

Clinico-immunologic Study on Immunotherapy with Mixed and Single Insect Allergens

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Abstract

Background Immunotherapy (IT) is practiced mainly with mixed and single allergen vaccines. But studies are rare with mixed allergen preparations.

Objective The objective of this study is to study mix and single insect allergen IT in patients of allergic rhinitis and asthma.

Methods We performed a double-blind placebo-controlled trial of mix and single allergen IT for 1 year in 99 patients of asthma or rhinitis or both. There were two groups, (1) active allergen IT ($n=61$) with three subgroups single insect extract (cockroach, housefly, or mosquito) and mix allergen IT (two or three insect extracts) and (2) placebo ($n=38$). Clinical (skin reactivity, airway reactivity, and symptom score) and immunological (IgE/IgG4 and IgG1/IgG4 ratio) parameters were assessed at baseline and after 1 year of IT. **Results** Eighty-five patients completed 1 year of IT. The active allergen IT group patients showed a significant improvement compared to baseline values ($p<0.05$) and

placebo group patients ($p<0.05$) with regard to symptom scores, FEV1 values, and immunological parameters (IgG4). No significant difference was found between mixed and single IT group patients for changes in clinical and immunological parameters. Positive correlation was observed between increase in IgG4 and clinical improvement. The changes in above parameters in placebo group were nonsignificant after 1 year of treatment.

Conclusion IT with two to three mix extract from the same allergen group is effective for insect hypersensitivity.

Keywords Insect allergy · immunotherapy · double-blind placebo-controlled trial · pulmonary function test · ELISA

Introduction

Insects play an important role in etiology of allergic bronchial asthma or rhinitis and have a significant bearing on the clinical characteristic of patients [1]. Cockroach, housefly, and mosquito are ubiquitous insects and induce respiratory allergies in a sizeable population [2, 3]. Mosquito and cockroaches derived particles are important inhalant allergens for IgE-mediated respiratory allergic disorders [4–7]. The whole-body insect extracts used for diagnosis or therapy contain both unique and cross-reactive allergenic and antigenic components [8, 9].

Recent studies in India show incidence of asthma in the range of 2.3–16.6% [10–12] and is significantly higher than reported (<1%) earlier [13]. Pharmacotherapy controls symptoms but has side effects on long-term use [14]. Immunotherapy (IT) is the only modality to gradually increase doses of allergens administered for the purpose of reducing or eliminating the patient's adverse clinical

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response on subsequent natural exposure to offending allergens [15]. Piero Mastrelli and colleagues reported that specific IT added to pharmacologic treatment and allergen avoidance provides statistically significant clinical benefits [16]. IT results in initial increase of specific IgE; however, levels of this antibody ultimately decrease and taken over by increased levels of IgG1 and IgG4 and the induction of suppressor T cells [17, 18].

In most of the studies, single allergen preparation(s) have been used for IT. In India, few systematic studies demonstrate effectiveness of IT with single allergen extract [5, 19, 20]. Immunotherapy is also prescribed with 2–5 allergen extracts [21, 22], but not many studies have proven its effectiveness. The present study was aimed to evaluate the benefits of IT with mixed and single insect allergen vaccine in the patients of asthma and rhinitis.

Methods

Study Design and Subjects The design of the study was double-blind and placebo-controlled. Two hundred sixteen patients were screened by history, skin test, and specific IgE estimation for diagnosis of allergy at outpatient department, V.P. Chest Institute, Delhi during the year 2004–2006. One hundred fifty patients with a history of exposure to insects (housefly, mosquito, cockroach, moth, and rice weevil) marked positive skin reactions with 1:500 w/v concentration, and raised specific IgE levels to insects were considered for the study. Of these, 99 patients who consented to participate in this study were allocated into two groups. One group ($n=61$) received the active IT with allergen vaccine and the other received placebo injections subcutaneously for 1 year ($n=38$). The patients in active IT group received pharmacotherapy also, whereas placebo group patients were treated with pharmacotherapy alone. Active IT group was further subdivided into three subgroups: (1) IT with one insect extract, i.e., either cockroach or housefly or mosquito ($n=12$ for each extract), (2) IT with any two insect extracts ($n=12$), and (3) IT with three insect extracts ($n=13$; Fig. 1).

This study was approved by the Ethics Committee of the Vallabhbhai Patel Chest Institute, Delhi and written consent from subjects was obtained (in Hindi and English) for inclusion in the study.

