

ORIGINAL ARTICLE

Time course of serum inhibitory activity for facilitated allergen–IgE binding during bee venom immunotherapy in children

Serum inhibitory activity for facilitated allergen–IgE binding during venom immunotherapy and its withdrawal

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Summary

Background Immunotherapy for bee venom allergy is effective and provides long-term protection. Venom-specific IgG4 levels are increased but with no correlation with clinical improvement. Following grass pollen immunotherapy, elevation of antigen-specific IgG4 is accompanied by increases in IgG-dependent serum inhibitory activity for IgE-facilitated binding of allergen–IgE complexes to B cells. As this ‘functional’ assay of inhibitory antibodies may be more predictive of clinical efficacy, we investigated the time course of serum inhibitory activity for IgE-facilitated antigen binding during venom immunotherapy (VIT) in children and following 2 years of VIT withdrawal.

Methods Ten bee venom-allergic children (mean age: 9.3 years; m/f, 7/3) with moderate to severe allergic reactions to bee stings received VIT. A separate group of seven children (mean age: 14 years; m/f, 5/2) were investigated 2 years after VIT withdrawal. Ten age- and gender-matched children served as non-allergic controls. Allergen-specific serum IgG4 and IgE levels were measured by ELISA at baseline, after 2 years of VIT and 2 years after VIT withdrawal. Serum inhibitory activity was assessed using the facilitated-allergen binding (FAB) assay.

Results Sera obtained during VIT significantly inhibited allergen–IgE binding to B-cells (pre-treatment = $104 \pm 23\%$; 2 years = $46 \pm 15\%$; $P < 0.001$) when compared with sera obtained after treatment withdrawal and sera from normal controls. In parallel to FAB inhibition during VIT, significantly higher IgG4 levels were noted after immunotherapy (pre-treatment = 8.6 ± 2.3 AU; 2 years = 26.7 ± 3.5 AU; $P < 0.001$) compared with those observed after withdrawal and in the controls. In contrast, progressively lower IgE concentrations were observed compared with pre-treatment (44 ± 7 AU) in sera obtained after 2 years of VIT (25 ± 5 AU; $P < 0.01$) and 2 years following the withdrawal of VIT (10 ± 3 AU; $P < 0.05$).

Conclusions In contrast to grass pollen immunotherapy, the persistent decline in venom-specific IgE levels, rather than serum inhibitory activity for FAB, may be more relevant for long-term clinical efficacy of VIT.

Keywords bee venom immunotherapy, children, facilitated allergen–IgE binding (FAB), serum inhibitory activity, time course

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Introduction

Immunotherapy has proved to be effective in patients with venom allergy-induced anaphylaxis. According to current guidelines, it is indicated both in children and adults, with a history of a severe systemic reaction including respira-

tory and cardiovascular symptoms and documented sensitization to the respective insect [1, 2]. Although children get frequently stung, only one-third develop a serious allergic reaction, whereas the majority show allergic skin manifestations only and reportedly has a very low risk of a systemic reaction after a re-sting [3]. This led to the

conclusion that venom allergic children with mild allergic reactions only, do not require immunotherapy.

In the latest survey of the long-term effects of venom immunotherapy (VIT) [4], Golden et al. studied the outcome of insect sting allergy in childhood up to 20 years after the first allergic reaction. In the group with a previous moderate to severe reaction, the reaction rate was significantly higher in the untreated group compared with the group with immunotherapy (32% vs. 5%). In patients with previous skin manifestations only, the risk of an allergic reaction was 13% in the untreated and 0% in the treated group. These data challenge the general view that children outgrow the allergy to insect stings, as almost one in five children reacted up to 32 years after the original reaction. This study provides evidence not only for prolonged effectiveness, but also indicates long-term immunomodulatory effects of VIT in children. However, the precise immunologic mechanisms are still not understood. Although transient increases in allergen-specific IgE levels during up-dosing and early maintenance have been shown to decline to pre-treatment levels after at least 3 years of treatment, they remain elevated in up to 30% of patients [5]. Additionally, an inverse correlation between the levels of allergen-specific IgG4 levels, so-called 'blocking antibodies', and allergic sensitization has been reported. However, elevation of venom-specific IgG4 levels during VIT, does not correlate with clinical improvement, which occurs very early in the course of rush VIT. One of the mechanisms responsible for clinical improvement early in the course of VIT, may be the induction of tolerance. Successfully treated patients develop a specific T cell unresponsiveness against bee venom allergens, which can be restored by addition of IL-2 and IL-15, thereby indicating T cell anergy and not deletion. The same results were also found in hyper-immune beekeepers [6] in whom IL-10 secretion by activated CD4⁺CD25⁺T cells, so-called T regulatory (Treg 1) cells, seems to be pivotal for maintenance of peripheral tolerance and suppression of effector cells.

