

Milk-responsive atopic dermatitis is associated with a casein-specific lymphocyte response in adolescent and adult patients

Thomas Werfel, MD,^a Gudrun Ahlers, MD,^a Petra Schmidt,^a
Martin Boeker, MD,^a Alexander Kapp, PhD,^a and Christine Neumann, PhD^b
Hannover and Göttingen, Germany

Background: Specific T-cell responses to food antigens have been described in children with atopic dermatitis (AD). However, a subgroup of adolescent and adult patients still experiences food-responsive AD.

Objective: This study was designed to evaluate lymphocyte responses to bovine casein in adult patients with AD.

Methods: The stimulatory capacity of lipopolysaccharide-depleted bovine casein was tested in proliferation assays and in limiting dilution assays. Casein-specific T-cell clones (TCCs) were generated and characterized.

Results: Higher proliferative responses to casein and modulation of cytokine receptors were observed in patients with milk-responsive AD compared with individuals without clinical reactions on milk ingestion. The results did not correlate with the amount of casein-specific IgE in the serum. The frequencies of T cells that grew in the presence of casein or house dust mite antigens were similar. Only 27% of CD4⁺ casein-specific TCCs from these patients, but the majority of house dust mite-specific TCCs, produced IL-4 on mitogen stimulation.

Interferon- γ , on the other hand, was produced by the majority of TCCs with both specificities.

Conclusion: A specific T-cell-mediated immune response to casein can be found in the blood of adolescent and adult patients with milk-related exacerbation of AD. In contrast to house dust mite-specific T cells, casein-specific T cells of adult patients who respond to cow's milk with worsening of AD produce little or no IL-4. (*J Allergy Clin Immunol* 1997;99:124-33.)

Key words: Atopic dermatitis, eczema, skin, T cells, casein, milk, cytokines, IL-4, interferon- γ

Atopic dermatitis (AD)¹ is a chronic eczematous skin disease, which often begins early in infancy and runs a course of remissions and exacerbations. At present, the pathogenesis of AD is not fully understood. Besides basophils, eosinophils, and cells of the mononuclear phagocyte lineage, skin lesions contain mainly CD4⁺ T lymphocytes.¹⁻³ Furthermore, studies of atopy patch test reactions have indicated that inhalant allergens may provoke

Abbreviations used

AD:	Atopic dermatitis
IFN:	Interferon
LPS:	Lipopolysaccharide
LTT:	Lymphocyte transformation test
mAb:	Monoclonal antibody
PBMCs:	Peripheral blood mononuclear cells
SI:	Stimulation index
TCC:	T-cell clone
TNF:	Tumor necrosis factor

From ^athe Department of Dermatology, Hannover Medical School; and ^bthe Department of Dermatology, University of Göttingen.

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Reprint requests: Thomas Werfel, MD, Department of Dermatology, Medical School Hannover, Ricklinger Str. 5, D-30449 Hannover, Germany.

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a cellular immune response in atopic skin, which is dominated by specific T_{H2} cells.^{4,5}

Food allergy seen with urticaria, asthma, and gastrointestinal symptoms is more often found in atopic children than in adolescents or adults. This has led to the assumption that eczematous skin lesions of atopic individuals might be provoked by

food antigens as well. A series of studies has been performed, including diet programs and oral provocations with various food antigens, in children with AD. Atherton et al.⁶ observed a significant improvement in atopic dermatitis in children during a period of placebo-controlled diet. Sampson and McCaskill⁷ observed cutaneous symptoms in a significant percentage of children with AD after some hours of exposure on oral provocation. Positive provocation test results correlated with skin prick test results, with specific IgE to the offending food antigen and with a rise of serum histamine. It was postulated that the repeated ingestion of food allergens by patients with AD and food allergy lead to a chronic inflammation and pruritus, which provokes frequent scratching and the consequent trauma-induced lichenified lesions.^{8,9}

Delayed eczematous reactions have been reported in other studies in which the suspected allergen was administered daily for several days.¹⁰⁻¹² Hill et al.¹² observed a delayed eczematous reaction (i.e., 24 hours or later after challenge) in 17 of 135 children with suspected cow's milk allergy. Ten of these 17 children had negative skin prick test responses to cow's milk antigens, suggesting a non-IgE-mediated pathogenesis.

