

J. Irsch
C. König
A. Löhndorf
H. Tesch
T. Krieg
H. Merk
A. Radbruch
N. Hunzelmann

The frequency of phospholipase A₂ binding of basophilic granulocytes does not decrease during bee-venom-specific immunotherapy

Authors' affiliations:

J. Irsch, A. Löhndorf, Department of Genetics, University of Cologne, Cologne
C. König, T. Krieg, N. Hunzelmann, Department of Dermatology, University of Cologne, Cologne
H. Tesch, Department of Internal Medicine, University of Cologne, Cologne
H. Merk, Department of Dermatology, RWTH Aachen
A. Radbruch, Deutsches Rheumaforschungszentrum, Berlin, Germany

Correspondence to:

Dr N. Hunzelmann
Department of Dermatology
University of Cologne
Joseph Stelzmann Str. 9
50924 Cologne
Germany

Key words: insect venom allergy; magnetic cell sorting; rush desensitization.

Background: The major allergenic component of bee venom is phospholipase A₂ (PLA₂).

Methods: In this study, PLA₂ was used to analyze and enrich PLA₂-binding cells from peripheral blood by high gradient magnetic cell sorting.

Results: In normal donors, the frequency of allergen (PLA₂)-binding cells among peripheral blood mononuclear cells (PBMC) as determined by flow cytometry is below 0.1%, whereas in bee-venom-allergic patients, PLA₂-binding cells are readily detectable at frequencies of up to 2.3%. In severely bee-venom-allergic patients, many basophilic granulocytes are present, as defined by anti-CD9, CD25, and CD38 mAb, comprising up to 95% of the PLA₂-binding cells. From blood of allergic and normal donors, about equal absolute numbers of allergen-binding CD19/21-positive B cells can be enriched. Severe anaphylactic reactions (Mueller grade IV) and failure of or adverse reactions during immunotherapy are associated with high numbers of circulating allergen-binding basophils. Interestingly, in the patients studied, the number of PLA₂-binding basophilic granulocytes did not markedly change during rush immunotherapy and up to 6 months of maintenance immunotherapy.

Conclusions: The specific and reproducible enrichment of PLA₂-binding cells provides a new tool for the analysis and monitoring of effector cells in bee-venom-allergic patients with immediate-type hypersensitivity.

Abbreviations. PLA₂: phospholipase A₂ from bee venom; MACS: magnetic cell sorting; DIG: digoxigenin; Ig: immunoglobulin.

Specific immunotherapy against bee-venom allergy induces tolerance in up to 95 % of these patients (1, 2). However, the

Date:

Accepted for publication 18 February 1999

To cite this article:

Irsch J., König C., Löhndorf A., Tesch H., Krieg T., Merk H., Radbruch A. & Hunzelmann N. The frequency of phospholipase A₂ binding of basophilic granulocytes does not decrease during bee-venom-specific immunotherapy. *Allergy* 1999; **54**, 742–747.

Copyright © Munksgaard 1999

ISSN 0105-4538

mechanism by which venom immunotherapy exerts its beneficial effect is unknown. The major allergens in bee venom have been identified and include phospholipase A₂ (PLA₂), a relevant allergen in up to 80% of the patients; hyaluronidase; mellitin; and a high-molecular-weight substance with acid phosphatase activity (2, 3). In previous studies, changes of serologic parameters during specific immunotherapy have been monitored intensively. It has been demonstrated that, after an initial increase, allergen-specific serum IgE levels decrease over a period of years, accompanied by a rise in concentrations of allergen-specific IgG1 and IgG4 (4, 5). However, in most studies, the serologic changes bear no relationship to the clinical response (2, 6, 7). In any case, serum IgE has a shorter half-life than cell-bound IgE and cannot reliably predict the sensitivity of a patient. We hypothesize that the frequency of allergen-binding effector cells is a more direct correlation of the clinical sensitivity of a patient to venom allergens. Basophilic granulocytes or mast cells, thought to play a prominent role in anaphylactic reactions, are able to bind the native antigen and can be stained and isolated via antigen (8).

In this study, we used PLA₂, the major allergen of bee venom, to isolate PLA-binding cells from the peripheral blood of bee-venom-allergic patients by high gradient magnetic cell sorting (MACS). The frequency of peripheral PLA₂-binding cells was determined before and during rush bee-venom therapy.

