

Antigen specific cytokine response in pediatric patients with atopic dermatitis

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The physiopathology of atopic dermatitis (AD) has still to be elucidated. T effector cells with cutaneous homing receptors or T-cell derived cytokines have been assumed to be implicated in the pathogenetic mechanisms in AD and to be responsible for the different immunologic responses of patients. In fact, the large majority of AD patients display high IgE levels while others do not develop an abnormal IgE response. Although, there are not significant clinical features characterizing the two different groups, patients with normal IgE belong to a younger age range, raising the possibility that the hypothesized dichotomy of AD might be due to age. In the present study we included 172 outpatient children attending the Pediatric Department of our institution. Serum IgE levels and percentages of peripheral T lymphocytes expressing the cutaneous homing antigen (CLA) were evaluated and results were analyzed in relation to the activity of the disease (SCORAD index) or age. In the overall patients, the IgE levels increased significantly with age (0–1 yr: 19.50 IU/ml; 1–3 yr: 62.0 IU/ml; 3–8 yr: 96.0 IU/ml; > 8 yr: 148.5 IU/ml; $p < 0.001$) and with the severity of the disease (SCORAD low: 46.80 IU/ml; medium: 42.90 IU/ml; high: 148.5 IU/ml; $p = 0.01$). Percentages of CLA+ peripheral T lymphocytes also increased with age (0–1 yr: 3.3; 1–3 yr: 4.85; 3–8 yr: 10.6; > 8 yr: 12.5; $p < 0.001$), although they were not significantly different in patients with different SCORAD ($p = 0.89$). We further investigated the cellular immune response to a specific antigen in 25 subjects, matched for age, SCORAD, and CLA+ T-cell percentages. Among them, 13 patients had casein serum specific IgE and 12 had no evidence of casein sensitization. Peripheral blood mononuclear cells were kept in short-term culture with endotoxin-free casein fractions and IFN- γ , TNF- α , IL-5, IL-10 cytokine-producing cells were detected by ELISpot. Statistical analysis showed significant higher numbers of TNF- α - or IL-10-producing cultures (stimulation index > 3) in the 'allergic' patients than in the milk tolerant subjects ($p = 0.01$ and 0.05). The analysis of individual responses confirmed this finding but also provide evidence of a significant increase in IFN- γ -producing cells ($p = 0.05$) induced by casein stimulation in the group of 'non-allergic' children. Our data showed that immunologic parameters as IgE levels or CLA+ T cells in AD pediatric patients are influenced by the age, confirming that age could represent a bias in the analysis of immune response in those patients. Although, we demonstrated in children with AD the existence of different cytokine patterns of the lymphocyte response that could account for the different immunologic features between the two hypothesized forms of AD, which are not dependent on age.

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Atopic dermatitis (AD) is an inflammatory skin disorder, and is very common among infants (1, 2). The disease is characterized by typically distributed eczematous lesions with lichenification, pruritic excoriations, and severely dry skin. It causes an increased susceptibility to cutaneous infections (3–6) and a course of remission and exacerbations. Food or environmental allergens and genetic determinants are considered to play an important pathogenetic role (7, 8). A variable percentage of AD patients have elevated serum IgE, food allergen specific IgE and food allergen positive Prick tests. This form of AD, often associated with other atopic diseases, has been defined as ‘allergic’ or ‘extrinsic’ AD (EAD) (9). The term ‘non-allergic’ or ‘intrinsic’ AD (IAD) has been adopted to identify a disease with the same clinical features, but without other associated atopic diseases, normal IgE levels and negative Prick tests. This form accounts for approximately 10–30% of all AD (10) suggesting that elevated IgE levels and IgE sensitization are not the only prerequisites in the pathogenesis of the disease (11, 12). Recent studies have focused on the role that activated T cells and T-cell derived cytokines play in the IgE or non-IgE associated forms of AD (13–17). Aeroallergens, food allergens and superantigens seem to be involved in the activation of T cells in AD (18–22).