Inclusion Criteria Patients of both sexes aged 15 to 45 years were included in the present study. The presence of airway obstruction and >12% reversibility with 200 ml increase in Forced expiratory volume in 1 s (FEV1) volume after 200 µg of inhaled salbutamol was considered to assign asthma as per American Thoracic Society guidelines [23]. The diagnosis of rhinitis was assigned to patients having

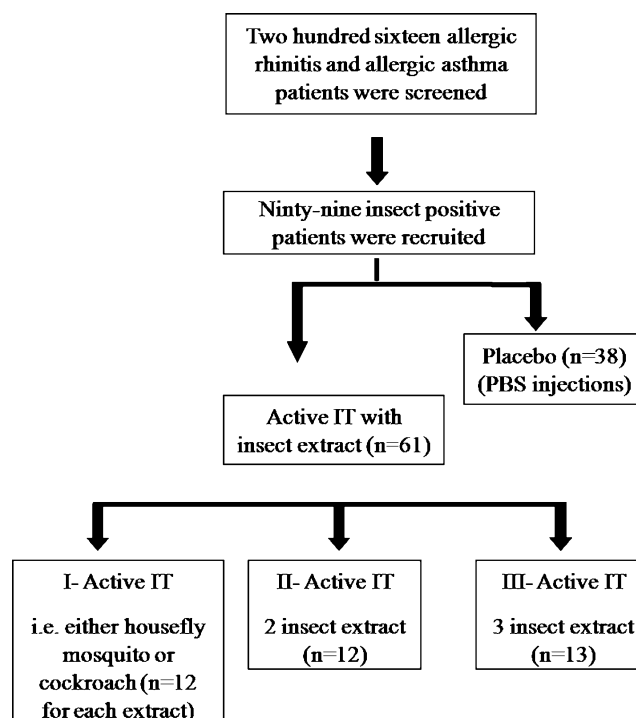


Fig. 1 Schematic representation of the number of patients who participated in the study

two or more of the symptoms—(1) post nasal drip, (2) watery nasal discharge, (3) sneezing, (4) itching of nose, and (5) nasal congestion, most of the times, for the last 2 years' period [24].

Exclusion Criteria Patients with associated cardiopulmonary disorders, infectious diseases, other respiratory or systemic diseases, steroid-dependent, noncooperative, pregnant or lactating females, and smokers were not included in the present study. The exclusion criteria those outlined in the WHO position paper on allergen IT [25] were also taken into consideration, and the patients who were on IT before the start of the study were excluded.

Allergen Extract The insects (cockroach, mosquito, and housefly) included in the study were freeze-dried, ground to a fine powder, and defatted with diethyl ether at 4°C. The antigens were extracted 1:50 (w/v) in 0.1 M phosphate-buffered saline (PBS), pH 7.2 containing 5 mM EDTA, and 1 mM phenyl methyl sulfonyl fluoride, by continuous stirring for 8 h at 4°C [26]. The supernatant collected after centrifugation at 13,000 rpm for 1 h at 4°C was dialyzed against distilled water. The extracts were lyophilized, stored at -20°C, and their protein concentration determined by modified Lowry's method. Reconstituted extract was filtered through 0.22 µm membrane and dispensed in 5-ml aliquots in sterile vials. For skin testing and IT, the extracts were prepared and standardized using protocols of

antigen laboratory, Institute of Genomics and Integrative Biology, Delhi, approved by drug controller of India.

Skin Test Intradermal skin testing was performed on the flexure aspect of the forearm in duplicate before the start and after completion of SIT. For intradermal (ID) tests, 0.02 mL of each insect extract (1:500 w/v) was injected raising a bleb of 2–3 mm, and reactions were graded after 20 min on the basis of wheal size of positive control, i.e., histamine diphosphate (100 µg/mL). The reactions with wheal size equal to the size of positive control were marked as 2+. Skin reactions greater than positive control with two pseudopodia were graded as 3+ and with multiple pseudopodia as 4+ [27]. Skin tests were also carried out with pollen and fungal aeroallergens to rule out other associated sensitivities in subjects. Blood was collected from patients showing marked positive skin reactions (wheal size equal to positive control and or more), sera separated and stored at -70°C for immunoassays. Blood was also collected from 10 non-allergic volunteers (negative ID tests) to serve as normal control.