If food allergens have a pathogenetic role in AD, food allergen-reactive T cells should be involved. Previously, Kondo et al.¹³ described a positive correlation of oral provocation tests with egg and milk with specific proliferative reactions of CD4⁺ blood T lymphocytes to ovalbumin or bovine serum albumin.¹³⁻¹⁵ In these studies T-cell responses did not correlate with specific IgE to the corresponding antigens. The previous finding that cutaneous lymphocyte antigen-positive mononuclear cells from children with milk-inducible AD can be expanded in vitro with casein¹⁶ points to a possible significance of food-specific T-cell responses in the pathogenesis of AD in these patients.

Until now, food-induced AD was mainly studied in infants and children,⁶⁻¹⁶ although many adolescent and adult patients with AD attribute worsening of their skin disease to the intake of certain foods. The significance of cellular immune responses in adults with food-reactive AD is not clear.

In this study we investigated the cellular immune response to food antigens of adolescent and adult patients with AD. Individuals who experienced worsening of AD caused by oral provocation with milk showed a higher incidence of casein-specific T-cell reactions when compared with control subjects. In contrast to *Dermatophagoides pteronyssini*-

specific T cells, casein-specific T cells of adult patients who responded to cow's milk with worsening of AD produced little or no IL-4.

METHODS

Patients and control subjects

Approximately 500 adult patients with AD were interviewed. Eight-eight patients fulfilling the criteria of Hanifin and Rajka¹⁷ attributed worsening of their eczema to the intake of milk and were included in the study. A milk-free elimination diet was maintained for 4 weeks, and a double-blind placebo-controlled oral food challenge (see below) was subsequently performed. According to the results of this clinical investigation, patients with AD were classified into two groups: P1, patients with AD without any deterioration of the skin caused by milk ingestion; and P2, patients with AD who reacted to food challenge with a worsening of eczema (i.e., increases of at least 30 SCORAD points after 24 or 48 hours from the beginning of the provocation procedure with milk). Nonatopic control subjects (C) did not have AD or hypersensitivity reactions to milk.

Oral provocation

The protocol described by Niggemann et al.¹⁸ for double-blind placebo-controlled provocation of children was modified for adult patients. A milk-free diet was followed by patients of groups P1 and P2 for 4 weeks, after which the skin was inspected and evaluated with the SCORAD score as described by Atherton et al.¹⁹ Oral provocation was performed with 5 ml of placebo (casein hydrolysate [Pregestimil; Mead Johnson, Neu Isenburg, Germany] with added banana flavor) or with 5 ml of milk (2.5 ml of milk mixed with 2.5 ml of placebo). After 1 hour, 50 ml of fluid was given. Because no immediate reaction occurred in adolescent or adult patients, a final volume of 500 ml of fluid (250 ml of milk plus 250 ml or 500 ml of placebo) was given on day 1 of the provocation procedure. No exanthematous rash, as described for children with AD,⁷ was detected in our adolescent or adult patients at any time. Because delayed eczematous reactions have been reported after daily administration of the suspected allergens for several days,¹⁰⁻¹² 500 ml of milk was given on days 2 and 3 of the week in which milk had been administered on day 1. A similar procedure was performed with placebo in another week. The order of administration was randomized and was not known by the patient or the clinical evaluator. The skin was inspected daily, and skin lesions were evaluated with the SCORAD score. The evaluators who classified the patients into groups P1 and P2 did not know the results of the in vitro tests (RAST and lymphocyte transformation test [LTT]).

Preparation of antigens for cell culture

Casein and conalbumin (Sigma Chemie, Deisenhofen, Germany) were found to be contaminated with lipopolysaccharides (LPSs). LPS was removed from the protein

