

Bee Venom Immunotherapy Results in Decrease of IL-4 and IL-5 and Increase of IFN- γ Secretion in Specific Allergen-Stimulated T Cell Cultures¹

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The mechanisms of bee venom immunotherapy (VIT) are largely unknown. The aim of this study was to follow the changes of T cell cytokine secretion during the course of VIT. Ten bee venom-allergic patients with a history of severe systemic reactions, positive skin tests, and bee venom (BV)-specific serum IgE Abs were treated as follows: on the first day, a cumulative dose of 111 μ g, starting with 0.1 μ g, was administered s.c. under intensive care conditions. Further injections of 100 μ g BV were given on day 7, day 21, and thereafter at intervals of 4 wk. Blood samples were obtained just before the initiation of VIT, after the last injection on the same day, and before the subsequent BV injections on days 7, 21, and 50 of VIT. Peripheral blood mononuclear cells (PBMC) were stimulated with phospholipase A (PLA), the major BV allergen, or with a control Ag tetanus toxoid (TT). Cytokine secretion was measured 24 h after restimulation of the cultures with solid-phase bound OKT3 F(ab')₂ mAbs after 7 days of culture. In PLA-stimulated cultures, VIT resulted in decreased IL-4 and IL-5 and increased IFN- γ secretion. In TT-stimulated cultures, we observed similar levels of cytokines before and during VIT. We conclude that ultra-rush VIT changes allergen-specific T cell reactivity. *The Journal of Immunology*, 1995, 154: 4187–4194.

Bee stings can result in serious allergic reactions, which may be life threatening in sensitive individuals. Venom immunotherapy (VIT)³ has been demonstrated to cause significant protection of these subjects (1–3). The principle of VIT is s.c. administration of increasing doses of BV up to a maintenance dose of 100 μ g BV. The mechanism of tolerance induction by VIT is largely unknown. The data suggesting the rise of blocking Abs, particularly IgG4, as the main reason for protection remain controversial (4–6).

Recent studies provide evidence that production of IgE Ab, which is believed to be decisive in the induction of immediate hypersensitivity, is largely dependent on the ratio between IL-4 and IFN- γ cytokines, which are secreted by activated T cells (7–23). Another cytokine, IL-5,

which is an important eosinophil differentiation and chemotactic factor also costimulates B cell growth and differentiation (23, 24, 46). CD4⁺ T cell populations are characterized by a marked variation of their cytokine secretion patterns (26, 27). They have been divided into type I (Th1) cells which secrete predominantly IL-2 and IFN- γ (28, 29), and type γ II (Th2) cells that secrete IL-4, IL-5, IL-6, and IL-10 (20, 30). Additionally, Th0 cells that are thought to be progenitors of Th1 and Th2 cells have been described to produce IL-2, IFN- γ , IL-4, and IL-5 simultaneously (31, 32). These functionally distinct T cell subsets, which were originally defined in mice, have also been shown to exist in humans (33–35). However, in humans, the expression of a few cytokines such as IL-2 and IL-10 may be less restricted than in mice (36).

Two recent studies provide some evidence that immunotherapy with mite or grass pollen allergens may act by induction of an altered pattern of cytokine synthesis by T cells (37–38). Thus, the aim of our study was to investigate whether VIT causes changes of cytokine secretion patterns by allergen-specific T cells.

Materials and Methods

Subjects

Ten bee venom-allergic subjects were selected on the basis of a history of severe systemic allergic reactions of grade III–IV according to H. L.

