

Clinico-immunological changes postimmunotherapy with *Periplaneta americana*

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ABSTRACT

Background Cockroach proteins induce allergies including asthma in predisposed individuals. Well-designed controlled studies are required to show the effect of cockroach immunotherapy (IT). This study is aimed to assess changes in clinical and immunological parameters post-IT with *Periplaneta americana* extract.

Materials and methods A double-blind, placebo-controlled trial of cockroach IT was performed for 1 year in 50 patients of asthma, rhinitis or both. The efficacy of IT was assessed by change in skin reactivity and clinical parameters such as symptom/drug score, airway reactivity and immunological parameters namely IgE, IgG1 and IgG4, IL-4 and IFN- γ by enzyme-linked immunosorbent assay and western blotting using patients' sera at baseline and after 1 year of treatment.

Results Immunotherapy with cockroach extract demonstrated significant improvement in clinical parameters of active group patients compared with baseline values and placebo group. Specific IgE levels showed a modest reduction, while IgG4 levels increased significantly in active IT group after 1 year. IgE immunoblotting demonstrated reduction in intensity and number of specific bands, whereas IgG4 binding showed more number and distinct bands following IT. Active group patients showed correlation between increase in IgG4/IgG1 ratio and reduction in symptom score post-IT.

Conclusions Immunotherapy with cockroach extract improved clinical and immunological status of asthma and rhinitis patients. Clinical improvement in patients after IT is associated with immunological changes.

Keywords Asthma, ELISA, immunoblot, immunotherapy, *Periplaneta americana*, rhinitis.

Eur J Clin Invest 2011

Introduction

Cockroaches are important allergen sources in most countries especially in the tropics. German cockroach (*Blattella germanica*) and American cockroach (*Periplaneta americana*) are the principal domiciliary species abundant in warm and humid tropical areas. The ubiquitous existence of cockroaches makes them a significant indoor allergen and a risk factor for asthma among inner-city residents [1,2]. Previous studies from India showed cockroach sensitization in 8–26% of allergic patients [3,4] but recent report shows it to be 53% based on skin tests [5].

Pharmacotherapy has improved in controlling symptoms but has side effects on long-term use [6]. Immunotherapy (IT) by repeated subcutaneous injections was demonstrated as an effective method for allergy treatment [7]. IT restricts seasonal increase in bronchial hyper-responsiveness and prevents onset of new sensitizations [8]. Immunological events in IT include

reduction in specific IgE antibodies and induction of IgG (blocking) antibodies and suppressor T cells [9]. Several well-designed placebo-controlled studies with mites, animal allergens and pollens have demonstrated therapeutic benefits of specific IT [10], but the indications in patients with asthma remain controversial [11,12]. Previous studies on cockroach IT reported beneficial changes in immunological and clinical parameters [13,14].

Immunological changes have been observed in IT studies but there is a need to study the changes in the sensitization profile against major and minor allergens also post-IT. It might have a better therapeutic potential to direct immune system to specific components. Therefore, this study was aimed to monitor immunological changes (IgE, IgG1, IgG4, IL-4 and IFN- γ response) post-IT with *P. americana* extract by enzyme-linked

immunosorbent assay (ELISA) and immunoblotting with patients' sera of allergic rhinitis and asthma. The effect of cockroach IT was also assessed by skin test reactivity, symptom/drug score and airway reactivity in patients.

Methods

Study design and subjects

This is a double-blind, placebo-controlled study of cockroach IT approved by the Human ethics committee of the V. P. Chest Institute, Delhi, and the written consent from subjects was obtained for participation in the study. The patients were included in this study following WHO inclusion and exclusion criteria [15]. The diagnosis of asthma and/or rhinitis was confirmed following ATS and ARIA guidelines [16,17]. One hundred and twenty patients with allergic asthma and rhinitis were screened by history, skin test and specific IgE estimation for diagnosis of cockroach allergy. Fifty cockroach (*P. americana*)-sensitized patients were included and allocated into two groups (one group received antigen treatment, i.e. active IT, and the other placebo injections of phosphate-buffered saline) as well as pharmacotherapy for 2 years. Clinical and immunological status of the active and placebo group patients was analysed after completion of 12 and 24 months of treatment.