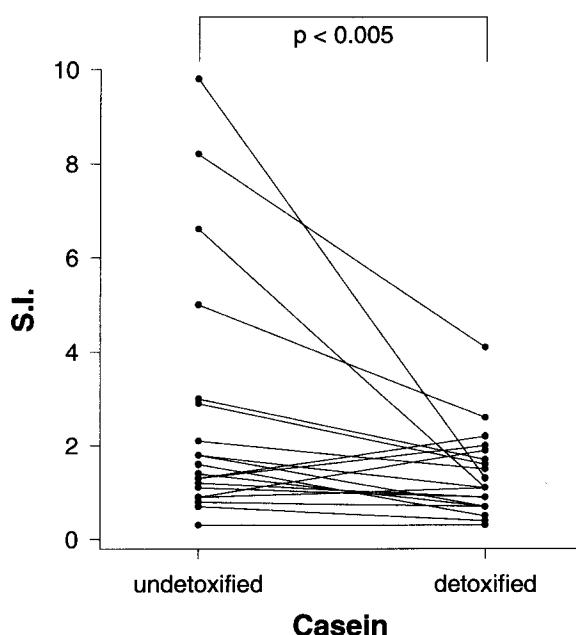


FIG. 1. Comparison of effects of crude and LPS-depleted casein fractions in LTT. Peripheral blood lymphocytes of nonatopic healthy donors were incubated for 5 days in the absence or presence of 25 μ g/ml casein. Proliferation was then determined by incorporation of labeled thymidine. Detoxified fractions had been incubated twice with solid-phase-bound polymyxin B (as described in Methods) before use in the stimulation assay. The Wilcoxon test was performed.

fractions by polymyxin B coupled to a solid phase as described previously.^{19a} The protein fractions were incubated on a shaker for 24 hours 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 supernatants were dialyzed twice against pyrogen-free 0.9% NaCl. LPS concentrations were determined by the Limulus assay (Coatest Endotoxin; Pharmacia, Freiburg, Germany) according to the manufacturer's protocol. The detection limit was 50 pg of LPS per milliliter.

LTT

The LTTs were performed before the elimination diets. No patient had been receiving steroids systemically 3 weeks before the tests were carried out. Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood samples by density centrifugation on Ficoll-Hypaque (Nycomed Pharma AS, Oslo, Norway) ($d = 1.077$ gm/ml). PBMCs (2×10^5) were cultured in Iscove's medium (Biochrom KG) supplemented with 4% heat-inactivated AB serum, glutamine, gentamycin, penicillin, streptomycin, and nonessential amino acids in the presence or absence of antigen. If not otherwise indicated, food proteins were used at a con-

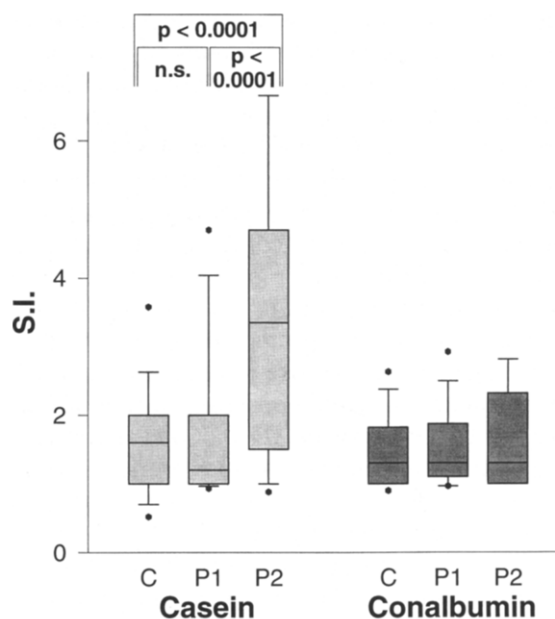


FIG. 2. LTT results with LPS-depleted casein and conalbumin. LTTs were performed with PBMCs of patients and control subjects by using LPS-depleted casein and conalbumin as stimuli. SIs obtained with PBMCs of different groups of patients and respective control subjects are shown. C, PBMCs of nonatopic control subjects; P1, PBMCs of patients with AD who did not respond to cow's milk; P2, PBMCs of patients with AD worsening after oral provocation with milk. Boxes contain 50% of all results. Vertical lines display the range of 90% of results. Horizontal lines show the median of results. For statistical analysis, the Mann-Whitney rank sum test was performed.

centration of 25 μ g/ml, *D. pteronyssinus* (Bencard LTD, Neuss, Germany) at a concentration of 3 μ g/ml, and tetanus toxoid (Behring, Marburg, Germany) at a concentration of 10 μ g/ml. Six replicate cultures were performed. After 5 days, the cultures were pulsed with tritiated thymidine (0.5 μ Ci/well for 4 hours), and the β decay was counted in a liquid scintillation counter. The stimulation index (SI) was defined by the ratio of mean counts per minute of stimulated to unstimulated cultures.