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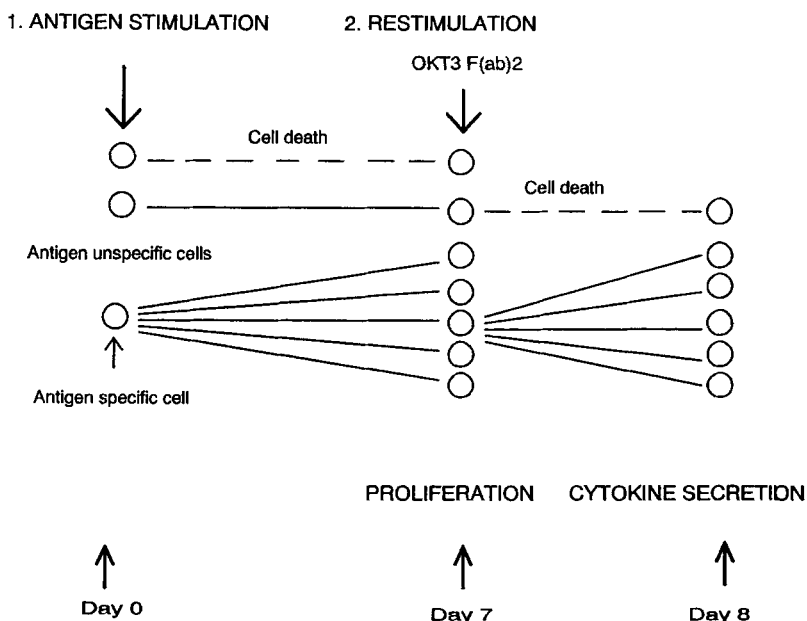
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³ Abbreviations used in this paper: VIT, bee venom immunotherapy; BV, bee venom; TT, tetanus toxoid; PLA, phospholipase; SI, stimulation index.

FIGURE 1. Experimental scheme of the stimulations *in vitro*. 1) Day 0: 3×10^5 PBMC/well were stimulated either with specific allergen PLA ($5 \mu\text{g/ml}$) or with control Ag TT ($5 \mu\text{g/ml}$) or left in medium alone (control cultures). Proliferation was measured after 7 days of culture. 2) Day 7: 2×10^5 cells/well were restimulated with plastic-bound OKT3 F(ab')₂; cytokine secretion was measured in supernatants 24 h later (day 8).



Mueller (39) after a honey bee sting and positive diagnostic tests to BV. The history of allergic reactions included such symptoms as urticaria, generalized pruritus, angioedema, laryngeal edema, hypotension, and shock. All patients had positive intracutaneous tests with honey bee venom (Pharmalgen venom, ALK, Horsholm, Denmark) at a concentration of $<10^{-4}$ g/l (5) and BV-specific serum IgE Abs (>1.0 ku_A/L) as estimated by Phadezym CAP (Pharmacia, Uppsala, Sweden).

Ultra-rush VIT

VIT according to Birnbaum et al. (40) was performed under intensive care conditions. BV (Pharmalgen venom, ALK) was administered s.c. in the upper arms. An initial venom dose of $0.1 \mu\text{g}$ was followed by 1, 10, and $20 \mu\text{g}$ at 30-min intervals and then 30 and $50 \mu\text{g}$ at 60-min intervals. The cumulative BV dose was $111 \mu\text{g}$. The following BV injections were performed on day 7 (two booster injections of $50 \mu\text{g}$ at an interval of 30 min), day 21, and day 50 (one booster each injection of $100 \mu\text{g}$). Thereafter, $100 \mu\text{g}$ boosters were given at intervals of 4 wk. Blood samples were obtained just before initiation of VIT, on the same day after injecting $111 \mu\text{g}$ BV, and before the following injections of BV on days 7, 21, and 50 and 270 of VIT.

Reagents

Tetanus toxoid (TT) was obtained from Dr. J. Cryz (Swiss Serum Institute, Bern, Switzerland). Phospholipase A₂ (PLA), the major allergen of bee venom, was obtained from Boehringer, Ingelheim, Germany and denatured as described before (41). OKT3 (anti-CD3) mAbs (Orthoclone) were kindly provided by Cilag, Schaffhausen, Switzerland. F(ab')₂ fragments were prepared by pepsin digestion, followed by purification over a protein A column. The F(ab')₂ preparation was unable to stimulate proliferation of PBMC even at high concentration ($>10 \mu\text{g/ml}$), indicating that the preparation contained less than 0.01% of the intact IgG2a molecule.