Symptom/Drug Score Day-to-day symptoms for the entire study were recorded on a diary card provided to patients. It was collected and assessed every 3 months. Symptoms were recorded on a scale from 0 to 4 (0 = no problem; 1 = morning/night; 2 = evening; 3 = daytime; 4 = most of the time) for nasal (sneeze, blockage, itching, and running nose) and bronchial symptoms (breathlessness, wheeze, chest tightness, coughing). The daily symptom score was calculated as the sum of all individual symptom scores. The patients were instructed to record the use of drugs for symptom treatment, and a specific score was given in relation to the class of drug. Drugs were scored as follows: 1 = Inhaled short acting β agonist (SABA)/Inhaled corticosteroid/Inhaled steroid nasal spray/oral antihistamines, 2 = Oral SABA/ Ipratropium/oral theophylline/oral steroid, 3 = LABA + inhaled steroid/high dose oral SABA, 4 = Injectible steroid/injectible theophylline/nebulization with LABA/antibiotics, 5 = hospitalization. The patients were evaluated weekly (at the buildup phase) and at every 3 months for 1 year (when maintenance dose was reached). After 1 year completion of IT, a comprehensive evaluation of the patients was done, and the medication was adjusted individually. Scores at 3-month preceding immunotherapy was taken as T0 value for both symptom and drug score. Patients with missing data were considered noncompliant and were dropped from the study.

Airway Reactivity It was assessed at baseline and after 1 year, according to American Thoracic Society guidelines [23] using histamine diphosphate. In brief, spirometric analysis was done to obtain FEV1 value of patients. Airway

reactivity was assessed following 2 min tidal breathing protocol where aerosolized histamine in PBS was administered in increasing 2-fold concentrations from 0.004 to 1.024 mg/mL. FEV1 was measured after 30 and 90 s and highest reading was recorded. The challenge test was stopped immediately when FEV1, decreased by 20% or more. Finally, patients were allowed to recover up to baseline value. The patients were kept under observation and checked regularly for at least 2 h. They were allowed to leave the clinic when their condition was normal. The results for each group were expressed as PC₂₀ FEV1, the concentration of histamine that produced a 20% fall in FEV1 and its increase from baseline to the end.

Serological Analysis Allergen-specific IgE and IgG4 levels were measured in serum by ELISA [28]. Briefly, microtiter plates (Maxisorp; Nunc™ Immunomodule, Roskilde, Denmark) were coated overnight with respective antigens at a concentration of 2 µg/100 µl in carbonate buffer (pH 9.6) at 4°C. Thereafter, the plates were blocked with 3% defatted milk in PBS (pH 7.2) at 37°C for 3 h. Individual patient serum samples were added in 1:10 dilutions for IgE/IgG4 after washing and incubated overnight at 4°C. The plates were incubated for 3 h with anti-human IgE-horse radish peroxidase (1:1,000 v/v; Sigma Chemical Co., USA) in PBS followed by washing. IgG4 plate was incubated for 2 h with biotinylated anti-human IgG4 (1:1,000 v/v BD Pharmingen, USA), washed, and incubated with streptavidin-horseradish peroxidase conjugate (1:1,000 v/v BD Pharmingen, USA) for 45 min. The plates were analyzed in ELISA reader (Benchmark Plus micro plate, Hercules, CA, USA) at 492 nm.

Immunotherapy IT was administered by a treatment team, which was also responsible for observation and treatment of adverse reactions. For mixed allergen IT, allergen extracts were mixed according to grading of skin reaction. Three consecutive numbered vials of allergen extract containing 10-fold increasing concentrations of allergen (0.03, 0.3, and 3 mg/mL) were given to the patients [5]. Briefly, treatment initiated with 0.03 mg/mL allergen extract concentration (or 0.003 mg/mL allergen extract concentration in patients with high skin reactivity) starting from 0.1, 0.2, up to 0.9 mL two times in a week. Highest allergen concentration 3 mg/mL was taken weekly from 0.1–0.5 mL for 5 weeks and further, 0.5 mL weekly was continued for 4 weeks. Same concentration (3 mg/mL) was used in subsequent vials with a slow increase from 0.6 to 1.0 mL. Next vials were prescribed as one injection in 2 weeks, then one injection in 3 weeks, and finally one injection in 1 month. Once the maintenance dose is achieved, it continued once a month. The first injection was given in the clinic under the supervision, and the patients were monitored for 1–2 h. In

case of systemic or a local reaction, the dose increment was stopped and the next prescribed dose was the second last dose, i.e., the dose given before the reaction.

