

Expression of Fas Ligand and CTLA4 in Adenoids Has a Predictive Value for Allergic Rhinitis Development in Children

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Key Words

FasL expression · CTLA4 expression · Adenoid · Allergic rhinitis

Abstract

Background: The balance of CD28/CTLA4-derived signals and Fas-dependent apoptosis activity is determined by the peripheral defense mechanisms and might play a role in the pathogenesis of allergy. **Objectives:** The aim of the study was to investigate the expression of costimulatory and pro- and antiapoptotic molecules in adenoid T cells of children suffering from allergic rhinitis and to find out which of these molecules have a predictive value in the development of allergic rhinitis. **Methods:** The adenoids of 60 children, removed because of nasal obstruction, chronic rhinitis and recurrent respiratory infection, were evaluated. Patients were divided into two groups: group 1, suffering from chronic allergic rhinitis, and group 2, suffering from chronic rhinitis, where no specific IgE was detected, including children with a positive family history of allergy (group 2a) and children with neither a personal nor a family history of allergy (group 2b). For immunohistochemical stainings anti-CD3, anti-CD19, anti-CD4, anti-CD8, anti-CD25, anti-CD28, anti-CTLA4 (CD152), anti-bcl-2, anti-Fas, and anti-FasL antibodies were used. The number of cells expressing these molecules was identified in adenoid interfollicular spaces. The results were

then analyzed in allergic and nonallergic children. During a 24-month follow-up children were re-examined for allergy and results were compared to previous immunohistochemical evaluations. **Results:** The expression of CD4, CD25, CD28, FasL, and CTLA4 was significantly increased in group 1 compared to group 2 ($p < 0.05$). However, the discriminant analysis confirmed that only CTLA4 and FasL expression fully discriminated allergic subjects from the others. During a 24-month period of observation 8 children from group 2a were also diagnosed with allergic rhinitis. All of them, especially those sensitized to mites, had an increased number of FasL+ and CTLA4+ in previously removed adenoids. **Conclusion:** An increased number of cells with intracellular expression of FasL and CTLA4, in interfollicular spaces of adenoids, seems to be a predictive factor of the development of allergic rhinitis.

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Introduction

Although atopy is a risk factor for both allergic rhinitis and IgE-dependent asthma, it is not the only factor necessary for the initiation of an allergic disease. Changes in the local tissue microenvironment, which induce the conversion of the immature lymphocytes into Th2 cells, seem to be crucial at the first step of allergy development.

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A successful interaction between CD28/CTLA4 on T cells and B7-1 or B7-2 on antigen-presenting cells is necessary for T cell activation [1–3]. The balance of CD28- and CTLA4-derived signals profoundly alters the outcome of T cell activation; CTLA4 plays a critical role in the downregulation of T cell responses and may play an important role in the pathogenesis of the increased IgE response [4–6].

The blocking effect for Fas expression, a cell surface molecule that mediates receptor-triggered apoptosis, of the Th-2 cytokine is important for allergic inflammation [7]. Local tissue inflammation due to Fas ligand (FasL) reaction usually precedes symptoms of allergic disease [8]. The increased expression of FasL might contribute to the inability of maintaining normal peripheral tolerance.

The ability of bcl-2 to protect autoreactive lymphocytes from apoptosis may be influenced by many factors, including the developmental stage of the lymphocyte as well as the nature of the antigen stimuli [9, 10]. The antiapoptotic members of the bcl protein family, which can prevent certain types of cell death, do not provide efficient protection from receptor-induced apoptosis.

Hypertrophy of the adenoids, part of the lymphoid tissue closest to the nasal mucosa, is often observed in many patients who develop allergic diseases of the respiratory tract, but the relationship between the hypertrophy of the adenoids and allergic disease has not been established [11, 12].

The aim of the present study was to identify adenoid-associated markers of the immune response that could serve as prognostic factors for the development of allergic rhinitis in children. We examined the expression of T cell antigens in adenoids removed from children because of chronic rhinitis and recurrent respiratory infections. The children were subsequently observed for 2 years for the development of allergic rhinitis.

Materials and Methods

Patients

The study group included 60 children (aged 5–8 years, median age 6.67 years, 41 boys and 19 girls) who qualified for adenoidectomy on the basis of nasal obstruction, chronic rhinitis and recurrent infections. We excluded children with ear inflammation, chronic tonsillitis, acute infection, treated with systemic and local corticosteroids or immunomodulatory drugs in preceding 4 weeks.