Culture method

The culture method is presented schematically in Figure 1. PBMC were freshly isolated by density gradient centrifugation on lymphoprep (Nycomed, Oslo, Norway). The cells were washed three times in HBSS and resuspended in medium composed of RPMI 1640 supplemented with 10% pooled AB serum (Swiss Red Cross, Bern, Switzerland), 25 mM HEPES buffer, 2 mM L-glutamine, 25 $\mu\text{g/ml}$ transferrin (Biotest, Dreieich, Germany), and 10 $\mu\text{g/ml}$ streptomycin and 100 U/ml penicillin (Amimed, Basel, Switzerland). Bulk cultures were set up in 96-well

flat-bottom tissue culture plates ($200 \mu\text{l}$) with 3×10^5 fresh PBMC in medium alone (control cultures) or together with either TT ($5 \mu\text{g/ml}$) or denatured PLA ($5 \mu\text{g/ml}$). After 7 days in culture, the cells were harvested, washed, and 1×10^5 cells were restimulated with solid phase-bound OKT3 F(ab')₂ in 96-well flat-bottom tissue culture plates. To coat culture plates, OKT3 F(ab')₂ was used at a concentration of $1 \mu\text{g/ml}$ as described (42). Briefly, OKT3 F(ab')₂ was diluted with 0.05 M Tris buffer (pH 9.5). $100 \mu\text{l}$ of diluted mAb was incubated in microtiter plates at room temperature for 60 min. Unbound mAb was removed by washing the plates three times with PBS and once with 1% FCS/PBS. Cell cultures were performed in similar mAb-coated plates. Supernatants were collected for cytokine measurements 24 h after stimulation of the cultures with OKT3 F(ab')₂. In our previous studies⁴ (43), we have shown that OKT3 F(ab')₂ was unable to activate resting T cells but restimulated Ag or allergen preactivated cells. The selective expansion of Ag or allergen-specific T cells was confirmed by a specificity test (restimulation of the cultures with either appropriate or control Ags or allergens). With this system we observed clear Th1-like cytokine secretion patterns in PPD-driven cultures and Th0-like patterns in TT- or PLA-stimulated cultures as well as preferentially Th2-like patterns to stimulation with Derp 1.

Proliferation assays

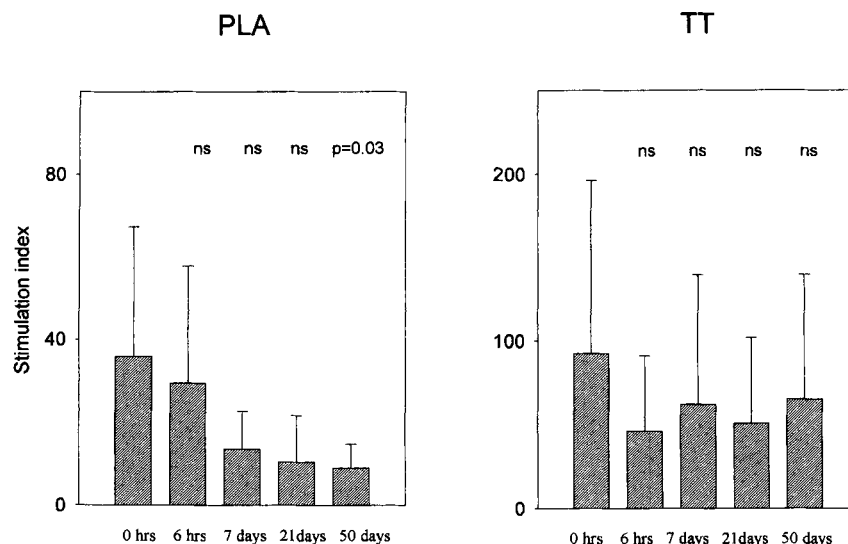
Proliferation assays were performed with 3×10^5 PBMC in 96-well flat-bottom tissue culture plates either with PLA ($5 \mu\text{g/ml}$) or TT ($5 \mu\text{g/ml}$), or in medium alone (control cultures). After 7 days of culture, the cells were pulsed for 6 h with [³H]-thymidine. The cultures were then harvested on glass fiber discs and counted in a microplate b-counter (Inotech Filter Counting System INB-384, Inotech, Dottikon, Switzerland). Stimulation index (SI) was calculated as a ratio between proliferative responses in Ag- or allergen-stimulated cultures and in medium alone.

Measurement of cytokines

Cytokine levels in supernatants were determined with sandwich ELISA using the solid-phase technique and the method described by Gallati et al. (44).