Material and methods

Patients

Nine bee-venom-allergic patients (aged 24–41 years) were studied on the basis of a history of severe systemic allergic reaction after a bee sting. Patients were classified as grades III–IV according to Mueller (9). IgE-mediated sensitivity was confirmed by skin testing (Table 1) and serum analysis (CAP, Pharmacia, Uppsala, Sweden), according to the manufacturer's instructions. Immuno-dot-blot analysis confirmed the presence of PLA₂-specific IgE in 8/9 patients. In one patient, PLA₂-specific IgE could not be detected by immunoblotting despite positive intracutaneous skin testing and specific IgE to commercially available bee-venom extract (Pharmacia, CAP), indicating that the IgE in this patient is directed exclusively to non-PLA₂ components of the bee venom. These results correspond to the inability to enrich PLA₂-binding cells from this patient (not shown). Immunotherapy was performed according to a modified

protocol of Glowania & Schulz (10). In brief, subcutaneous injections were performed on 5 consecutive days starting at 10⁻⁵ mg/l bee venom on day 1, leading to daily cumulative doses of 10⁻² mg/l on day 1; 1.2 µg on day 2; 12 µg on day 3; 90 µg on day 4; and 170 µg on day 5. This protocol is followed by maintenance therapy of 100 µg/month over 3–5 years. Peripheral venous blood samples were obtained after informed consent just before initiation of immunotherapy in nine patients. In addition, blood samples were obtained from two patients, 1 h after the last injection on day 3, and 1 h after the last injection on day 5. In four patients, samples were obtained during maintenance therapy 3–9 months after initiation of immunotherapy, shortly before the monthly dose was given. For control, peripheral venous blood samples were taken from five healthy donors.

Skin testing

For quantitative skin testing, a stock solution of 100 mg/l bee venom (Reless, Scherax, Hamburg, Germany) was used to obtain stepwise decreasing venom concentrations (1–10⁻² mg/ml) by dilution with commercial skin test diluent containing 0.03% human serum albumin. A wheal formation of at least 5 mm in diameter with erythema was classified as a positive reaction to determine the minimal dilution provoking a skin reaction. Histamine and the albumin diluent were used as positive and negative controls, respectively.

Immuno-dot-blot analysis

Unconjugated PLA₂ (100 ng, 1 µg, 2 µg) or bovine serum albumin were blotted onto nitrocellulose filters (Hybond C Extra, Amersham, Braunschweig, Germany) with the microsample filtration manifold (Schleicher-Schuell, Dassel, Germany). After washing with PBS, the filters were blocked for 1 h with Trizma base, NaCl, pH 7.6, containing 3% Tween 20 and 5% dry milk powder. After incubation with the patient's serum (1:500) for 1 h, the filters were washed three times for 5 min in TBS/Tween 20. Thereafter, the filters were incubated with HRPO conjugates of anti-IgE antibodies (SBA, Birmingham, AL, USA) diluted in TBS/Tween/dry milk for 40 min at room temperature. Filters were developed with the ECL system (Amersham, Braunschweig, Germany) and radiosensitive films (Kodak, X-Omat, AR).

Table 1. Correlation of clinical and experimental data of bee-venom-allergic patients. Note difference in percentage of PLA2-binding cells in peripheral blood before MACS in patients with Mueller grades III (severe respiratory symptoms; e.g., dyspnea [d], stridor [s]) and IV (severe cardiovascular symptoms; e.g., hypotension [h], collapse [c], unconsciousness [u] [9]) and high percentage of CD38-positive cells after MACS enrichment

Patient no.	Age (years)	Mueller grade (7)	Skin test (µg/ml)	Specific IgE (CAP class)	% PLA2 binding before MACS	%CD38 positive after MACS
1	23	IV (u)	10 ⁻³	3	1.1	76
2	16	IV (h, c)	10 ⁻³	5	2.3	99
3	49	IV (h, c)	10 ⁻⁴	4	0.7	90
4	49	IV (u,c)	10 ⁻³	4	2.1	96
5	13	III (d)	10 ⁻⁴	3	0.56	82
6	30	III (d)	10 ⁻¹	1	0.2	10
7	42	III (d)	10 ⁻³	3	0.2	8
8	33	III (d, s)	10 ⁻³	3	0.37	10

Antigen-specific MACS

PLA₂ from bee venom was purchased from Sigma, Freiburg, Germany (P9279) and digoxigenized with the DIG labeling kit (Boehringer Mannheim, Germany). PLA₂ (0.5–1.0 mg) was incubated with digoxigenin succinimid ester for 1 h at room temperature, and unbound digoxigenin was then removed with a PD10 column (Pharmacia, Freiburg, Germany). The prepared conjugates were titrated out on peripheral blood mononuclear cells (PBMC) for their ability to stain specifically distinct populations of the cells, but not monocytes or macrophages.