All children were evaluated according to the EAACI protocol [13]. They were divided into two groups. Group 1 included 20 children suffering from chronic allergic rhinitis, 5 of them also from asthma (aged 6–8 years, median age 7.54, 14 boys and 6 girls).

Group 2 included 40 children suffering from chronic rhinitis but without allergen-specific IgE in skin and serum. In addition group 2 was subdivided according to their family history of allergy. Children with a positive family history were classified as group 2a (20 children aged 5–7 years, median age 6.26, 13 boys and 7 girls) and children with no family history of allergy as group 2b (20 children aged 5–8 years, median age 7.05, 14 boys and 6 girls). In all examined children adenoidectomy was performed out of the pollen season (from October to February). Skin prick tests with common allergens (Allergopharma, Germany) were performed in all patients, but in some cases a sensitization was also confirmed by identification of specific serum IgE (Pharmacia CAP System, Sweden).

Tissue Examination

The excised adenoids were snap-frozen in liquid nitrogen and stored at -80°C . The frozen tissue was cut on the cryostat. The tissue sections were subsequently stained with hematoxylin and eosin or underwent immunohistochemical staining with one of the following antibodies: anti-CD3, anti-CD4, anti-CD8, anti-CD19, anti-CD25, anti-CD28, anti-Fas, anti-FasL, anti-CTLA4(CD152) or anti-bcl-2 (Novocastra and PharMingen). Eleven tissue sections were analyzed for every adenoid, 1 of them was histomorphologically and 10 sections were immunohistochemically evaluated.

Marker-positive cells (expressing examined molecules on the surface and/or intracellularly) were quantified with the computer image analysis system MultiScan Base 8.08 software (CSS, Warsaw, Poland). The number of 'positive' cells in interfollicular spaces was calculated in each field at the magnification $400\times$ in Nikon Microplat-FXA with the video camera CC20P. A mean value from three fields per one section was taken for further analysis.

Follow-Up Observation

Children were followed up for 24 months after adenoidectomy. Group 1 was treated according to the WHO recommendations on Allergic Rhinitis and Its Impact on Asthma Guidelines [14]. Group 2 (19 children from group 2a and 15 children from group 2b) was examined for any signs of allergy (symptoms of rhinitis, asthma and allergic skin disease as well as the presence of specific IgE in the skin and/or in serum).

Statistical Analysis

At first the Tukey-type multiple comparison tests for normally and abnormally distributed variables were used [15]. Results were considered to be statistically significant at $p < 0.05$.

Subsequently, the stepwise discriminant function analyses were performed in order to evaluate correlations of the above-mentioned statistically significant values with positive allergy diagnosis. Then the variables were defined on the basis of the respective F value and partial Wilk's lambda, which was associated with the unique contribution of the respective variable to the discriminatory power of the model, and then approximated to the logic of multiple regression coefficients. The scores of a given discriminant function were calculated for each individual and served as the coordinates in a graphical presentation of a cluster of the points representing the separation of the studied groups. The relative resemblance between the examined groups of subjects was expressed as the squared Mahalanobis distances (d_M) between the centroids of the examined group in figure 2. It made it possible to establish whether the clinical classification of patients agreed with the results of the immunohistochemical evaluation of the adenoids [16].

Table 1. Quantity of CD3- and CD19-expressing cells

Expression in groups examined		Mean $\pm$ SD	Range	Statistical comparison		
				group 1 to 2a	group 1 to 2b	group 2a to 2b
CD3	Group 1	156.7 $\pm$ 20.9	120–186	0.59	0.48	0.56
	Group 2a	148.3 $\pm$ 28.7	128–178			
	Group 2b	149.3 $\pm$ 31.3	119–176			
CD19	Group 1	63.9 $\pm$ 10.4	56–72	0.87	0.68	0.95
	Group 2a	62.1 $\pm$ 9.3	52–69			
	Group 2b	62.3 $\pm$ 10.8	53–71			

Table 2. Mean value, standard deviation and statistical analysis of the cells expressing molecules evaluated in examined groups