A subset of effector T-cells expressing a skin-homing receptor, the cutaneous lymphocyte antigen (CLA) (23–25), represents more than 90% of cellular infiltrate in AD eczematous skin lesions and up to 10% of peripheral T cells of patients. In the process of T-cell homing to the skin, CLA interacts with its vascular counter-receptor E-selectin (CD62E), expressed on inflamed superficial dermal post-capillary venules (26). The CLA expression has been associated *in vitro* to high Th2-cytokine production and implicated in regulating IgE synthesis mainly by IL-13 secretion (27) in AD after allergen exposure (28) as well as polyclonal activation (29). However, it has become evident that allergen specific responses are not only caused by Th2 cytokines and that the polarization of T helper cells is more complex than what was first believed (30). The increasing prevalence of IgE sensitization and allergic diseases during childhood has stressed the need to find ways to predict allergic disease early in life, but so far the search has been unsuccessful. A better understanding of the immune events during fetal life and childhood is necessary in order to clearly understand the specific role of T cells and help us be able to predict and prevent such

diseases. For a better understanding of the immunoregulatory mechanisms involved in AD and confirming the existence of different forms of this pathology, in this study we first analyzed the percentage of peripheral T/CLA+ cells in AD pediatric patients in relation to the activity of the disease, serum IgE levels or age. Then we investigated whether peripheral blood mononuclear cell (PBMC) antigen reactivity was related to IgE sensitization. For this purpose we evaluated, using the ELISpot technology, the frequency of IFN- γ , TNF- α , IL-5, and IL-10 casein-induced producing cells from two clinically homogeneous groups of young children with AD with or without milk allergy, as assessed by general accepted laboratory routine tests.

Patients and methods

Patients

A total of 172 consecutive patients (mean age: 62.6 months; range: 1–564 months) with AD defined by the criteria of Hanifin and Rajka (1) were included in this study. Informed consent was obtained from all patients' parents.

Patients were classified into the following four groups according to age. Group 1 comprised 54 young children < 1 yr [mean age: 7.5 months; range: 1–12 months; female:male (f:m), 29:25]. Group 2 comprised 57 children between 1 and 3 yr (mean age: 24.5 months, range: 13–36 months; f:m, 22:35; mean age of onset: 1.5 yr. Group 3 comprised 33 children between 3 and 8 yr (mean age: 65.7 months, range: 42–96 months; f:m, 22:11; mean age of onset: 2.5 yr). Group 4 comprised 28 adults more than 8 yr (mean age: 146.7 months, range: 98–216 months; f:m, 11:17; mean age of onset: 3 yr). Data that characterize patients are summarized in Table 1.

None of them had other atopic conditions (asthma, rhinitis, or conjunctivitis) nor received antihistamines, systemic, or topical corticosteroids during the 4 wk before clinical evaluation.

The severity of AD was measured by the Scoring Atopic Dermatitis index (SCORAD) of the European Task Force on Atopic Dermatitis (31). Briefly, it is defined by the following parameters: (a) extent, (b) intensity of skin lesions, and (c) subjective symptoms like pruritus and sleep loss. The final score was calculated by using the following equation: $a/5 + 7b/2 + c$. In this study patients were grouped into low, mild, and severe disease, according to SCORAD values ranging between 0 and 25, 25 and 50, or > 50, respectively.

Table 1. Summary of the clinical characteristics of the patients divided into four groups according to the age

Age group	No. of patients	Sex (female:male)	Age (months)†	Total serum IgE (IU/ml)†	CD3/CLA+†	SCORAD†
Group 1 (<1 yr old)	54	29:25	7.5 (1–12)	19.50 (1.6–565)	3.3 (0.31–16.0)	39 (11.2–81)
Group 2 (1–3 yr old)	57	22:35	24.5 (13–36)	62.00 (7.2–3827)	4.85 (0.8–16.50)	38.8 (7.9–80)
Group 3 (3–8 yr old)	33	22:11	65.7 (42–96)	96.00 (8.3–4100)	10.6 (2.6–20.3)	40 (18.4–70)
Group 4 (>8 yr old)	28	11:17	146.7 (98–216)	148.5 (8–2645)	12.5 (4.3–21.7)	42 (20.2–80)
				p < 0.001*	p < 0.001*	p > 0.05*

p-Values are calculated according to the increase in age.

*Kruskal–Wallis nonparametric test.

