

Evaluation of Receptor Expression on Immune System Cells in the Peripheral Blood of Asthmatic Children Undergoing Food Challenges

Aneta Krogulska^a Krystyna Wąsowska-Królikowska^a Ewa Polakowska^b
Sławomir Chrul^b

Departments of ^aPediatric Allergology, Gastroenterology and Nutrition, and ^bPediatrics, Medical University of Lodz, Poland

Key Words

Asthma • Children • Food allergy • Food challenge •
Receptor expression

Abstract

Background: The role of food allergens in the induction of allergic reactions in the airways is not completely understood. The aim of the present study was to evaluate fluorocytometric assays of the peripheral blood during food challenge tests in children with asthma and food allergy. **Patients and Methods:** 22 children with asthma and concomitant food allergy and 18 children with asthma without food allergy participated in the study. Oral challenge tests were performed using double-blind, placebo-controlled food challenge. Blood samples were collected before and 4 and 24 h after the challenge. CD25 and CD23 antigen expression was determined with monoclonal antibodies using a FACS-Calibur flow. **Results:** The evaluation of the CD25+ T subpopulation and CD19+CD23+ B lymphocytes revealed statistically significant differences between the study group and the control group. In children with asthma and food allergy, the cell pool consisted (on average) of $9 \pm 2.8\%$ of CD3+CD25+ cells before the challenge and of $10.3 \pm 3.8\%$ (mean delta: 1.623; $p = 0.01$) after the provocation. However,

placebo challenge did not significantly change the number of this T-lymphocyte subpopulation (mean delta: -0.121 ; $p > 0.05$). The highest increase in the CD25+ T-subpopulation expression was found in patients with respiratory reactions during the positive food challenge (mean delta: 4.065; $p < 0.004$). **Conclusions:** An increase in CD25+ T-lymphocyte and CD23 B-lymphocyte populations after food allergen challenge may indicate their significant role in the pathogenesis of the active phase of the immunoinflammatory process in children with asthma and concomitant food allergy.

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Introduction

Unlike inhaled allergens, which have been known to exacerbate asthma, food allergen involvement in the induction of an allergic response in the airways has not been completely elucidated. Usually, the effect of food allergens is associated with atopic skin inflammation, urticaria, vasomotor edema, anaphylactic reactions, oral allergy syndrome or digestive tract ailments. However, there is evidence that each part of the respiratory tract may be an effector organ of food-challenged allergic re-

sponses. Food-induced symptoms in the airways are not common, nevertheless their role in a certain group of patients suffering from asthma has been emphasized [1, 2]. In the study by Wood et al. [3], 73% of asthmatic patients reported asthma exacerbation after food ingestion, and therefore 61% of them were placed on restrictive diet. In a study by Warner [4], the prevalence of asthma exacerbation was associated with food intake in 21% of the patients. A detailed evaluation of the prevalence of food-induced symptoms in the respiratory tract is difficult. Individual perceptions reported by patients are much more common than research data, for which challenge tests are necessary for confirmation of the relationship between symptoms and food ingestion. Published data on the results of food challenge tests indicate that food allergens are responsible for the induction of symptoms in the respiratory tract in 2–29% of children [5, 6]. It is estimated that 2–9% of asthmatics, both children and adults, have a concomitant food allergy, provoking exacerbations in the respiratory tract [7, 8]. In addition, allergic reactions to food ingestion can vary in patients with asthma, depending on its frequency (occasional or routine). This response can be associated with a wide range of complaints, e.g. itching, rhinitis, xerostomia, cough, shallow/wheezing breathing, dyspnea and finally generalized anaphylactic reactions. Exacerbation of food-challenged asthma varies and, in mild cases, can be manifested by persistent cough or effort-induced asthma. These symptoms are usually associated with an effect of inhaled allergens rather than food allergens. An early reaction after food intake provides a greater chance of noticing the food allergen that has induced it, compared to a late reaction, when the connection between ailments and the provocative factor is difficult to be seen. Considering the complex pathogenesis of asthma exacerbations and their diversified course, the search for methods that might evaluate the impact of food allergens on asthma seems to be justified. Double-blind, placebo-controlled food challenge (DBPCFC) appears to be one of the commonly recognized procedures used for this purpose. However, food does not always clinically manifest its influence within the respiratory tract, i.e. causing dyspnea, cough or wheezing, which can be observed during the provocative test. In some patients with asthma, reactivity of the respiratory tract to food challenge has been shown to increase without distinct symptoms of dyspnea or changes in forced expiratory volume in 1 s (FEV₁). Therefore, the DBPCFC has been combined with bronchial hyperreactivity tests to assess the effect of food on asthmatic patients. The cytofluorimetric evaluation of immune cell

reactivity is another method to estimate possible effects of food allergens on the course of asthma.

Based on these considerations, the aim of the present study was to determine whether food challenge in children with asthma and food allergy changes the number and activity of immune system cells involved in the inflammatory process underlying these diseases via the determination of CD25 (IL-2R) antigen expression on T lymphocytes and CD23 (FcεRII) antigen expression on B lymphocytes before and after food challenge. An attempt was also made to assess what association (if any) was between the activation of immune system cells and clinical reactions in children with asthma and concomitant food allergy after food challenge tests. Studies on the activity of lymphocytes in asthmatic children undergoing food provocation have not been published to date.

