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Tolerance induction after specific immunotherapy with pollen allergoids adjuvanted by monophosphoryl lipid A in children

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Summary

Specific immunotherapy (SIT) is a well-established and clinically effective treatment for allergic diseases. A pollen allergoid formulated with the T helper type 1 (Th1)-inducing adjuvant monophosphoryl lipid A (MPL) facilitates short-term SIT. Little is known about mechanisms of tolerance induction in this setting. In a prospective study, 34 patients allergic to grass pollen (25 male, nine female, median age 10·2 years) received a total of 44 SIT courses (20 in the first, 24 in the second) with MPL-adjuvanted pollen allergoids. Immunogenicity was measured by levels of specific immunoglobulin G (IgG_{grass}) and $IgG4_{grass}$ by antibody blocking properties on basophil activation, and by induction of $CD4^+$, $CD25^+$ and forkhead box P3 ($FoxP3^+$) regulatory T cells (T_{reg}). Specific IgG and IgG4 levels increased only slightly in the first year of SIT. In the second year these changes reached significance ($P < 0{\cdot}0001$). In keeping with these findings, we were able to show an increase of T_{reg} cells and a decreased release of leukotrienes after the second year of treatment. In the first year of treatment we found little evidence for immunological changes. A significant antibody induction was seen only after the second course of SIT. Short-course immunotherapy with pollen allergoids formulated with the Th1-inducing adjuvant MPL needs at least two courses to establish tolerance.

Keywords: allergoids, blocking antibodies, monophosphoryl lipid A (MPL), regulatory T -cells, ultra-short-course specific immunotherapy

Introduction

Immunoglobulin (Ig)E-mediated allergic disorders such as asthma and allergic rhinitis have become a growing epidemic in industrialized countries, affecting 20–30% of the population [1]. Atopic disease nowadays is a major burden to public health, demanding an ongoing research to prevent and treat allergic conditions. Modern pharmacotherapy can only mitigate the symptoms of allergic diseases; to date, specific immunotherapy (SIT) is the only safe and efficacious treatment with the potential to similarly ameliorate symptoms and alter the natural course of the disease. Gradually increasing quantities of specific allergen are given to induce immunotolerance and reduce symptoms to that allergen upon subsequent exposure [2–4]. Tolerance to allergens can be defined as persistence of efficacy after discontinuation of treatment, implying an altered allergen-specific memory T and B cell response [5,6]. Ever since the introduction of SIT in 1911, continuing development has improved safety and efficacy. The vaccines are now better standardized and characterized, according to new regulatory requirements [7]. This goal was achieved by addressing manufacturing quality (better characterized and standardized allergen extracts), optimal dosing, allergen modification (to reduce allergenicity while maintaining immunogenicity), adjuvant adsorption (to control release) and adjuvant activity (to assist immunomodulatory action).

It was shown that L-tyrosine is a safe adjuvant for human use [8] and has both an adsorptive role (as a short-term depot) and a favourable immunological effect [9,10]. Monophosphoryl lipid A (MPL®) is an adjuvant derived from the lipopolysaccharide of *Salmonella minnesota* R 595. The lipid A portion of the endotoxin has long been known to be a potent adjuvant, but unacceptable toxicity has limited its clinical use. The removal of a phosphate and fatty acid group from lipid A produced a molecule, MPL®, that retained the adjuvant properties of lipid A but reduced its toxicity significantly [11,12]. The adjuvant activity of MPL® promotes primarily a T helper type 1 (Th1) response [13–15]. MPL® has been shown to be well tolerated and to enhance both humoral and cellular immune responses.

One formulation (Pollinex® Quattro) is employing glutaraldehyde modified pollen (allergoids) adsorbed onto L-tyrosine and MPL®. This provides a vaccine that is efficacious with only four doses, in contrast to longer administration schedules in use for other allergy vaccines [16]. Short-term SIT offers a convenient option and supports compliance in children and adolescents.