†Median value (minimum and maximum).

We selected a group of 25 AD patients to study the cytokine-producing cells, by ELISpot assay. They included 13 patients with and 12 subjects without evidence of milk allergy, strictly homogeneous regarding age (1–3 yr), SCORAD index, and percentage of CLA+ T lymphocytes. Children with cows' milk allergy had a specific IgE response to casein (RAST > 0.35 kU/ml), the Prick test positive to the same protein and the ingestion of milk caused the worsening of their clinical symptoms (pruritus).

Determination of total and specific IgE antibodies

Patients sera were collected and analyzed for concentrations of total IgE (IMX System; Abbott Diagnostics, IL, USA) and specific IgE levels (UniCAP-FEIA RAST Specific IgE; Pharmacia Diagnostics, Uppsala, Sweden) vs. a mix of food allergens (casein, α -lactalbumin, β -lactoglobulin, egg yolk, egg white, wheat, fish, and soybean) or aeroallergen (*Dermatophagoides pteronyssinus*). Total IgE lower than 120 IU/ml and specific IgE lower than 0.35 kU/l were considered normal.

Flow cytometry

EDTA-whole blood samples (100 μ l) were incubated with (20 μ l) anti-human CLA (HECA-452)-fluorescein isothiocyanate (Pharmingen, San Diego, CA, USA) and with anti-human CD3-PECy7, CD4-PECy5, or CD8- PECy7 monoclonal antibodies (5 μ l) (Caltag, Burlingame, CA, USA). After lysis of erythrocytes, T/CLA+ lymphocytes were analyzed by FACSCalibur, flow cytometer (BD Biosciences Immunocytometry Systems, San José, CA, USA) performing a morphologic gate using CELL-QUEST software (BD Biosciences, San Jose, CA, USA; non-specific fluorescence regions were established by staining cells with isotype control monoclonal antibodies.

Results were expressed as a percentage of lymphocytes CLA+/CD3+, CLA+/CD4+, and CLA+/CD8+.

PBMC

The PBMC were isolated from 5 to 10 ml of heparinized blood by standard Ficoll density gradient centrifugation (using Lympholyte-H steril solution; Cederlane, Hornby, ON, Canada), washed twice with PBS, frozen in 90% heat-inactivated fetal bovine serum (FBS; Euroclone, Milano, Italy) and 10% dimethylsulfoxide (DMSO; Sigma, St Louis, MO, USA) and kept in liquid nitrogen until tested for ELISpot assay.

Preparation of casein for cell cultures

As casein was the major represented protein of cow's milk positive routine test reactions in our 'allergic' children, a mix of commercially available casein preparations of a α - β - and κ -casein was chosen as 'model antigen'.

α - β - and κ -casein (Sigma Chemie, Deisenhofen, Germany) were used to stimulate antigen specific cells for cytokines analysis by ELISpot. LPS contamination was detected by the *Limulus* assay (BioWhittaker, Cambrex Company, NJ, USA) according to the manufacturer's protocol and was removed from the protein fractions by Detoxi-Gel Endotoxin Removing Gel Columns (Pierce Biotechnology, Rockford, IL, USA). Casein preparations were used if LPS was < 0.5 EU/ml by the *Limulus* assay.

ELISpot assay

ELISpot analyses were undertaken according to the manufacturing procedures with some modifications. Briefly, flat bottom 96-well microtiter plates (Multiscreen MAIPS4510, Millipore Corp, Bedford, MA, USA) were coated overnight at 4°C with 100 μ l/well of specific capture antibody anti-IFN- γ or anti-TNF- α (5 μ g/ml, 100 μ l/well; BD Biosciences, San Diego, CA, USA), anti-IL-10, or -IL-5 (10 μ g/ml, 100 μ l/well, Endogen Woburn, MA, USA). The plates were washed three times and incubated 1 h at 37°C with