Patients and Methods

Patients

The initial study group consisted of 304 subjects who were admitted to the Department of Pediatric Allergology, Gastroenterology and Nutrition of the Medical University of Lodz for the evaluation of food hypersensitivity. Only patients who were able to adequately perform pulmonary function testing were enrolled into the study between 2004 and 2007. Lack of data or cooperation resulted in exclusion of 59 cases. Therefore, a group of 245 children was subjected to further investigations. Criteria for asthma diagnosis and evaluation of disease severity were in accordance with the recommendations of the Global Initiative for Asthma. Based on completed questionnaires, food allergy was suspected in 164 children. Skin prick tests and/or specific IgE revealed sensitization to food allergens in 70 patients, suspecting a concomitant IgE-dependent food allergy. This group of children underwent further examinations: DBPCFC, pulmonary function tests and methacholine inhalation challenges. Food allergy was confirmed in 24 children with asthma. In this group, food allergy was verified by performing DBPCFC in conjunction with blood sample collection to determine CD25 (IL-2R) antigen expression on T lymphocytes and CD23 (FcεRII) antigen expression on B lymphocytes. Due to lack of cooperation, 2 children were excluded from the study. Thus, the study group comprised 22 children with atopic asthma and a concomitant IgE-dependent food allergy. Eighteen asthmatic children with food allergy and a negative DBPCFC result constituted the control group. Characteristics of the study patients are shown in table 1. Allergens responsible for positive results of the challenge included: egg in 13 children, milk in 5, wheat flour in 1, celery in 1 and peanuts in 2 children. The eliciting dose for egg ranged between 0.5 and 15 g (mean 5.6 g) and for cow milk 3–100 ml (mean 30.6 ml), for wheat 3.1 g, celery 5.6 g, and peanut 0.0625 and 0.135 g. Of the 22 children with positive food challenge tests, 8 children developed mainly respiratory symptoms, 7 gastric symptoms and 7 skin lesions.

Table 1. Characteristics of asthmatic children with and without food allergy (FA)

	Children with asthma and FA		Children with asthma without FA	
Children, n	22		18	
Age, years	5.5 – 18		6 – 17	
Mean $\pm$ SD	9.5 $\pm$ 3.34		10.2 $\pm$ 3.2	
Males, n (%)	13 (59.1)		14 (77.7)	
Clinical symptoms in anamnesis, n (%)				
AD in anamnesis	14 (63.6)		10 (55.5)	
AD recently	18 (81.8)		6 (33.3)	
Urticaria	10 (45.4)		1 (5.6)	
Vasomotor edema	2 (9.1)		0	
Digestive symptoms	14 (63.6)		9 (50)	
FA in anamnesis	21 (95.4)		16 (88.9)	
Family history of allergic diseases, n (%)	17 (77.3)		10 (55.6)	
Asthma severity, n (%)				
Mild	9 (40.9)		10 (55.6)	
Moderate	12 (54.5)		8 (44.4)	
Severe	1 (4.6)		0	
tIgE, IU/ml (mean $\pm$ SD)	612 $\pm$ 304		426 $\pm$ 313	
	before provocation	after provocation	before provocation	after provocation
FEV ₁ (mean SDS $\pm$ SD)				
Provocation with allergen	-0.25 $\pm$ 2.19	-0.55 $\pm$ 1.62	-0.5 $\pm$ 1.98 ^a	-0.6 $\pm$ 1.69 ^b
Provocation with placebo	-0.4 $\pm$ 1.83	-0.8 $\pm$ 1.55	-0.4 $\pm$ 1.83 ^c	-0.65 $\pm$ 1.76 ^d
PC20, mg/ml (mean $\pm$ SD)				
Provocation with allergen	1.41 $\pm$ 1.12	0.86 $\pm$ 0.71	1.93 $\pm$ 1.68 ^e	2.02 $\pm$ 1.75 ^f
Provocation with placebo	1.37 $\pm$ 1.08	1.42 $\pm$ 1.1	2.29 $\pm$ 2.98 ^g	2.34 $\pm$ 2.96 ^h

SDS = Standard deviation score. Before allergen challenge, ^a $p > 0.05$, ^e $p > 0.05$; after allergen challenge, ^b $p > 0.05$, ^f $p = 0.007$; before placebo challenge, ^c $p > 0.05$, ^g $p > 0.05$; after placebo challenge, ^d $p > 0.05$, ^h $p > 0.05$.

Methods

The studies were performed on asthma patients who had been in a stable condition for ≥ 6 weeks. Regarding skin prick tests, standardization of the method was in line with the position papers of the European Academy of Allergology and Clinical Immunology [9]. Standard allergen extracts were provided by Allergopharma (Reinbek, Germany). The positive control was 10 mg/ml histamine (Allergopharma). As a negative control 50% glycerosaline solution was used. Reactions to each allergen were measured 15 min after the pricks. Skin prick test positivity was defined as a wheal of at least 3 mm being larger than the negative control. The specific serum IgE levels were measured using the UniCAP 100 (Pharmacia Upjohn). Results >0.71 kU/l ($>$ class 2) were considered positive. Oral challenge was performed by means of the DBPCFC method. The trials were carried out in hospitalized patients according to the recommendations of the European Academy of Allergology and Clinical Immunology, after an eliminating diet of at least 2 weeks [10]. Native forms of samples were applied. In the provocative procedures, the following food allergens were used: egg, milk, wheat flour, celery and peanuts. Prior to each test, administration of antihistamine drugs was discontinued

for 2 weeks, and there was a 48-hour break in taking inhalants. The patients were observed for at least 48 h. The food challenge results were scored as negative, mild/moderate or severe using a clinical reference table adapted from Sampson [11] and Benhamou et al. [12].

Blood samples were collected before, and 4 and 24 h after completion of the oral challenge test. They were collected using anticoagulant (EDTA) and labeled with adequate monoclonal fluorochrome-coupled antibodies CD3FITC, CD25PE, CD19FITC, CD23PE and CD56PE-Cy (BD Biosciences). For individual antibodies, parallel controls with isotype-matched diagnostic antibodies, i.e. IgG1FITC for CD3FITC and CD19FITC, IgG2aPE for CD25PE and CD23PE, and IgG2aPE-Cy for CD56PE-Cy, were made. Labeling was carried out for 30 min at room temperature, protecting the samples against light. After incubation, erythrocytes were lysed using a lysing solution reagent (BD Biosciences). The lymphocytes were stained immediately after the blood was obtained. The samples were collected and analyzed using a FACS-Calibur flow cytometer, and analysis was performed with the Cell Quest program (Becton-Dickinson). Before reading, calibration of the cytometer was carried out using Calibrite beads (BD Biosci-

Table 2. Lymphocyte subsets CD3+, CD3+CD25+ and CD19+CD23+ before the challenge, and 4 and 24 h after the challenge with allergen or placebo in the group of children with asthma with and without food allergy (FA)