As a consequence of the disparity between the induction of elevated allergen-specific IgG4 levels during immunotherapy and the onset of clinical improvement, rather than solely focusing on qualitative changes in allergen-specific IgG4 antibody levels, the functional properties of IgG antibodies during allergen immunotherapy are now being evaluated. In a study of grass pollen immunotherapy, Wachholz et al. [7] have investigated the inhibitory properties of IgG by using a novel flow cytometric assay, based on detection of allergen-IgE binding to the low-affinity IgE receptor on B-cells. Inhibition of allergen-IgE binding to B-cells, as well as subsequent antigen presentation, was found in all actively treated patients, but not in the placebo group, and co-purified with grass pollen allergen-specific IgG. In a recent study [8], the same authors were able to show that serum

allergen-specific IgG4 inhibitory activity for IgE-FAB and histamine release from basophils during grass pollen immunotherapy, occurred after 2 months of immunotherapy and coincided with inhibition of early skin responses. In addition, a recent study of birch pollen immunotherapy confirmed inhibition of facilitated antigen presentation and histamine release from basophils during the first and second year of treatment [9]. Therefore, studying the functional changes in allergen-specific IgG4 antibody levels during allergen immunotherapy [10] may be more relevant for predicting clinical efficacy and may even serve as a biomarker for estimation of optimal immunotherapy duration.

We were interested to investigate the time-course of serum inhibitory activity for IgE-facilitated allergen binding (FAB) in children during bee VIT and two years after treatment withdrawal, to assess the potential of functional changes in venom-specific IgG4 serum antibody levels for induction of long-term clinical efficacy and tolerance (persistent efficacy after withdrawal of treatment).

Materials and methods

Subjects

Ten children (mean age: 9.7 years; m/f, 7/3; Table 1) with moderate to severe allergic reactions to bee stings were hospitalized to receive a daily rush up-dosing schedule (Reless aqueous bee venom, ALK-Abelló, Hørshoem, Denmark) for 4 days, followed by monthly maintenance injections (Alutard SQ bee venom, ALK-Abelló) for 3 years. Blood samples were taken at baseline, after 2 years of treatment and, from a separate group of seven

Table 1. Demographic data and clinical characteristics of subjects

	SIT-group baseline	SIT-group withdrawal	Non-allergic controls
Number (n =)	10	7	10
Age (mean years)	9.7	14	9.9
Gender (m/f)	7/3	5/2	9/1
History of clinical reaction:	Pre- treatment	2 years withdrawal	Control day
Urticaria	10/10	0/7	0/10
Asthma	9/10	0/7	0/10
Anaphylaxis	3/10	0/7	0/10
Local swelling	10/10	1/7	6/10
Skin prick test positivity	7/10	2/7	ND
Re-sting in study period without allergic reaction	2/10	2/7	ND
Re-sting in study period with allergic reaction	0/10	0/7	ND

children (mean age: 14 years; m/f,5/2), 2 years after VIT withdrawal. In this group, there was no difference at baseline, regarding age, severity of allergic reaction and male/female ratio, as compared with the baseline characteristics of the group during VIT. Ten age- and gender-matched non-allergic children served as non-allergic controls during routine blood tests (Table 1). Informed consent was obtained from all participants and/or their parents and the study was approved by the local ethics committee.