So far there is no consensus regarding the mechanism of successful SIT, but it is thought to involve a redressed Th1/Th2 cell balance [17] by depressing the Th2 cellular response [interleukin (IL)-4, IL-5 and IL-13] in favour of a more Th1-like response [interferon (IFN)-γ, IL-2 and tumour necrosis factor (TNF)-α], the induction of regulatory T cells [18] and formation of specific IgG, especially IgG4 antibodies [19–22].

IgG antibodies induced by means of immunotherapy block allergen-induced IgE-dependent histamine release by basophils [23] and suppress allergen-specific T cell responses *in vitro* by inhibiting binding of IgE allergen complexes to antigen-presenting cells [24,25]. It has been shown that blocking IgG antibodies, which have been induced by allergen-specific immunotherapy, inhibit IgE-facilitated allergen presentation and can result in decreased T cell proliferation and reduced production of IL-4 and IL-5 [26]. Furthermore, IL-10 and transforming growth factor (TGF)-β cooperate in the regulatory T cell response to mucosal allergens in short-term immunotherapy (SCIT) via an antigen-specific suppressive activity in $CD4^+CD25^+$ T cells of allergic individuals. Regulatory T cells (T_{regs}) are able to inhibit the development of allergic Th2 responses and their up-regulation plays a major role in allergen-specific immunotherapy (SIT) [27,28]. This association was found because forkhead box P3 (FoxP3)-deficient subjects suffer among other findings from atopic disease [29]. Subsets of T_{reg} cells are the thymus-selected $CD4^+CD25^+FoxP3^+$ T cells [30,31]. FoxP3 is a member of the forkhead/winged-helix family of transcription factors and acts as the master regulator for the development and function of T_{regs} [32]. T_{regs} selectively expressing FoxP3 [33] show suppressive properties on effector T cells [34,35]. This suppressive capacity of T_{regs} is impaired in atopic patients, especially during the pollen season [36,37]. The demonstration of local $FoxP3^+CD25^+$ T cells in the nasal mucosa and their increased numbers after immunotherapy supported the role of T_{reg} cells in the induction of allergen-specific tolerance in human subjects [38]. Their increased numbers correlate with clinical efficacy and suppression of seasonal allergic inflammation. Therefore, they have become a prime target for strategies aimed at inducing tolerance. Children who outgrew cow's milk allergy had increased numbers of circulating T_{regs} compared with children with persistent active allergic disease [39]. Consequently, the formation of specific IgG antibodies and the induction of regulatory T cells can be used as surrogate markers for successful SIT. In our study we investigated the time–course of specific IgG and IgG4 antibodies, their property to act as blocking antibodies and the induction of $CD4^+$, $CD25^+$ and $FoxP3^+$ T cells (T_{reg}) in the first and second years of a specific immunotherapy.

Methods

Participants

Thirty-four patients [25 male, nine female, 6–17 years old, mean age 10·2 years, standard deviation (s.d.) 3·4] were enrolled into the study (Table 1). Criteria for inclusion were as follows: signed informed consent, participants ≥ 6 and < 18 years of age who gave a clinical history of troublesome symptoms of seasonal allergic rhinoconjunctivitis (SAR) and/or allergic asthma grades I–II according to the Global Initiative for Asthma (GINA) guidelines [40] during the grass pollen season, raised serum allergen-specific IgE [radioallergosorbent test (RAST) ≥ II, ≥ 0·7 kU/ml] to grass pollen and no significantly abnormal findings on physical examination.

Reasons for exclusion included the following: forced expiratory volume in 1 s (FEV_1) less than 70% of the predicted value prior to enrollment, concomitant therapy with inhaled corticosteroids (ICS) of > 500 μg/day of budesonide or beclomethasone or 250 μg/day of fluticasone for at least 3 months, and the use of systemic steroids. Further patients with serious underlying conditions were excluded from the study.