Preparation of specific T-cell clones (TCCs)

Cloning was performed by limiting dilution in the presence of antigen and IL-2 as previously described.^{4,5} Responder cells from six patients of group P2 who were older than 18 years and casein (25 μ g/ml) or *D. pteronyssinus* extract (3 μ g/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 (PBMCs irradiated with 55 Gy cesium 37). The cultures were kept in Iscove's medium supplemented with 4% heat-inactivated AB serum, glutamine, gentamycin, penicillin, streptomycin, nonessential amino acids, and 10 U/L recombinant (r)IL-2. After

TABLE I. Allergen-specific IgE and lymphocyte proliferation in patients with milk-responsive AD

Patient	Casein		<i>D. pteronyssinus</i>	
	SI	RAST class	SI	RAST class
AL	1.5	4	42.0	6
BO	2.7	2	3.2	3
CO	3.4	4	6.3	4
FR	6.6	0	4.7	0
GI	3.9	0	8.2	0
HI	8.1	0	23.6	0
HN	1.0	6	3.4	2
HU	0.7	0	8.9	2
KA	2.9	0	5.6	0
KE	4.9	0	1.2	0
LI	1.0	2	12.8	3
MO	4.5	0	13.0	0
NI	4.4	0	3.0	0
OR	2.4	0	1.0	3
PI	3.3	0	5.0	0
SC	1.2	0	13.6	3
ST	4.2	0	2.7	0
TE	4.7	3	42.0	6
TI	1.0	0	4.7	0
WA	6.8	2	4.8	0
WE	2.6	0	15.0	4
WI	6.3	0	23.6	0

The LTT was performed with 25 µg/ml casein or 3 µg/ml *D. pteronyssinus* extract as described in Methods.

12 to 14 days, wells that demonstrated growth of more than 50 lymphoblasts were scored as positive. Frequencies of responding CD3⁺ T cells were determined by the minimum chi square method according to the method of Taswell²⁰ and with a special computer program.²¹ Because cultures were expanded only from dilutions with 0.03 proliferating cells or less, there was a high probability of clonal cell growth.

Culture conditions for TCCs

After 14 days and every 8 to 10 days thereafter, expansion of the growing cultures was performed in 24-well flat-bottom plates (Nunc) with allogenic, irradiated PBMCs as feeder cells (1×10^6 cells per well), phytohemagglutinin (10 µg/ml), and rIL-2 (20 U/ml) in Iscove's medium.

Restimulation assay

For assessment of the antigen specificity of TCCs, an 8-day 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, 3 µg/ml *D. pteronyssinus* antigen, 25 µg/ml conalbumin (negative control), or phytohemagglutinin (positive control) in the presence of 7.5×10^4 irradiated autologous PBMCs. On day 5 the

TABLE II. Flow cytometric analysis of casein-stimulated blood lymphocytes

Antigen	Patient no.	Medium*	Casein
TNF-R (p75)	1	8.7	29.3
	2	5.9	14.3
	3	7.0	14.2
IFN-αR	1	3.6	18.7
	2	nt	nt
	3	4.7	11.3
IL-2R (p75)	1	10.7	52.2
	2	5.2	14.3
	3	4.3	12.4
IL-7R	1	80.4	63.8
	2	68.4	57.6
	3	77.7	72.0

Peripheral blood lymphocytes from patients of group P2 were cultivated for 8 days in the absence ("medium") or presence ("casein") of 25 µg/ml casein. Indirect immunofluorescence was performed with stimulated and unstimulated cells and investigated by flow cytometry. The SI in the LTT was greater than 3.5 for all patients.

*The percentage of positive cells is shown.

cultures were pulsed with tritiated thymidine. The β decay was counted in a liquid scintillation counter, and results of triplicate cultures were expressed as mean counts per minute.

Flow cytometry

Established TCCs were stained with fluorescein isothiocyanate- or phycoerythrin-labeled CD3, CD4, CD8, and anti-T-cell receptor α/β antibodies (Immunotech, Hamburg, Germany). Casein-stimulated mononuclear cells were investigated with antibodies directed to interferon (IFN)-αR (monoclonal antibody [mAb] IFN-αR3), tumor necrosis factor (TNF)-R [p75] (mAb BU63), IL-2R [p75] (mAb Mik-β3), and IL-7-R (mAb M20). These mAbs had been obtained from the 5th International Workshop on Leukocyte Differentiation Antigens (Boston 1993). Flow cytometry was performed with a FACSCAN (Becton Dickinson, Heidelberg, Germany).

Cytokine assays

Washed TCCs (1×10^6 /ml) were cultured with Iscove's medium, 10% fetal calf serum, and concanavalin A (Con A) (10 µg/ml, Sigma Chemie) in 24-well plates. Supernatants were collected at 24 hours for assessment of IL-4 (ELISA, Biotrack; Amersham, Braunschweig, Germany; 30 pg/ml detection limit). IFN-γ was tested after 48 hours of culture (ELISA; Holland Biotechnology, Leiden, The Netherlands, 50 pg/ml detection limit).