	CD3+, %		CD3+CD25+, %		CD19+CD23+, %	
	with FA	without FA	with FA	without FA	with FA	without FA
Values before allergen challenge						
Min – max	59.6 – 78.2	56.6 – 78.2	5.3 – 15.2	4.5 – 15.9	5.38 – 16.3	4.6 – 14.2
Mean $\pm$ SD	68.4 $\pm$ 6.1	67.5 $\pm$ 5.7	9 $\pm$ 2.8	9.2 $\pm$ 2.8	10.2 $\pm$ 3.1	9.3 $\pm$ 2.5
Values 4 h after allergen challenge						
Min – max	59.1 – 76.7	49.1 – 76.7	4.6 – 19.3	5.4 – 12.4	5.05 – 23.15	5.9 – 17.4
Mean $\pm$ SD	67.9 $\pm$ 6.1	67.5 $\pm$ 7	10.3 $\pm$ 3.8	8.3 $\pm$ 2.1	12.1 $\pm$ 4	9.6 $\pm$ 3.1
Values 24 h after allergen challenge						
Min – max	59.3 – 76.8	61.1 – 76.5	4.6 – 20.4	5.1 – 14.5	5.1 – 18.6	5.8 – 15.2
Mean $\pm$ SD	68.7 $\pm$ 6	67.9 $\pm$ 4.9	10 $\pm$ 3.8	8.4 $\pm$ 2.5	11.4 $\pm$ 3.6	9.5 $\pm$ 2.2
Values before placebo challenge						
Min – max	56.5 – 75.7	48 – 75.3	4.5 – 16.1	4.6 – 16.1	5.6 – 17.1	5.6 – 15.9
Mean $\pm$ SD	68 $\pm$ 6.5	67.8 $\pm$ 6.5	9.3 $\pm$ 3.4	9.5 $\pm$ 2.8	11.3 $\pm$ 3.7	10.01 $\pm$ 2.8
Values 4 h after placebo challenge						
Min – max	57.6 – 77	50.6 – 76.9	4.3 – 15.8	4.3 – 14.3	4.6 – 26.3	5.4 – 16.1
Mean $\pm$ SD	67.8 $\pm$ 6.2	68.2 $\pm$ 6.2	9.2 $\pm$ 3.4	9.3 $\pm$ 3.1	12.4 $\pm$ 4.8	11.4 $\pm$ 2.8
Values 24 h after placebo challenge						
Min – max	59.8 – 76.8	48.4 – 76.8	4.5 – 15.5	4.5 – 13.9	5.6 – 18.1	5.6 – 16.1
Mean $\pm$ SD	67.8 $\pm$ 6.4	67.8 $\pm$ 7	8.7 $\pm$ 2.9	8.8 $\pm$ 2.8	11.9 $\pm$ 3.9	10 $\pm$ 2.2

ences). The voltage amplification on a photomultiplier and the compensation of various fluorochrome colors were set. Cellular recruitment occurred in a previously determined window with a constant setup of sensitivity parameters and cytofluorometer compensation. For the analysis, 20,000 cells were collected from each test tube containing suspension with the cells studied. Lymphocytes were gated on the two-dimensional diagram with coordinates corresponding to cell size (FSC) and grain density (SSC). Simultaneously, the gate was set so that the total percentage of cells with CD3, CD19 and CD56 antigen expression in the lymphocyte field was at least 96%. The percentage of cells with surface expression of a given determinant was defined by means of the quadrant analysis on a two-dimensional dot diagram with coordinates corresponding to FL1 and FL2 fluorescence. For separation of cells indicating autofluorescence and unspecific luminescence, the above-mentioned isotypic controls were used.

All children and/or their parents gave informed consent. The study protocol obtained approval from the Ethics Board.

Statistical Analysis

The mean values of measurable features (arithmetic means and medians), variable ranges (minimum to maximum), the mean delta as well standard deviations were calculated. At first, the F-Snedecor test was used. To compare mean values, the Mann-Whitney test (in case of two groups) or the Wilcoxon test or Student's t test for paired samples (in case of results in the same group at two different time points) was used. Spearman's rank correlation coefficient was used to evaluate relationships between variables. The value of $p \leq 0.05$ was considered statistically significant. The programs Statgraphics Plus 5.1 and Statistica 5.0 were used for calculations.

Results

In the pediatric patients with asthma and food allergy, the T-lymphocyte percentage before food challenge ranged between 59.6 and 78.2% (mean: 68.4%) of all cells studied. The respective values before the placebo challenge were: 56.5–75.7% (mean 68%; table 2). No statistically significant differences were found prior to food and placebo challenge ($p > 0.05$). Four hours after the allergen challenge, the T-lymphocyte number did not change and ranged between 59.1 and 76.7% (mean: 67.9%) of all the cells studied; after the placebo challenge it was 57.6–77% (mean 67.8%; $p > 0.05$). The number of T lymphocytes did not significantly differ 24 h after allergen or placebo challenge, and ranged between 59.3 and 76.8% after allergen challenge (mean: 68.7%); after placebo, it was 59.8–76.8% (mean 67.8%; $p > 0.05$). Thus, allergen or placebo challenge did not alter the number of the T-lymphocyte (CD3+) population in the study group.

In the asthmatic children without food allergy, the T-lymphocyte percentage ranged between 56.6 and 78.2% before food challenge (mean: 67.5%) of all cells studied. The respective percentages before the placebo challenge were 48–75.3% (mean 67.8%; table 2). No statistically significant differences were found prior to the food and placebo challenge ($p > 0.05$). Four hours after the allergen

Table 3. The mean delta lymphocyte subsets CD3+, CD3+CD25+ and CD19+CD23+ before the challenge, followed by 4 and 24 h after the challenge with allergen or placebo in the group of children with asthma with and without food allergy (FA)

Mean delta lymphocyte subsets	Time					
	0–4 h		0–24 h		4–24 h	
	with FA	without FA	with FA	without FA	with FA	without FA
After allergen challenge						
CD3+	–0.481	–0.014	0.383	0.471	0.864	0.485
CD3+CD25+	1.623 ^a	–0.775	1.324 ^b	–0.721	–0.299	0.054
CD19+CD23+	1.817 ^c	0.361	1.234 ^d	0.207	–0.583	–0.154
After placebo challenge						
CD3+	–0.184	0.299	–0.174	–0.018	0.01	–0.318
CD3+CD25+	–0.121	–0.264	–0.550	–0.701	–0.428	–0.437
CD19+CD23+	1.182 ^e	0.409	0.606	–0.002	–0.576	–0.411

^a $p = 0.01$; ^b $p = 0.014$; ^c $p = 0.0001$; ^d $p = 0.01$; ^e $p = 0.04$.

challenge, the T-lymphocyte number did not change and ranged between 49.1 and 76.7% (mean: 67.5%) of all the cells studied; after the placebo challenge it was 50–76.9% (mean 68.2%; $p > 0.05$). The number of T lymphocytes did not significantly differ 24 h after allergen or placebo challenge and ranged between 61.1 and 76.5% after allergen challenge (mean: 67.9%); after placebo, it was 48.4–76.8% (mean 67.8%; $p > 0.05$). Thus, allergen or placebo challenge did not exert an effect on the alteration in the number of T lymphocytes (CD3+) in the control group. There were no statistically significant differences between the number of T lymphocytes at the time points studied in the group of children with asthma and food allergy compared to asthmatic children without food allergy.

