Original Article

Allergen immunotherapy decreases specific allergen-induced expression of Fas and FasL on $CD4^+$ and $CD8^+$ cells

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Abstract. Apoptosis is programmed cell death without induction of an inflammatory response. It is mediated by Fas – a cell surface protein which is expressed on activated lymphocytes. Interaction with its counterpart – the Fas ligand induces the apoptosis of Fas bearing cells. The mechanism underlying successful immunotherapy has not been identified. The aim of this study was to investigate whether specific immunotherapy (SIT) might affect Fas and FasL expression after stimulation with specific allergen.

The study was conducted on 8 allergic subjects and 8 healthy volunteers as controls. The allergic patients were treated with conventional SIT (Pollinex). Blood samples were collected before the first day and 7 days after the last injection. Isolated $CD4^+$ and $CD8^+$ cells were incubated in various concentrations of specific allergen (1, 10, 100 ng/ml) or in medium alone. Indirect immunofluorescence test with rabbit IgG against human Fas and FasL was performed. The percentage of positive cells was determined under fluorescence microscope.

The expression of Fas and FasL before SIT was significantly increased on $CD4^+$ and $CD8^+$ cells under the influence of specific allergen (10, 100 ng/ml). After SIT, significant decrease in the expression of both molecules was observed, although elimination of allergen-reactive cells was not complete and their number was still higher than in the controls.

Conclusion. The exposure of $CD4^+$ and $CD8^+$ cells on allergen may induce the Fas – FasL apoptotic pathway. Significant decrease in number of allergen-reactive $CD4^+$, $CD8^+$ cells after SIT suggests participation of the phenomenon in deletion of clones, which may be a part of the allergen tolerance mechanism achieved naturally or during SIT.

Key words: Specific allergen immunotherapy, apoptosis, $CD4^+$ and $CD8^+$

Introduction

Specific allergen immunotherapy (SIT) is nowadays the only causal treatment of IgE-dependent allergic diseases, related to type 1 allergy, such as allergic rhinitis, rhinoconjunctivitis, atopic asthma and sting-induced anaphylaxis. Despite the fact that injection immunotherapy was introduced by L. Noon as early as in 1911 it still provokes numerous controversies. Conventional SIT is based on subcutaneous injections of gradually increasing doses of allergen vaccine. The optimal period of SIT duration is not established and the patients with clinical improvement are recommended to continue therapy for 3 – 5 years [1, 2]. The purpose of SIT is the decrease or withdrawal of clinical symptoms related to exposure to an allergen or to restrain the natural development of the disease. Controlled studies have shown that SIT is an effective treatment in pollinosis,

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hypersensitivity to the venom of hymenopterous insects and atopic asthma. High efficacy of SIT measured by the scale of clinical symptoms and consumption of drugs was observed in subjects sensitive to grass pollen [1, 3]. No correlation was found so far between clinical improvement after SIT and many parameters of an immunological response. Unacquaintance with the precise mechanism of action of SIT causes the lack of standard parameters of its monitoring and prognosis [1, 2, 4, 5].

It is known that $CD4^+$ T cells have a key role in initiation and regulation of the course of an allergic reaction also by an influence on the synthesis of IgE and various cytokines affecting the function of other cells [6, 7]. Regarding the profiles of produced cytokines $CD4^+$ cells, were divided into subpopulations – Th1 (IL-2, IFNγ) and Th2 (GM-CSF, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13). Shifting of balance in synthesis of cytokines IL-4 and IFNγ, acting antagonistically on the synthesis of IgE in favour of IL-4 is observed in atopy. SIT was found to lead a decrease in production of IL-4 by $CD4^+$ cells [8] and to increase in synthesis of IFNγ [9].

T lymphocytes through T cell receptor (TCR) have the ability of allergen recognition. Stimulation of TCR dependent on concentration, time, frequency of interactions, functional status of a cell, participation of antigen-presenting or co-stimulating molecules may lead to proliferation or apoptosis [10].

Elimination of activated T lymphocytes through apoptosis is defined as activation-induced cell death [11]. Apoptosis induced by antigen-specific receptors occurs both in central (thymus, bone marrow) and peripheral lymphoid organs. Elimination of these lymphocytes from the periphery enables immunological tolerance and also limitation of an immunological response [11, 12].