Statistical analysis

The effect of LPS depletion on lymphocyte proliferation was evaluated with the Wilcoxon test. The compar-

TABLE III. Frequencies of T cells proliferating in the presence of casein or *D. pteronyssinus* and IL-2

Patient No.	Casein + IL-2	<i>D. pteronyssinus</i> + IL-2	IL-2 alone
1	1:153	1:339	1:2288
2	1:1138	1:1538	1:4065
3	1:213	1:246	1:2740
4	1:1420	1:393	1:7874
5	1:176	1:371	1:8264
6	1:619	1:789	1:9714
Average	1:309	1:423	1:4289

Lymphocytes of the peripheral blood of patients (P2) with SI greater than 3.5 in the LTT were cloned by limiting dilution in the presence of 25 µg/ml casein and 10 U/ml IL-2, 3 µg/ml *D. pteronyssinus* and 10 U/ml IL-2, or 10 U/ml IL-2 alone. The frequencies of growing cells were calculated as described.^{20,21}

ison of LTT results of different groups of tested individuals was performed with the Mann-Whitney rank sum test.

RESULTS

Clinical evaluation of patients with AD and control subjects

Eighty-eight patients with AD who attributed worsening of their eczema to the intake of milk were investigated. Sixty-six patients (age, 16.0 to 66.6 years; median, 27.8 years) did not react to milk (P1). The mean SCORAD values were 51 ± 20 before diet, 49 ± 15 after diet, and maximal 52 ± 19 during the provocation phase with cow's milk. Twenty-two patients with milk-responsive eczema (median age, 29.2 years; range, 17.3 to 47.0 years) were identified (P2). The mean SCORAD values were 46 ± 19 before diet, 12 ± 3 after diet, and maximal 62 ± 20 after 24 or 48 hours from the beginning of the provocation procedure with serum during provocation with verum. The non-atopic control group C (median age, 30.3 years; range, 20.9 to 67.7 years) was not challenged.

Effect of LPS depletion in food antigen preparations on lymphocyte proliferation

Contamination with LPS was 1 ng/mg casein and 2.2 ng/mg conalbumin. After the detoxification procedure, the LPS concentrations in the protein fractions that were finally used in the LTT were below 5 pg/ml. In contrast to higher LPS concentrations, this amount did not induce any proliferation of PBMCs from healthy donors (not shown). As shown in Fig. 1, significantly lower SIs were obtained when PBMCs of healthy individuals were

cultured with LPS-depleted casein. Because detoxified casein still caused nonspecific proliferation of PBMCs of healthy donors at concentrations greater than 200 µg/ml, 25 µg/ml was chosen as standard concentration in the LTT.

Casein-specific proliferation of PBMCs and IgE in patients with milk-responsive AD

Significantly higher proliferative responses to casein, but not to chicken egg conalbumin, were observed in PBMCs from patients with AD of group P2 as compared with PBMCs from patients with AD of group P1 or control subjects (Fig. 2). With *D. pteronyssinus*, median SIs were higher (SI >4) in all groups compared with casein and its subfractions. No significant differences between groups P1, P2, and C were observed when PBMCs were stimulated with phytohemagglutinin or tetanus toxoid (results not shown).

Casein-specific IgE was detectable in the serum of 41% and *D. pteronyssinus*-specific IgE was detectable in 50% of patients with AD belonging to group P2 (Table I). There was no correlation between specific IgE and the SI in the LTT performed with the corresponding allergens. Casein-specific IgE was found in 5.5% and *D. pteronyssinus*-specific IgE was detected in 63.6% of the sera from patients with AD of group P1 (not shown).

Flow cytometric evaluation of casein-specific lymphocyte activation

There was an increase of cells expressing IFN-αR, IL-2R, or TNF-R. IL-7R⁺ cells were decreased after stimulation of PBMCs (P2) with casein for a week (Table II and Fig. 3). No qualitative differences were observed when casein-, *D. pteronyssinus*-, or tetanus toxoid-stimulated lymphocytes were compared by flow cytometry (results not shown).