Skin tests and in vitro allergy tests

Skin prick tests (Soluprick, ALK-Abelló, at 10, 100 and 300 µg/mL of bee and wasp venom) were performed at baseline, after 2 years and 2 years after VIT withdrawal on the lower arms of the venom-allergic children only. Weal sizes of ≥ 3 mm in the presence of a positive (histamine) and a negative (diluent) control were considered positive. In venom allergic children, bee venom-specific IgE levels were measured by Phadia[®] CAP (Phadia AB, Uppsala, Sweden) with a cut-off level of ≥ 0.35 kU/L indicating sensitization to bee venom.

Serum antibody measurements

Serum allergen-specific IgE and IgG levels were measured by means of ELISA (coating allergen concentration, 5 µg/mL; 1 : 100 or 1 : 20 dilution of sera, respectively; detection with biotinylated anti-human IgG4 or IgE mAb; BD PharMingen, San Diego, CA, USA). For ELISA measurements, the same allergen was used as for rush immunotherapy (Reless aqueous bee venom, ALK-Abelló) containing a mixture of all relevant bee venom allergens. The standards for IgG4 and IgE ELISAs consisted of a pool of equal volumes of sera from 10 subjects receiving SIT or untreated allergic subjects, respectively. The highest standard serum was given an arbitrary value of 100 U and the corresponding units for the test serum are read from the standard curve as arbitrary units per millilitre (AU/mL).

Serum inhibitory activity (FAB assay)

IgE-facilitated allergen binding to B-cells was performed as previously described [11]. Specifically, stock indicator serum (20 µL) containing high concentration of bee venom-specific specific IgE (> 100 IU/mL), was pre-incubated with 1 µg/mL allergen (Reless aqueous bee venom, 5 µL) at 37 °C for 1 h to form allergen-IgE complexes. To test for inhibition of facilitated allergen binding, we mixed an equal volume of indicator serum (20 µL) with test serum (20 µL) or an equal volume of RPMI as a control. EBV-transformed B cells (5 µL) were then added to the allergen-IgE mixture and incubated for 1 h at 4 °C. Cells were washed and bound complexes were revealed

using a polyclonal human anti-IgE antibody and detected by flow cytometry (FACS Calibur; BD Biosciences, Rockville, MD, USA). B cells were gated using forward scatter/side scatter parameters and a positive marker was set using cells incubated with indicator serum only. Five thousand cells were analysed and all samples were measured in triplicate. Data was expressed as the percentage relative binding to allergen-IgE complexes where binding with indicator serum alone was normalized to 100%.

Statistics

Differences from baseline measurements were assessed by Friedman's non-parametric repeated measures test. *P*-values < 0.05 were considered statistically significant.

Results

Serum obtained during VIT, significantly inhibited allergen-IgE binding to B-cells (pre-treatment = $104 \pm 23\%$; 2 years = $46 \pm 15\%$; $P < 0.001$). However, inhibition of allergen-IgE binding was not sustained after VIT withdrawal, without any statistical difference from pre-treatment results. Serum collected from non-allergic controls, did not display any inhibition of allergen-IgE binding (Fig. 1a). This serum inhibitory activity observed after 2 years of VIT was accompanied by significantly higher IgG4 levels (pre-treatment = 86 ± 23 AU/mL; 2 years = 267 ± 35 AU/mL; $P < 0.001$). In parallel to the observed lower levels of serum inhibitory activity observed in the 2-year-withdrawal group, venom-specific IgG4 levels were at approximately the same level as pre-treatment values (Fig. 1b). Low levels of venom-specific IgG4 antibody levels were observed in the non-allergic control group. Significantly lower levels of allergen-specific IgE were observed after VIT (pre-treatment = 44 ± 7 AU/mL; 2 years = 25 ± 5 AU/mL; $P < 0.05$) and levels were observed to be even lower, 2 years following treatment withdrawal (10 ± 3 AU/mL; $P < 0.01$) (Fig. 1c). These findings of persistent and progressively lower venom-specific IgE levels during and 2 years following withdrawal from immunotherapy were confirmed by measuring venom-specific IgE levels in the CAP assay (pre-treatment = 55 ± 13 kU/L; 2 years = 25 ± 8 kU/L; withdrawal group = 11 ± 3 kU/L). Additionally, a significantly higher IgG4/IgE ratio was seen during VIT as compared with baseline ($P < 0.001$) but was not maintained after treatment withdrawal (Fig. 1d).