In the asthmatic food-allergic children, evaluation of T-lymphocyte subsets (CD3+CD25+) revealed statistically significant differences. Before the allergen challenge, the cell pool comprised on average 9% of CD3+CD25+ cells, ranging between 5.3 and 15.2%. Four hours after the challenge, an increase in their number was observed, the mean delta being 1.623 ($p = 0.01$), similar to 24 h later (mean delta: 1.324; $p = 0.014$; table 3). However, the placebo challenge did not significantly affect the amount of this T-lymphocyte subset (CD3+CD25+). The percentage of T lymphocytes (CD3+CD25+) before the challenge was 4.5–16.1% (mean: 9.3%), after 4 h the mean delta was –0.121, and after 24 h the mean delta was –0.550 ($p > 0.05$).

In the control group, the food challenge did not result in significant differences in the T-lymphocyte subsets (CD3+CD25+; table 2, 3).

The mean values of CD3+CD25+ in children with food allergy were $\geq 10.3\%$ 4 h after allergen challenge and 10% 24 h after allergen challenge, while in the group without food allergy they were 8.3 and 8.4%, respectively, however the differences were not statistically significant ($p = 0.053$ and $p = 0.143$).

The differences in time between the parameters studied in the study and control groups were also assessed using delta mean values. The changes in CD3+CD25+ expression 0–4 and 0–24 h after allergen challenge were statistically greater in children with food allergy (1.623 and 1.324) than in children without food allergy (0.775 and 0.721; $p = 0.003$ and $p = 0.008$, respectively).

In the asthmatic food-allergic children, the CD19+CD23+ B-lymphocyte subset before the allergen challenge comprised, on average, 10.2% of all the cells studied. The respective values before the placebo challenge were: 5.6–17.1% (mean 11.3%). No statistically significant differences before the allergen and placebo challenge ($p > 0.05$) were noticed. Four hours after the allergen challenge, the CD19+CD23+ B-lymphocyte subset number increased (mean delta: 1.817; $p = 0.0001$) and after placebo challenge the CD19+CD23+ B-lymphocyte subset percentage was 4.6–26.3%, the mean delta being 1.182 ($p = 0.04$). The CD19+CD23+ lymphocyte subset ranged between 5.1 and 18.6% of all cells 24 h after allergen challenge (mean delta: 1.234; $p = 0.01$); after placebo administration, the mean delta was 0.606 ($p > 0.05$). Thus, the allergen challenge significantly affected the number of CD19+CD23+ lymphocytes 24 h after allergen challenge compared with placebo.

However, no such differences were noted in the group of asthmatic children without food allergy (table 2, 3). Food challenge in children with asthma and food allergy caused significant differences in the level of CD19+CD23+ lymphocytes, contrary to the results of food challenge tests in asthmatic children without food allergy. The mean values of CD19+CD23+ in children with food allergy were 12.06 and 11.474 at the time points 4 and 24 h after allergen challenge, being 9.647 ($p = 0.043$) and 9.492 ($p = 0.04$) in the group without food allergy, respectively. The differences between the study group and the control group were also assessed using mean delta values. The changes in CD19+CD23+ after allergen challenge (0–4 h) were statistically greater in children with (1.817) than in children without food allergy (0.361; $p = 0.045$).

In addition, the correlation between the changes in CD3+CD25+ and CD19+CD23+ expression and the changes in PC20 (the provocative concentration of a substance producing a 20% decline in FEV₁) was analyzed. Statistical analysis shows that the higher the increase in CD3+CD25+ and CD19+CD23+ expression after the food allergen challenge in asthmatic food-allergic children, the lower the PC20 value, i.e. the more elevated bronchial hyperreactivity; however, this dependence was not statistically significant (for CD3+CD25+ $p = -0.264$, $p > 0.05$; for CD19+CD23+ $p = -0.170$, $p > 0.05$). No correlation was observed in the case of placebo ($p = 0.137$, $p = -0.114$, $p > 0.05$).

We also assessed whether there was any relationship between the changes in CD3+CD25+ and CD19+CD23+ expression and a type of reaction (effector organ) in the course of the oral provocation test. It was shown that the type of reaction during the test was associated with the changes in CD3+CD25+ expression, both 4 and 24 h after the challenge. An increase in the expression was most frequently observed in children with respiratory reactions, less frequently in those with skin lesions (after 4 h: $p = 0.034$, $p = 0.040$; after 24 h: $p = 0.009$, $p = 0.014$) and rarely in case of gastric symptoms ($p > 0.05$).