Frequency analysis of proliferating cells and generation of casein and *D. pteronyssinus*-specific TCCs

Casein- or *D. pteronyssinus*-specific TCCs were generated by a direct limiting dilution protocol with antigen and IL-2 from six adult patients of group P2 who reacted in the LTT to casein with an SI greater than 3.5 (SI = 5.3 ± 0.6). Specific IgE to both casein and *D. pteronyssinus* were detected in four patients. As shown in Table III, the mean value of frequencies of T cells proliferating in the presence of casein was in the same range as the mean value of frequencies of T cells growing in the presence of *D. pteronyssinus*. In P1 patients ($n = 2$), the frequencies

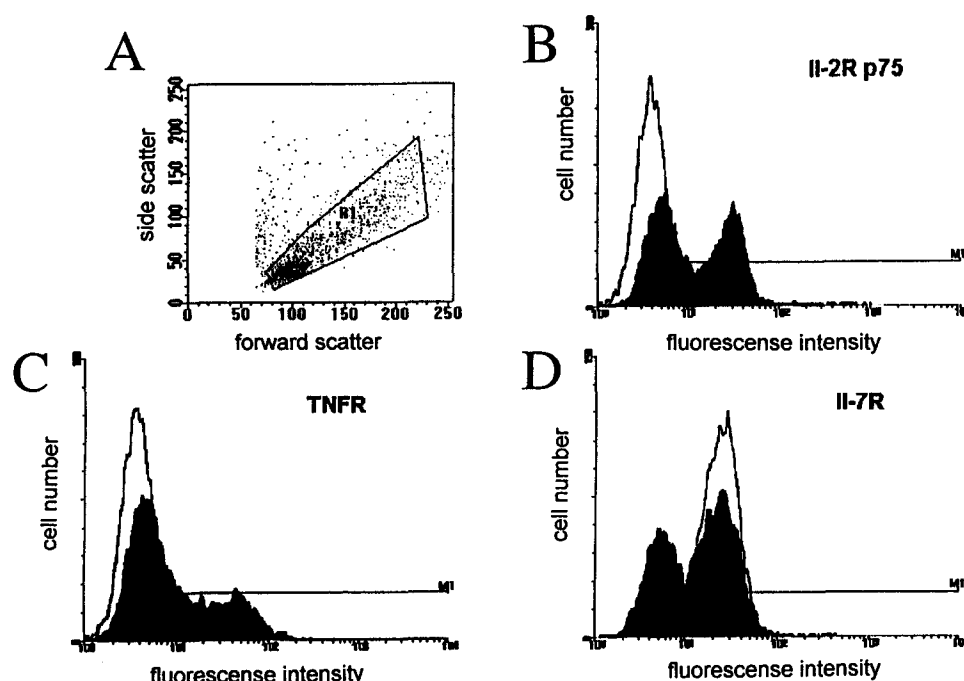


FIG. 3. Expression of cytokine receptors on unstimulated and casein-stimulated PBMCs (fluorescence-activated cell sorter analysis). PBMCs of a patient (group P2), who had reacted to casein with an SI of 8 in LTT, were cultured in the absence or presence of 25 μ g/ml casein for 6 days. Indirect immunofluorescence was performed and analyzed by flow cytometry. **A**, Dot plot of light scatter characteristics of casein-stimulated PBMCs. Analysis of mAb binding to cultured PBMCs was performed, setting a large gate to include blast cells. **B-D**, Overlay histograms are shown to compare membrane expression of given antigens on unstimulated cells (*open histograms*) and casein-stimulated cells (*filled histograms*). **B**, Staining of cultured PBMCs with mAb Mik- β 3 (anti-IL2R, p75, CD122). **C**, Staining of cultured PBMCs with mAb BU63 (anti-TNF, p75). **D**, Staining of cultured PBMCs with mAb M20 (anti-IL-7R).

of T cells growing in the presence of casein were in a similar range as the frequencies in cultures containing only IL-2 (not shown).

Fifty-three TCCs, which reacted positively in the restimulation test with casein (SI >5), and 61 TCCs reactive with *D. pteronyssinus* were generated from the limiting dilution cultures. TCCs, which had been established in the presence of casein, responded exclusively to this antigen but not to *D. pteronyssinus* and vice versa (Table IV).

Characterization of casein and *D. pteronyssinus* TCCs

The majority of allergen-specific TCCs expressed the CD4 antigen. Thirty-four percent of the casein-specific TCCs and 7% of the *D. pteronyssinus*-specific TCCs were CD8⁺ (Table V).