Molecule	Group 1	Group 2a ¹	Group 2b	Statistical comparison		
				1/2a	1/2b	2a/2b
CD4	135.0 $\pm$ 30.6	129.5 $\pm$ 39.6	114.6 $\pm$ 28.4	0.6100	0.0090	0.0250
CD8	96.8 $\pm$ 32.9	113.3 $\pm$ 40.5	114.0 $\pm$ 33.9	0.1450	0.2870	0.9500
CD25	123.7 $\pm$ 32.9	121.6 $\pm$ 20.5	114.2 $\pm$ 20.7	0.1590	0.0160	0.0580
CD28	163.8 $\pm$ 36.7	139.2 $\pm$ 30.3	113.4 $\pm$ 31.5	0.0250	0.0030	0.0450
CD95	144.1 $\pm$ 15.2	139.2 $\pm$ 21.9	146.9 $\pm$ 35.7	0.5440	0.7850	0.6510
FasL	138.7 $\pm$ 19.9	108.2 $\pm$ 12.5	90.0 $\pm$ 7.9	0.0001	0.0001	0.0003
CD152	149.0 $\pm$ 13.7	120.6 $\pm$ 9.5	104.3 $\pm$ 8.1	0.0012	0.0001	0.0015
Bcl-2	149.4 $\pm$ 11.9	134.0 $\pm$ 19.5	129.9 $\pm$ 30.1	0.4760	0.1570	0.6460

¹ Group 2a includes the children who after 24 months' follow-up were diagnosed with allergic rhinitis.

Results

Examination of Adenoid Tissue

The morphological analysis revealed follicular hypertrophy with a chronic inflammation in all tested adenoids. The number and the size of secondary adenoid follicles were similar in all the examined groups.

The number of CD3+ cells in interfollicular spaces was up to 3-fold higher than the number of CD19+ cells. No statistical differences were found between the evaluated groups. The tissue sections with a comparable number of CD3+ and CD19+ cells were chosen for further analysis (table 1).

The number of CD4+ cells in the interfollicular spaces of adenoids in the group of allergic children was significantly increased ($p = 0.0067$). In contrast, the number of CD8+ cells did not differ in all examined groups.

The number of cells expressing CD25, CD28, CTLA4(CD152) and FasL was significantly higher in group 1 and 2a than in group 2b. The number of Fas+ or

bcl-2+ cells was similar in all the examined groups (table 2).

Discriminate Analysis of Expression of Evaluated Molecules for Allergic Rhinitis Diagnosis

Changes in the expression of CD25, CD28, CTLA4 and FasL were examined as important factors in children suffering from allergic rhinitis. Thus, the statistical discriminant analysis was used to evaluate molecules important for allergic rhinitis in children's adenoids. Only values for the expression of CTLA4 and FasL fully discriminated allergic subjects from other groups and were retained in the model ($p < 10^{-5}$, partial Wilk's lambda = 0.46, and $p = 0.03$, partial Wilk's lambda = 0.88, respectively, for CTLA4 and FasL). This computational approach revealed significant differences in the examined groups. The highest discrepancies were noticed between group 1 and groups 2a ($p < 10^{-5}$, $d_M = 14.6$) and 2b ($p < 10^{-5}$, $d_M = 31.6$). The distance in multivariate space between groups 1 and 2a or 2b was less significant ($p = 0.0001$, $d_M = 3.3$).