⁴ Jutel, M., T. Wyss-Coray, J. M. Carballido, K. Blaser, U. R. Müller, and W. J. Pichler. Selective restimulation of Ag or allergen preactivated T-cells using OKT3 F(ab')₂ results in the secretion of Th1 or Th2 like cytokine patterns. Submitted for publication.

FIGURE 2. Changes of proliferation of PBMC from 10 VIT patients to specific allergen (PLA) and control Ag (TT) during 50 days of VIT. PBMC were isolated from blood samples obtained just before initiation of VIT, 6 h later after injecting 111 μ g bee venom, and on days 7, 21, and 50 before subsequent BV injections. [^{3} H]thymidine incorporation was measured after 7 days of culture. SI was calculated as a ratio between proliferative responses in Ag- or allergen-stimulated cultures and in medium alone. Proliferation to TT is not significantly changed during 50 days of VIT. Proliferation to PLA is reduced (statistical significance after 50 days of treatment). Columns give arithmetic mean values of SI, bars SD.



Measurement of BV-specific IgE and IgG Abs in serum

BV-specific IgE serum Abs were estimated by the CAP method (Pharmacia AB, Uppsala, Sweden). The results are given in ku_A/L . BV-specific IgG serum Abs were detected by an ELISA technique as described previously (5). The results are given in $\mu\text{g}/\text{ml}$.

Statistical analysis

Statistical significance of differences between results obtained at various time points of VIT was analyzed by a paired Student's *t*-test. The tests with *p* values of less than 0.05 were considered as significant.

Results

VIT results in decreased proliferation of PBMC to specific allergen but not to control Ag

Changes of proliferation of PBMC from 10 VIT patients to specific allergen (PLA) and control Ag (TT) during 50 days of VIT are shown in Figure 2. PBMC were obtained from blood samples collected just before initiation of VIT, 6 h later after injecting 111 μg BV, and on days 7, 21, and 50 of VIT. [^{3} H]thymidine incorporation was measured after 7 days of culture with either TT or PLA, or in medium alone. The results are expressed as SI, which is the ratio between proliferative responses in Ag- or allergen-stimulated cultures and in medium alone. Proliferation in medium alone ranged between 56 and 536 cpm. During VIT, we observed a reduction of the mean SI to PLA, which was statistically significant after 50 days of treatment ($p = 0.03$). Proliferation to TT remained at a similar level up to day 50 of VIT (no statistically significant differences were observed).

Secretion of IL-4 and IL-5 from allergen-specific T cells is significantly decreased because of VIT

To assess whether secretion of cytokines from allergen-specific T cells is affected by VIT, we applied our previ-

ously established protocol to induce an enhanced cytokine secretion in specifically activated T cells (43). PBMC obtained from 10 patients at various time points of VIT were stimulated either with specific allergen (PLA) or control Ag TT and after 7 days of culture restimulated with solid phase-bound OKT3 F(ab')₂ mabs to potentiate cytokine secretion from allergen- or Ag-specific T cells. Changes of IL-4 secretion in such cultures during 50 days of VIT are demonstrated in Figure 3. In PLA-stimulated cultures, strikingly decreased IL-4 levels were observed. The statistical significance of the differences between IL-4 levels before and during VIT was demonstrated already after 7 days of VIT ($p = 0.0001$). On the contrary, secretion of IL-4 in TT-stimulated cultures remained at a similar level during 50 days of VIT (no statistical differences were demonstrated). Secretion of IL-5 was similarly decreased in PLA-stimulated cultures (Fig. 4). Statistical significance of reduction of IL-5 secretion was also observed after 7, 21, and 50 days of VIT ($p = 0.04$, 0.03, and 0.02 respectively). No significant differences were demonstrated in TT-stimulated cultures.

VIT results in a significant increase of IFN- γ secretion from allergen-specific T cells

Secretion of IFN- γ was also measured in the same cultures (Fig. 5). The increase of IFN- γ secretion in PLA-stimulated cultures reached statistical significance after 7 days of VIT ($p = 0.006$) and remained significant up to day 50 of VIT ($p = 0.00003$). No significant differences in IFN- γ secretion were demonstrated in TT-stimulated cultures.