The main mediator of apoptosis is the system of surface molecules Fas – Fas ligand (FasL), which may be expressed after activation of cells by specific allergen. Activated lymphocytes may have simultaneous expression of Fas and FasL or these molecules may be expressed separately. As a result of interaction between them, the cell with the expression of Fas undergoes apoptosis.

The aim of our work was the investigation of the influence of SIT on specific allergen-induced expression of Fas and FasL on $CD4^+$ and $CD8^+$ cells.

Subjects, materials and methods

The studies were performed on 8 control, non-atopic volunteers aged 22.8 ± 0.9 years (3 males, 5 females) and on 8 grass pollen sensitive subjects, with clinical diagnosis of allergic rhinitis/conjunctivitis, aged 24.8 ± 3.1 years (2 males, 6 females). Skin-prick test with grass pollen allergens (B2–Bencard–Beecham, UK) was negative in the controls and positive in the allergic patients (wheal diameter > 5 mm). Prior to the pollen season, the allergic patients were subjected to SIT (all of them for the first time) with grass pollen vaccine (Pollinex, Bencard, UK). First injections of Pollinex were done in February and subsequent 3 repeated at 7-day intervals. Blood samples were collected before the first day and 7 days after the last injection. Clinical efficacy of SIT was indicated by reported reduction in drug intake (by 50-70%) and reduction in nasal and eye symptoms based on the cards of patients self evaluation during the nearest grass pollen season in comparison to the previous one.

Isolation of peripheral blood lymphocytes was performed on Gradisol gradient [13]. $CD4^+$ and $CD8^+$ cells were obtained by solid phase-panning method [14], using monoclonal antibodies to human CD4 and CD8 antigens (DAKO A/S, Denmark). The viability of cells determined by Trypan blue exclusion was > 98%. The isolated cells were suspended in phosphate buffered saline (PBS) containing1.5% human serum of AB group and 40 μg/ml gentamicin (Polfa, Poland).

After estabilishing the preliminary experimental parameters of the test, 2 x 10^6 cells in PBS containing 1, 10 or 100 ng/ml grass pollen allergens or PBS alone (control) were incubated for 18h at 4° C. Next, the cells were washed 3 times in PBS and after rechecking viability (usually about 98%) indirect immunofluorescence test was performed with rabbit affinity purified polyclonal IgG against Fas and FasL of human origin, following the protocol of the manufacturer (Santa Cruz Biotechnology, USA). Fluorescein-conjugated swine anti rabbit immunoglobulin secondary antibody was from DAKO A/S, Denmark. All tests were performed in duplicate. The percentage of positive cells was determined under fluorescence microscope (Olympus BX-50).

Mean and standard deviations were calculated. Differences were considered statistically significant at $p < 0.05$ with the Student's t test.

Results

Grass pollen allergens did not change the expression of Fas and FasL on cells of healthy subjects in concentrations 1 and 10 ng/ml, whereas in concentration 100 ng/ml the expression of Fas only was significantly increased on $CD4^+$, from 0.12 ± 0.33 to 1.5 ± 1.3% ($p < 0.02$) and $CD8^+$ cells from 0.12 ± 033 to 0.75 ± 0.66% ($p < 0.05$). Expression of FasL on both types of cells remained unchanged (Fig.1).

In allergic subjects before immunotherapy the expression of Fas and FasL on $CD4^+$ cells was not changed at the concentration of allergen 1 ng/ml, whereas at concentrations 10 and 100 ng/ml, they significantly increased ($p < 0.001$). It rose from 0.5 ± 0.5% in PBS alone to 4.25 ± 0.2% at 10 ng/ml and to 6.62 ± 2.1% at 100 ng/ml. The expression of FasL in