Both groups received pharmacologic treatment consisting of antihistamines, loratadine, salbutamol, and topical (nasal and bronchial) glucocorticosteroids (budesonide), injectable steroid and nebulization with long-acting β -agonist/Antibiotics as per requirement. All patients were evaluated weekly at the build-up phase and every 3 month, once maintenance dose was reached. After 1 year of IT, patients were evaluated and medication was adjusted on individual patient basis.

Allergen extracts. The antigens were extracted from cockroach powder as described previously [5]. The extract was lyophilized, stored at -20°C and the protein concentration determined by modified Lowry's method. For skin testing and IT, the extract was prepared using good manufacturing practice followed at antigen laboratory, Institute of Genomics and Integrative Biology, Delhi, approved by drug controller of India for supply of diagnostic and therapeutic extracts. The cockroach

extract was standardized by skin test, ELISA and immunoblot using patients' sera. The nine major allergens of 23, 28, 35, 38, 40, 49, 72, 78 and 97 kDa were demonstrated in cockroach extract similar to our previous studies [5,18].

Skin tests. For intradermal tests, 0.02 mL of the allergen extract (1 : 500 *w/v*) was injected into the forearm of the patient raising a bleb of 2–3 mm and reactions were graded after 20 min [18]. Histamine diphosphate (100 $\mu\text{g mL}^{-1}$) was used as positive control to grade the skin reactions. Skin tests were repeated with a lower concentration (1 : 5000 *w/v*) for establishing sensitization against *P. americana*. Blood was collected from patients showing marked positive skin reactions for immunological assays.

Treatment schedule. The active IT treatment consisted of subcutaneous administration of an aqueous solution of standardized cockroach extract. The qualitative and quantitative composition of the placebo was identical to the experimental product but without allergen extract. Three consecutively numbered vials containing 0.03, 0.3 and 3 mg mL^{-1} of allergen (protein) concentrations were given to the patients according to schedule described in Table 1.

Symptom/drug score. Day-to-day symptoms related to lower (bronchial) and upper respiratory tract (nasal) were recorded on a diary chart provided to patient(s) and assessed every 3 months for 2 years [19]. Score at 3 month preceding IT was taken as ' T_0 ' value for both symptom and drug score. The patients were allowed to take anti-allergic/anti-asthmatic drugs and instructed to record the use of drugs during the study. A specific score was given in relation to the class of drug [19]. Patients who failed to appear twice or more at the scheduled call for monitoring and evaluation were considered noncompliant and were dropped from the study.

Airway reactivity. It was assessed following 2-min tidal breathing protocol, where aerosolized histamine in PBS was administered in increasing twofold concentrations from 0.004 to 1.024 mg mL^{-1} at baseline and after 1 year of IT [20]. The results were expressed as $\text{PC}_{20}\text{FEV}_1$, the concentration of histamine that produced a 20% fall in FEV1.

Table 1 Immunotherapy schedule for the active group patients

	Vial 1	Vial 2	Vial 3						
Conc. (mg mL^{-1})	0.03	0.3	3	3	3	3	3	3	3
Doses (in mL)	0.1–0.9	0.1–0.9	0.1–0.4	0.5	0.6	0.7	0.8	0.9	1
Dose frequency	Twice a week	Twice a week	Once a week	Once a week (8 weeks)	Once in 2 week (16 weeks)	Once in 3 week (21 weeks)	Once in 4 week (24 weeks)	Once in 4 week (24 weeks)	Once in 4 week (24 weeks)

Serological analysis. Specific IgE, IgG1 and IgG4 antibodies were determined in patients' sera against cockroach extract using ELISA at baseline, after 1 and 2 years of IT [18,21]. To rule out nonspecific binding, patients' sera were checked by ELISA with an irrelevant pollen allergen *Prosopis juliflora*.