Safety Side reactions were recorded and classified as local and systemic. Local and systemic reactions were further categorized to immediate and late. Immediate reactions appeared within 30 min. If immediate local reactions exceeded 5–10 mm in size, they were regarded by many clinicians as a warning sign of impending immediate systemic reactions. Late reactions began to develop 4–6 h after initial type-I reaction and persisted for 1–2 days. Systemic reactions were the symptoms elicited by immunotherapy and were categorized into (1) focal symptoms, e.g., mild rhinitis and mild asthma; (2) urticaria, itching or flushing, itching of the throat, and angio-edema; and (3) multiple symptoms terminating in anaphylactic shock [29].

Statistical Methods Statistical analysis was performed using GRAPH PAD version 5 software. ANOVA (nonparametric) was used for group comparisons. Among nonparametric tests Kruskal–Wallis was used. The Spearman correlation test was used for correlation analysis. Wilcoxon test taking log-transformed values was performed for airway reactivity. P value ≤ 0.05 was considered significant.

Results

Skin Reactivity and Specific IgE Out of 99 patients, 85 completed 1 year of IT. All these patients were sensitized to insect extracts as revealed by marked positive skin tests and elevated specific IgE by ELISA. Eight (8/61) patients dropped out of the active group (two because of adverse events in family and six were noncompliant) and 6/38 of patients from placebo group (because of personal reasons). Intradermal testing on patients with insect extracts carried out after 1 year of treatment in mix as well as single allergen active IT groups showed reduction in skin reactivity compared to their baseline values. In mixed allergen IT group, 65% patients showed reduction in IgE levels whereas 70% of patients in single allergen active IT group showed reduction in IgE levels. In placebo group, six of the 32 (19%) patients showed increased sensitization and increased specific IgE levels; five showed decreased skin reactivity and decrease in specific IgE levels (15% of patients); however, 21 showed no change in skin reactions or specific IgE than basal level. After 12 months of IT, intergroup comparison of active IT groups and placebo group showed significant reduction in wheal size ($p < 0.05$); however, intragroup comparison of the active groups (mixed vs. single IT) did not show much difference ($p > 0.05$, Fig. 2). None of the patients in active IT groups

showed increased sensitization to insect extracts unlike placebo group.

Symptom/Drug Score The mean values for symptom score of the patients in active IT and placebo group was 237 and 234, respectively, at baseline. After 1 year, the mean values in active IT and placebo group was 132 and 210, respectively. There was improvement in symptom score in 16/25 (64%) patients receiving mix allergen active IT. In the single extract active IT group, 29/36 (80%) patients showed reduction in symptom score. Two patients in mix allergen IT and five in single allergen IT group had no symptoms of asthma or rhinitis after 1 year of treatment. While in placebo group, 17/32 (53%) patients showed some reduction in symptom score. After 12 months of IT, intergroup comparison of active IT groups with placebo group showed significant reduction in symptom score ($p < 0.05$) but intragroup comparison of the active groups (mixed vs. single IT) did not show significant difference ($p > 0.05$, Fig. 3).

Medication or drug score was not significantly different between the groups at the start of the study. A change of 10–15% constituted reduction in drug use. After 1 year of IT, 13/25 (52%) patients in mix and 25/36 (70%) patients in single allergen active IT group demonstrated reduction in drug use; however, it was not statistically significant ($p > 0.05$). While in placebo group drug reduction was seen in 20% of patients.

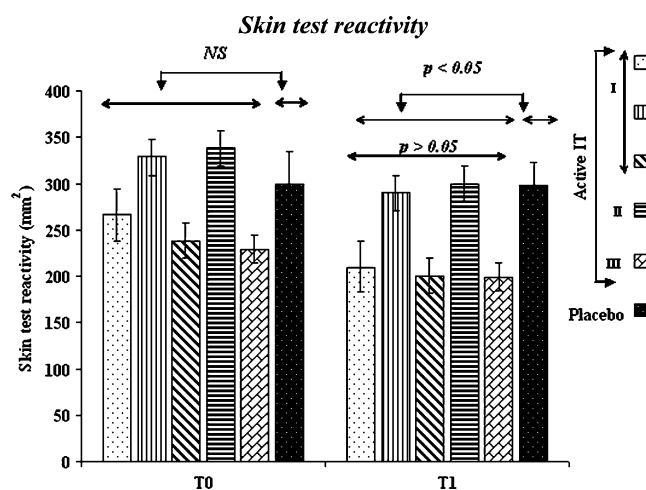


Fig. 2 Skin test reactivity (average wheal size expressed in $\text{mm}^2 \pm \text{SEM}$) of the active IT group (I, II, III subgroups) and placebo group patients at “T0” (baseline, i.e., before IT) and “T1” (after 1 year of IT). T time, I subgroup of patients getting IT with single insect extract, II subgroup of patients getting IT with two insect extracts—represent average of skin reactivity of two insects, III subgroup of patients getting IT with three insect extracts—represents average of skin reactivity of three insects. After 1 year of IT, statistically significant difference was observed between active IT and placebo group patients ($p = 0.01$, Kruskal–Wallis; nonparametric ANOVA) and no significant difference was recorded between active IT subgroups ($p = 0.9813$, Kruskal–Wallis; nonparametric ANOVA), NS not significant