Serum levels of allergen-specific IgE and IgG

BV-specific IgE serum Abs before initiation of VIT and after 2 and 9 mo of treatment are shown in Figure 6A.

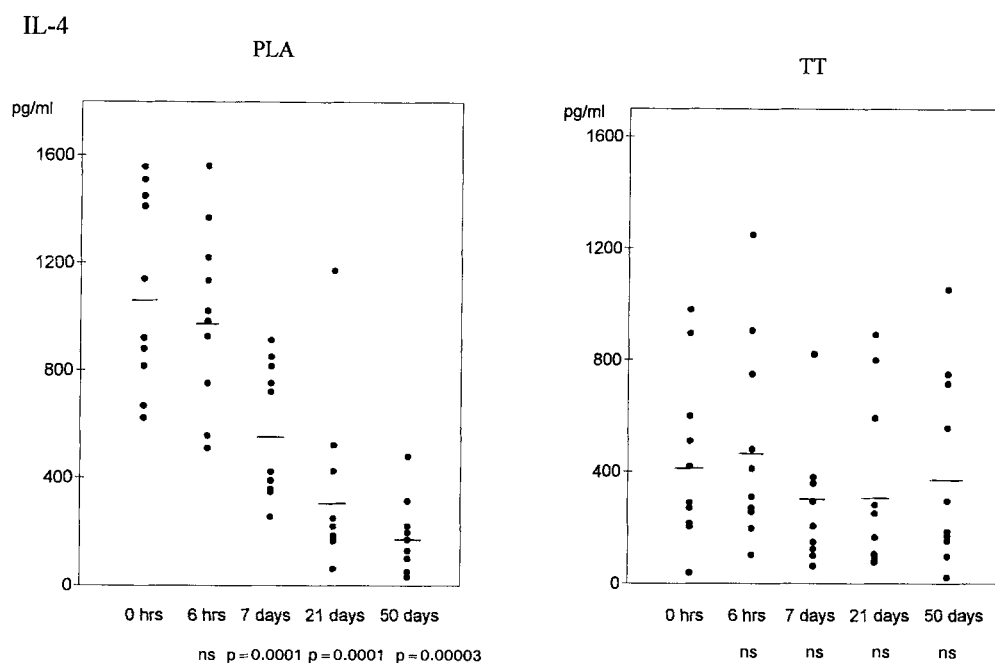


FIGURE 3. Changes of IL-4 secretion in specific allergen (PLA)- and control Ag (TT)-driven cultures during ultra-rush VIT. Decreased IL-4 levels were observed in PLA-stimulated cultures. The decrease is statistically significant from day 7. IL-4 secretion in TT-stimulated cultures remains at a similar level during 50 days of VIT. Horizontal bars give arithmetic mean values.