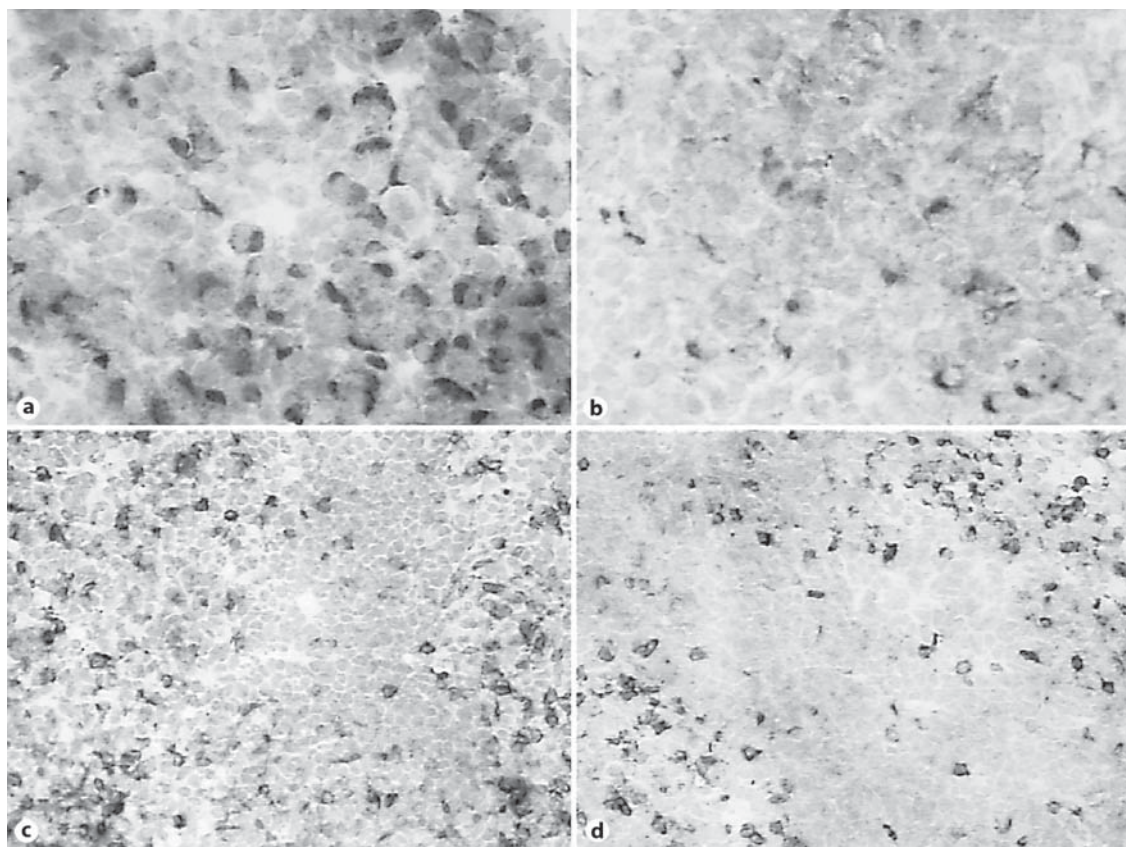


Fig. 1. The interfollicular space of the adenoids. Expression of FasL: in allergic children (a) and in nonallergic children (b). Magnification $\times 400$. Expression of CTLA4(CD152): in allergic children (c) and in nonallergic children (d). Magnification $\times 200$.

The expression of CTLA4 and FasL in adenoids of allergic and nonallergic children was presented in figure 1.

Using the estimated discriminant analysis coefficients (for CTLA4 and FasL) we calculated the scores of discriminant functions for each evaluated individual. The distribution of the points (given by the resultant coordinates) which show the clusters of the points representing the different groups of subjects is presented in figure 2.

The compatibility of the results of the discriminate stepwise analysis and the clinical classification of children

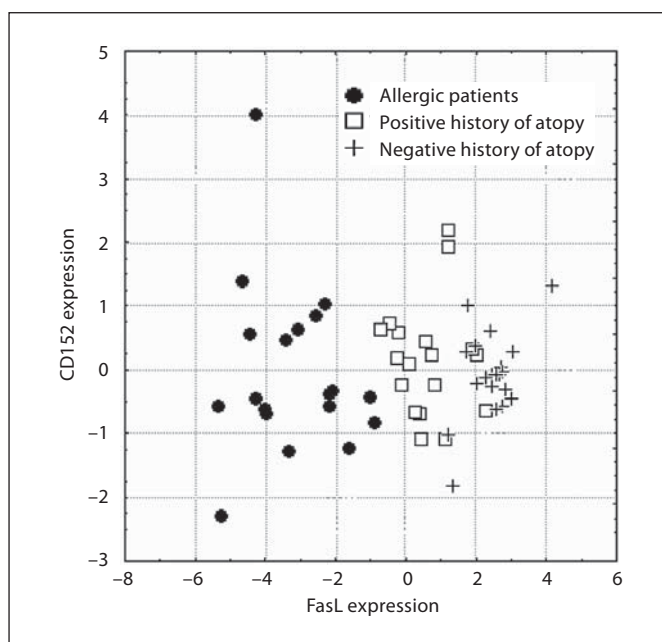


Fig. 2. Stepwise model of discriminant analysis of expression of FasL and CD152 antigens in adenoids in groups of patients with chronic allergic rhinitis, chronic rhinitis without allergy but with a positive family history of atopy and chronic rhinitis without allergy and negative family history of atopy before follow-up.