Human guidelines of good clinical practice, the German Drug Act and the declarations of Helsinki (1964) and Edinburgh (2000) were followed in the conduct of the trial. The study was approved by the local ethics committee. All parents and all patients older than 14 years of age provided written informed consent.

Study design

All patients received a standardized allergy vaccine containing L-tyrosine and the adjuvant monophosphoryl Lipid A (MPL®), to which a mixture of pollen allergoids from grasses were adsorbed (Pollinex® Quattro; Bencard Allergie, Munich, Germany). The treatment cycles were conducted off-season between September and March. Included patients received four injections subcutaneously prior to the pollen

Table 1. Patient characteristics.

Gender (male : female)	25 : 9
Age (years)	10·2 (5–17)
Lung function	
Vital capacity	95·4 (70–147)
Forced expiratory volume (mean %)	102 (79–158)
Exhaled nitric oxide (ppB)	24.1 (4–125)
Total immunoglobulin E (kU/l)	460 (49–2000)
Immunoglobulin E specific to grass (kU/l)	105 (1–525)

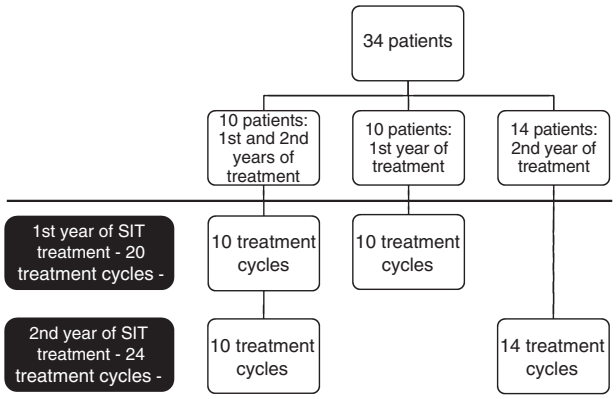

Fig. 1. Flowchart of the patients in the conduct of this study.

season comprising a total of 5100 standardized grass pollen units [visit 1: 300 standardized units (SU); visit 2: 800 SU; visit 3: 2000 SU; visit 4: 2000 SU] per treatment cycle. The injections were administered on a weekly basis.

Prior to the first injection (week 1), directly after the fourth injection (week 4) and – in a subset of patients – four weeks later (week 8), serum levels of specific IgE to grasses (IgE_{grass}), specific IgG to grasses (IgG_{grass}) and specific IgG4 to grasses ($IgG4_{grass}$) were determined as described below. T_{regs} were analysed by flow cytometry. In a subset of patients we analysed further the induction of blocking antibodies.

This open trial encompassed 34 patients receiving a total of 44 treatment cycles (consisting of four injections each) over a time-period of 2 years. Ten patients were followed over two treatment cycles (first and second years of treatment), 10 over the first year of treatment only, and 14 over the second year of treatment only (the first treatment cycle was given before the patients were enrolled into this study). In total we were able to analyse 20 treatment cycles of the first year and 24 of the second year of treatment (Fig. 1.)

Clinical procedures

In order to characterize our subjects, at visit 1 a skin prick test was performed using biological standardized allergens including *Dermatophagoides pteronyssinus* and farinae, cat, dog, mould mix, grass- and tree-pollen-mix (Allergopharma, Reinbek, Germany). Pulmonary function tests were performed with the MasterScreen® bodyplethysmograph by VIASYS Healthcare GmbH (Höchberg, Germany).

During the 4 weeks of therapy patients underwent weekly visits for updosing and maintenance therapy. All injections were administered in the upper arm, and patients were observed for half an hour after each injection.

Specific antibodies

Serum was collected at weeks 1, 4 and 8 and stored at −80°C until testing. Samples were analysed for specific antibodies against *Phleum pratense* L. (IgE_{grass}, IgG_{grass}) using a two-sided chemiluminescent assay (Immulite DPC, Bad Nauheim, Germany). Specific $IgG4_{grass}$ was detected by enzyme-linked immunosorbent assay (ELISA) technique (DST, Schwerin, Germany). Analysis was performed according to the manufacturer's instructions.