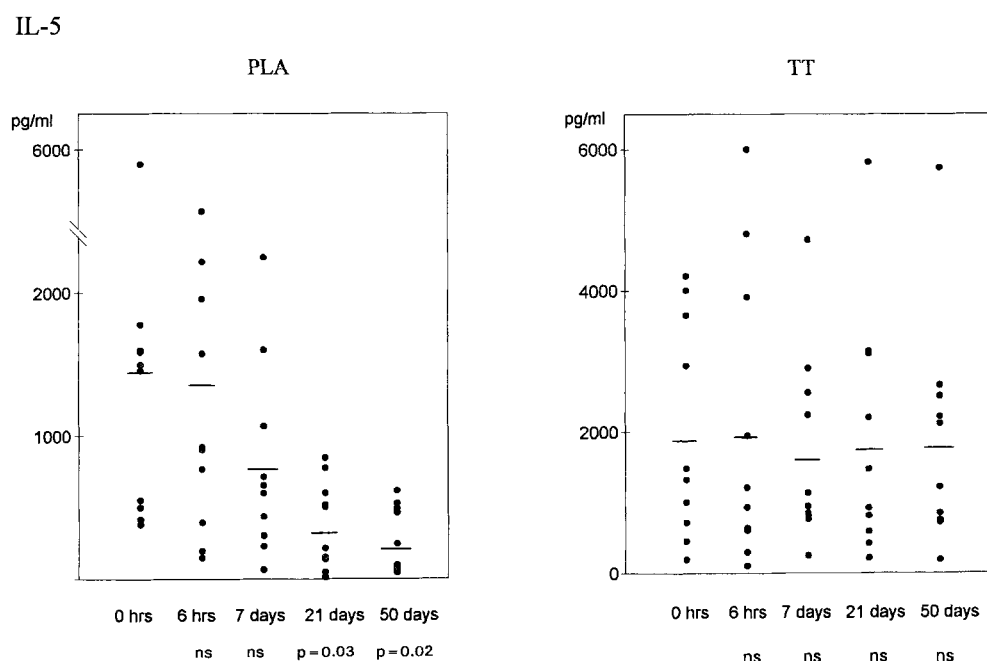


FIGURE 4. Changes of IL-5 secretion in specific allergen (PLA)- and control Ag (TT)-driven cultures during ultra-rush VIT. IL-5 levels decreased in PLA-stimulated cultures. The decrease is statistically significant from day 7. No significant differences were demonstrated in TT-stimulated cultures. Horizontal bars give arithmetic mean values.

After 2 mo, we observed a tendency to increased IgE concentrations in serum, which did not reach statistical significance ($p = 0.21$). After 9 mo of VIT serum, IgE concentrations were decreased in seven patients, increased in two

patients, and remained at a similar level in one subject. The tendency to decreased IgE concentrations was not statistically significant ($p = 0.5$). Figure 6B shows levels of BV-specific IgG. We observed a marked tendency to increased IgG

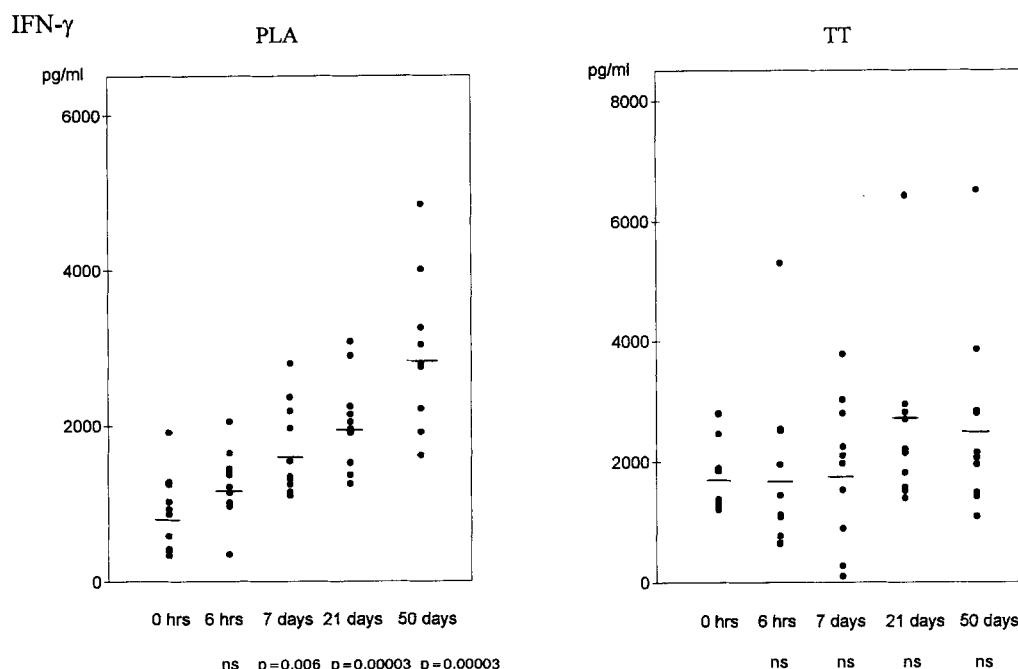


FIGURE 5. Changes of IFN- γ secretion in specific allergen (PLA)- and control Ag (TT)-driven cultures during ultra-rush VIT. IFN- γ levels increased in PLA-stimulated cultures. The increase is statistically significant from day 7. No significant differences were demonstrated in TT-stimulated cultures. Horizontal bars give arithmetic mean values.

concentrations already after 2 mo of treatment, which reached statistical significance after 9 mo of VIT ($p = 0.001$).