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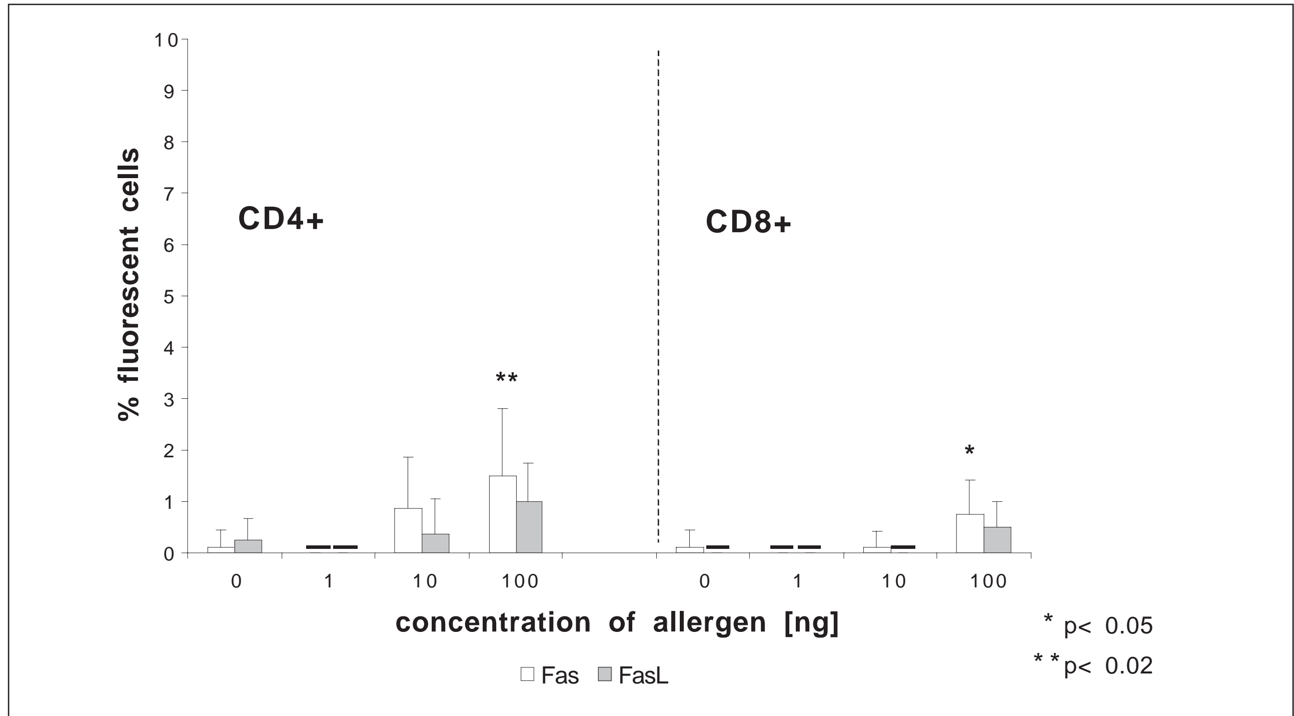

Figure 1. Expression of Fas and FasL on CD4+ and CD8+ T cells in response to grass pollen allergen in healthy subjects. CD4+ and CD8+ subpopulations isolated from peripheral blood lymphocytes were incubated with grass pollen allergen at the concentration indicated above. The number of Fas and FasL positive cells was determined using affinity purified polyclonal IgG against Fas and FasL antigens in indirect immunofluorescence test. Specific allergen induced significantly increased expression of Fas only at the 100 ng/ml concentration.