The sortings were essentially carried out as described elsewhere (8). After removal of RBC, eosinophils, and neutrophils by Ficoll separation, the resulting PBMC were incubated for 10 min on ice with 2 mg/ml of polyclonal human IgG antibodies (Behring, Marburg, Germany) to block unspecific binding to Fc receptors. The digoxigenized PLA₂ was added at a final concentration of 10 µg/ml, and cells were incubated for an additional 10 min on ice. After washing with PBS/0.5 % BSA, the cells were labeled with the anti-DIG superparamagnetic microbeads (Miltenyi Biotec), the stock diluted at 1/5 v/v, and were incubated for 10 min at room temperature. The fluorescent staining was achieved by incubating the cells with 1/50 volume of phycoerythrin (PE)-coupled anti-DIG antibodies, resulting in a final concentration of 2 mg/ml. After another washing step, the cells were applied onto the prepared MiniMACS columns (Miltenyi Biotec, Bergisch Gladbach, Germany). After washing with 5 ml of PBS/BSA, the columns were removed from the magnetic field, and bound cells were washed off the matrix with 1 ml of PBS/BSA.

The separation was controlled by flow cytometry, with FACScan and FACScan Research or Cellquest software (Becton Dickinson, San Jose, CA, USA). For cytometric analysis, dead cells were excluded by electronic gating, according to relative Fl2 vs Fl3 fluorescence, with propidium iodide. Debris was excluded by electronic gating according to forward vs side-scatter. For counterstaining of the enriched cells, FITC-conjugated CD9 and CD38 antibodies (Immunotech, Dardilly, France) and CD19, CD21, and CD25 antibodies (Becton Dickinson, San Jose, CA, USA) were used.

Results

PLA₂-binding cells were isolated from the peripheral blood of bee-venom-allergic patients and normal controls by a previously published protocol (8). In bee-venom-allergic patients, PLA₂-binding cells could be stained at frequencies of up to 2.3 % before enrichment (Table 1). Allergen-binding cells were enriched by MACS from PBMC to frequencies of up to 80%. In nonallergic persons and in one of the bee-venom-allergic patients, whose serum was negative for PLA₂-specific IgE, the frequency was below 0.1% (not shown). For phenotypic analysis, indirect staining of the bound digoxigenized allergen with anti-DIG PE and subsequent counterstaining with αCD38 and αCD19 was performed on the enriched cells (Fig. 1). Furthermore, the isolated basophilic granulocytes were counterstained with αCD9, αCD25, αCD21, and αCD19 and αIgE-specific antibodies (not shown). Patients with a history of Mueller grade IV reactions revealed a higher percentage of PLA₂-

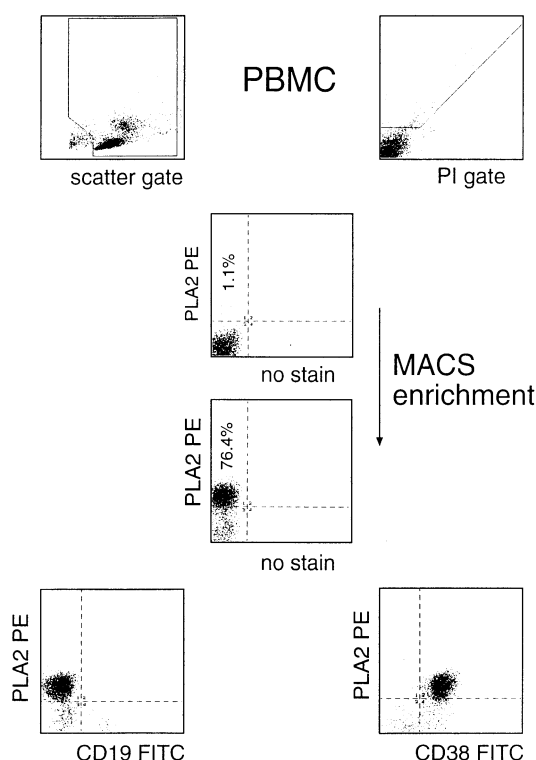


Figure 1. Enrichment and counterstaining of PLA₂-binding cells from PBMC of one patient (no. 5) 6 months after successful rush hyposensitization. Gates used in analysis are indicated. Even before enrichment, 1.1% of cells could be stained with PE-conjugated α -DIG antibody recognizing DIG-coupled PLA₂. With MiniMACS, PLA₂-binding cells could be enriched to 76.4%. As could be demonstrated by counterstaining with FITC-conjugated α CD19 or α CD38 antibodies, most cells binding PLA₂ express CD38, but only very few can be stained with α CD19 antibody. This shows that most cells binding allergen even after hyposensitization are basophilic granulocytes, and that only very few are B cells.