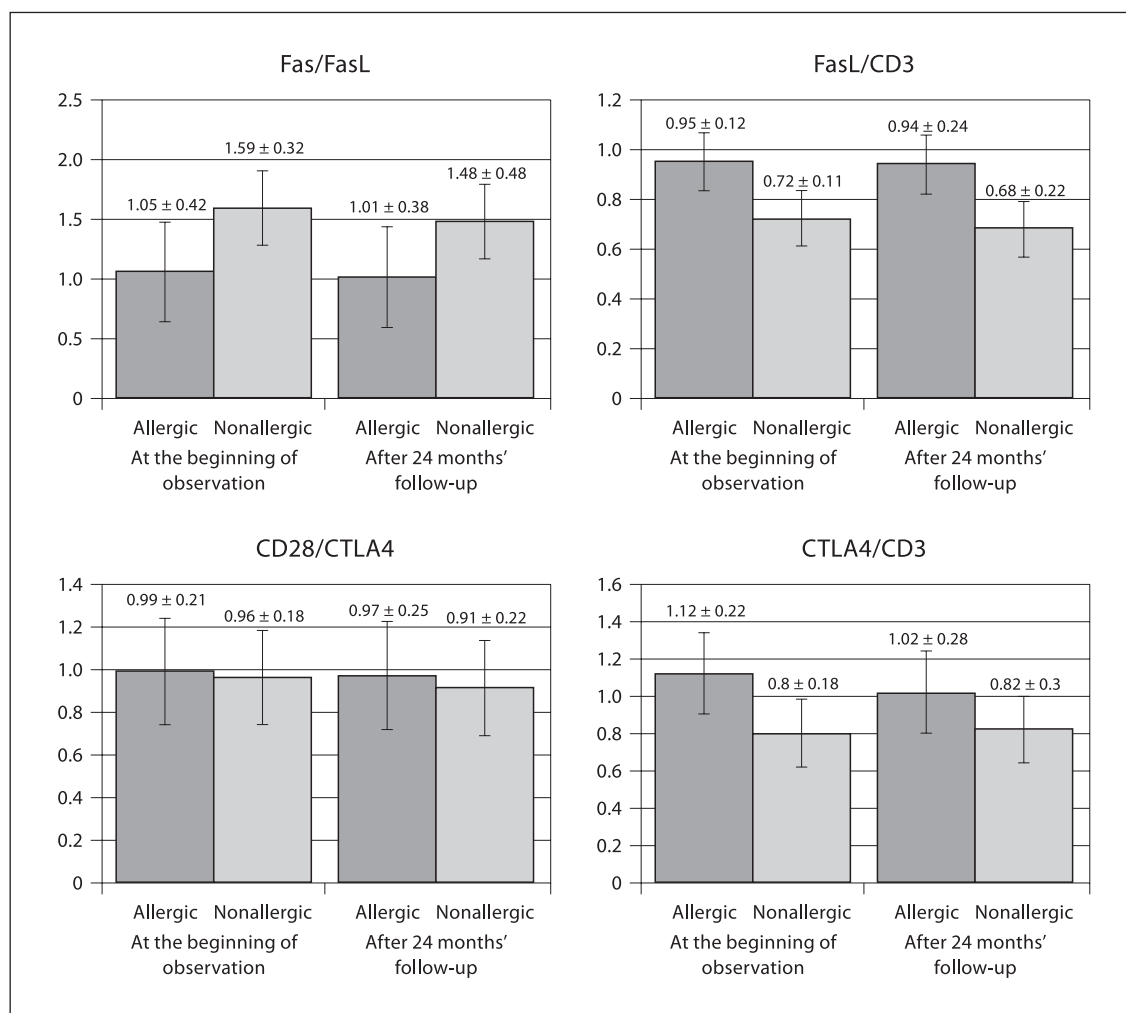


Fig. 3. Comparison of Fas+/FasL+, FasL+/CD3+, CD28+/CTLA4+ and CTLA4+/CD3+ cell ratios in the interfollicular spaces of adenoids of allergic and nonallergic children before and after 24 months' follow-up. Differences between allergic and nonallergic children in Fas+/FasL+, FasL+/CD3+ and CTLA4+/CD3+ cell ratios are statistically significant.

to the study groups was 89.5% in group 1, 78.8% in group 2a and 95.0% in group 2b. Quite a high incompatibility in group 2a ($100\% - 78.8\% = 21.2\%$) was caused by the patients who were classified as allergic during follow-up.