complete medium (RPMI 1640, 10% FBS, 1% penicillin–streptomycin–L-glutamine; GIBCO Invitrogen Corp, Paisley, Scotland, UK). Vital PBMC from cryopreserved aliquots were employed in a single run for detection of cytokine release. Cells (2×10^5 /well) were plated to a final volume of 200 μ l/well of complete medium. Casein (α - β - and κ -casein in equal amounts) was added in triplicate wells at a final concentration of 250 μ g/ml. Control duplicate wells contained PBMC with medium alone or with phytohaemagglutinin (PHA, SIGMA, St Louis, MO, USA) (1 μ g/ml; Sigma). After 20 h for IFN- γ - and TNF- α -coated wells, or after 40 h for IL-10- and IL-5-coated wells, the plates were washed and biotinylated detection antibodies (anti-IFN- γ and anti-TNF- α , 2 μ g/ml, 100 μ l/well; BD Biosciences/anti-IL10 and -IL5 1 μ g/ml, 100 μ l/well; Endogen, Woburn, MA, USA) were added for 2 h at room temperature. Subsequently to specific substrate addition, the spots were developed using AEC-chromogen solution (Sigma) and evaluated by the Automated ImmunoSpot Image Analyzer Software (AEL-VIS Technologies, Hannover, Germany). The number of spot-forming cells per 2×10^5 PBMC plated was considered to calculate the stimulation index (SI), ratio between stimulated and unstimulated cultures for each subject. We arbitrarily assumed as stimulated a culture with SI > 3.

Statistical analysis

Statistical analysis was performed with Graph-Pad PRISM4 version software (San Diego, CA, USA). Nonparametric tests were used to elaborate data because it was not possible to assume normal distribution for variables. Differences among age groups were analyzed by one-way ANOVA; differences between groups of patients were established using the Mann–Whitney (MW) rank sum; correlations were evaluated with the Spearman rank correlation coefficient. The chi-squared test was used to evaluate contingency tables. Results were considered significant at $p < 0.05$.

Results

Serology

All patients described in Table 1 completed the study. Serum IgE levels were normal in 124 (72%) of the 172 patients. According to the age groups showed in Table 1, the percentage of patients with normal IgE levels decreased in each

group in the percentage of 90.7% in group 1, 71.9% in group 2, 63.6% in group 3, and 39.2% in group 4, as age increased. Analyzing IgE serum concentration in the different age groups (from 1 to 4) IgE levels increased with age ($p < 0.001$) (Table 1). The analysis of IgE levels in patients with low, mild, or severe disease activity also showed higher values in more severely affected patients ($p = 0.01$) (Table 2).

CLA expression

Investigating circulating lymphocytes expressing the tissue-selective homing receptor CLA, we found that the percentage of CLA+ T cells increased with age ($p < 0.001$) (Table 1). The expression of CLA antigen on CD3 lymphocytes and the severity of disease were not significantly associated ($p = 0.89$) (Table 2). Otherwise, the percentage of CD3/CLA+ and IgE levels were significantly correlated in all patients ($p < 0.001$, $r = 0.32$). Considering patients according to their normal (group A) or elevated (group B) IgE levels, percentages of CLA-positive T cells were significantly higher in patients with elevated IgE ($p = 0.02$), due to the increase of the T-helper (CD3+/CD4+) rather than the suppressor (CD3+/CD8+) subpopulation ($p = 0.017$ and 0.05, respectively) (Table 3).

ELISpot assay

To study the specific cytokine production upon antigen stimulation in AD patients, PBMC from children with (B) or without (A) evidence of milk

Table 2. Analysis of IgE levels and percentages of CLA+ T lymphocytes according to severity of the disease

SCORAD	Total serum IgE (IU/ml)†	CD3/CLA+†
Low (0–25)	46.80 (6–565)	6.8 (2.6–17.3)
Medium (25–50)	42.90 (2–1460)	6.9 (3.2–17.4)
High (>50)	148.5 (5–4100)	7.4 (0.4–21.7)
	$p = 0.01^*$	$p = 0.89^*$

*Kruskal–Wallis nonparametric test.

†Median value (minimum and maximum).