Cytokine analysis in PBMC's cell culture supernatant. Lymphoproliferation assay was carried out as described elsewhere [18]. Briefly, peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood using Ficoll Hypaque (Sigma, St. Louis, MO, USA). For assay, 10^5 cells mL^{-1} were cultured in RPMI-1640 medium (supplemented with 10% heat-inactivated foetal calf serum, 10 mM HEPES buffer, 2 mM L-glutamine, 100 U mL^{-1} penicillin and 100 $\mu\text{g mL}^{-1}$ streptomycin; Sigma) in a 96-well plate and stimulated with 1 μg protein of cockroach. Phytohemagglutinin (Bangalore Genei, Bangalore, India) was used as positive control, and no stimulant was added to negative control. The culture plates were placed in a CO_2 incubator, and the supernatant was collected for cytokine assay after 72 h. Cytokines IL-4 and IFN- γ (BD Pharmingen, San Diego, CA, USA) were determined in PBMC's culture supernatants by ELISA following manufacturer's instructions [18]. The detection limit for IL-4 and IFN- γ was 7.8 and 15.6 pg mL^{-1} , respectively.

Immunoblotting. SDS-PAGE-separated cockroach proteins were electrophoretically transferred onto nitrocellulose membrane in Tris-glycine buffer containing methanol and probed with individual patients' sera [5]. Briefly, the membrane with cockroach proteins was cut into strips, blocked with 3% defatted milk and incubated overnight at 4 °C with active IT group ($n = 24$) patients' sera (1 : 10 v/v) collected at baseline (T_0) and after 1 year of IT (T_1). The strips were probed with anti-human-IgE peroxidase (Sigma) (1 : 1000 v/v) for 3 h at 37 °C. For IgG4, strips were incubated for 2 h with biotinylated anti-human IgG4 (1 : 500 v/v; BD Pharmingen), washed and probed with streptavidin-horseradish peroxidase (1 : 1000 v/v; BD Pharmingen) for 45 min. Changes in IgE or IgG4 binding against cockroach extract were assessed by visual evaluation of density of bands in immunoblots with sera before and after 1 year of IT.

Adverse reactions. 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, and if they exceeded 5–10 mm in size, they were regarded 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 IT and were categorized into (i) focal symptoms, e.g. mild rhinitis and mild asthma; (ii) urticaria, itching or flushing, itching of the throat and angioe-

dema; and (iii) multiple symptoms leading to anaphylactic shock.

Statistical analysis

Parametric analysis of data was performed using student's two-tailed 't' test for skin tests, symptom/drug score and immunological parameters. Wilcoxon test taking log-transformed values was carried out for airway reactivity. Paired 't' test was used for intragroup (before and after IT of same group) and unpaired 't' test was used for intergroup (active and placebo) comparisons. P value ≤ 0.05 was considered significant.

Results

Of 50 cockroach-sensitized patients, 42 (24 in active and 18 in placebo) completed 1 year of study (Tables 2 and 3). Two patients dropped out of the active group (noncompliant) and six patients from placebo group (personal reasons). Twenty-four active group patients were followed up for next year also but only 12 of them co-operated in the follow-up. The effect of IT was assessed based on changes in skin test reactivity and various clinical and immunological parameters assessed at different time period.

Skin test reactivity

Intradermal testing with cockroach extract carried out after 1 year of IT showed reduction in skin reactivity in 21 of 24 patients of active IT group compared with the baseline values (Mean values at T_0 $297 \pm 59.6 \text{ mm}^2$ and T_1 $138 \pm 116 \text{ mm}^2$; Table 2). The baseline values for wheal size did not differ significantly between active and placebo groups. However, three patients in active IT group showed increased sensitization to cockroach extract after 1 year. In placebo group, 11 of the 18 patients showed increased sensitization and seven showed decrease in skin reactivity (Mean value at T_0 $240 \pm 167 \text{ mm}^2$ and T_1 $222 \pm 162 \text{ mm}^2$; Table 3).

Clinical parameters

Airway reactivity. In active IT group, 18 of 24 patients showed significant increase in $\text{PC}_{20}\text{FEV}_1$ compared with baseline values and placebo group ($P < 0.05$) and 8 of 24 patients tolerated highest dose, i.e. 1.024 mg mL^{-1} of histamine after 1 year of IT. However, in placebo group, 15 of 18 patients showed decrease in $\text{PC}_{20}\text{FEV}_1$ indicating rise in airway reactivity and three showed increase in $\text{PC}_{20}\text{FEV}_1$ values (Fig. 1).