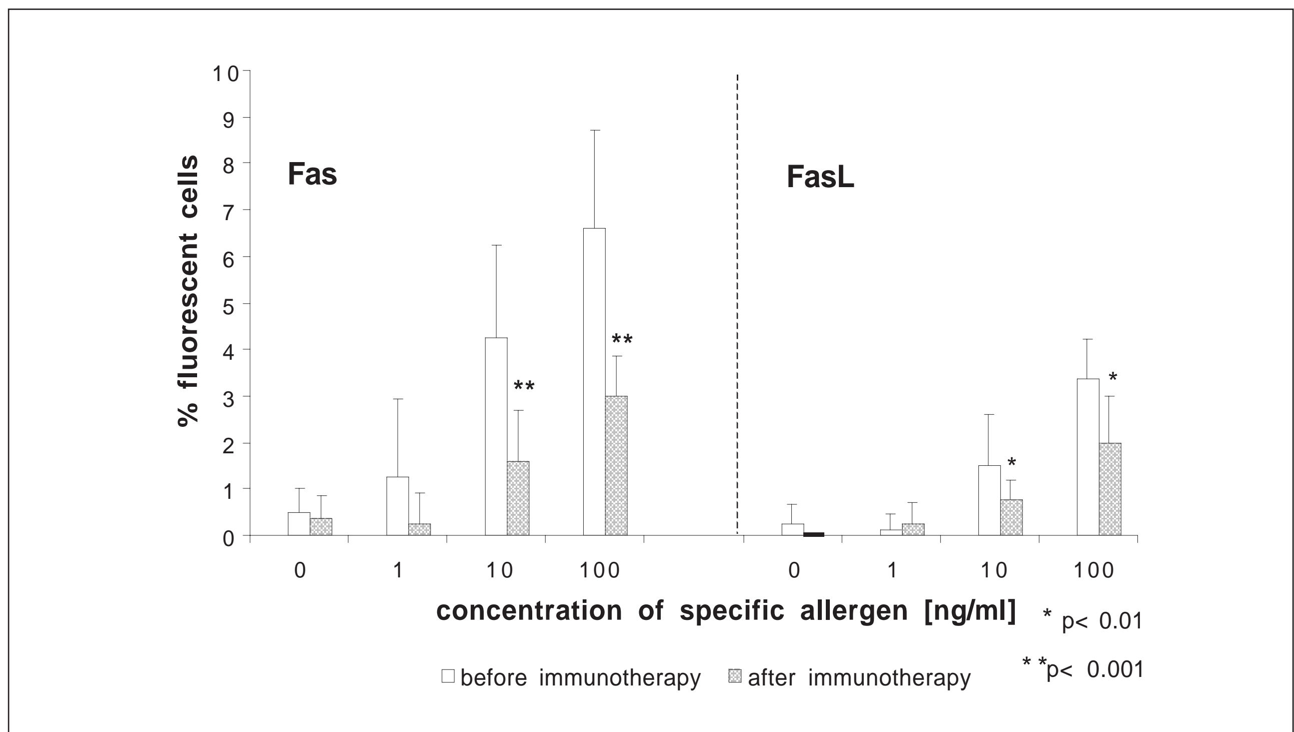

Figure 2. The effect of immunotherapy on expression Fas and FasL of CD4+ T cells in response to specific allergen. Using the same methodology as in Fig. 1 we observed significant decrease of Fas and FasL expression after immunotherapy at concentrations of allergen 10 and 100 ng/ml (Fas - $p<0.001$, FasL - $p<0.001$ and 0.01 respectively).

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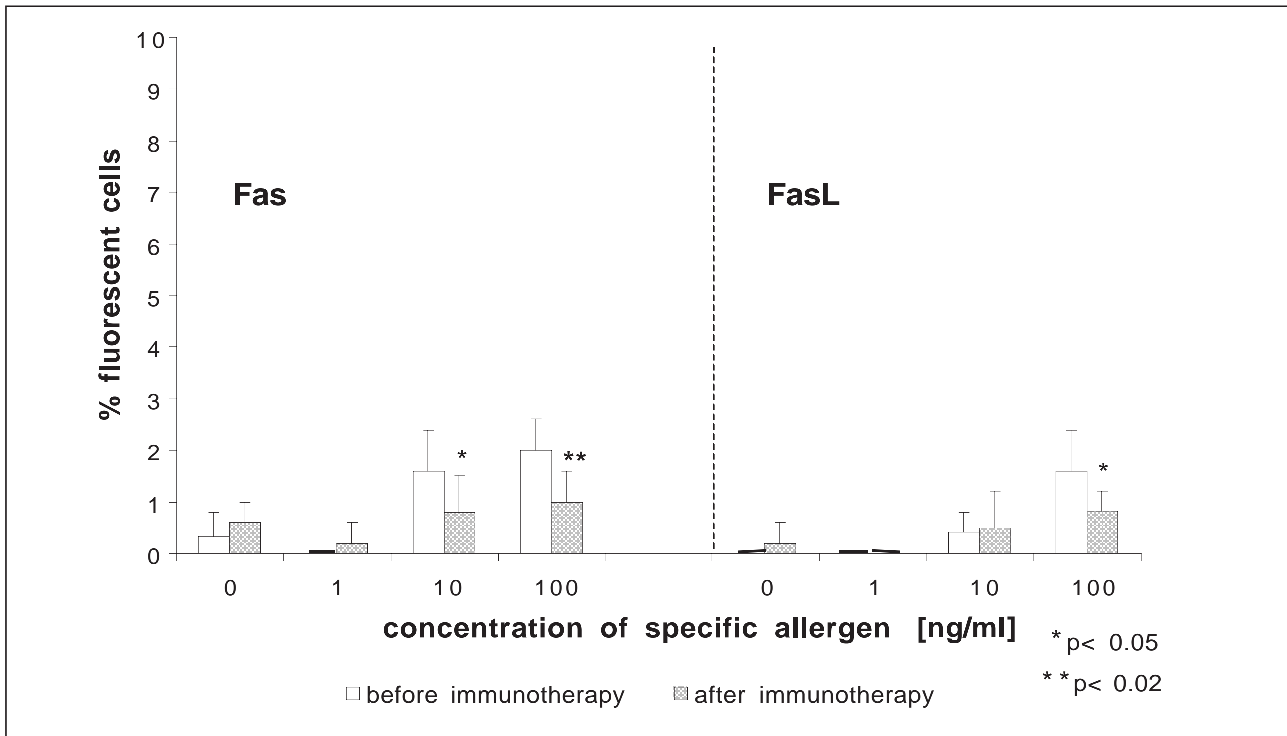