In a sub-group of three children, serum samples were available to longitudinally assess FAB inhibition, allergen-specific IgG4 and IgE levels at baseline, after 2 years of VIT and 2 years post VIT withdrawal. Similar trends were observed compared with the grouped data. After 2 years of VIT inhibition of allergen-IgE binding was seen in all three patients. Sera from two patients obtained at baseline already showed an inhibitory activity between

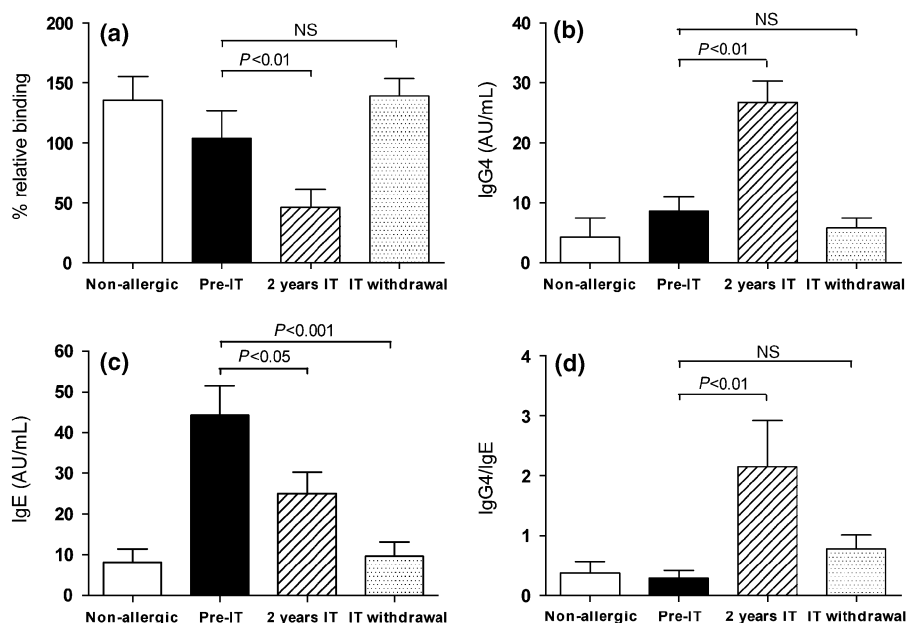


Fig. 1. Assessment of facilitated allergen-binding (FAB) and allergen specific-IgG4 and IgE in serum samples obtained from patients before SIT, 2 years after SIT, 2 years after treatment withdrawal and in normal controls. Serum was tested for inhibitory activity using the facilitated-allergen binding assay (a) where values of $< 100\%$ represent inhibitory activity and values $> 100\%$ as enhanced allergen-IgE binding presumably due to IgE present in test serum. Venom-specific IgG4 (b) and IgE (c) were measured by means of ELISA. The ratio of allergen-specific IgG4/IgE was calculated (d). Differences from pre-treatment measurements were assessed using Friedman's non-parametric repeated measures test. P values < 0.05 were considered statistically significant.