Symptom/drug score. The median values of symptom score at the baseline for active IT and placebo groups were 154 and 157, respectively. After 1 year of IT, the median values for active and placebo groups were 47 and 96, respectively. The

Table 2 Skin test results, specific IgE and IgG4 values of active group patients at T_0 , T_1 and T_2

Patient No.	Sex/age (year)	Clinical history	Intradermal skin test		Specific IgE OD at 492 nm			Specific IgG4 OD at 492 nm		
			T_0	T_1	T_0	T_1	T_2	T_0	T_1	T_2
1	F/18	BA/AR	216	196	0.545	0.410	0.355	0.161	0.210	0.290
2	F/32	BA/AR	315	210	0.540	0.500	0.313	0.119	0.168	0.198
3	M/25	BA/AR	180	210	0.294	0.299	0.237	0.156	0.309	0.480
4	F/39	BA/AR	225	25	0.400	0.390	0.337	0.177	0.248	0.262
5	M/32	AR	168	30	0.216	0.324	0.215	0.183	0.233	0.187
6	M/43	BA/AR	378	576	0.257	0.300	0.222	0.149	0.256	0.300
7	M/22	BA/AR	324	36	0.425	0.450	0.407	0.191	0.220	0.241
8	F/27	BA/AR	121	49	0.384	0.340	0.279	0.151	0.284	0.282
9	M/15	BA	169	64	0.364	0.340	0.572	0.175	0.428	0.750
10	F/18	AR	324	289	0.380	0.287	0.258	0.102	0.215	0.220
11	F/29	BA/AR	270	20	0.483	0.400	0.335	0.141	0.310	0.423
12	M/22	BA	345	195	0.393	0.287	0.259	0.190	0.253	0.331
13	M/35	BA/AR	225	100	0.326	0.228		0.387	0.550	
14	M/21	BA/AR	225	180	0.650	0.343		0.252	0.395	
15	M/15	BA/AR	225	168	0.467	0.470		0.433	0.515	
16	M/19	BA/AR	180	9	0.308	0.212		0.198	0.278	
17	F/27	BA/AR	270	56	0.239	0.267		0.158	0.253	
18	M/29	AR	225	100	0.369	0.316		0.156	0.119	
19	M/23	AR	182	121	0.432	0.312		0.186	0.243	
20	F/22	BA/AR	195	182	0.738	0.680		0.170	0.310	
21	M/21	BA	324	196	0.503	0.530		0.161	0.168	
22	M/19	BA/AR	216	99	0.579	0.620		0.119	0.209	
23	M/28	BA/AR	180	441	0.631	0.629		0.256	0.248	
24	F/19	BA/AR	676	49	0.537	0.732		0.147	0.173	

BA, bronchial asthma; AR, allergic rhinitis; NA, not applicable; OD, optical density; T_0 , baseline [i.e. before immunotherapy (IT)]; T_1 , after 1 year of IT; T_2 , after 2 year of IT.

Mean values for Skin test at $T_0 - 297 \pm 59.6 \text{ mm}^2$ and at $T_1 - 138 \pm 116 \text{ mm}^2$; Specific IgE at $T_0 - 0.435 \pm 0.135$ and $T_1 - 0.402 \pm 0.144$; Specific IgG4 at $T_0 - 0.188 \pm 0.077$ and $T_1 - 0.274 \pm 0.104$.

active IT group patients showed statistically significant reduction in symptoms when compared with baseline or placebo ($P < 0.05$). The change in symptom score after 2 years of IT in active group was highly significant with a P value of 0.0001 compared with baseline (Fig. 2).

Medication/drug score was similar between the active IT and placebo groups at the start of the study. Most of the active group patients showed reduction in drug use after 1 year of IT but it was not statistically significant ($P > 0.05$). However, after 2 years of IT, highly significant reduction in drug score was

observed ($P < 0.0001$) in active IT group. Also few patients (20%) in placebo group showed some reduction in drug use (Data not shown).