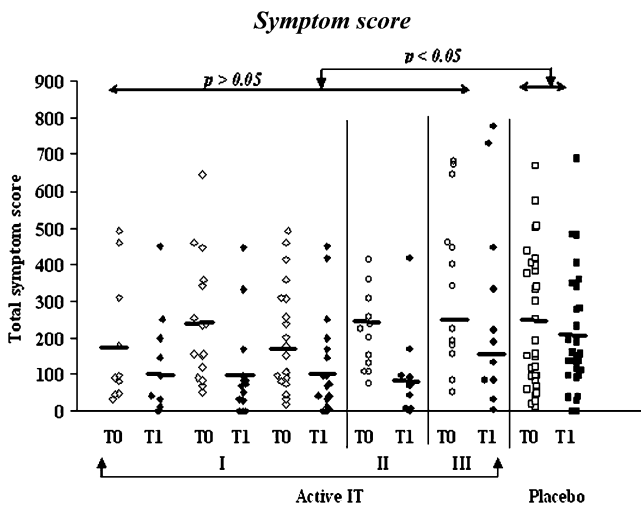


Fig. 3 Total symptom score of the active group (I, II, III subgroups) and placebo group patients at “T0” (baseline, i.e., before IT) and “T1” (after 1 year of IT); T time, I subgroup of patients getting IT with single insect extract, II subgroup of patients getting IT with two insect extracts, III subgroup of patients getting IT with three insect extracts. After 1 year of IT, statistically significant difference was observed between active IT and placebo group patients ($p=0.04$, Kruskal–Wallis; nonparametric ANOVA) and no significant difference was recorded between active IT subgroups ($p=0.2737$, Kruskal–Wallis; nonparametric ANOVA). Bars represent average values for each of the groups and subgroups

Airway Reactivity In the active IT groups, 15/25 (60%) patients in mixed insect extract and 25/36 (70%) patients in single insect extract IT showed significant increase in PC₂₀ FEV1 values compared to baseline values. In the placebo

group, 25/32 (78%) patients showed decrease in PC₂₀ FEV1 values. There was significant increase in PC₂₀ FEV1 value in active allergen IT group compared to placebo group ($p < 0.05$), but the subgroups in the active IT group did not show significant difference ($p > 0.05$, Fig. 4A). Out of the total 53 patients in active IT groups, 25 required more than twice the amount of histamine than baseline to reach 20% fall in FEV1 and approximately 25% of the patients tolerated highest dose of histamine on challenge and showed no fall in FEV1 values. The results were also expressed as the percentage of patients who showed improvement showed no change and/or deteriorated (Fig. 4B).

IgE and IgG4 After 1 year, there was a reduction of specific IgE levels ($p=0.02$) in active IT compared to placebo group while there was no significant difference within the subgroups ($p > 0.05$, Fig. 5). Significant increase in specific IgG4 levels ($p < 0.05$) were observed in mix as well as single allergen active IT group patients compared to placebo group (Fig. 6), but no significant difference was observed within the subgroups ($p > 0.05$). IgG4/IgG1 ratio correlated well with symptom score in mix as well as single allergen active IT groups (Fig. 7).

Treatment-Related Adverse Events

Local reactions were observed with higher doses or concentration of antigen in 12/61 (20%) active allergen IT