Figure 3. The effect of immunotherapy on expression Fas and FasL of CD8+ T cells in response to specific allergen. SIT reduced allergen-induced expression of Fas at allergen concentrations 10 and 100 ng/ml ($p<0.05$, $p<0.02$ - respectively) and FasL at the allergen concentration 100 ng/ml ($p<0.05$).

PBS control was 0.25 ± 0.43% and at concentrations of allergen 10 and 100 ng/ml – 1.5 ± 1.1 and 3.37 ± 0.85% respectively. Following SIT the significant decrease in allergen-induced expression of Fas was observed at the concentration 10 ng/ml, from 4.25 ± 0.2 to 1.6 ± 1.1% ($p < 0.001$) and at the concentration 100 ng/ml from 6.62 ± 2.1 to 3.0 ± 0.86% ($p < 0.001$). The expression of FasL was also significantly decreased from 1.5 ± 1.1 to 0.75 ± 0.43% at the concentration 10 ng/ml and from 3.37 ± 0.85 to 2.0 ± 1.0% at 100 ng/ml ($p < 0.001$, $p < 0.01$, respectively, Fig. 2).

However, despite substantial decrease in the expression of these proteins after SIT, the allergen still induced them above control level. The expression of Fas in PBS control – 0.37 ± 0.48% was higher at 10 ng/ml of allergen (1.6 ± 0.8%, $p < 0.01$) and at 100 ng/ml (3.0 ± 0.86%, $p < 0.001$). The expression of FasL was 0 in PBS control and at allergen concentrations 1, 10 and 100 ng/ml was 0.25 ± 0.45%, 0.75 ± 0.43% and 2.0 ± 1.0% respectively.

The expression of Fas on CD8+ cells before immunotherapy was increased from the value 0.33 ± 0.47% in PBS control to 1.6 ± 0.8% ($p < 0.01$) at 10 ng/ml and to 2.0 ± 0.6% ($p < 0.001$) at 100 ng/ml. The expression of FasL being 0 in PBS control and at allergen concentration 1 ng/ml increased to 0.4 ± 0.4% at 10 ng/ml and to 1.6 ± 0.8% at 100 ng/ml. After SIT the changes in specific-allergen-induced expression of Fas were non significant at concentration 1 ng/ml, whereas at concentrations 10 and 100 ng/ml the expression of Fas was significantly decreased from 1.6 ± 0.8 to 0.8 ± 0.7% ng/ml ($p < 0.05$) and from 2.0 ± 0.6 to 1.0 ± 0.6% ($p < 0.02$) respectively. The expression of FasL after SIT was decreased at the concentration of allergen 100 ng/ml only from 1.6 ± 0.8 to 0.83 ± 0.37% ($p < 0.05$). In comparison to PBS control 0.2 ± 0.4%, the expression of FasL at allergen concentration 100 ng/ml still remained statistically significant (0.83 ± 0.37%, $p < 0.02$, Fig. 3).

Discussion

In the studies presented above, the induction of Fas expression by grass pollen allergens was observed on CD4+ and CD8+ cells of healthy (not displaying any clinical symptoms) volunteers. However, in contrast to allergic subjects, the increase occurred at the highest concentration (100 ng/ml) of allergen only.

In allergic patients before SIT the allergen concentration-dependent increase in expression of Fas and FasL was found on CD4+ cells (at the concentration 10

and 100 ng/ml, $p < 0.001$). After SIT, the induction of both proteins was significantly lowered ($p < 0.001 – 0.01$), although the expression was still higher than in medium alone.

Specific allergen caused also concentration-dependent increase of Fas and FasL on $CD8^+$ cells at higher concentrations. The expression of Fas, after SIT was significantly lower at the concentrations 10 and 100 ng/ml and expression of FasL at the concentration 100 ng/ml only.