Blocking antibodies

Blocking antibodies were analysed in a subset of nine patients. Blood samples were taken before SIT and after the fourth injection in the first year and before SIT, after the fourth injection, and 4 weeks after the fourth injection in the second year of treatment. Cellular antigen stimulation test (CAST; Buehlmann Laboratories, Allschwill, Switzerland) was used to analyse inhibitory properties of serum antibodies. Briefly, leucocytes were isolated by dextrane sedimentation from ethylenediamine tetraacetic acid (EDTA) blood. Simultaneously, patient's serum was incubated with grass extract (2 ng/ml) for 1 h at room temperature. This mixture of patient's serum and grass extract was then used for cellular stimulation for 40 min. Supernatants were decanted and stored at −80°C. Ionomycin and anti-IgE-receptor antibody solutions were used as positive controls and patient's serum without grass extract was used as negative control. Cysteinyl leucotrienes (cysLT) were determined by ELISA technique according to the manufacturer's instructions.

Detection of T_{regs}

Peripheral blood mononuclear cell (PBMCs) lymphocytes from patients were obtained by centrifugation on Ficoll-Hypaque (Pharmacia, Uppsala, Sweden). Isolated PBMCs were surface-stained with a cocktail of anti-CD4-fluorescein isothiocyanate (FITC) (OKT-4) and anti-CD25-phycoerythrin (PE) (BC96) monoclonal antibodies. After fixation and permeabilization, the cells were blocked with 2% normal rat serum and intracellular staining was performed using anti-human FoxP3 allophycocyanin (APC) (PCH101) antibody (Natutec, Frankfurt am Main, Germany). On each sample, 10 000 events were analysed by a flow cytometer (Becton Dickinson, San Jose, CA, USA). Data analysis was performed with CellQuest software (Becton Dickinson, San Jose, CA, USA).

Recording of side effects

Participants were given diary cards and were asked to record side effects directly after administration and each of the following 4 days after administration of specific immunotherapy. We asked specifically for local swelling and pain, asthma symptoms, urticaria and other signs of anaphylaxis. Of the 176 injections, the diary data of 167 participants are available. The participants recorded their local and systemic side effects as being present (1) or not (2).

Table 2. Symptom scores following subcutaneous administration of specific immunotherapy.

	Redness	Diameter of swelling		Local pain
		< 5 cm	> 5 cm	
Visit 1: Injections with symptoms	15	17	8	11
% positive	35	40	19	26
Visit 2: Injections with symptoms	13	15	10	18
% positive	31	36	24	43
Visit 3: Injections with symptoms	11	14	9	11
% positive	27	34	22	27
Visit 4: Injections with symptoms	5	10	4	10
% positive	12	24	10	24
% positive (all injections)	26·3	33·5	18·5	30·0

The symptoms rated were local redness, diameter of swelling, local pain, urticaria, asthma, tissue irritation and symptoms of anaphylaxis. The results of each visit (visit 1 equals first injection) are shown in Table 2. In case of allergic symptoms, subjects had free access to relief medication (dimetindenmaleate locally, oral cetirizine, inhaled formoterol, oral prednisone and subcutaneous adrenaline) in a stepwise fashion, depending on the persistence and severity of the symptoms.

Statistical analysis

Data were analysed using the statistical software SPSS version 11·0 (SPSS Inc., Chicago, IL, USA). Within-group comparison was performed using a two-sided Wilcoxon matched-pairs test, and differences between baseline and other time-points were assessed by the Friedmann non-parametric repeated-measures test. Comparisons between groups were performed using a Mann–Whitney *U*-test. Induction of blocking antibodies was calculated by unpaired Student's *t*-test. Values of $P < 0{\cdot}05$ were considered statistically significant.