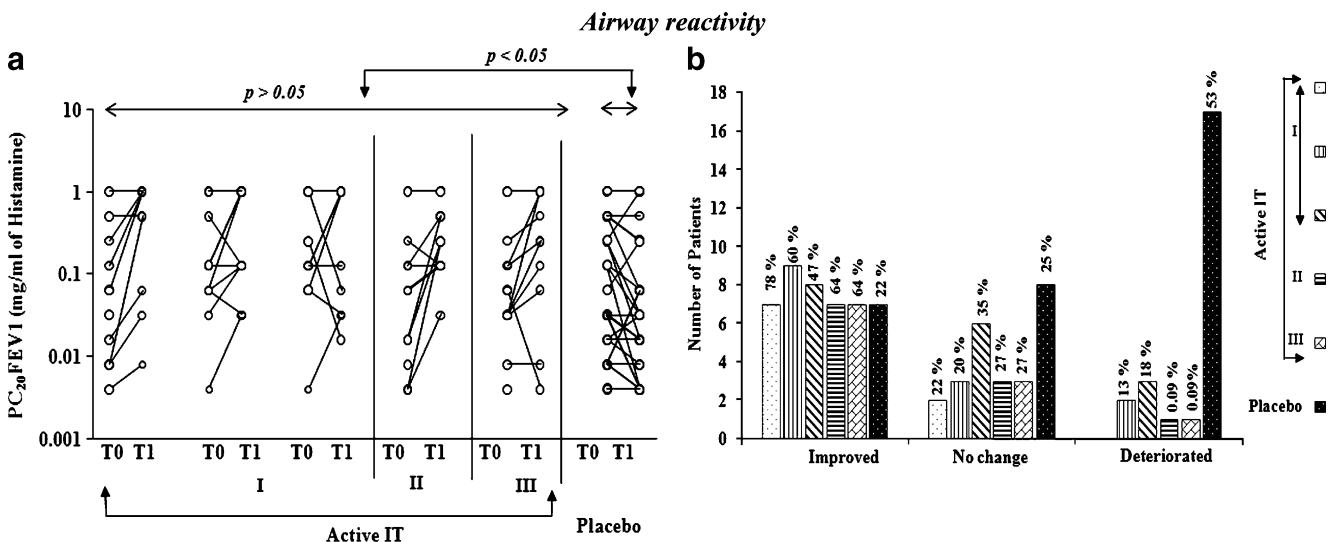


Fig. 4 **A** Airway reactivity (PC₂₀FEV1) of the active group (I, II, III subgroups) and placebo group patients at T0 (baseline, i.e., before IT) and T1 (after 1 year of IT). T time, I subgroup of patients getting IT with single insect extract, II subgroup of patients getting IT with two insect extracts, III subgroup of patients getting IT with three insect extracts. After 1 year of IT, statistically significant difference was observed between active IT and placebo group patients ($p=0.01$,

Kruskal–Wallis; nonparametric ANOVA) and no significant difference was recorded between active IT subgroups ($p=0.9813$, Kruskal–Wallis; nonparametric ANOVA). **B** Analysis of BHR in terms of reduction (improvement), no change, and increase in BHR (deterioration). I subgroup of patients getting IT with single insect extract, II subgroup of patients getting IT with two insect extracts, III subgroup of patients getting IT with three insect extracts

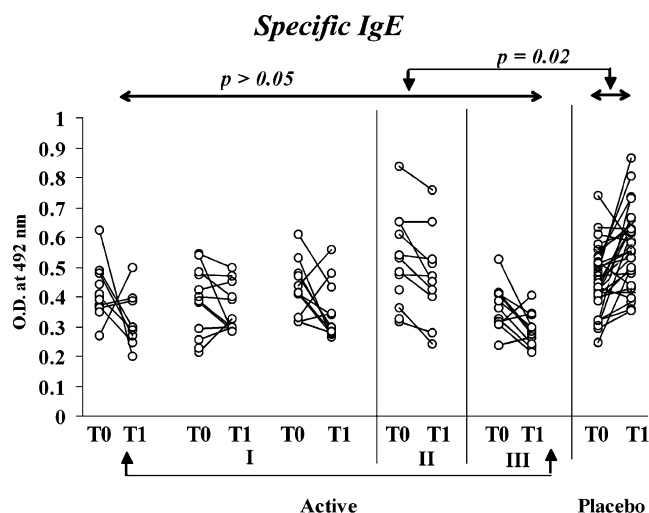


Fig. 5 Analysis of specific IgE in active IT group (three subgroups) and placebo group patients at T_0 (baseline, i.e., before IT) and T_1 (after 1 year of IT); T time, I subgroup of patients getting IT with single insect extract, II subgroup of patients getting IT with two insect extracts—represents average of specific IgE values against two insects, III subgroup of patients getting IT with three insect extracts—represents average of specific IgE values against three insects. After 1 year of IT, statistically significant difference was observed between active IT and placebo group patients ($p=0.0001$, Kruskal–Wallis; nonparametric ANOVA) and no significant difference was recorded between active IT subgroups ($p=0.1706$, Kruskal–Wallis; nonparametric ANOVA)

