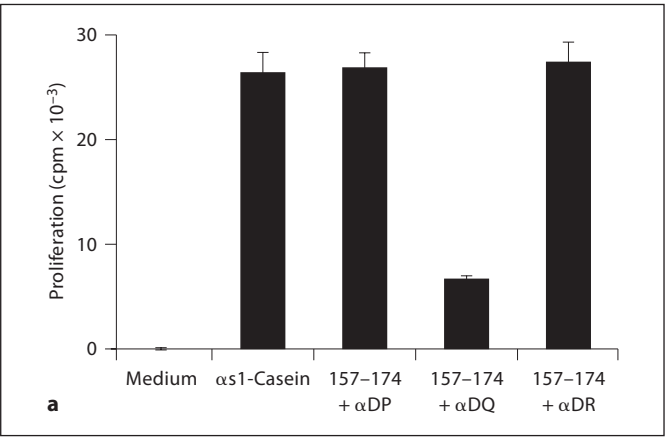
HLA Restriction of TCCs in Relation to HLA Genotypes

The data above indicated that HLA-DQ plays an important role in the presentation of T cell epitopes in α s1-casein. This led us to focus on the HLA genotypes of our children group. Sequencing-based typing of the loci encoding the β -chains of HLA class II (HLA-DPB1, HLA-DQB1 and HLA-DRB1) was performed using genomic DNA from 8 children with persistent CMA, 9 children with transient CMA, 13 atopic children and 10 non-atopic children. These groups included the children of whom an α s1-casein-specific TCC was tested. The epitope specificity and HLA restriction of the TCCs and the HLA genotypes of the corresponding children are summarized in table 1. Because subjects 6 and 8 were homozygous for DQB1*0201, it could be concluded that T cell epitopes present in AA residues 49–66 and 133–144 (the overlapping sequence between the peptides spanning AA residues 127–144 and 133–150) were restricted to this genotype (table 1; fig. 1b). Also the region spanned by AA residues 139–150 likely contained DQB1*0201-restricted epitopes. This sequence was recognized by TCCs from subjects 10 and 18 who were both heterozygous for this genotype. Moreover, a DPB1*0401-restricted epitope was present in AA residues 139–156, which was recognized by TCCs from the homozygous subject 12 and the heterozygous subject 1. Probable HLA restrictions could further be found for T cell epitopes in AA residues 169–180 (DQB1*0202, subjects 9 and 11) and 151–162 (DRB1*1501, subjects 15 and 20).

HLA-DQB1 Allele Frequencies in Cow's Milk Allergic, Atopic and Non-Atopic Children

The frequencies of the HLA-DQB1 alleles in the entire children group and in two reference populations are shown in table 2. The reference populations have been described in literature and consisted of 2,440 Dutch blood donors (RP1) [23] and 1,899 European-American bone marrow donors (RP2) [24], who were non-selected with respect to CMA. The HLA genotype frequencies have been determined by serological methods in RP1 and by sequence-specific DNA typing in RP2. Although numbers of subjects were relatively small in our children group, a significant difference was found in the occur-

Fig. 1. HLA restriction and epitope specificity of α s1-casein-specific T cell clones. **a** Antibodies against HLA-DQ inhibit proliferation of an α s1-casein-specific TCC. The TCC, derived from subject 13, proliferated to whole α s1-casein and to a synthetic peptide spanning AA residues 157–174 of this protein. Antibodies against HLA-DQ, but not HLA-DP or HLA-DR, reduced recognition of the peptide by the TCC and thereby inhibited proliferation (shown as mean cpm + SEM). **b** Epitope specificity and HLA restriction of 20 α s1-casein-specific TCCs. TCCs from 6 children with CMA, 9 atopic children without CMA and 5 non-atopic children were assessed for proliferation to overlapping synthetic peptides (18-mers), spanning the AA sequence of α s1-casein. Four TCCs responded to peptides presented by HLA-DP, 10 TCCs were restricted to HLA-DQ and 6 TCCs to HLA-DR.



rence of the DQB1*0501 genotype in children with CMA compared with non-atopic children, but not with atopic children. This genotype was negatively associated with CMA (persistent CMA vs. non-atopic children, $p = 0.04$; persistent + transient CMA vs. non-atopic children, $p = 0.02$). The DQB1*0501 allele frequency in children with CMA also tended to be lower than in RP2 ($p = 0.07$), and

the total frequency of DQ5 alleles was lower in children with CMA than in RP1 ($p = 0.006$). No significant differences were found between the subject groups for the other HLA-DQB1 alleles. Moreover, allele frequencies of HLA-DPB1 and HLA-DRB1 showed no differences between children with CMA, atopic and non-atopic children (data not shown).

