

Detection of a κ -casein-specific lymphocyte response in milk-responsive atopic dermatitis

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Summary

Background Bovine casein leads to an expansion of lymphocytes expressing the cutaneous lymphocyte antigen and to specific lymphocyte proliferation in a subgroup of patients with milk-responsive atopic dermatitis (AD). The casein fraction is composed of different proteins with defined and completely different sequences.

Objective To define the stimulatory capacity of the major casein protein (α , β and κ) in lymphocyte proliferation assays with cells from milk-allergic and non-allergic individuals.

Methods Proliferative responses of peripheral blood mononuclear cells to lipopolysaccharide-depleted casein subfractions were measured by thymidine incorporation. Lymphocytes from milk-responsive patients with AD were compared with cells from non-responsive patients with AD and to non-atopic individuals. Atopic individuals with immediate symptoms following consumption of cow's milk were included as positive controls. Casein-specific T-cell clones (TCC) from four patients with milk-responsive AD were restimulated with unfractionated casein and κ -casein.

Results Higher proliferative responses to unfractionated casein and α -, β - and κ -casein were observed in milk-responsive patients compared with non-responders. Unfractionated casein and κ -casein discriminated best between the milk-responsive patients with AD and non-responders. Twenty-five of 31 TCC from patients with milk-responsive AD reacted to the mixed casein preparation and κ -casein.

Conclusion A pronounced κ -casein-specific T-cell-mediated immune response is found in the blood of many patients with a history of milk-related exacerbation of AD.

Keywords: atopic dermatitis, milk allergy, cow milk, casein, κ -casein, T-lymphocyte, lymphocyte proliferation test.

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Introduction

Atopic dermatitis (AD) represents 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. Food antigens can provoke both immediate symptoms and eczema in atopic patients. A series of studies has been performed including diet programmes and oral provocations with various food antigens in patients with AD. Atherton *et al.*

[2] observed a significant improvement of the dermatitis in children during a period of placebo controlled diet. Sampson and McCaskill [3] observed an itching exanthema in a significant percentage of individuals after some hours of exposure following oral food challenge in children. Positive provocation tests correlated with skin-prick tests, with specific IgE to the offending food antigen and with a rise of serum histamine [4,5].

Cutaneous lymphocyte antigen-positive mononuclear cells from children with milk-inducible AD can be expanded *in vitro* with casein [6]. Casein-specific T-cell clones (TCC) were generated from the blood of patients with milk-responsive AD [7,8]. These findings point to a

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possible significance of food-specific T-cell responses in the pathogenic process of AD in these patients. Casein is composed of different protein fractions. In this study we investigated the cellular immune response to the major casein subfractions (α , β and κ) in patients with milk-responsive AD and compared the results with non-responders and patients with immediate symptoms to cow's milk. Our results indicate a pronounced response to the κ -casein fraction in milk-responsive patients with AD.

Materials and methods

Oral provocation

The protocol described by Niggemann *et al.* [9] for double-blind placebo-controlled provocation was modified to evaluate the effects of cow's milk on the late eczematous reaction in AD. A milk-free diet was performed for 3 weeks, followed by 250 ml pasteurized cow's milk or placebo given on three consecutive days. The skin was inspected daily and skin lesions were scored as described [10]. An increase of 30 SCORAD points was considered to be significant. The clinical evaluators who classified the patients into P1 and P2 (see below) were 'blinded' and did not know the results of the *in vitro* tests (radio allergosorbent test (RAST) and lymphocyte proliferation test (CPT)).

Casein-specific antibodies

Casein-specific IgE and casein-specific IgG were determined with the CAP system (Pharmacia, Freiburg, Germany).

Patients and controls

Patients and control individuals were classified into the following four groups.

P1. Patients with AD fulfilling the criteria of Hanifin & Rajka [11] without any deterioration of the skin by milk ingestion. Peripheral blood mononuclear cells (PBMC) of 86 patients from this group (median 21.9 (range 2.5–66.6) years) were investigated with the LPT as described below. No specific IgE to casein was detected in individuals from this group.

P2. Patients with AD who had clearly improved during a milk-free diet and who reacted upon oral provocation with worsening of the eczema ($n = 32$; median 21.9 (range 0.7–47.0) years). Casein-specific IgE (> 0.7 kUI, CAP RAST class ≥ 2) was detected in 31% of this group.