Expression of CTLA4 and FasL Was Subsequently Evaluated as a Prognostic Factor for Allergic Rhinitis

The CD28+ cell/CTLA4+ cell ratio, Fas+ cell/FasL+ cell ratio and both CTLA4+/CD3+ and FasL+/CD3+ ratios were calculated in all evaluated adenoids.

The number of CD28+ and CTLA4+ cells was higher in allergic rhinitis than in nonallergic subjects, but the ratio of CD28+/CTLA4+ cells was similar in allergic

(164/149) and nonallergic (114/104) children. The mean ratio of Fas+ cells/FasL+ cells in allergic and nonallergic children directly after adenoidectomy was 1.05 ± 0.42 (144/136) and 1.59 ± 0.32 (147/90), respectively ($p < 0.05$).

Eight children from group 2a, who did not show positive skin prick tests, and specific serum IgE at the time of adenoidectomy, were diagnosed as allergic after a 24-month follow-up (at periods of 6–24 months). Specific IgE to house dust mite was detected in the serum in 3 of them. In 5 children specific serum IgE to pollens was identified; 1 of the children suffered from allergic rhinitis from February to September.

Interestingly, the mean ratio of Fas+/FasL+ cells in these children was 1.01 ± 0.38 (140/136). In the other 10 children from group 2a no specific IgE after 24 months was found, but 1 of them demonstrated symptoms of allergic rhinitis when exposed to house dust and 3 of them after exposure to grass pollens. The mean value of the ratio of Fas+/FasL+ adenoid cells in children without specific IgE after follow-up was 1.48 ± 0.48 (142/101) and did not differ from children of group 2b: 1.59 ± 0.32 (147/90). The ratio of Fas+/FasL+ in the adenoids of children suffering from allergic rhinitis was compatible with the ratio in children who were diagnosed with allergic rhinitis after the follow-up.

In children who were diagnosed as allergic after the follow-up the ratio of CD28+/CTLA4+ cells was 149/138 and in nonallergic patients it was 140/122 ($p > 0.05$).

The differences between the ratio of FasL+ and CTLA4+ to CD3+ cells were significant in allergic and nonallergic children diagnosed directly after adenoidectomy as well as in children who were diagnosed as allergic and nonallergic after a 24-month follow-up. The ratio of FasL+/CD3+ in allergic children diagnosed before adenoidectomy was 0.96 ± 0.12 (139/150) and in children who were diagnosed as allergic after follow-up it was 0.94 ± 0.24 (136/148).

In nonallergic patients this ratio was 0.72 ± 0.11 (90/140) and 0.68 ± 0.22 (100/148), respectively. The ratio of CTLA4+/CD3+ was 1.12 ± 0.22 (150/148) in initially allergic patients and 1.02 ± 0.28 (139/132) in those diagnosed after follow-up. The ratio of CTLA4+/CD3+ was 0.8 ± 0.18 (104/146) and 0.82 ± 0.3 (116/147) in nonallergic patients, respectively. The differences between allergic and nonallergic children were statistically significant ($p < 0.05$). Data are presented in figure 3.

Discussion

The activation and expansion of lymphocytes which take place in the peripheral lymphoid organs in response to antigens are primarily regulated by apoptosis and an anergic state of the cells [17–19]. The interaction between T cells and antigen-presenting cells takes place in the interfollicular spaces of the adenoids [18–20]. Therefore, we examined the number of costimulatory expressing T cells and apoptosis-associated molecules in the interfollicular spaces of the adenoids. In the absence of costimulation lymphocytes fail to respond effectively and become anergic. Hyporesponsive cells require a costimulation to the response to an antigen [21].

Our results confirmed the increased number of CD4+, CD25+, CD28+, FasL+ and CTLA4(CD152)+ cells in the interfollicular spaces of adenoids in allergic subjects. Their expression was associated with symptoms of allergic rhinitis. The stepwise discriminant analysis showed that only the expression of CTLA4 and FasL in interfollicular regions of adenoids fully discriminated allergic subjects from other groups of patients (without specific IgE and positive family history of atopy as well as negative history of atopy).