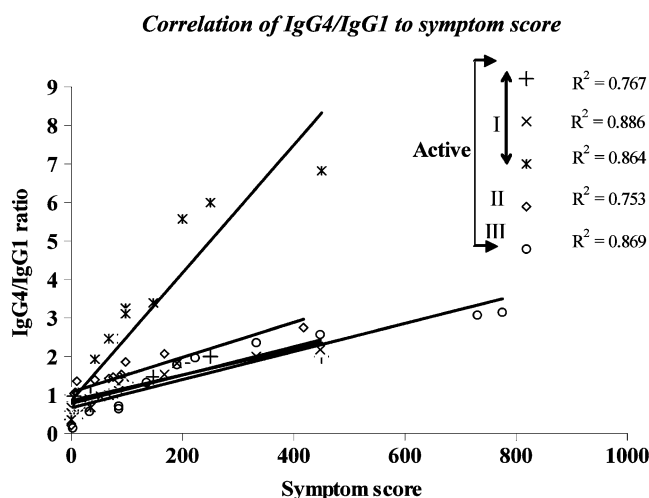


Fig. 7 Correlation of IgG4/IgG1 ratio with symptom scores in active IT group (three subgroups); I subgroup of patients getting IT with single insect extract, II subgroup of patients getting IT with two insect extracts, III subgroup of patients getting IT with three insect extracts

group patients. Local reactions were more frequently recorded during the buildup phase and the first maintenance week. These reactions were managed by decreasing the dose or concentration and use of appropriate medication. Apparent systemic reactions (SRs) to injections occurred in three of the 61 patients in the active IT group (5%). The SRs occurred during the buildup phase consisted of skin reaction (itching) in one and digestive symptoms (diarrhea) in two patients. All the SRs were of mild intensity and subsided itself or with oral antihistamines, antacids, and/or omeprazol.

Discussion

Allergen vaccines from both mixed and single allergen source have been recommended for IT with defined potency, composition, stability, and have shown beneficial effects in asthma or allergic patients [25]. Studies on multi-allergen IT are rare, and a few studies have demonstrated that immunotherapy containing higher doses of mix allergens is effective [30–32]. But a study in children did not document efficacy, indicating that the concept of mixing allergens may not prove useful [22]. Some of the reasons which can be attributed to the failure of immunotherapy are inadequate dose of allergen in the allergy vaccine, missing allergens not identified during the allergy evaluation, high levels of allergen in environment (i.e., inadequate environmental control), or significant exposure to non-allergic triggers (i.e., tobacco smoke). The present study was undertaken to assess the efficacy of IT with mixed and single insect allergen extracts in the patients of asthma and rhinitis.

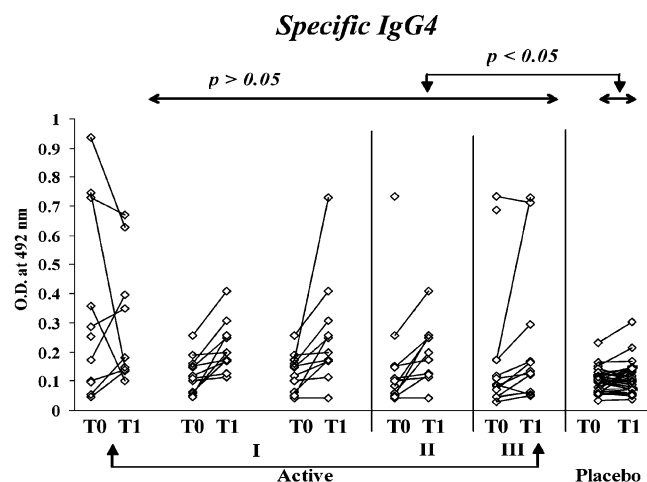


Fig. 6 Analysis of specific IgG4 in active IT group (three subgroups) and placebo group patients at T_0 (baseline, i.e., before IT) and T_1 (after 1 year of IT); T time, I subgroup of patients getting IT with single insect extract, II subgroup of patients getting IT with two insect extracts—represents average of specific IgG4 values against two insects, III subgroup of patients getting IT with three insect extracts—represents average of specific IgG4 values against three insects. After 1 year of IT, statistically significant difference was observed between active IT and placebo group patients ($p=0.0001$, Kruskal–Wallis; nonparametric ANOVA) and no significant difference was recorded between active IT subgroups ($p=0.1706$, Kruskal–Wallis; nonparametric ANOVA)

Changes in skin-test reactivity have been a controversial aspect in allergen specific IT. Studies demonstrated decrease in wheal size after 1–2 years of allergen IT while some of them showed no change in skin reactivity [33]. In the present study, skin-test reactivity decreased after 1 year in both mixed and single allergen IT groups. However, it did not show significant difference between the active IT subgroups ($p=0.3607$). There was a significant reduction in skin reactivity in active IT group compared to placebo group ($p=0.0008$). Approximately 25/53 (48%) of patients in the active groups showed $\geq 50\%$ reduction in skin reactivity than baseline.