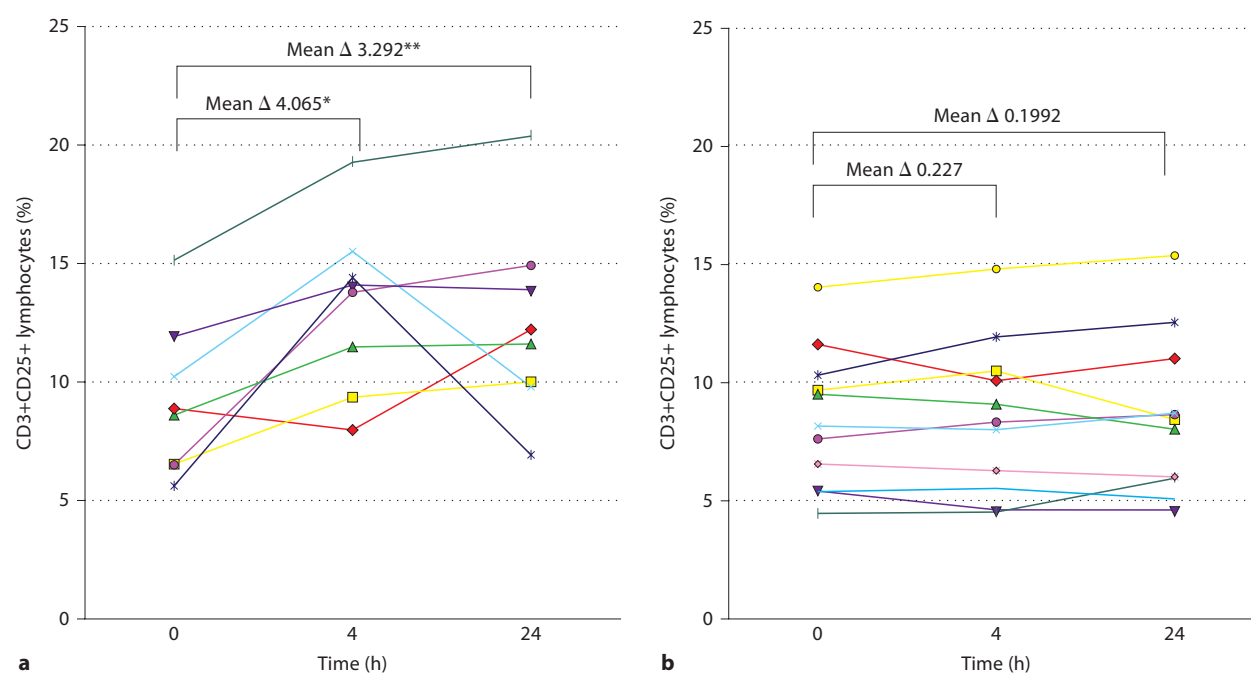
Four hours following the food challenge test, CD3+CD25+ expression was significantly higher in children reacting with respiratory symptoms compared to children with the remaining types of reactions (after 4 h in the respiratory group the mean delta was 4.065, in the remaining group it was 0.227; $p < 0.004$; fig. 1). Similar alterations were absent following the placebo challenge (after 4 h the mean delta was -0.793 in the respiratory group, being 0.227 in the remaining group; $p > 0.05$; fig. 2). Likewise, 24 h after the food provocation, the CD3+CD25+ expression significantly differed between

children with respiratory reactions and those with the remaining types of reactions (mean delta: 3.292 and 0.199, respectively; $p < 0.003$), while no significant differences were found after the placebo provocation (mean delta: -1.168 and -0.32 , respectively; $p > 0.05$). Moreover, there was no correlation between changes in the CD19+CD23+ expression and type of clinical response ($p > 0.05$) either after allergen or placebo (4 h after the allergen challenge, the mean delta was 2.046 in the respiratory group and 1.686 in the other group; $p > 0.05$, and 24 h after allergen challenge, it was 1.93 in the respiratory group and 0.835 in the other group; $p > 0.05$; fig. 3, 4).

Correlations between the changes in CD3+CD25+ and CD19+CD23+ expression and the allergen dose were also analyzed. CD3+CD25+ expression was found to be more frequently elevated in case of a reaction after administration of a low dose of allergen ($p = 0.04$) than after a medium or a high dose ($p = 0.3$, $p = 0.2$). Similar correlations were not detected in relation to CD3+CD25+ after placebo provocation and to CD19+CD23+, both in the case of allergen as well as placebo.

An attempt was made to check whether CD3+CD25+ and CD19+CD23+ expression was associated with the severity of the reaction following the challenge test. Severe reactions occurred in 9 children, moderate reactions in 8 children and mild reactions in 5 children. In patients with severe reactions, they affected the respiratory tract ($n = 6$), skin ($n = 2$) and the gastrointestinal tract ($n = 1$). Moderate reactions were detected in 2 children with respiratory reactions, 2 with skin and 4 with gastrointestinal reactions. In patients with mild reactions, 2 concerned the gastrointestinal tract and 3 the skin. A statistically significant correlation between the changes in the CD3+CD25+ and CD19+CD23+ expression and the severity of the reaction following the allergen challenge test was found. The mean value of CD3+CD25+ was 12.9% in the patients with severe reactions 4 h after allergen challenge, being -8.76% ($p = 0.015$) in patients with moderate reactions and 8% ($p = 0.028$) in patients with mild reactions. In addition, the increase in CD3+CD25+ 4 h after allergen challenge was greater in patients with severe reactions (mean delta: -3.8) than in patients with moderate reactions (mean delta: -0.01 ; $p = 0.003$) and in patients with mild reactions (mean delta: -0.2 ; $p = 0.004$). Moreover, the increase in CD19+CD23+ expression 4 h after allergen challenge was more significant in patients with severe reactions (mean delta: 2.6) than in patients with moderate reactions (mean delta -0.65 ; $p = 0.04$). CD3+CD25+ and CD19+CD23+ expression was greater in patients with severe reactions than in those with moderate/mild reactions.