positive cells before MACS than patients with Mueller grade III reactions, and all showed a high percentage of CD38⁺, IgE⁺ cells (70–95%) among the enriched cells. Phenotypic analyses by flow cytometry revealed that most of the PLA₂⁺ cells expressed CD9, CD25, and CD38, a pattern known to be expressed by basophilic granulocytes.

Further evidence of the nature of the cells was achieved by isolating cells according to surface-bound IgE. The isolated cells were stained for the κ and the γ immunoglobulin light chains. Nearly all IgE⁺ cells from peripheral blood of allergic patients expressed both Ig κ and Ig γ . Thus, these cells are not IgE-expressing B cells, but basophilic granulocytes which unspecifically took up a mixture of IgE from the serum. This was also confirmed by May-Grünwald/Giemsa cytospin staining, demonstrating the typical nuclear structure and the dark granules of these cells (not shown). In contrast, no major difference in the frequency of PLA₂-binding B cells among CD19⁺ B cells was found between allergic and

normal donors (not shown), although a more detailed analysis of these cells may reveal functional differences.

Despite similar reactivity in quantitative skin testing (Table 1), patients with Mueller grade III reactions had fewer PLA₂-binding cells among PBMC than patients with Mueller grade IV reactions. In these patients (Mueller grade III), the enriched cells also stained less bright for PLA₂-PE, suggesting less PLA₂-binding IgE per cell (not shown). Most of the CD38-negative PLA₂-binding cells were B cells, which could be identified by CD19/CD21.

When subsequent determinations were performed 6–9 months after institution of specific immunotherapy, the percentage of PLA₂-binding cells among PBMC remained unchanged in three out of four patients examined (i.e., patient 3: 0.7%; patient 7: 0.3%; patient 8: 0.5%). Comparison of the staining intensities of the PLA₂-binding cells during and after rush desensitization revealed that the cells were stained equally bright, as shown in Fig. 2. In one patient (Figs. 1 and 2), an increase (from 0.6% to 1.09%) in the frequency of PLA₂-binding cells was observed.

Discussion

Diagnosis of bee-venom allergy relies on the determination of specific IgE antibodies by skin testing and serum analysis accompanied by a careful history. However, the severity of a prospective allergic reaction in a given donor cannot be related to the serum titer of allergen-specific IgE (2). The same is true for the concentration of bee venom required to obtain a positive skin test (11). To date, no method is available except taking the history of the patient to estimate the clinical severity of the sensitization, and performing a skin test, as well as serum analysis, to confirm the presence of specific IgE (2, 11, 12). Furthermore, these methods give no information on the amount of IgE bound to the surface of circulating basophilic granulocytes, a factor which may play an important role in determining the severity of generalized anaphylactic reactions in these patients (13, 14). The major allergen of bee venom in up to 80% of the patients is PLA₂. It constitutes about 10% of the total protein of bee venom (4, 15). Here we have directly analyzed the PLA₂-binding compartment of circulating effector cells; i.e., basophilic granulocytes in peripheral blood. We found that the frequency of PLA₂-binding cells is markedly higher in bee-venom-allergic patients than normal controls and is most pronounced in patients with severe anaphylactic reactions; i.e., Mueller grade IV. Interestingly, in the patient with the highest percentage of PLA₂-binding cells (patient 2; 2.3% before and 99% basophilic