Results

Specific IgE

In total, 44 therapy courses with patients allergic to grass pollen were monitored. The median IgE_{grass} was 33·4 (range 1–310, $n = 20$) before the start of the therapy; immediately after the SCIT (after the fourth injection) the median was 48·3 IU/ml (range 1–493, $n = 20$; $P < 0{\cdot}0001$). Four weeks later the median was 14·8 (range 1–96·7, $n = 7$). This change was not statistically significant (Fig. 2).

Before the second year of treatment the median of specific IgE_{grass} was 85·05 kU/l (1–525, $n = 20$) and 100 (0·9–493; $n = 20$, $P < 0{\cdot}001$) after the fourth injection. Four weeks later the median was 48·4 ($n = 7$; 1–436). This again did not reach statistical significance.

Specific IgG

The median of specific IgG_{grass} was 9 KU/l (2·9–31, $n = 20$) before SIT and 11 ($n = 19$; 2·8–47; $P < 0{\cdot}05$) after the fourth injection. Four weeks later the median was 9·7 [$n = 7$; 2·8–23·8; not significant (n.s.)]. Before the second year of treatment the median of specific IgG_{grass} was 13 KU/l ($n = 24$; 2·8–23) and 33·5 ($n = 24$; 3·8–101; $P < 0{\cdot}001$) immediately after the fourth injection. Four weeks later the median was 39·5 ($n = 11$; 6–86·7; $P < 0{\cdot}05$).

The medians 4 weeks after immunotherapy between years 1 and 2 differed significantly (cycles 1 and 2: $P < 0{\cdot}001$; Fig. 3).

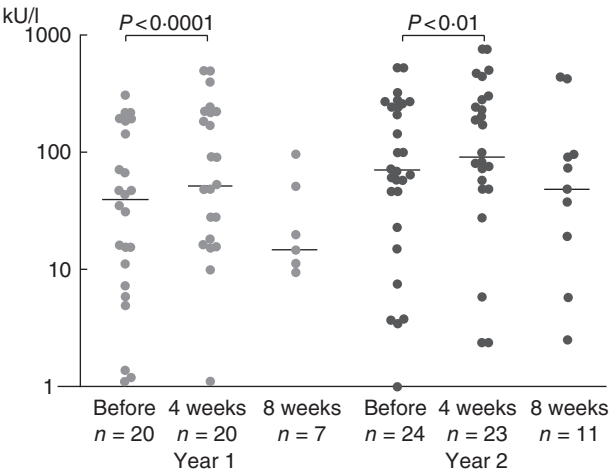

Fig. 2. Levels of specific immunoglobulin (Ig)E to grass pollen before and after specific immunotherapy.

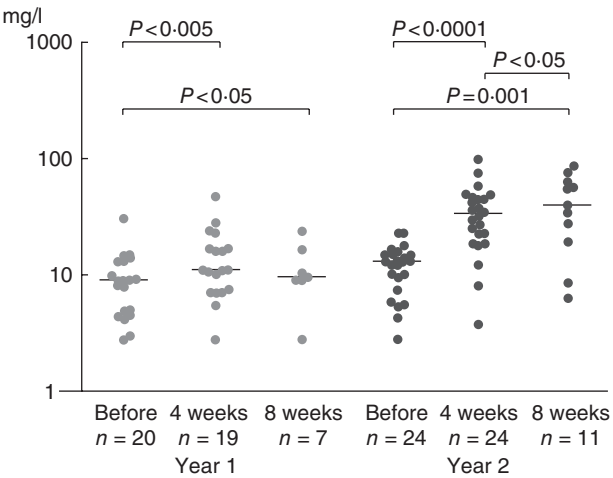

Fig. 3. Levels of specific immunoglobulin (Ig)G to grass pollen before and after specific immunotherapy.