The production of IFN- γ and IL-4 was assessed in 33 CD4⁺ casein- and 56 CD4⁺ *D. pteronyssinus*-specific TCCs (Fig. 4). No significant differences in the production of IFN- γ were detected in either group of TCCs ($p = 0.103$), whereas casein-specific

TCCs secreted significantly less IL-4 (median, 0.08 ng/ml) compared with *D. pteronyssinus*-specific TCCs (median, 1.43 ng/ml; $p < 0.0001$). By means of IFN- γ and IL-4 secretion more casein-specific TCCs were classified as T_H1-like cells when compared with *D. pteronyssinus*-specific TCCs ($p < 0.005$, Table VI). All CD8⁺ TCCs produced IFN- γ . Four CD8⁺ TCCs secreted IL-4 in addition to IFN- γ (Table VI).

DISCUSSION

In this study we characterized a specific T-cell-mediated immune response to casein in the blood of patients with milk-related exacerbation of AD. We found a higher incidence of casein-specific T-cell reactions in patients who experienced worsening of AD caused by cow's milk when compared with control subjects. In contrast to *D. pteronyssinus*-specific T cells, casein-specific T cells of adult patients who responded to cow's milk with worsening of AD produced little or no IL-4.

Early studies of passively sensitized individuals

TABLE IV. Restimulation assay of TCCs

Name of TCC	Medium alone	Casein	Conalbumin	<i>D. pteronyssinus</i>
cas-6	33.0 ± 0.4*	2131 ± 516	43.2 ± 6.9	37.2 ± 1.1
cas-11	32.0 ± 6.0	2619 ± 85	49.0 ± 4.6	39.0 ± 5.1
der-36	30.1 ± 1.6	37.5 ± 2.1	47.0 ± 4.1	7921 ± 723
der-38	32.5 ± 4.7	30.0 ± 2.8	32.4 ± 1.3	1244 ± 148

Representative results of a restimulation assay performed with TCCs, autologous irradiated PBMCs, and the given stimuli. The TCCs had been generated from the same donor. TCCs cas-6 and cas-11 were generated from limiting dilution cultures with casein and IL-2. TCCs der-36 and der-38 had been initially established in the presence of *D. pteronyssinus* and IL-2.

*Mean of counts per minute ± SD.

TABLE V. Phenotypes of casein- and *D. pteronyssinus*-specific TCCs

Antigen	n	TCR2	CD3	CD4	CD8	CD4+CD8
Casein	53*	53	53	33	18	2
<i>D. pteronyssinus</i>	61†*	61	61	56	4	1

Direct immunofluorescence was performed and analyzed by flow cytometry.

TCR, T-cell receptor.

*Total number of TCCs tested.

†Number of positive TCCs.

TABLE VI. Cytokine profile of TCCs from patients with milk-responsive eczema

Specificity	Phenotype	No. of TCCs	Cytokine pattern 0	Cytokine pattern 1†	Cytokine pattern 2‡
Casein	CD4	33	9 (27.3%)	24 (72.7%)	0
	CD8	18	2 (11.1%)	18 (88.9%)	0
<i>D. pteronyssinus</i>	CD4	56	30 (56.6%)	21 (39.6%)	5 (9.4%)
	CD8	4	2 (50%)	2 (50%)	0

The secretion of IFN-γ and IL-4 of concanavalin A-stimulated antigen-specific TCCs was determined.

*Cytokine pattern 0 was defined by a secretion of >1.0 ng/ml IFN-γ and >0.4 ng/ml IL-4.

†Cytokine pattern 1 was defined by a secretion of >1.0 ng/ml IFN-γ and <0.4 ng/ml IL-4.

‡Cytokine pattern 2 was defined by a secretion of <0.4 ng/ml IFN-γ and >1.0 ng/ml IL-4.

demonstrated that immunologically active food proteins can enter the circulation and are distributed throughout the body, including skin sites.^{22, 23} Such food antigens may well cause a T-cell-mediated immune response, which leads to worsening of AD. Kondo et al.¹³⁻¹⁵ were the first to directly address the question of lymphocyte proliferation in response to food antigens in AD. Using ovalbumin and bovine serum albumin, the authors described a positive correlation between food-specific activation of IFN-γ and IL-2-secreting CD4⁺ peripheral blood T lymphocytes in children whose AD worsened in response to oral provocation.¹³⁻¹⁵

We focused on immune responses to food antigens in adolescent and adult patients with milk-responsive AD who often attribute worsening of their skin disease to the intake of certain foods. Bovine serum albumin, a minor cow's milk com-

ponent, did not result in enhanced proliferation of PBMCs from milk-responsive adults in preliminary experiments (not shown). Therefore caseins that are the major proteins of cow's milk and the main protein source for many individuals were chosen as "model antigens" in this investigation.