1



2

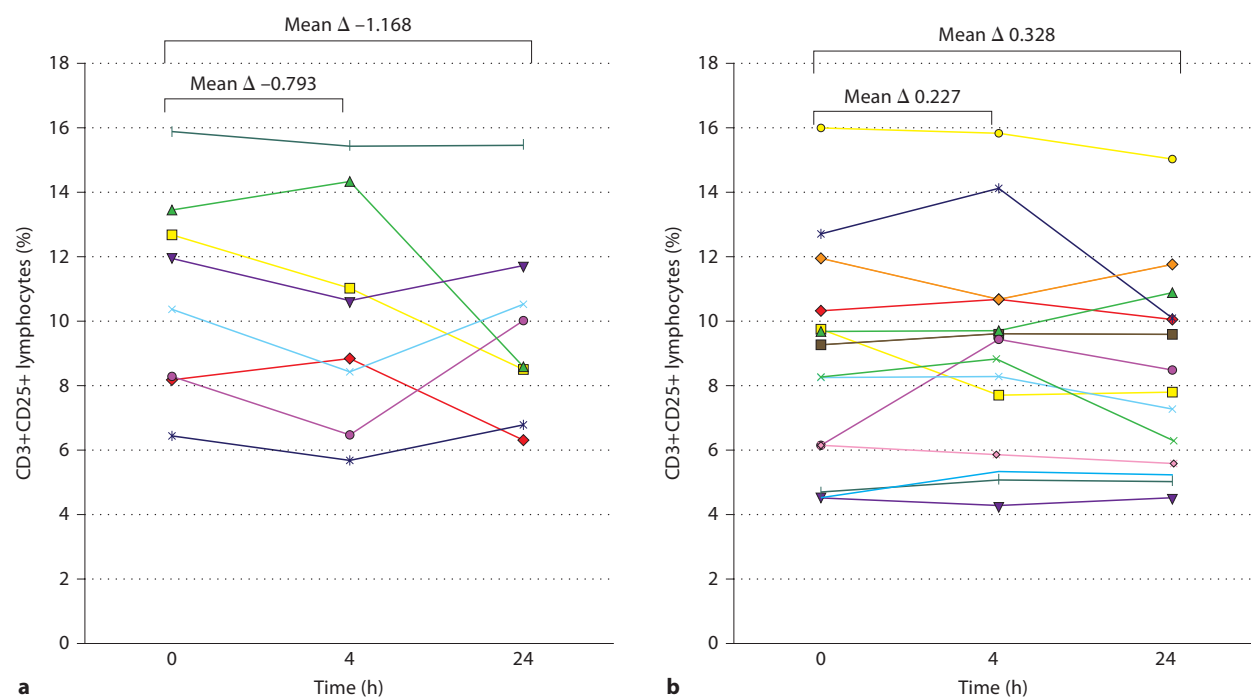
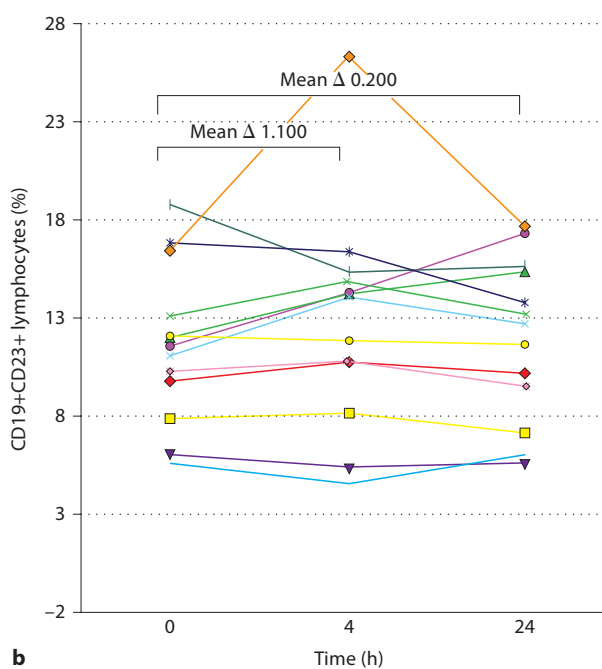
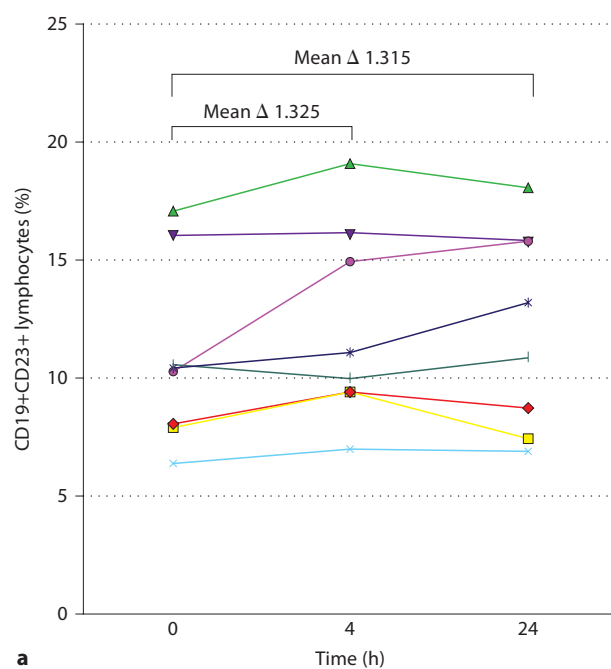
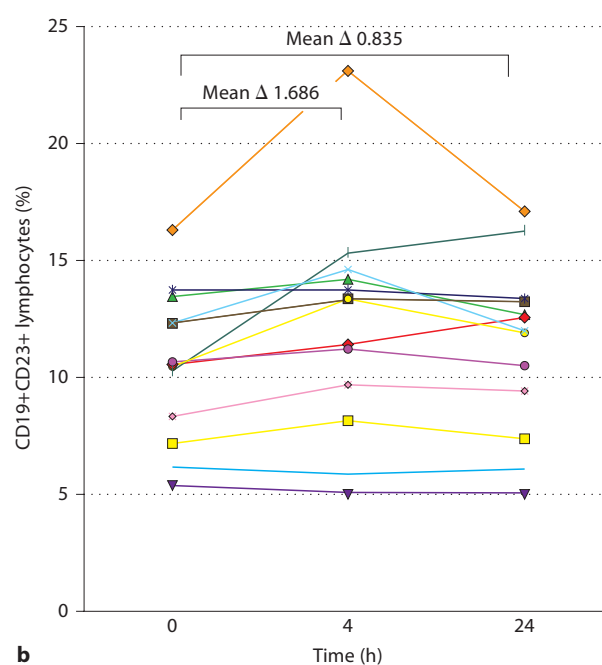
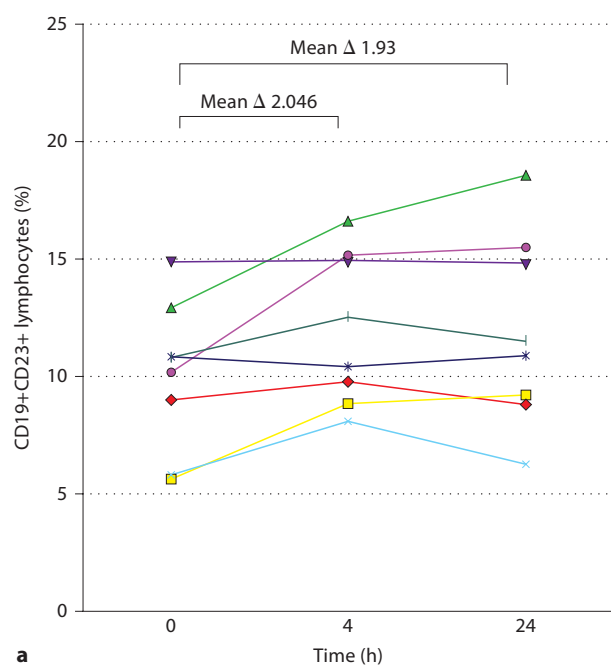