Specific IgG4

The median of specific $IgG4_{grass}$ was 0·4 U/ml ($n = 20$; 0·4–0·4) before SIT and 0·4 ($n = 20$; 0·4–93·4; n.s.) after the fourth injection. Four weeks later the median was 0·4 ($n = 7$, 0·4–197; n.s.).

Before the second year of treatment the median of specific $IgG4_{grass}$ was 2·1 U/ml ($n = 24$, 0·4–59·7) before the SIT and 48·8 ($n = 24$, 0·4–1362; $P < 0·001$) immediately after the fourth injection. Four weeks later the median was 23·8 ($n = 11$, 0·4–705; $P < 0·05$). These changes reached statistical significance ($P < 0·001$, Fig. 4).

The antibody levels (IgE_{grass}, IgG_{grass}, and $IgG4_{grass}$) of those patients who had been observed for 2 years demonstrated a similar course to the overall values.

T_{regs}

T_{regs} were detected by the simultaneous expression of CD4, CD25 and FoxP3, comparing the percentage of cells before and 24 h after the fourth injection in the first and second years (Fig. 5). The percentage of T_{regs} raised significantly after the fourth injection in the second year (before: 2·1%, 1·27–4; 24 h after: 3·6%, 2·0–4·5; $P < 0·005$), but not in the first year of treatment (before: 2·1%, 0·5–3·0; 24 h after: 2·7%, 0·3–4·63).

Induction of blocking antibodies

In a subset of nine patients, we looked further for blocking activity of the specific antibodies by analysing the levels of cysLT before and 4 weeks after treatment in the first and second years (Fig. 6). We found a continuous decrease in cysLT release during the whole treatment period, which became significant after 4 weeks after the last injection in the second year compared to the time before SIT (before: 1383 pg/ml, ± 121; last injection: 1198 pg/ml, ± 133; $P < 0·05$).

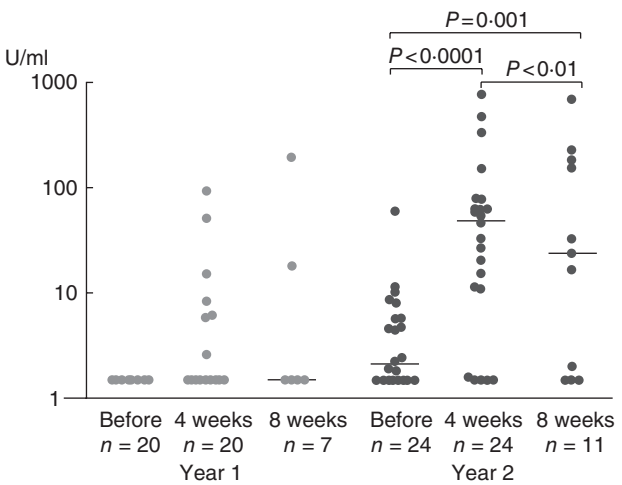

Fig. 4. Levels of specific immunoglobulin (Ig)G4 to grass pollen before and after specific immunotherapy.

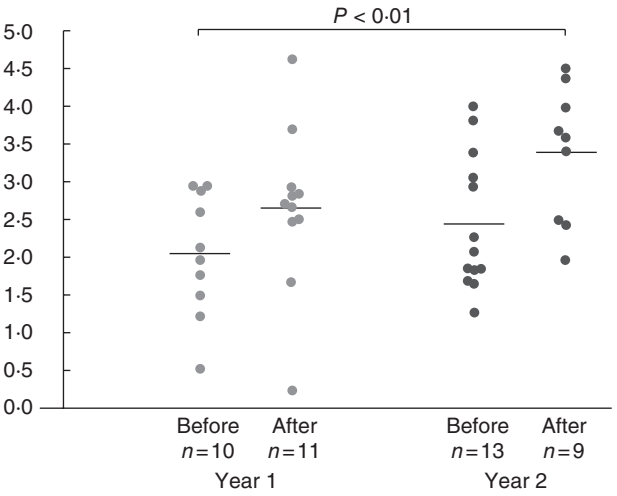

Fig. 5. Percentage of $CD4^+CD25^+$forkhead box $P3^+$ regulatory T cells of all T cells before and after specific immunotherapy.