Holgate and other investigators [4, 22–25] described that the balance of CD28- and CTLA4-derived signals profoundly alters the outcome of T cell activation and might play an important role in the pathogenesis of the increased IgE-associated responsiveness in atopic diseases.

We identified a higher number of cells expressing CTLA4 in interfollicular spaces of the adenoids in allergic children. However, in allergics we found an increased number of cell-expressing CD28, too. The immunohistochemical stainings did not exactly demonstrate whether the expression was only intracellular or both intracellular and on the surface. The quantity of cells with cytoplasmic expression of CTLA4 was distinctly increased in adenoid T cells of allergic children.

Many authors believe that the high intracellular expression of CTLA4 represents evidence that the T cells were not activated [26–31]. Munthe-Kaas et al. [32] found that the inhibitory effect of CTLA4 was partly mediated by the ability of the extracellular domain to compete for ligands. The cytoplasmic domain of CTLA4 was necessary for the complete inhibitory function of the receptor and for the regulation of in vivo Th cell differentiation [33].

The high expression of both intracellular CTLA4 and membrane CD28 points out the predominant CD28 pathway of T cell activation in children suffering from allergic rhinitis.

In our statistical analysis neither CD25 nor CD28 expression discriminated allergic subject from other groups, despite the fact that differences in the CD25 and CD28 expression between allergic and nonallergic children were significant. There were no differences in the CD25 expression between allergic subjects and children without specific IgE but with a positive history of atopy. The CD28 expression, increased in children suffering from allergic rhinitis, was lower in subjects without specific IgE and did not differ between children with a positive and negative family history of atopy. This observation seems to be important since CD28 has been proved to control the development and maintenance of regulatory T cells, possibly by

stimulating the expression of antiapoptotic proteins or the production of autocrine growth and survival factors [34]. Our sample is relatively small; it is likely that some of our findings did not reach significance because of a lack of statistical power to detect a difference. Thus, further studies with larger sample sizes may generate a more accurate model for the discrimination between the allergic and nonallergic status.

We observed that neither bcl-2 nor Fas expression in adenoid T cells differed between children with and without allergic rhinitis. The FasL expression in human tonsil follicles was higher than in interfollicular spaces [35–37]. The expression of Fas antigen did not differ in studied groups, but the FasL expression in interfollicular spaces was significantly higher in allergic as compared to nonallergic children. Zhang et al. [38] confirmed the induction of T cell tolerance via antigen-presenting cells with a higher expression of FasL. The tolerance depends on the apoptotic elimination of cells initiating immunological responses to antigens. Membrane binding FasL induces signals that greatly depend on both Fas receptor numbers and signal transduction [39]. According to our data the number of cells expressing Fas and FasL in the adenoids of allergic children was similar, but in nonallergics Fas+ cells were predominant. The number of Fas+ and FasL+ cells in children with allergic rhinitis at the time of adenoidectomy was similar to the proportion of cells in the adenoids of children with allergic disease diagnosed during the 24-month follow-up. Thus, allergic inflammation begins before there are clinical symptoms, which confirms previous observations that remodeling of the airways de-

velops for a long time before symptoms of asthma appear [40–42].

With the use of the statistical model based on the estimated discriminant analysis coefficients for CTLA4(CD152) and FasL expression we were able to find parameters discriminating groups of allergic from nonallergic subjects.

On this basis we propose the use of ratios of Fas+ to FasL+, FasL+ to CD3+ and CTLA4+ to CD3+ cells in the interfollicular adenoid spaces as prognostic factors of the development of allergic rhinitis in children. The routine histomorphologic evaluations complemented by immunohistochemical analysis of Fas+, FasL+, CTLA4 (CD152)+ cells in interfollicular spaces of the adenoid after adenoidectomy can be a useful diagnostic procedure at an early stage of allergic disease. We proved that the relation between the number of Fas+/FasL+, FasL/CD3+ and CTLA4+/CD3+ cells in the interfollicular space of the adenoid differed between allergic and nonallergic children. However, further studies are necessary to prove the potential usefulness of this evaluation in the early diagnosis of allergy.

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