Cochrane review of 54 specific immunotherapy randomized controlled trials confirmed the efficacy of immunotherapy by emphasizing the clinically useful outcomes of decreased symptom scores, medication requirements, improved allergen-specific, and nonspecific airway hyper-responsiveness [34]. Symptom and drug score self-evaluated by patients then combined with physicians' evaluation is considered to be the most convenient way of assessment [25]. In the present study, symptoms improved significantly in the active IT group patients as compared to placebo group ($p=0.04$). But the difference in the mixed and single allergen IT subgroups was not significant ($p=0.2737$). Few patients (7/53) in both mixed as well as single IT group showed no symptoms after 1 year of IT. Most of the patients in placebo group showed exacerbations in both symptom and drug score.

Airway reactivity ($PC_{20} FEV_1$ values) is an important parameter to assess the outcome of immunotherapy trials [34]. A mite immunotherapy trial considered reduction in bronchial hyper-reactivity (BHR) as the main outcome to document clinical efficacy [33]. In the present study, active IT-group patients showed significant reduction in BHR as compared to placebo group ($p=0.01$). But there was no significant difference ($p=0.9813$) in mixed and single allergen IT subgroups. A comparative study of inhaled corticosteroids (ICS) and IT demonstrated that on cessation the benefits declined faster for ICS group while it lasted longer in IT group [20]. Immunotherapy prevents the worsening of the specific bronchial hyper-reactivity which is observed in the placebo group; hence, the early use of immunotherapy in correctly selected patients should be considered.

Allergen-specific IgE increases frequently during the first few months of immunotherapy and shows a slow decline on long-term treatment [35, 36]. The immunotherapy-induced early increase in specific IgE is nonpathogenic and may be a result of a reduced affinity to the Fc receptor or to a switch from IgE^+ to IgE^- [37]. Durham showed modest reduction in allergen-specific IgE levels and induction of allergen-specific IgG subclasses by IT [38]. In the present study, moderate downward trend in specific IgE was seen after 1 year in

active IT groups, which was not significant. While an increase in IgG4 antibodies was observed in mixed and single IT groups. IgG has been proposed as blocking antibody and IgE compete with IgG for allergen binding to mast cells, basophils, and other IgE receptor-expressing cells [17].

IT has been shown to modulate allergen-specific antibody responses with a decrease in the IgE/IgG4 ratio leading to reduction in the recruitment and activation of pro-inflammatory cells in target mucosa. The prolonged IT results in a shift from IgG1 to IgG4 syntheses. Furthermore, positive correlation has been recorded in specific IgG4 levels with clinical improvement [39]. In the present study, significant improvement was observed in the active IT-group patients, with decrease in IgE/IgG4 ratio. IgG4 showed significant increase in active IT subgroups as compared to placebo group ($p<0.05$) and clinical improvement correlated with increase in IgG4.

Stability of antigens is an important concern which needs to be addressed properly during extraction, storage, transport, and mixing of allergen vaccines. The extract of fungi, insects, and house dust mite contain proteases that can cleave self or other extracts' proteins in vaccines [40–42]. Studies suggest that potency of timothy grass, rye grass, white oak, box alder, Russian thistle, and rye grass reduced substantially when mixed with *P. americana* and or *Helminthosporium interseminatum* extracts rich in proteases [43, 44]. Hence, it has been suggested to avoid mixing of unrelated extracts in vaccines. In the present study, mix allergen vaccine has been prepared by mixing two or three insect extracts, where cockroach (*P. americana*) is known to contain proteases [45]. The effect of cockroach proteases can be overcome by adding 0.25 M ϵ -amino caproic acid or 0.25 M sucrose or combination of both in extract [26]. However, this requires detailed study with mixed allergen preparations and subsequent permission from drug regulatory authorities.

In conclusion, IT with mixed and/or single insect allergen [2, 3] for 1 year demonstrated significant improvement in clinical (skin reactivity, airway reactivity, and symptom score) and immunological (IgE/IgG4 and IgG1/IgG4 ratio) parameters.

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