P3. Patients with immediate reactions (gastrointestinal symptoms, urticaria, asthma) to milk were included in this

study as 'positive' control persons ($n = 13$; median 13.3 (range 0.8–44.9) years). Casein-specific IgE (≥ 0.7 kU/l, CAP RAST $\geq$ class 2) was detected in 61% of this group.

C. Non-atopic control persons ($n = 52$; median 30.0 (range 9.1–67.7) years) who did not suffer from AD or hypersensitivity reactions to milk.

Preparation of antigens for cell culture

Casein preparations (Sigma Chemie, Deisenhofen, Germany) were found to be contaminated with lipopolysaccharides (LPS). They were removed from the protein fractions by polymyxin B coupled to a solid phase as described [12]. Briefly, casein fractions were incubated on a shaker for 24 h at 4°C with polymyxin B agarose (Serva, Heidelberg, Germany) in the presence of octyl- β -D-glucopyranoside (Calbiochem-Novabiochem, Bad Soden, Germany). After centrifugation the whole procedure was repeated and the supernatant fluids were dialysed twice against pyrogen-free 0.9% NaCl. Lipopolysaccharide concentrations were determined by the *Limulus* assay (Coatest Endotoxin, Pharmacia) according to the manufacturer's protocol. The detection limit was 50 pg LPS/ml.

Lymphocyte proliferation test

No patient had been receiving steroids systemically 3 weeks before the tests were carried out. Peripheral blood mononuclear cells were isolated from heparinized blood samples by density centrifugation on Ficoll Hypaque (Nycomed Pharma AS, Oslo, Norway) ($d = 1077$ g/ml). Peripheral blood mononuclear cells (2×10^5) were cultured in Iscove's medium (Biochrom KG, Berlin, Germany) supplemented with 4% heat-inactivated AB serum, glutamine, gentamycin, penicillin, streptomycin, and non-essential amino acids in the presence or absence of antigen. If not indicated otherwise food proteins were used at a concentration of 25 μ g/ml. Hexaplicate cultures were performed. After 5 days the cultures were pulsed with ^{3} H-thymidine (0.5 μ Ci/well for 4 h) and the β decay counted in a liquid scintillation counter. The stimulation index (SI) was defined as the ratio of mean counts per minute (cpm) of stimulated to unstimulated cultures.

Preparation of specific T-cell clones from peripheral blood

Cloning was performed by limiting dilution in the presence of antigen and interleukin 2 (IL-2) as described [7,13]. Responder cells from six patients from group P2 and casein (25 μ g/ml) or *Dermatophagoides pteronyssinus* Der p extract (Smith Kline Beecham, Munich, Germany; 3 μ m/ml) were added to

limiting-dilution wells (96-well round bottom trays; Nunc GmbH, Roskilde, Denmark) together with 2×10^4 autologous, antigen-presenting cells (PBMC, irradiated with 55 Gy, ^{37}Cs). The cultures were kept in Iscove's medium supplement with 4% heat-inactivated AB serum, glutamine, gentamycin, penicillin, streptomycin, non-essential amino acids and 10 U/l rIL-2. Frequencies of growing CD3⁺ T-cells were determined by the minimum chi-square method according to Taswell [14]. Since cultures were only expanded from dilutions with 0.03 proliferating cells or less, there was a high probability of clonal cell growth. After 14 d and every 8–10 d thereafter, expansion of the growing cultures was performed in 24-well flat bottom plates (Nunc) with allogenic, irradiated PBMC as feeder cells (1×10^6 cells per well), phytohaemagglutinin (PHA) (10 µg/ml and rIL-2 (20 U/ml) in Iscove's medium. T-cell clones were phenotyped with monoclonal antibodies directed to CD3, CD4, CD8 and TCR2 (α/β) and evaluated by flow cytometry with a FACSCAN (Becton Dickinson, Heidelberg, Germany).

Restimulation assay

For assessment of the antigen specificity of TCC an 8-d interval between the last expansion step was allowed before the cells were used in the restimulation assay. After repeated washings, 5000 T-cells were stimulated with 25 µg/ml casein, κ -casein, *Der p* extract (Smith Kline Beecham, Munich, Germany) or 10 µg/ml PHA (positive control) in the presence of 7.5×10^4 irradiated autologous PBMC. On day 5 the cultures were pulsed with ^3H -thymidine. The β decay was counted in a liquid scintillation counter and results of triplicate cultures were expressed as mean cpm.

Statistical analysis

The effect of LPS depletion on lymphocyte proliferation was evaluated with the Wilcoxon test. The comparison of LPT results of different groups of tested individuals was performed with the Mann–Whitney rank sum test.