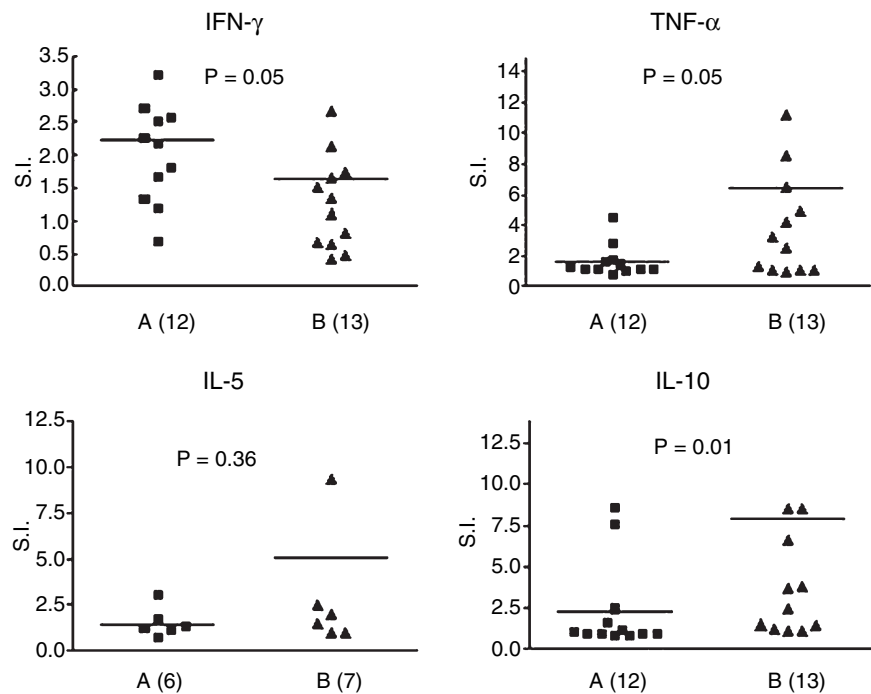
Table 3. Evaluation of the percentages of CLA+ T cells in relation to normal (group A) or elevated (group B) IgE levels

Group	CD3/CLA+†	CD4/CLA+†	CD8/CLA+†
Group A	4.50 (0.4–17.3)	2.60 (0.5–13.4)	1.70 (0.5–7.7)
Group B	6.75 (0.6–21.7)	4.05 (0.4–17.4)	2.60 (0.4–10.6)
	$p = 0.02^*$	$p = 0.017^*$	$p = 0.05^*$

*Mann–Whitney test.

†Median value (minimum and maximum).

Fig. 1. Elispot detection of cytokine-producing cells upon stimulation of PBMC with 250 μ g/ml of (α - β - and κ -casein fractions) in AD patients with normal (A) or elevated (B) serum IgE levels. Results were expressed as SI.



allergy were kept in short-term cultures in the presence of casein fractions (α , β , and κ , endotoxin free) and subsequently analyzed by the ELISpot assay. To overcome the variability of immunologic lymphocyte response because of age or severity of disease, we retrospectively analyzed PBMC from patients belonging to the same age group (1–3 yr), with the same clinical evaluation (SCORAD > 50) and CD3/CLA+ between 3% and 5%.

Contingency analysis of positive casein stimulated PBMC cultures (SI > 3) in patients with evidence of milk allergy (B) or age-matched milk-tolerant subjects (A) showed that the subjects of group B had a significantly higher number of positive responses to casein than group A, with TNF- α - and IL-10-producing cells ($p = 0.01$ and 0.05 , respectively), while the IFN- γ and IL-5 responder patients were not different in the two groups. The comparison of individual responses (SI) to casein revealed a significant increase in IFN- γ -producing cells in patients without other evidence of sensitivity to milk proteins. Subjects with significantly higher SI in TNF- α - and IL-10-producing cells were confirmed as significantly higher in the milk allergy group (Fig. 1). A positive correlation was found between TNF- α and IL-10 production ($r = 0.74$, $p = 0.003$) while a negative correlation was found between IFN- γ and IL-10 even if it was not statistically significant because of the low number of subjects with IL-10-producing cells ($r = -0.34$, $p = 0.25$). These results suggest that IFN- γ or TNF- α

response could identify different pathways of immune response. PHA stimulated cultures invariably produced SI > 3, and no differences could be observed in PBMC of 'allergic' and 'non-allergic' children. This indicated that there were no intrinsic differences among lymphocytes of atopic children in their capacity to respond to a stimulation and that the procedure of cryopreservation did not damage them.