Using these proteins in the LTT, we found significant differences in the proliferative response of blood lymphocytes between patients who reacted to milk with worsening of AD and the control groups, which indicates a casein-specific cellular immune response in milk-reactive patients. As shown, LPS may substantially influence the proliferation of PBMCs. Therefore we used casein preparations that had been pretreated with polymyxin B to eliminate contaminating LPSs.

Specificities and sensitivities of the LTT in milk



FIG. 4. Cytokine production by CD4⁺ casein and *D. pteronyssinus* TCCs. TCCs (10⁶/ml) were stimulated with Con A, and supernatants were tested by ELISA for IFN- γ and IL-4 activity. Results obtained with 33 casein-reactive and 56 *D. pteronyssinus*-reactive CD4⁺ TCCs of milk-responsive patients (P2) are shown. Each bar represents one TCC.

allergy have been investigated before.^{13-16, 24-28} Contaminating LPSs have not been discussed in any of these publications and may well have led to the poor discriminations between patients allergic

to milk and control subjects, which have been described for the LTT in some studies.^{16, 24-27}

Cow's milk casein has been previously studied by two groups. Räsänen et al.²⁸ reported a high

sensitivity and diagnostic specificity for the LTT in milk allergy, having performed a limited number of LTTs with an α -casein preparation, which is no longer commercially available. In contrast, a mixed casein fraction, which was obtained from the same source as used in this study, led to equally high proliferative responses of milk-reactive children and control subjects.¹⁶ These conflicting results may be due to different characteristics of patients and control subjects (e.g., children vs adults) or methodological differences (e.g., antigen concentrations in the LTT).

Casein-specific IgE was found in only 41% of adult patients with milk-responsive AD. Moreover, no correlation between specific lymphocyte proliferation and specific IgE was found. A lack of correlation between specific IgE and the clinical response to food and the specific lymphocyte proliferation has been reported for children with AD before.^{6, 12, 13} This indicates that an IgE-independent mechanism may be involved in the eczematous reaction to food in some patients with AD. On the other hand, Sampson et al.⁷⁻⁹ found a high correlation between food-induced cutaneous symptoms and specific IgE. In contrast to the adult patients described here, most children with AD investigated by Sampson et al.⁷⁻⁹ had additional clinical symptoms with early onset on oral provocation.

Casein-stimulated blood lymphocytes of patients with milk-responsive AD expressed cytokine receptors such as IFN- α R, IL-2R, and TNF-R; and fewer stimulated cells were IL-7R⁺ when compared with unstimulated cells. In this respect, the casein-induced lymphocyte response resembles the response to well-established T-cell antigens, such as tetanus toxoid.²⁹ Our frequency analysis revealed similar growth rates of lymphocytes in limiting dilution assays in the presence of casein compared with lymphocytes growing in the presence of *D. pteronyssinus* antigens, which were chosen as established allergens eliciting T-cell responses in AD.^{3, 4}

Several specific TCCs were generated by a protocol, which had been established for the generation of specific TCCs reactive to inhalant allergens.^{4, 5} The fact that casein- and *D. pteronyssinus*-specific TCCs were generated under the same culture conditions from the blood of the same individuals enabled us to compare these cells with respect to their cytokine secretion pattern. Most casein-specific TCCs generated from the blood of our patients displayed a T_{H1}-like response. In contrast, most *D. pteronyssinus*-specific TCCs from

the blood of the same patients displayed a T_{H0}-like response, secreting more IL-4 as casein-specific TCCs.

Former studies had revealed that T_{H2}-like immune reactions to inhalant allergens are a characteristic feature in early patch test reactions of patients with AD.^{4, 5} However, the T_{H2} response is almost confined to the skin, whereas specific T cells in the blood of the same patients are mainly of the T_{H0} type, secreting both IFN- γ and IL-4 and other cytokines.^{4, 30, 31} Later, it became evident that in older patch test lesions and in chronic lesions of AD, type 2 cytokines such as IL-4 are downmodulated and type 1 cytokines such as IFN- γ may be predominant.³²⁻³⁵

We therefore suggest that T_{H1}-like casein-specific T cells characterized in this study may be of clinical relevance for milk-inducible eczema in adult patients with AD. To prove a functional role of these cells in the pathogenesis of AD, a quantitative comparison of these cells in skin and blood of milk-responsive patients is being performed.

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