Results

Lymphocyte proliferation tests with casein proteins and PBMC from healthy non-atopic control individuals

Contamination with endotoxin was 1 ng/mg casein, 0.5 ng/mg α -casein, 0.2 ng/mg β -casein and 3.5 ng/mg κ -casein as detected by the *Limulus* assay. Since SIs up to 6 were observed with 0.1–10 ng/ml LPS in the LPT performed with PBMC of healthy donors, polymyxin B coupled to a solid phase was used to eliminate LPS. Endotoxin concentrations of the detoxified casein fractions which were finally

used in the LPT were below 5 pg/ml. This amount of LPS did not induce any proliferation of PBMC of healthy donors. As shown in Table 1 significantly lower SIs were obtained when PBMC of healthy individuals were cultured with LPS-depleted casein (mixed fraction) or with κ -casein as compared with crude fractions.

Comparison of LPT results between milk-responsive and non-responsive individuals

When compared with healthy controls (C), significantly higher proliferative responses were obtained in the LPT with casein and its subfractions with PBMC of patients who had shown exacerbation of their dermatitis (group P2) (Fig. 1a). Moreover, differences between results obtained with group P2 compared with group P1 (atopic individuals without evidence of food-aggravated AD) were evident as shown in Fig. 1(b). The best statistical discrimination between P2 and the control groups C or P1 was obtained when the effects of casein (mixed fraction) or of κ -casein were compared. As described for the mixed casein fraction [7] there was no correlation between the level of casein-specific serum IgE and the proliferative response to any subfraction (not shown). Moreover, we did not detect significant differences in the levels of circulating casein-specific IgG in the sera of individuals from group C or P2 (Table 2).

Significantly higher proliferation was also observed with PBMC of the positive control group P3 in response to the

Table 1. Comparison of the effects of crude and lipopolysaccharide-depleted casein fractions in the lymphocyte proliferation test (LPT)

Protein	Crude fractions	Purified fractions	$n\ddagger$	$P\text{\S}$
Casein	$2.93 \pm 0.65^{*\ddagger}$	1.34 ± 0.18	22	<0.005
α -casein	1.29 ± 0.17	1.50 ± 0.22	21	N.S.
β -casein	2.27 ± 0.37	2.12 ± 0.21	22	N.S.
κ -casein	2.56 ± 0.48	1.31 ± 0.24	22	<0.005

*Peripheral blood lymphocytes of non-atopic healthy donors were incubated for 5 ds in the absence or presence of casein. The proliferation was then determined by the incorporation of labelled thymidine. Purified fractions had been incubated twice with solid phase-bound polymyxin B before being used in the stimulation assay. Counts per minute in the negative control (medium only) were 602 ± 150 .

$\ddagger$ Stimulation index mean $\pm$ S.E.M.

$\ddagger$ Number of LPTs.

$\text{\S}$ Wilcoxon test.

N.S., Not significant.