Fig. 1–4. CD3+CD25+ (1, 2) and CD19+CD23+ T lymphocytes (3, 4) in children after allergen (1, 3) and placebo challenge (2, 4) with (a) and without respiratory reaction (b). 1 * $p < 0.004$, ** $p < 0.003$. 2–4 $p > 0.05$. (For figures 3 and 4 see next page.)



Discussion

The pathomechanism of asthma and food allergy is complex and not well understood. An essential feature of inflammatory reactions and immune responses involved in allergy is excessive activation of cells such as T lymphocytes, eosinophils, mast cells, basophils, mononuclear phagocytes and blood platelets. While the role of eosinophils is indisputable, increasing interest is attributed to lymphocytes in the modulation of IgE-mediated reactivity.

Flow cytometry is a diagnostic method used to determine cell population ratios and percentages of cell subsets and cells exhibiting surface expression for a determinant examined. Quantitative and qualitative evaluation improves the understanding of activation mechanisms and functions of immune cells in the pathogenesis of inflammatory diseases as well as in the regulation of systemic immune responses [13]. Numerous studies have been published to date using flow cytometry to evaluate the activity of individual immune cells in different allergies. In seasonal allergic rhinitis, for example, a correlation between an increase in the number of various inflammatory cells infiltrating the nasal mucosa and the intensity of symptoms and nonspecific nasal hyperreactivity has been observed together with the rise in the number of CD4⁺T lymphocytes during the pollen period. In the course of allergic rhinitis, inflammatory changes in the mucosa are accompanied by the presence of activated CD4⁺ and CD25⁺ T lymphocytes [14]. According to Hallstrand et al. [15], activated Th2 lymphocytes play a dominant role in initiating and maintaining airway inflammation in asthmatic patients, manifested by CD25⁺ and CD23⁺ increases in peripheral blood cells of asthmatics compared to healthy controls. Domínguez Ortega et al. [16] reported a higher expression of activation markers on immune cells, i.e. CD25 on T lymphocytes and CD23 on B lymphocytes, in induced sputum of stable mild asthmatics. On the other hand, Majori et al. [17] found decreased CD25 expression on T cells and decreased CD23 expression on B cells in asthmatic patients sensitized to pollen 1 year after immunotherapy in comparison to healthy controls. The aim of the present study was to determine whether food challenge in children with asthma and food allergy changes the number and activity of immune system cells involved in the inflammatory process underlying these diseases, via the determination of CD25 (IL-2R) antigen expression on T lymphocytes and CD23 (FcεRII) antigen expression on B lymphocytes before and after food challenge.

Activated T lymphocytes exhibit CD25 antigen expression (receptor for IL-2R). A relationship between asthma severity and lymphocyte activation manifested by CD25 expression (IL-2R) has been found [18, 19]. On the basis of the study on bronchial epithelial biopsies in asthmatics, an elevated number of CD25⁺ lymphocytes was detected compared to controls [20]. Moreover, statistically significant differences in T-lymphocyte activation, i.e. a higher CD25⁺ expression, were determined in the bronchoalveolar lavage (BAL) of patients with asthma. No differences in the populations of peripheral blood cells were observed in asthmatics compared to healthy subjects [21].

Data on activated T lymphocytes exhibiting IL-2R expression in patients with asthma are not consistent. In a study by Hashimoto et al. [22], peripheral blood CD25⁺ lymphocytes were increased in asthma, being in contrast to the results of Wilson et al. [21], who stated that their rise can be observed only in BAL. A considerable increase in peripheral blood CD3 and CD25 lymphocytes was revealed in asthmatic children at the time of disease exacerbation, whereas their level dropped during remission [18]. There were no data concerning these problems in children suffering from asthma concomitant with food allergy.

In the study on the CD3 lymphocyte subset in peripheral blood, we have not detected any statistically significant difference after the food allergen challenge in children with asthma and food allergy as well as in children with asthma without food allergy. The studied lymphocytes originated from the blood, and it is likely that CD3 assessment in the bronchial epithelium, BAL, intestinal epithelium or in the skin would be more appropriate. However, the results indicate a significant rise in the subpopulation of CD3⁺CD25⁺ lymphocytes, 4 as well as 24 h after allergen challenge in comparison to the period before the challenge, and contrary to placebo in children with asthma and positive DBPCFC. It may suggest a significant involvement of this cell subpopulation in the pathogenesis of the inflammatory process in patients suffering from asthma concomitant with food allergy, being further evidence of the effect of food on the immune system of the patient allergic to this type of food. Thus, apart from a food challenge test in which clinical reactions can be observed, cytometry appears to be an additional method to confirm the importance of a type of food for the food-allergic patient. Similar correlations were not detected in relation to asthmatic children with a negative DBPCFC.

Although the changes in CD3⁺CD25⁺ expression were statistically significant in the study group after al-

lergen challenge, contrary to placebo challenge, there were no statistically significant differences between mean values in the study and control groups. Probably, this is due to the relatively small number of patients and the wide range of data. The significant differences in mean delta values of CD3+CD25+ 4 and 24 h after allergen challenge between the study group and the control group indicate that this cell type can play a crucial role in patients with asthma and food allergy.