Discussion

According to recent epidemiologic data showing that AD is mainly a childhood disease, 64% of patients with AD attending our institution in the last 2 yr, belonged to the age range between 0 and 3 yr.

A study from Falk (32) reported a prevalence of 13% in individuals under the age of 20 yr and only of 2% in those older than 20 yr old. Moreover, the prevalence among adults over the age of 40 yr is approximately 0.2%, as reported by Herd et al. (2). In the same study, the authors found a prevalence point of 8% in children < 2 yr, and of 2.6% in children from 2 to 11 yr. Furthermore, in the majority of children the eczema disappears in the age range of 7–11 yr (33, 34) and only a small percentage (0.2–2%) maintains the disease in adulthood (2). These results supported our choice to make an age-related evaluation of immunologic parameters in our patients. We studied the IgE levels and CLA+ T lymphocytes, in 172 subjects aged

0–18 yr. IgE levels were elevated in only 28% of patients and were significantly related to age. The low frequency (18%) of high IgE levels in patients under 3 yr of age is concordant with data reported by Bohme et al. (34) and by Schmid et al. (10) and confirms that non-allergic AD accounts for most of AD in infancy (35). Moreover, a direct significant correlation was found between IgE levels and the severity of disease, which is in agreement with clinical observations (36–38).

CLA antigen expression on peripheral T cells of patients was also studied and a statistically significant increase in the proportion of CLA⁺ T cells was found with an increase of age differently from what is reported by other authors in AD children (25). However, CLA is reported to be expressed exclusively on memory T cells and memory T cells increase in proportion throughout childhood (39). No association statistically significant between CLA⁺ peripheral T cells and the severity of disease ($p = 0.89$) could be demonstrated. One possible explanation is that peripheral T cells do not have a direct role in the clinical expression of the disease. Moreover, analysis of data according to IgE levels, we found higher percentages of peripheral CD3/CLA⁺ cells in patients with elevated IgE patients, because of the T-helper subpopulation. The correlation between CLA⁺ T cells and IgE levels fits the hypothesis that these cells can help, via IL-13 production, the B lymphocytes to produce large amounts of IgE (27, 40, 41).

The controversial data on the different T-cell behaviour in the two forms of AD, could be due to the fact that the age of the patient population or the clinical expression of the disease are biases which might influence the analysis of data (15, 19) as well as the T-cell stimulation procedure. In a selected group of patients, matched for age, SCORAD, and CLA⁺ T cells, but with different allergic response to milk proteins we detected, by the ELISpot technology, the increase of TNF- α , IL-10 casein-induced producing cells in the 'allergic' group, and an increase in IFN- γ -producing cells in patients without evidence of sensitivity to milk proteins, measured by the routine testing. The positive correlation found between TNF- α and IL-10 production and the negative correlation found between INF- γ and IL-10 suggested different pathways of immune response in AD. It is accepted that atopic diseases are caused by Th2 cytokine response to allergens in affected tissues (42, 43) or to increased levels of IL-4 (44, 45), IL-5 (46, 47), and IL-13 (46, 47), associated with decreased (48), similar (44, 46), or

increased (49) levels of IFN- γ in allergen stimulated cultures with PBMC. In fact, INF- γ is considered to be a central player in atopic diseases, preventing overproduction of IgE via suppression of the IL-4-induced IgE production (50, 51). However, TNF- α could act through upregulation of the expression of E-selectin on activated endothelium (52), and it has been proposed as a marker discriminating between active vs. recovered cows' milk allergy (53). Similarly to what we found, several investigators described an increased allergen-specific Th1 activity, with synthesis of IFN- γ , among non-sensitized individuals, while an increased Th2 response was detected in the sensitized subjects (16), also if results from other authors were controversial (54, 55). In conclusion, our data sustain the existence of the two clinical forms of AD, IAD and EAD based on different immunologic features of patients, associated with TNF- α /IL-10 or IFN- γ allergen-specific cytokine response. For the different pathogenetic mechanisms the immunoregulatory role of IL-10 in driving an IFN- γ or TNF- α mediated response (56) should be considered.

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