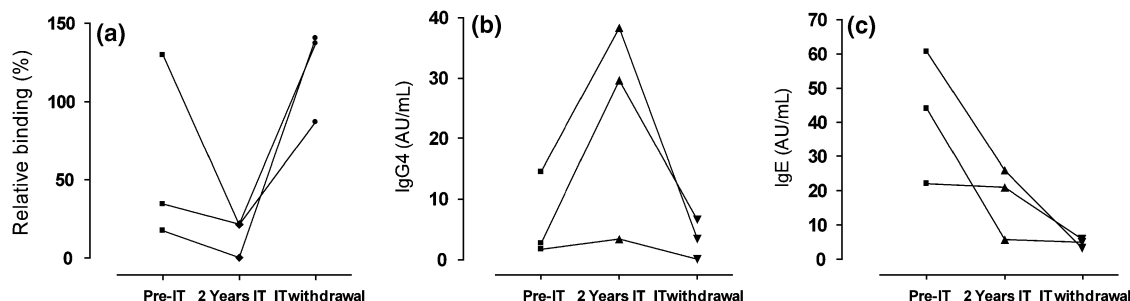


Fig. 2. Longitudinal assessment of venom-specific IgG4 and IgE antibody levels and of serum inhibitory activity (FAB) in three venom-allergic children before venom immunotherapy, after 2 years treatment and after withdrawal. Serum was tested for inhibitory activity using the facilitated-allergen binding assay (a), and for venom-specific IgG4 (b) and IgE (c) by means of ELISA.

20% and 40%, which further increased after VIT withdrawal (Fig. 2a). These patients had been repeatedly stung before becoming venom-allergic, but never after VIT withdrawal. Compared with baseline measurements, higher venom-specific IgG4 levels were observed after VIT but were not sustained after VIT withdrawal (Fig. 2b). In contrast, a marked decline in venom-specific IgE was noted during VIT, with subsequent further decreases after VIT withdrawal (Fig. 2c). A very low re-sting rate was observed in our study, as only two children were stung during VIT and another two, after VIT withdrawal (Table 1). We therefore could not address the possibility of treatment failure and a potential correlation with serum inhibitory activity for FAB. Apart from local swelling at

the sting site, no systemic allergic reactions were reported in either group.

Discussion

This is the first study to assess the time course of the functional properties of serum IgG antibodies, so called 'blocking antibodies', during VIT in children. Induction of these antibody isotypes has long been regarded as the characteristic immunological feature induced by VIT, although no correlation with clinical improvement could be observed. In this study of bee VIT in children, we confirmed the finding reported after grass pollen

immunotherapy [7] that serum inhibitory activity for allergen-IgE binding to B-cells, as well as subsequent antigen presentation, is induced by allergen immunotherapy. In parallel, we observed significant increases in venom-specific serum IgG4 antibody and a significant decline in venom-specific IgE antibody levels during VIT. However, following a VIT withdrawal of 2 years, high levels of allergen-specific IgG4 serum antibody levels and inhibition of facilitated allergen-IgE binding were not sustained.

Although subcutaneous allergen immunotherapy has been shown to induce a range of immunological changes irrespective of the allergen, from induction of CD4⁺CD25⁺(Treg 1 cells) with up-regulation of the anti-allergic cytokines IL-10 and TGF- β [12, 13] and T lymphocyte switching to down-regulation of pro-inflammatory cytokines from effector cells [14], persistent induction of functional changes in serum IgG4 antibodies may be influenced by the associated allergic disease. For example, in contrast to grass pollen immunotherapy, with continued seasonal exposure to the offending allergen, venom allergic patients are only randomly exposed to bee stings following VIT. In our study, the majority of children were not stung after initiation of immunotherapy and after treatment withdrawal, possibly due to greater awareness of the need for insect sting avoidance. Regular and repeated allergen exposure may, however, be pivotal for the persistence of a proportion of venom-specific IgG4 antibodies with functional properties to inhibit allergen-IgE binding and thereby facilitated antigen presentation.

Whether our findings may indicate a risk of a renewed allergic reaction to bee stings, could only be addressed by bee sting challenges, which, at least in our clinical setting, are not performed in children. Together with the decline in serum inhibitory activity after a VIT withdrawal of 2 years, we also observed a continued decrease in venom-specific serum IgE antibody levels. Allergen immunotherapy has been shown to induce significant decreases in allergen-specific IgE levels following immunotherapy withdrawal, as compared with pre-treatment levels [15]. In the absence of a sustained serum inhibitory activity for allergen-IgE binding after VIT withdrawal, this persistent decrease in allergen-specific IgE levels can only be speculated on. Whether this decrease is induced by VIT *per se* or simply represents the lack of allergen exposure, cannot be answered by our study. Therefore, prospective allergen immunotherapy withdrawal studies with grass pollen and venom allergen are needed to establish potential differences between mucosal and injected allergens, in regard of a sustained serum inhibitory activity for facilitated

antigen presentation and the correlation with long-term clinical efficacy.

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