HLA-DQB1 Allele Frequencies in Cow's Milk Allergic, Atopic and Non-Atopic Adults

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

Discussion

For a better comprehension of the immune response towards allergens, it is essential to determine the genetic background underlying this response. To date, little is known about the role of genetic factors in allergy towards cow's milk proteins. In the present study, 20 α s1-casein-specific TCCs, derived from children with CMA, atopic and non-atopic children, were tested for epitope specificity and HLA restriction. The peptides recognized by the TCCs were comparable with those that were immunogenic to the polyclonal T cell lines tested earlier [16] and were not markedly different between the subject groups. Ten TCCs were restricted to HLA-DQ, 6 TCCs to HLA-DR and 4 TCCs to HLA-DP. To our knowledge, this is the first time that a predominant HLA-DQ restriction is shown for T cell epitopes in an allergen, using TCCs from a substantial number of different subjects. This finding is interesting, because the expression of HLA-DQ in APCs is at least 3- and 5-fold lower than that of HLA-DP and HLA-DR, respectively [1]. Previously, expression of HLA-DQ in intestinal epithelium cells was shown to be even lower than in conventional APCs [25]. This renders it unlikely that the predominant HLA-DQ restriction of α s1-casein epitopes is caused by alternative HLA expression at the site of allergen take-up. T cell epitopes in other (food) allergens were previously found to be predominantly restricted to HLA-DR [9–13]. In contrast, the food antigen gliadin is well known for its HLA-DQ-restricted epitopes. Gliadin plays a key role in the pathogenic response leading to coeliac disease [26]. This disease is strongly correlated with the presence of HLA-DQB1*0201,

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

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

¹ Subject numbers correspond to those in figure 1b.

² (Probable) HLA restrictions are given in italics.

DQB1*0202 and DQB1*0302 genotypes, which are involved in the presentation of gliadin-derived T cell epitopes. However, coeliac disease is not an IgE-mediated disorder and is therefore not fully comparable with food allergy.

HLA genotyping of our children group showed that the DQB1*0501 allele was less frequently present in children with persistent or transient CMA than in non-atopic children, which suggested a protective effect (table 1). In line with these findings, DQB1*0501 was reported earlier to be negatively associated with hazelnut allergy [6]. Nevertheless, HLA genotyping in adult subjects revealed no differences in DQB1*0501 allele frequency between adults with CMA and non-atopic adults. This discrepancy between the two age groups is unlikely to be caused by a different mechanism underlying CMA in children

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

Subjects: Alleles (2n):	Persistent CMA 16	Transient CMA 18	Atopic 26	Non- atopic 20	RP1 4,880	RP2 3,798
0201 (DQ2)	25.0	22.2	15.4	20.0	20.8	13.2
0202 (DQ2)	6.3	11.1	7.7	0		11.2
0301 (DQ7)	18.8	27.8	11.5	5.0	15.1	17.9
0302 (DQ8)	12.5	11.1	15.4	10.0	10.7	10.5
0303 (DQ9)	0	0	3.8	0	4.0	4.5
0401 (DQ4)	0	0	0	0	1.6	n.d.
0402 (DQ4)	0	0	0	10.0		2.4
0501 (DQ5)	0*	5.6*	11.5	25.0*	19.1	11.6
0502 (DQ5)	0	0	3.8	0		1.3
0503 (DQ5)	0	0	3.8	0		2.1
0602 (DQ6)	18.8	5.6	19.2	15.0	29.4	14.3
0603 (DQ6)	12.5	11.1	7.7	15.0		5.9
0604 (DQ6)	6.3	0	0	0		3.4

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

* $p = 0.02$, combined CMA groups versus non-atopic children.