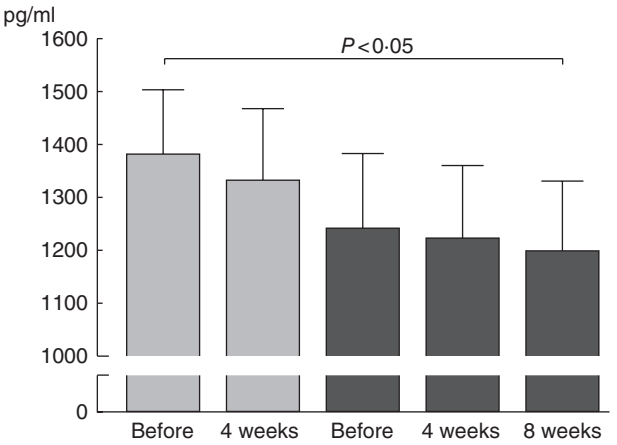

Fig. 6. Levels of leukotrienes in supernatant as a surrogate marker for the induction of blocking antibodies.

Local and systemic side effects following subcutaneous specific immunotherapy

Participants showed local redness, swelling and pain in approximately 30% of all injections. These side effects were transient and did not require any systemic treatment. However, two patients experienced a local urticarial rash. One patient suffered from a mild form of anaphylaxis after the first injection with mild symptoms of asthma and urticaria. After administration of prednisone orally the symptoms resolved within 30 min. Further injections showed only local side effects.

Overall, we were not able to show any correlation between antibody induction and local or systemic side effects following injection (data not shown).

Discussion

Allergen-specific immunotherapy (SIT) leads to significant clinical improvement of allergic rhinitis, conjunctivitis and asthma in affected patients [41]. These effects last for at least 10 years after discontinuation of therapy in conventional SIT [42]. The immunological mechanisms underlying these effects are being clarified continuously. A rise in specific IgG and IgG4 antibodies, a reduction in tissue numbers and/or decrease in mediator release of eosinophils, basophils and mast cells are involved similarly in successful SIT as a switch from a Th2 to a more Th1 allergen-specific immune response with the induction of T_{regs} [5].

SIT has the disadvantage that its efficacy only builds up with time. In the data published so far there is controversy about when and to what extent the clinical effects can be expected [43–46]. Especially in the first year of treatment, the clinical effects are limited. In a study by Rolinck-Werninghaus *et al.* [47], treatment with SIT alone before the first grass pollen season was unable to reduce either symptom score or the use of rescue medication significantly in children when compared with the reference group of SIT with an irrelevant allergen.

Consistent with the weak clinical effects after the first treatment cycle, we only found a modest increase of specific IgG and IgG4 antibodies in this study.

However, we could demonstrate a pronounced increase in specific IgG and IgG4 antibodies after the second course of SIT without a persistent rise of total IgE levels, as reported in successful conventional SIT [48]. Interestingly, the antibody levels rose quickly and were already detectable after 3 weeks of therapy. The fast and profound rise in the second and third years of treatment suggests immunological priming. These IgG antibodies were described first as blocking antibodies in 1935 [49]. In particular, IgG4 induced by means of immunotherapy blocks allergen-induced IgE-dependent histamine release by basophils and suppresses allergen-specific T cell responses *in vitro* by inhibiting binding of IgE–allergen complexes to antigen-presenting cells [24].

There are contradictory reports on the role of IgG on the efficacy of the immunotherapy [50,51]. The issue of whether there is a cause-and-effect relationship between the increase in IgG levels and the symptomatic improvement conferred by immunotherapy, or if this is just a surrogate marker of exposure to the SIT, remains unresolved. However, higher IgG concentrations are associated independently with symptomatic improvement in many studies, implying a linkage between immunological and clinical changes [48,52].