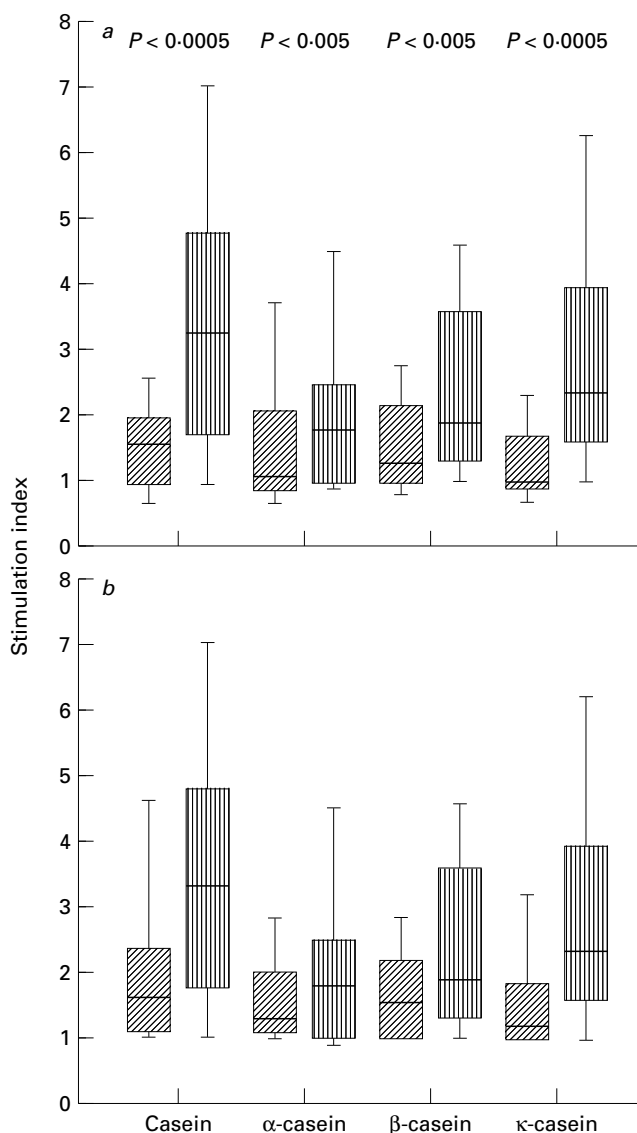


Fig. 1. Lymphocyte proliferation test (LPT) results with lipopolysaccharide (LPS)-depleted casein and its subfractions. The LPT was performed with peripheral blood mononuclear cells (PBMC) of patients and control individuals using LPS-depleted casein and its subfraction as stimuli. Stimulation indices obtained with PBMC of different groups of patients and control individuals are shown. Counts per minute of the negative controls were 819 ± 199 in group C, 891 ± 243 in P1 and 796 ± 176 in P2. (a) LPT results obtained with PBMC of atopic dermatitis (AD) patients showing worsening following oral provocation with milk (P2) (■) as compared with non-atopic controls (C) (▨). (b) LPT results obtained with PBMC of P2 (■) as compared with patients with AD not responding to milk (P1) (▨). Boxes contain 50% of all results, vertical lines display the 90% range of the results and horizontal lines show the median of the results. For statistical analysis, the Mann-Whitney-Wilcoxon test was performed.

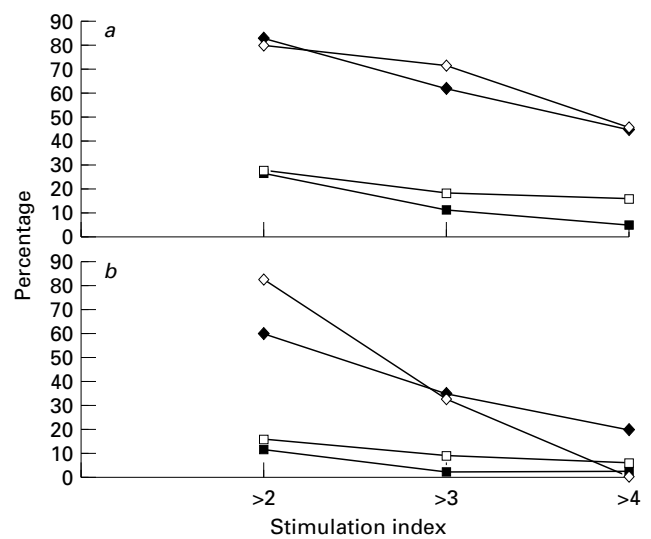


Fig. 2. Lymphocyte proliferation test (LPT) results with lipopolysaccharide (LPS)-depleted casein and κ-casein. The LPT was performed with peripheral blood mononuclear cells of patients and control individuals using LPS-depleted casein and κ-casein as stimuli. The percentages of all individuals of a given group tested with the LPT who responded with a stimulation index >2 are shown. Counts per minute of the medium control were 819 ± 199 in group C, 891 ± 243 in P1, 796 ± 176 in P2 and 815 ± 301 in P3. (a) Casein (mixed fraction (25 μg/ml); (b) κ-casein (25 μg/ml). (c) Non-atopic controls (■); P1 atopic dermatitis (AD) not worsening following challenge (□); P2, AD worsening following challenge with milk (◆) P3, immediate symptoms after ingestion of milk (◇).

mixed casein preparation and the subfractions compared with P1 or C in the LPT ($P < 0.01$): median SIs in P3 were 3.3 for the mixed casein fraction, 3.4 for α-casein, 2.8 for β-casein and 2.8 for κ-casein. The rate of all patients (P1, P2 and P3) and control subjects (C) whose lymphocytes reacted to casein (mixed fraction) or κ-casein with SIs >2 in the LPT is shown in Fig. 2. From these data the sensitivity of the LPT (SI > 2) in detecting milk-responsive patients (groups P2 and P3) was calculated to be 70% with casein and 67% with κ-casein. The specificity was 77% with both

Table 2. Casein-specific IgG values of patients (P2) and control individuals (C)

Group	Median (μg/ml)	25–75% percentiles
P2	3.18	1.95–5.33
C	3.46	1.53–4.30

Casein-specific IgG was determined with the CAP system. The difference between P2 and C was not significant ($P = 0.84$).

protein fractions. The specificities were $>70\%$ with α - or β -caseins and thus in the same range as with the mixed casein fraction or κ -casein. In contrast, the sensitivities of the LPT (SI > 2) with α - and β -caseins were below 50%.