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

Subjects: Alleles (2n):	CMA 30	Atopic 20	Non-atopic 24	RP1 4,880	RP2 3,798
0201 (DQ2)	13.3	15.0	12.5	20.8	13.2
0202 (DQ2)	6.7	5.0	0		11.2
0301 (DQ7)	13.3	30.0	12.5	15.1	17.9
0302 (DQ8)	10.0	5.0	4.2	10.7	10.5
0303 (DQ9)	0	5.0	12.5	4.0	4.5
0401 (DQ4)	3.3	0	0	1.6	n.d.
0402 (DQ4)	3.3	10.0	8.3		2.4
0501 (DQ5)	13.3	0	8.3	19.1	11.6
0502 (DQ5)	0	0	0		1.3
0503 (DQ5)	0	5.0	8.3		2.1
0602 (DQ6)	16.7	5.0	12.5	29.4	14.3
0603 (DQ6)	6.7	5.0	8.3		5.9
0604 (DQ6)	10.0	5.0	4.2		3.4

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

and adults, because 2 of the 4 adult CMA subjects carrying the DQB1*0501 allele had become allergic before the age of 3 years. Therefore, these subjects could be considered comparable with children with persistent CMA. Moreover, a difference in age does not influence the re-

sults of genotyping. Although our subject groups were relatively small, these data suggest that CMA is not significantly associated with certain HLA genotypes. The previously reported positive correlation of CMA with HLA-DQB1*0301/0304 (DQ7) [8] could not be confirmed in our study. This association was found by determining population frequencies of HLA-DQ genotypes. In contrast, allele frequencies of HLA-DQB1 genotypes were calculated in the present study, which correct for homo- or heterozygosity. Although a slightly higher DQB1*0301 allele frequency was present in children with CMA compared with RP1 ($p = 0.07$), this effect was completely absent in adults with CMA.

The protein fraction in cow's milk is comprised of at least 6 different antigens, which are all potentially allergenic in individuals with CMA [14]. Like α s1-casein, the other milk proteins probably contain several different T cell epitopes, which can be assumed to be presented by a broad range of HLA types. In this light, the influence of a single DQB1 haplotype on the maintenance of tolerance towards cow's milk can be expected to be moderate. Therefore, a significant association with certain HLA genotypes is less likely for CMA than for allergies that are caused by proteins containing few T cell epitopes, such as the major mugwort pollen allergen [7].

Thinking of potential therapeutic approaches, it could be considered to induce tolerance towards α s1-casein in subjects with CMA by immunotherapy (IT) with short

peptides (12–18 AA residues in length). Peptide IT has been successfully applied in subjects allergic to bee venom [27] and cat allergens [28]. To develop cow's milk-specific peptide IT, it is necessary to identify the epitopes recognized by T cells of subjects with CMA and to assess which of these epitopes are suitable for treating a population of allergic subjects with different HLA genotypes. In our previous work [16] and in the present study, we showed that T cells from children with CMA most frequently recognized α s1-casein epitopes in the region spanned by AA residues 133–168. This sequence contained various T cell epitopes that were presented by several HLA genotypes (fig. 1b; table 1), amongst others DQB1*0201 (AA 133–144, AA 139–150), DPB1*0401 (AA 139–156) and DRB1*1501 (AA 151–162). These genotypes were abundantly present in children with CMA (23.5, 38.2 and 14.7%, respectively; data not shown), as well as in the normal population [24, 29]. Therefore, peptides spanning the α s1-casein AA residues 133–168 seem promising for application of peptide IT in the majority of subjects with CMA. A combination of peptides spanning the most immunogenic sequences of α s1-casein and other important milk allergens, like β -lactoglobulin [13], may well be effective in inducing tolerance to cow's milk in CMA patients.

In conclusion, we found that T cell epitopes in α s1-casein, a major allergen in cow's milk, were predominantly restricted to HLA-DQ. However, no major differences could be found in allele frequencies of HLA genotypes between subjects with CMA, atopic individuals without CMA and non-atopic subjects. The sequence spanned by AA residues 133–168 in α s1-casein is highly immunogenic to T cells from allergic children. Epitopes in this region are presented by various HLA genotypes, which are likely to be abundantly present in subjects with CMA in general. Therefore, this sequence may be a suitable target for peptide IT.

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