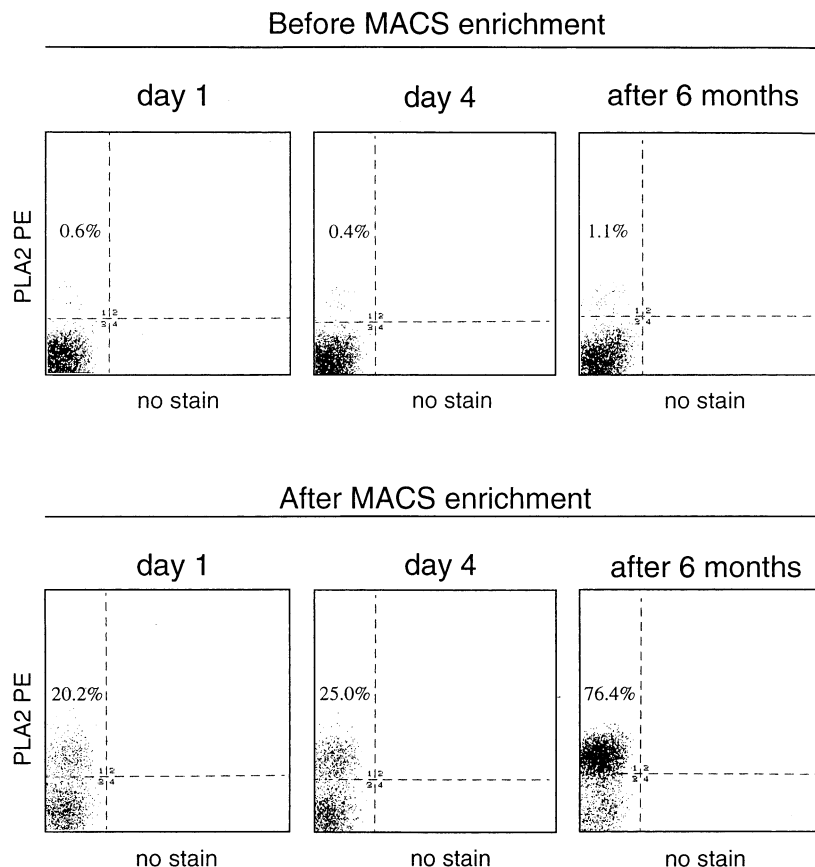


Figure 2. Comparison of staining intensities of PLA₂-binding cells during (days 1 and 4) and after rush desensitization (after 6 months) of one patient. Cells were stained equally bright on these three time points, and before as well as after MACS enrichment. In all experiments, same amount of conjugate and staining antibody was used. This shows that cells had bound comparable numbers of PLA₂-specific IgE onto their surface receptors.

granulocytes after MACS), two trials of immunotherapy failed due to severe anaphylactic reactions of the patient.

Basophilic granulocytes constitute the majority of PLA₂-binding cells in the bee-venom-allergic patients investigated, and the percentage of PLA₂-binding basophils can be correlated with the history of clinical sensitization. However, during rush immunotherapy and short-term follow-up, no major changes in the frequencies of basophils binding PLA₂ were found, suggesting that arming of the effector cell with allergen-specific IgE is not changed during the early phase of immunotherapy and thus may not be a reliable indicator of clinical sensitivity. The unchanged frequency of basophils binding PLA₂ is in line with previous studies indicating that specific immunotherapy exerts its effects directly after initiation and during the first months of treatment, presumably via a modification of the potential of the allergen-specific cells to release certain mediators, and not by a systemic decrease in the number of allergen-specific effector cells (16). We also found no evidence of a postulated inhibition of IgE binding to the high-affinity receptor via antigen excess (7, 14, 17, 18). Preliminary studies also suggest that there are no major alterations of IgG4 bound on the surface of these cells (unpublished observation). It is not

yet known whether an altered potential to release mediators may be due to the recently reported altered cytokine pattern observed in CD4⁺ T cells or antigen-specific T cells (19–21) during specific immunotherapy.

The results presented here diverge from those published by Donohoe et al. (22), who stained IgE-positive cells in allergic patients and described them as highly differentiated B cells expressing CD38, but not CD19 or CD21. However, the methods used in our study not only characterize the CD38⁺, IgE⁺ allergen-binding cells by surface markers but also by conventional means, i.e., cyto-spin staining, confirming that the vast majority of the IgE-positive PLA₂-binding cells in the highly allergic patients are indeed basophilic granulocytes. The enrichment of the basophilic granulocytes in the patients shows that a major proportion (at least 10 000 molecules per cell) of the FcR-bound immunoglobulin has PLA₂-specificity.

In summary, this study indicates that the number of allergen-binding basophils does not change during the early phase (up to 6 months) of immunotherapy. Therefore, during this phase, immunotherapy exerts its effects via a modification of the releasability of effector cells rather than by the number or antigen-binding capacity of

these cells. The direct analysis of circulating basophilic granulocytes carrying allergen-specific IgE, as described in this study, provides a new tool to investigate the complex

mechanisms involved in immediate-type hypersensitivity and its treatment.