Besides assessing CD25 antigens on T lymphocytes, we have also analyzed antigens on B lymphocytes. The CD23 receptor (FcεRII) plays an important role in IgE-related reactions, in the control of IgE synthesis, phagocytosis and in the presentation of antigens to different cells. Stable CD23 expression on human intestinal epithelial cells has been shown as well as increased expression in patients with inflammatory bowel disease and cow milk protein-induced enteropathy. CD23 antigen also indicates an intensified expression in the tonsils and lymph nodes of allergic children. In patients with atopic dermatitis (AD), a higher CD23 expression on B lymphocytes and monocytes in the peripheral blood and also in antigen-presenting cells of the skin has been observed [23]. With subsiding changes in case of AD therapy, decreased CD23 expression can be noticed in the skin. Higher percent values of CD23 lymphocytes have been reported in children with AD, however, there were no statistically significant differences in comparison to healthy subjects.

Changes in immune cell activity and receptor expression are a very valuable method to assess exacerbating factors in the course of allergic disease. Studying the effect of specific challenge trials on cellular activity in asthma, CD4/CD8 activation did not differ between the peripheral blood of asthmatics and healthy subjects. These negative observations concerning lack of T-lymphocyte activation in the peripheral blood following allergen challenge indicate that T-cell activation may be limited to the target organ of the allergic reaction. The results of T-lymphocyte activation in the peripheral blood or BAL after the provocation are diverse, showing not only an increased CD4/CD8 ratio, but also their reduction [24–26]. This is arguably why Schmitt et al. [24] did not find any differences in T-lymphocyte activation in asthmatics 24 h after the allergen provocation, being in opposition to *in vitro* studies by others who, after allergen challenge, demonstrated elevated CD25 expression in lymphocyte cultures of asthmatics compared with healthy subjects. Furthermore, according to Schmitt et al. [24], a higher CD23 expression on CD19 takes place in asthmatic chil-

dren compared to controls, although no changes in their expression were noted after the challenge [25]. Also, Gargro et al. [27] demonstrated an increase in CD23 receptor expression in children with atopic asthma challenged with inhaled allergens. Hallstrand et al. [15] reported increased activation of CD23 B lymphocytes and CD25 T lymphocytes after an exercise test in asthmatic children.

There are some publications assessing cytometry in food allergy. Clinical reactions to food allergens have been known to be associated with alterations in different cell populations. Beyer et al. [28] observed a drop in leukocytes and T and B lymphocytes in the peripheral blood of children sensitized to food allergens, as well as a CD4/CD8 increase, irrespective of the type of clinical reaction at the time of the positive food challenge. The results of these studies signify a crucial role of cellular mechanisms in the response to food allergens; however, they are not useful for monitoring the results of DBPCFC [28]. It has also been shown that the number of CD23+ B lymphocytes decreases during allergen challenge. Part of these cells is assumed to become memory B lymphocytes which are characterized by the loss of CD23 expression.

In animal models, challenge of food-sensitized animals does not affect the total number of T cells, but increases the CD25 subpopulation [29], in accord with our findings. Previously, it was also reported that CD69 lymphocyte expression increases after stimulation of children with cow milk allergy. However, after an eliminating diet in these children, a slight reduction in CD69 expression can be observed. It is suggested that CD69 can be used for monitoring therapy in children with cow milk allergy. Interesting outcomes of the study were presented by Hoffmann et al. [30] who demonstrated a higher CD25 expression on CD4+ T lymphocytes after oral challenge with placebo, and a lower one after the allergen challenge. Our results reveal a significant increase in CD19 CD23 expression in the children examined 4 h after positive food challenge. This significant difference remained for 24 h following the challenge, however, the percentage of CD19 CD23 antigens was lower at that time. CD19+CD23+ expression differed significantly after allergen challenge in the study group compared with the control group.

However, we observed a significant rise in CD19 CD23 antigens 4 h after placebo administration. This finding was unexpected but probably induced by the stress associated with blood collection leading to the increased subpopulation of T lymphocytes, eosinophils and natural killer cells [31]. This is likely to be explained in a similar way as Hoffman et al. [30] have done in their study, in which they demonstrated an increase in CD25+ on T cells

after placebo, and they associated it with the recruitment of activated T cells from the circulation to the site of allergen exposure. Moreover, if CD23 receptor expression depends on the phase of cellular development and involves only antigen-activated B lymphocytes before their differentiation into immunoglobulin-producing plasma cells, perhaps our results depend on another phase of differentiation.

Furthermore, our study revealed the highest increase in CD3+CD25+ expression in patients with asthma and food allergy, who developed respiratory reactions during food challenge. It is likely that children with asthma and food allergy reacting with asthma exacerbation after food intake constitute a consecutive asthma phenotype with considerable bronchial hyperreactivity, in which special activation of CD3+CD25+ takes place.

The study has not revealed a statistically significant correlation between changes in the expression and eliciting doses, however expression was found to be more frequently elevated in case of a reaction after administration of a low dose of allergen than after a medium or a high dose. The highest increase in expression was shown in patients with respiratory reactions after food challenge. Thus, a change in immune cell activity was associated with reaction severity during the provocative test; the more severe the reaction, the greater the change observed in expression. It can also be stated that the more severe the reaction, the lower the eliciting dose.

Data concerning the correlation between the eliciting dose and reaction severity are diversified. Perry et al. [32] found no positive correlation between reaction severity and the eliciting dose, though more severe reactions were

seen in subjects who reacted to lower doses. Benhamou et al. [12] reported no statistically significant relationship between the threshold dose and IgE values. The latter, however, revealed a significant correlation with reaction severity.

Chen et al. [33] reported T-lymphocyte activation and an increase in CD25 and CD23 expression leading to bronchial reactivity after specific provocations in asthmatics. Maciorkowska et al. [34] demonstrated significantly elevated numbers of T (CD3 and CD8) and B lymphocytes (CD20) in the gastric mucosa of patients with food allergy compared to controls.

The results of the present study indicate a significant role of CD25 T lymphocytes and CD23 B lymphocytes in the activation of inflammatory processes in children with asthma concomitant with food allergy. It is reasonable to speculate that it would be useful to monitor the effect of treatment (elimination diet) in these patients.

Conclusions

Firstly, increases in CD25 T-lymphocyte and CD23 B-lymphocyte populations after food allergen challenge may indicate their significant role in the pathogenesis of the active phase of the immunoinflammatory process in children with asthma concomitant with IgE-mediated food allergy. Secondly, there is a correlation between the activity of the CD25+ T-lymphocyte population and an allergic reaction to food in the respiratory tract in children with asthma and food allergy.

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