Discussion

The understanding of tolerance induction by VIT is of great interest both for the explanation of basic mechanisms of allergy and for practical reasons, including monitoring and modification of VIT. Recent developments suggest an important role of T cell-derived cytokines in induction and maintenance of the allergic status (reviewed in Refs. 45, 46). Because T cells are stimulated to cytokine secretion upon Ag recognition, our initial hypothesis was that allergen immunotherapy influences the immune response by the induction of alterations in cytokine secretion patterns by allergen-specific T cells. In fact, in this study, we demonstrate that VIT is associated with a significant reduction in allergen-induced IL-4 and IL-5 secretion and a simultaneous increase of IFN- γ secretion by allergen-specific T cells in vitro. This is the first description of the effect of allergen immunotherapy on cytokine secretion in bee venom allergy. Additionally, this is the first study showing the association of allergen immunotherapy with decreased IL-4 and IL-5 secretion simultaneous with increased IFN- γ , a finding which suggests clearly a shift towards a more Th1-like response resulting from allergen immunotherapy. We could not, however, demonstrate that VIT was associated with any significant changes in secretion of

other cytokines such as IL-2, IL-10, GM-CSF, and TNF- α (not shown).

Our data are in agreement with two recent studies, which also indicated that allergen IT modifies cytokine secretion by T cells from atopic subjects. Secrist et al. (37) demonstrated reduction of IL-4 secretion and unaffected production of IFN- γ by in vitro-stimulated CD4⁺ T cells from grass pollen and mite allergic subjects receiving allergen IT. Varney et al. (37), who analyzed the influence of grass pollen immunotherapy on cytokine mRNA expression in skin biopsies during allergen-induced late-phase cutaneous responses, indicated enhanced expression of mRNA for Th1-type cytokines, but found no effect on Th2 cytokine secretion pattern (38). However, in contrast to mite and pollen allergy, Hymenoptera venom allergy, although IgE mediated, is not an atopic disease. It is commonly accepted that true atopic allergies are characterized by multiple positive skin tests to inhalant allergens and a history of hay fever, allergic bronchial asthma, or atopic dermatitis. These allergic disorders are not observed more frequently in patients with venom allergy than in the general population (47). In fact, it has been demonstrated that PLA-specific cells are able to secrete both IL-4 and IFN- γ -like Th0 cells (48), whereas Derp I or Lol p-specific cells express a clear Th2 phenotype (45). Th1 and Th2 cells most probably differentiate from Th0 cells. Because PLA-specific Th0-like cells are less differentiated, they may be particularly susceptible to signals affecting