Immunological parameters

Immunoglobulins. Serum IgE concentration in the active IT and placebo groups at baseline were (mean value) 0.435 ± 0.135 and 0.457 ± 0.124 , respectively. After 1 year of IT, slight reduction (Mean value 0.402 ± 0.144 ; Table 2) was

Table 3 Skin test results, specific IgE and IgG4 values at T_0 and T_1 of placebo group patients

Patient No.	Sex/age (year)	Clinical history	Intradermal skin test		Specific IgE OD at 492 nm		Specific IgG4 OD at 492 nm	
			T_0	T_1	T_0	T_1	T_0	T_1
1	F/22	BA/AR	157	170	0.483	0.666	0.082	0.129
2	M/18	AR	437	434	0.300	0.456	0.102	0.080
3	F/32	BA/AR	404	398	0.447	0.500	0.234	0.305
4	M/15	BA/AR	340	360	0.559	0.609	0.099	0.070
5	M/22	BA	194	188	0.320	0.380	0.152	0.216
6	M/17	BA/AR	70	159	0.579	0.620	0.121	0.120
7	F/25	BA/AR	50	78	0.503	0.530	0.104	0.099
8	M/42	BA/AR	44	49	0.738	0.580	0.132	0.106
9	F/23	BA/AR	499	506	0.415	0.416	0.106	0.089
10	M/24	BA	504	689	0.515	0.435	0.145	0.111
11	F/23	BA/AR	398	279	0.250	0.350	0.109	0.088
12	M/21	AR	98	134	0.631	0.629	0.117	0.096
13	M/18	BA/AR	250	248	0.403	0.470	0.060	0.133
14	M/27	BA/AR	84	88	0.415	0.450	0.105	0.069
15	F/18	BA/AR	667	483	0.537	0.632	0.270	0.280
16	F/30	AR	574	480	0.430	0.330	0.147	0.195
17	F/28	BA	380	387	0.383	0.468	0.159	0.159
18	M/29	BA/AR	157	159	0.320	0.433	0.150	0.179

BA, bronchial asthma; AR, allergic rhinitis; NA, not applicable; OD, optical density; T_0 , baseline [i.e. before immunotherapy (IT)]; T_1 , after 1 year of IT. Mean values for Skin test at $T_0 - 240 \pm 167 \text{ mm}^2$ and at $T_1 - 222 \pm 162 \text{ mm}^2$; Specific IgE at $T_0 - 0.457 \pm 0.124$ and $T_1 - 0.497 \pm 0.103$; Specific IgG4 at $T_0 - 0.133 \pm 0.050$ and $T_1 - 0.140 \pm 0.069$.

observed in specific IgE levels in active group patients when compared with baseline. However, placebo group patients did not show reduction in specific IgE levels post-IT (Mean value 0.497 ± 0.103 ; Table 3). Specific IgG4 levels increased significantly in the group receiving allergen IT (Mean value at $T_0 - 0.188 \pm 0.077$, $T_1 - 0.274 \pm 0.104$; $P < 0.01$; Table 2), but there was not much change in the placebo group (Mean value at $T_0 - 0.133 \pm 0.050$, $T_1 - 0.140 \pm 0.069$; Table 3). There has been a significant reduction in specific IgE (Mean value 0.315 ± 0.099 ; $P = 0.009$) and increase in IgG4 (Mean value 0.330 ± 0.158 ; $P = 0.002$) levels compared with baseline after 2 years of active IT (Table 2).

IgG1 value demonstrated some increase from the baseline (Mean at $T_0 - 0.200$ and $T_1 - 0.260$). IgG4/IgG1 ratio was calculated and it showed increase post 1 year of IT in active IT group (Mean at $T_0 - 0.503$ and $T_1 - 1.153$). The graph was plotted by taking IgG4/IgG1 ratio at T_1 on one axis and change in symptom score, i.e. $T_0 - T_1$, on another axis (Fig. 3). Correlation

($r^2 = 0.8865$) was observed between reduction in symptom score and increase in IgG4/IgG1 ratio in the active IT group.