As there is a lack of correlation between IgG4 antibody levels and the degree of clinical improvement it is suggested rather to measure the blocking activity of allergen-specific IgG than the crude levels in sera [53]. In our work we were able to show a significant increase of blocking activity in the serum measured by reduced leukotriene release in treated patients.

Another attractive hypothesis for the immunological basis of specific immunotherapy is its effect on T_{regs} [54]. T_{regs} are able to inhibit the development of allergic Th2 responses and play a major role in allergen SIT [55].

IL-10 derived from T_{regs} generates tolerance in T cells, regulates specific isotype formation and skews the specific response from an IgE- to an IgG4-dominated phenotype. Description of T_{reg} goes back to Groux *et al.* in 1997 [56]. They are critical players in peripheral immune tolerance [57]. Further, they have the ability to suppress allergen-specific T cell responses and to regulate allergen-specific IgG4 and IgE synthesis [27,58]. Normally, 5–10% of peripheral T lymphocytes are T_{regs} [59]; our study revealed a diminished basal frequency of T_{regs} of 2–3·6%. Previous studies have shown that the capacity of $CD4^+$, $CD25^+$ and $FoxP3^+$ T cells from allergic individuals are reduced or even absent [37,60]. Hartl *et al.* found a significant increase in $CD25^-$ high $CD4^+$ T cells in peripheral blood and in bronchoalveolar lavage fluids after corticoid therapy [61]. In our work a significant increase in the proportion of $CD4^+$, $CD25^+$ and $FoxP3^+$ cells was seen after the second treatment cycle compared to baseline. This finding is in keeping with a study by Pereira-Santos *et al.*, who showed a significant increase in $FoxP3^+$ T cells 6 and 12 months after venom immunotherapy in adolescents and adults (mean age 47, range 16–65) [62].

Due to the relevant allergen challenge in SIT, IgE levels rise frequently at the beginning. This effect is transient, and is followed by a gradual decrease in the course of the therapy [63]. These changes are also found in our study with short-term immunotherapy. The IgE levels increase slightly during the therapy and decrease 4 weeks after discontinuation in the first and second years of treatment.

The success of SIT led to extensive research to improve efficacy and convenience for the patients. Ultra short-term immunotherapy (uSCIT) demonstrates some shortcomings in convenience, as one-third of our patients suffered from local side effects. In comparison to other types of SIT, this increase of side effects could be caused by MPL®. Other immunomodulators, such as cytosine-guanine dinucleotide (CpG) motifs, are in development, while new targets, such as the Notch protein/receptor interaction, may eventually give further improvement [64]. The excellent safety profile of the sublingual route of administration of allergy vaccines could lead to the wider use of SIT, and locally active immunomodulators could make SIT a therapy of choice for many more patients than at present. Besides clinical outcomes, i.e. bronchial hyperreactivity, or symptom scores, IgG- and IgG4 antibodies could similarly be helpful tools to evaluate efficacy of SIT. The same applies to the amount of T_{regs} and the induction of blocking antibodies. The long-term efficacy of new therapy regimens has to be elucidated in further studies.

Conclusion

Our study demonstrates immunological changes after short-term immunotherapy in childhood and adolescence. In the first year of treatment we found only moderate evidence for immunological tolerance. A significant induction of blocking antibodies was seen only after the second course of SIT. Short-course immunotherapy with pollen allergoids formulated with the Th1-inducing adjuvant MPL needs at least two courses to establish tolerance in allergic patients.

Disclosure

Stefan Zielen has disclosed receiving a research grant from Allergy Therapeutics and MSD; he also received fees for speaking and consulting for Novartis, Allergy Therapeutics, Symbio Vaccines, HAL, MSD, GSK and Wyeth. All the other authors have disclosed that they have no relevant financial interests.

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