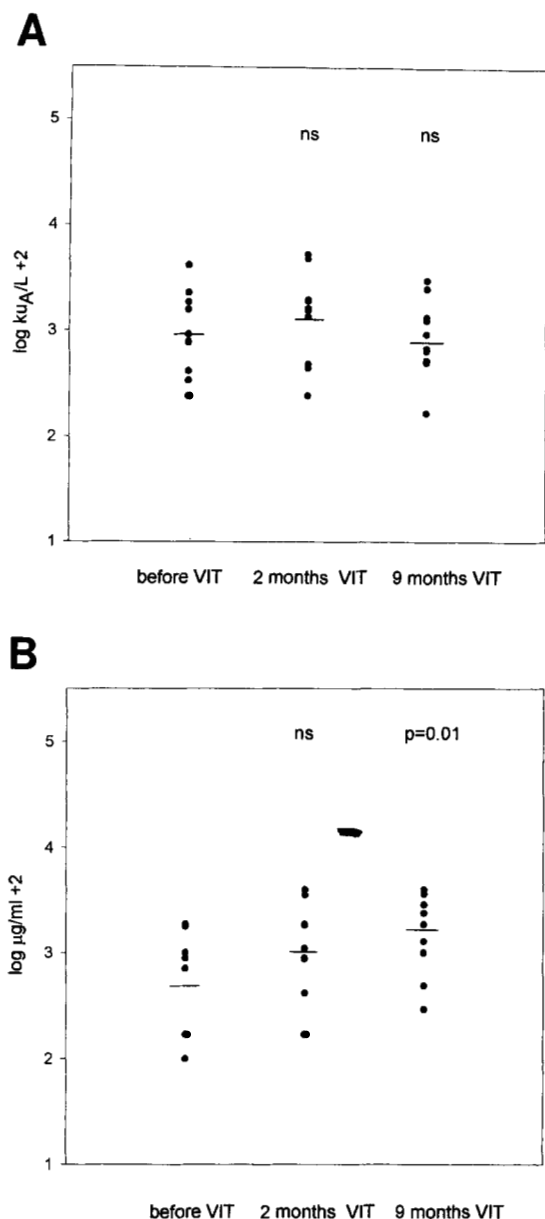


FIGURE 6. A) BV-specific IgE serum Abs before initiation of VIT and after 2 and 9 mo of treatment ($n = 10$). A tendency to increased IgE concentrations after 2 mo is statistically insignificant. The tendency to decreased IgE concentrations after 9 mo of VIT is also statistically insignificant. B) BV-specific serum IgG. A marked tendency to increased IgG concentrations after 2 mo of treatment reached statistical significance after 9 mo of VIT. Horizontal bars give geometric mean values.

cytokine secretion. This might explain the better efficacy of VIT, compared with immunotherapy with other allergens.

The alteration of cytokine patterns in VIT can be achieved by various mechanisms. Experiments *in vitro* have shown that cells at Th0 stage stimulated in the absence of a costimulatory molecule (B7/CD28) on the sur-

face of APC, differentiate to Th2 phenotype (49). On the contrary, cells of Th1 phenotype may develop when Th0 cells are stimulated in the presence of a costimulatory signal, under influence of IL-12 and IFN- α cytokines (50, 51). It is likely that cytokine secretion profiles are affected by high cumulative doses of Ag, such as those administered during allergen immunotherapy. Carballido et al. (48) demonstrated in PLA-specific clones that an increase in Ag concentration correlated with a significant increase of IFN- γ secretion, which might result from the increased panel of stimulated T-cell clones for which a threshold for IFN- γ production is reached. Another possibility is the involvement of different APC depending on Ag concentration (52), which in turn might affect cytokine secretion (53). Thus, our clinical model seems to confirm the *in vitro* observations. However, we also observed a decrease of T cell proliferation to the specific allergen (PLA) during VIT. This could be caused by a clonal anergy or the generation of new subsets of allergen-specific cells with altered reactivity to PLA. Thus, VIT might affect the cytokine secretion not only by influencing the reactivity of existing, individual cells, but also by changing the whole population of allergen-specific cells.

Another factor that could direct stimulation of T cells to different cytokine secretion upon stimulation might be the manner of allergen administration. Allergen is injected intracutaneously during an insect sting, whereas VIT injections are applied subcutaneously, where the mast cell density is considerably lower. Thus, VIT could influence immune response with less participation of mast cells, which may be a source of regulatory cytokines (54, 55). In fact, it has been postulated that the route of allergen exposure might influence cytokine profiles in allergen-specific T cells (56).

In many previous studies, changes of serologic parameters under VIT have been intensively investigated. It has been demonstrated that levels of allergen-specific serum IgE, after initial increase, decrease over a period of months to years (4, 5, 6). In our group of patients, the decreased values of IL-4/IFN- γ ratio were not closely correlated with decreased levels of allergen-specific IgE. We observed a marked decrease of BV-specific serum IgE levels in only four of ten patients after 2 mo of VIT and in seven patients after 9 mo. These data are consistent with recent reports suggesting that cytokine receptor agonists can hardly affect ongoing IgE production *in vitro* by terminally differentiated B-cells (57). In the current study we also confirmed the observation that VIT is associated with increased concentrations of "blocking" IgG Abs in serum. However, most studies demonstrate that changes of serologic parameters bear no relationship to the clinical response. Indeed, recent experiments demonstrating active anaphylaxis in IgE-deficient mice suggest the existence of non-IgE-dependent pathways of hypersensitivity reactions (58). Therefore, we would rather interpret our data as evidence that the effect of VIT might be attributed to changes

of T cell reactivity as assessed by modification of cytokine secretion patterns, which in turn could alter the reactivity of effector cells. The exact mechanism of protection induced by VIT remains to be investigated.

Acknowledgments

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