Chicken egg conalbumin served as negative control and tetanus toxoid as positive control in the LPT. No significant differences in the proliferation of PBMC from the four groups were detected with the antigens: median values for the SI were 1.5 (C), 1.5 (P1), 1.6 (P2) and 1.3 (P3) with conalbumin and 14.4 (C), 19.5 (P1), 22.0 (P2) and 16.8 (P3) with tetanus toxoid.

Restimulation of casein-specific T-cell clones with κ -casein

Thirty-one $CD3^+ TCR2(\alpha/\beta)^+$ casein-specific TCC which were from adult patients from group P2 [7] were restimulated with casein and κ -casein in this study. Twenty-five of these TCC were positive (SI > 5) in both test systems (Fig. 3, Table 3). Reactivity to κ -casein was observable both in $CD4^+$ and $CD8^+$ TCC (Fig. 3, Table 2). Fifteen *Der p*-specific TCC from the same patients served as negative controls. No specific proliferation was detected upon stimulation with casein and κ -casein with these cells (not shown), thus excluding a mitogenic effect of casein preparations on TCC.

Discussion

Worsening of AD by food may be associated with a food-specific, T-cell-mediated immune response which is triggered by ingested food antigens. In favour of this hypothesis it was recently found that the extravasation of casein-specific T-lymphocytes into lesional skin of AD might be mediated by the appearance of specific homing receptors on their membranes such as the cutaneous lymphocyte antigen [6]. Casein is the major protein fraction of cow's milk and the main protein source for many individuals. We recently observed a casein-specific response both in children and adults with clinical reactions to cow's milk [8]. The present study focuses on the more precise cellular immune response to bovine casein and its subfractions of patients with milk-responsive AD.

Significant differences in the proliferative response of blood lymphocytes between patients who reacted to milk with worsening of AD or with immediate symptoms and the control groups indicate a casein-specific cellular immune response in milk-responsive patients. Interestingly, the proliferative response of circulating lymphocytes to casein and the serum levels of casein-specific IgG or IgE did not correlate. This may be due to the compartmentalization of the specific immune response. Casein-specific antibodies are produced and secreted mainly in lymphoid organs in the presence of specific T-cells. These T-cells may well belong