These findings are opposite to our previous observation showing significant increase in Fas and FasL expression on PBMC of untreated grass pollen allergic subjects [15].

Immunological response of T lymphocytes is preceded by their activation depending on antigen recognition by TCR. Activation of T lymphocytes through TCR/CD3 complex may, in turn, lead to proliferation or apoptosis [16].

Many data indicate that SIT caused the decrease in T cell lymphoproliferation in response to specific allergens [17 – 20]. TCR-induced apoptosis of activated T cells was also found to be mediated by Fas – FasL proteins and this process was regulated by IL-2 [16]. Repeating antigenic stimulations of $CD4^+$ cells may also lead to their apoptosis [21]. The Fas receptor (CD95) was found only on a small percentage of resting T lymphocytes and on $CD4^+$, $CD8^+$ subpopulations in peripheral blood. Its expression was not detected on peripheral naïve cells. It is assumed, therefore, that the expression of Fas on lymphocytes reflects their activation status. FasL is almost exclusively present on activated T cells. Interaction Fas – FasL induces apoptosis of a cell with the expression of Fas [22].

Clonal deletion of antigen-specific T lymphocytes through the activation-induced cell death (AICD) pathway may play an important part in generating peripheral tolerance. It was found that, as a result of AICD, the small population of antigen-specific cells resistant to apoptosis appeared on the periphery. These cells had a much higher level of synthesis of cytokines of Th2 type. In the opinion of the authors, these cytokines had a suppressive influence on proliferation of other antigen-specific T cells, promoting in effect tolerance [23]. It is assumed that elimination of mature, activated lymphocytes through apoptosis is an important regulator of an immunological response, controlling a number of immunocompetent cells [24].

Observations of other authors concerning the relations between apoptosis and SIT are also in favour of participation of apoptosis in an efficient SIT. Guerra et al. [25] showed that lymphocytes of patients desensitized for 2 years, incubated for 48h in cell culture with specific allergen underwent apoptosis and that most of them were $CD4^+$, $IL\text{-}4^+$ cells (expression of IL-4 was 84%) and only a small fraction (4%) had the expression of IFNγ. According to the authors, these results suggest the possibility of change of the type of immunological response from Th2 to Th1 through elimination of Th2 cells by AICD leading, in consequence, to prevalence of Th1 cells.

Esteban et al. [26] studied, at weekly intervals, the apoptosis of peripheral blood mononuclear cells in patients subjected to SIT for 4 weeks. They found significant, linear increase in apoptosis of T lymphocytes from initial value 0.35% to 1.64% in the fourth week of therapy.

The reports concerning beneficial effects of SIT are based mainly on decrease in intensity of allergic symptoms and reduction in the consumption of drugs. Among many changes observed in immunological response evoked by SIT, particular attention is concentrated on its influence on T cells, the main participants in regulation of a response to an allergen. One of the suggested mechanisms explains the efficacy of SIT at decreasing Th2 response and increasing Th1 type which causes quantitative changes in production of IL-4 and IFNγ [1].

SIT is burdened with risk of unwanted symptoms of various intensities, including life threatening systemic reactions [1, 20]. However, one may obtain the minimalization of side effects using antihistamine pretreatment before the injections [3, 27, 28]. Moreover, Muller et al. [28] observed that the antagonist of type 1 histamine receptor (H1R) – terfenadine applied to increase safety of SIT, significantly enhanced its efficacy. This effect may be caused by modulation of membrane expression of H2R, through which histamine suppresses immunological response. In atopy, lowered suppressor function of T lymphocytes, lower number of cells with membrane expression of H2R and lower expression H2R mRNA were demonstrated [29, 30]. In earlier studies, we had shown that lymphocytes of pollinotic subjects bound significantly less H2R antagonist than the lymphocytes of healthy ones. It was also demonstrated that SIT in these patients caused significant increase in binding H2R ligand and this increase correlated with clinical improvement [31]. We had also found that treatment with H1R antagonist – ebastine caused an increase in H2R mRNA, and so not only by blocking H1R but also by increasing membrane representation of H2R histamine might enable the suppression of the immunological response [32].

The observations of many authors indicate the complexity of mechanism of allergen immunotherapy. On the basis of results submitted one may assume that the elimination of allergen-specific T lymphocytes through Fas – FasL pathway may contribute to clinically confirmed efficacy of the therapy.

Acknowledgement

The authors thank Mrs. Barbara Rzadkowska for her excellent technical assistance.

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