References

- Hunt JK, Valentine MD, Sobotka AK, Benton AW, Amodio FJ, Lichtenstein LM. A controlled trial of immunotherapy in insect hypersensitivity. *N Engl J Med* 1978;**299**:157–161.
- BSACI Position paper on allergen immunotherapy. *Clin Exp Allergy* 1993;**23 Suppl 3**:11–14.
- King TP, Sobotka AK, Kochoumian C, Lichtenstein LM. Allergens of honeybee venom. *Arch Biochem Biophys* 1976;**172**:661–671.
- Sobotka AK, Franklin RM, Adkinson NF, et al. Allergy to insect stings. Phospholipase A: the major allergen in honeybee venom. *J Allergy Clin Immunol* 1976;**57**:29–40.
- Golden DBK, Meyers DA, Kagey-Sobotka A, Valentine MD, Lichtenstein LM. Clinical relevance of the venom specific IgG antibody level during immunotherapy. *J Allergy Clin Immunol* 1982;**69**:489–493.
- Müller U, Heibling A, Bischof M. Predictive value of venom specific IgE, IgG and IgG subclass antibodies with honeybee venom. *Allergy* 1989;**44**:412–418.
- Stämpfli MR, Rudolf M, Miescher S, Pachlopnik JM, Stadler BM. Antigen-specific inhibition of IgE binding to the high-affinity receptor. *J Immunol* 1995;**155**:2948–2954.
- Irsch J, Hunzelmann N, Tesch H, et al. Isolation and characterization of allergen-binding cells from normal and allergic donors. *Immunotechnology* 1995;**1**:115–125.
- Mueller HL. Diagnosis and treatment of insect sensitivity. *J Asthma Res* 1966;**3**:331–336.
- Glowania HJ, Schulz KH. Zur Hyposensibilisierungsbehandlung mit Bienen- und Wespengift. *Allergologie* 1981;**4**:60–66.
- Reisman RE. Insect stings. *N Engl J Med* 1994;**331**:523–527.
- Nüsslein HG, Baenkler HW. Spontaneous loss of hypersensitivity in patients allergic to bee or wasp stings; detection by venom induced histamine release. *Ann Allergy* 1985;**54**:516–520.
- Serafin WE, Austen KF. Mediators of immediate hypersensitivity reactions. *N Engl J Med* 1987;**317**:30–34.
- Eberlein-König B, Ullmann S, Thomas P, Przybilla B. Tryptase and histamine release due to a sting challenge in bee-venom-allergic patients treated successfully or unsuccessfully with hyposensitization. *Clin Exp Allergy* 1995;**25**:704–712.
- Müller UR, Dudler T, Schneider T, et al. Type I skin reactivity to native and recombinant phospholipase A₂ from honeybee venom is similar. *J Allergy Clin Immunol* 1995;**96**:395–402.
- Jutel M, Müller UR, Fricker M, Rihs S, Pichler WJ, Dahinden C. Influence of bee venom immunotherapy on degranulation and leukotriene generation in human blood basophils. *Clin Exp Allergy* 1996;**26**:1112–1118.
- Stephen V, Kühr U, Urbanek R. Relevance of basophil histamine release changes during venom immunotherapy. *Allergy* 1989;**44**:453–459.
- Reisman RE, Lantner R. Further observations of stopping venom immunotherapy: comparison of patients stopped because of a fall in serum venom specific IgE to insignificant levels with patients stopped prematurely by self choice. *J Allergy Clin Immunol* 1989;**83**:1049–1054.
- Jutel M, Pichler WJ, Skribic D, Urwyler A, Dahinden C, Müller UR. Bee venom immunotherapy results in decrease of IL-4 and IL-5 and increase of IFN- γ secretion in specific allergen stimulated T cell cultures. *J Immunol* 1995;**154**:4187–4194.
- Segura JA, Assenmacher M, Irsch J, Hunzelmann N, Radbruch A. Systemic T-cell unresponsiveness during rush bee-venom immunotherapy. *Allergy* 1998;**53**:233–240.
- Secrist H, Chelen CJ, Wen Y, Marshall JD, Umetsu DT. Allergen immunotherapy decreases interleukin 4 production in CD4 T cells from allergic individuals. *J Exp Med* 1993;**178**:2123–2130.
- Donohoe PJ, Heddle RJ, Sykes PJ, Fusco M, Flego LR, Zola H. IgE cells in the peripheral blood of atopic, nonatopic and bee venom hypersensitive individuals exhibit the phenotype of highly differentiated B-cells. *J Allergy Clin Immunol* 1995;**95**:587–596.