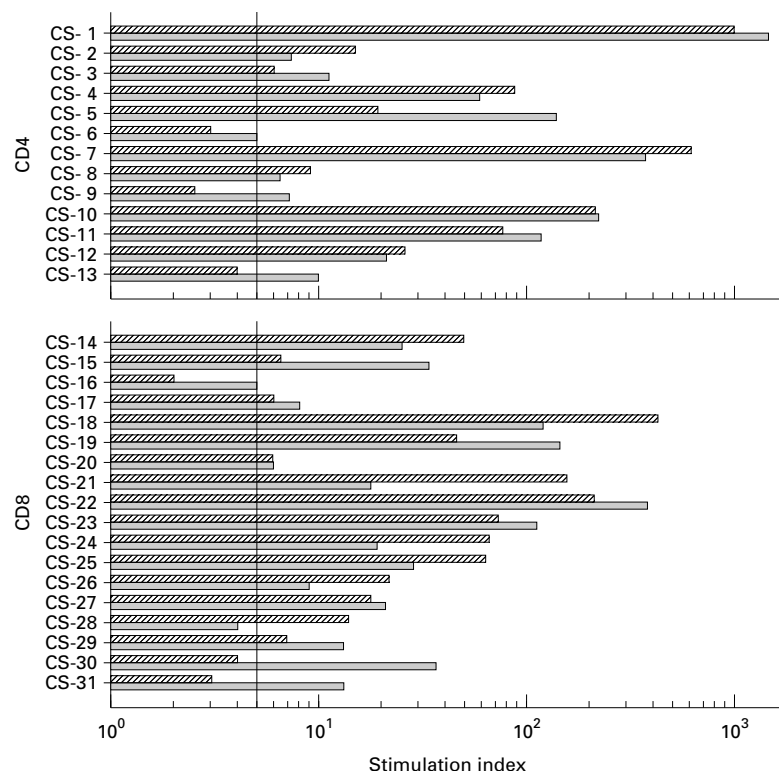


Fig. 3. Restimulation assay. $CD3^+ TCR2^+$ casein-specific T-cell clones (TCC) from patients with milk-responsive atopic dermatitis were restimulated with $25 \mu\text{g/ml}$ of the mixed casein (▨) or the κ -casein fraction (▩). The tests were performed with TCC and autologous irradiated peripheral blood mononuclear cells as described in Materials and methods. CS x indicates the results obtained with casein-specific T cell clone x. A stimulation index > 5 was considered as positive. Counts per minute of the negative controls were 184 ± 140 .

Table 3. Restimulation assay with casein, κ -casein and *Dermatophagoides pteronyssinus* (*Der p*) with casein-specific T-cell clones (TCC)

Name of TCC	Medium alone	Casein	κ -casein	<i>Der p</i>	PHA
CS-4	35 $\pm$ 18*†	2121 $\pm$ 1437	3125 $\pm$ 1033	NT	131 189 $\pm$ 20 124
CS-5	74 $\pm$ 9	10 434 $\pm$ 523	1406 $\pm$ 649	45 $\pm$ 20	NT
CS-7	66 $\pm$ 53*	25 086 $\pm$ 840	41 290 $\pm$ 4522	NT	99 682 $\pm$ 14 644
CS-23	85 $\pm$ 18	9690 $\pm$ 971	6437 $\pm$ 710	199 $\pm$ 93	NT

* Representative results of a restimulation assay performed with TCC, autologous irradiated peripheral blood mononuclear cells and the given stimuli.

† Mean counts per minute $\pm$ SD.

PHA = phytohaemagglutinin; NT = not tested

to different subgroups to the circulating and possibly skin-infiltrating casein-specific T-cells in AD which have been studied here and previously been shown to express the cutaneous lymphocyte antigen [6]. The most pronounced differences between milk-responsive patients with AD and control individuals could be induced by the 'mixed' and κ -casein fraction in the LPT. κ -casein is composed of 169 residues and has a molecular weight of 18 kD. It is the only glycosylated casein and contains various amounts and types of carbohydrates [15]. It is the only major casein component which is soluble in cow's milk in the presence of natural concentrations of Ca^{2+} . Since only 9% of the total milk protein consists of κ -casein this result suggests that κ -casein is highly immunogenic in patients with milk-responsive AD.

Patients with immediate symptoms of hypersensitivity were included as the positive control group in this study. Their PBMC proliferated well in the presence of all casein fractions. In this group we did not detect a pronounced response to κ -casein or any other casein subfraction.

The specificity and sensitivity of the LPT in milk allergy have been investigated previously by other groups. To our knowledge, only two studies have so far focused on different casein fractions. Räsänen *et al.* [16] reported a high sensitivity and diagnostic specificity for the LPT with an α -casein fraction which had been derived from a different source from the α -casein used in our study. In contrast to our findings blood lymphocytes from milk-reactive patients with AD and control individuals proliferated equally well in response to a mixed casein fraction in another study [6]. However, high concentrations of casein had been used in the LPT and contaminating LPS may have led to unspecific proliferation of PBMC of control donors. As LPS substantially influences the specificity of the LPT, its removal from protein antigens appears to be essential.

In order to study casein-specific T-cell responses in milk-responsive patients with AD in more detail we have generated a number of specific TCC from the blood of these

patients. A protocol was used which had been established for the generation of specific TCC reactive to inhalant allergens [13]. Our previous results indicated that casein-specific T-cells circulate in high frequencies in the blood of patients with milk-responsive AD and that the majority of casein-specific TCC produces IFN- γ upon stimulation either in combination with IL-4 (type 0 cytokine pattern) or alone (type 1 cytokine pattern) [7,8]. Similar cytokine patterns have been described for house dust mite-specific TCC from the blood of atopic patients whereas a preferential type 2 pattern has been found in patch test reactions in a model of 'acute' eczema [13]. In this study we detected proliferative responses to κ -casein in the majority of casein-specific TCC. A mitogenic effect of casein was excluded since *Der p*-specific TCC from the same patients did not respond to casein at all. Our findings underline the pathophysiological importance of the bovine κ -casein subfraction in patients with milk-responsive AD.

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