Cytokines. Cytokine IL-4 decreased after 1 year of active IT (Mean at $T_0 - 99 \pm 0.095 \text{ pg mL}^{-1}$ and $T_1 - 72 \pm 0.110 \text{ pg mL}^{-1}$; $P > 0.05$), while IFN- γ did not show much change compared with baseline and placebo (Data not shown).

Immunoblotting

Changes in IgE binding against cockroach allergens. Previously, proteins of 23, 28, 35, 38, 40, 49, 72, 78 and 97 kDa were identified as major allergens of *P. americana* [5]. In the present study, IgE blots showed apparent decline in both intensity and number of bands following 1 year of IT with cockroach extract than baseline. Before the start of IT, cockroach-sensitive patients' sera showed several IgE binding components with MW 23-, 28-, 35-, 38-, 40-, 49-, 72-, 78- and 97-kDa regions on

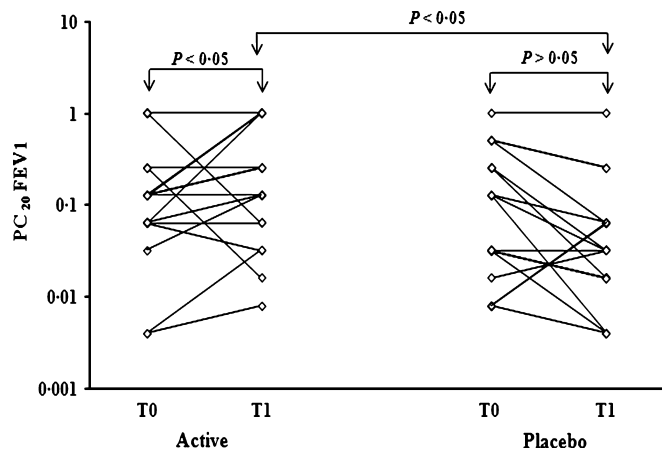


Figure 1 Airway reactivity (PC₂₀FEV₁) of active immunotherapy (IT) and placebo group patients at T₀ (baseline i.e. before IT) and T₁ (after 1 year of IT). 'T' stands for time. PC₂₀FEV₁ values were analysed for active IT group patients in comparison with baseline value and placebo group patients after 1 year of treatment. $P < 0.05$ was considered statistically significant.

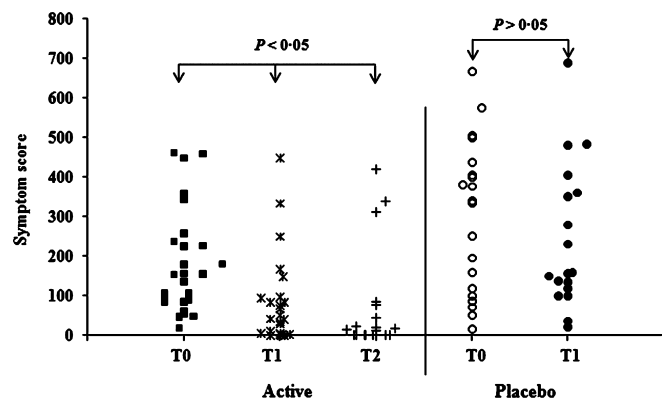


Figure 2 Scattered diagram of the total symptom score of active group 'T₀' [baseline i.e. before immunotherapy (IT)], 'T₁' (after 1 year of IT) and 'T₂' (after 2 year of IT) and placebo group at 'T₀' (baseline i.e. before IT) and 'T₁' (after 1 year of IT). 'T' stands for time. Symptom score was analysed for active IT group patients in comparison with baseline value and placebo group patients after 1 year of treatment. $P < 0.05$ was considered statistically significant.

immunoblot. Here, 72- and 78-kDa components are representing Per a 3 of *P. americana* identified earlier. Per a 9 separated at 43 kDa and Per a 7 at 37 kDa in previous studies, whereas in the present study, they separated at 40 and 38 kDa, respectively (Table 4, Fig. 4). Of 24 patients, 16 (67%) reacted to Per a 10 (~28 kDa), a major cockroach allergen at baseline. After 1 year of IT, IgE binding to Per a 10 reduced in 6 of 16 (38%) patients.

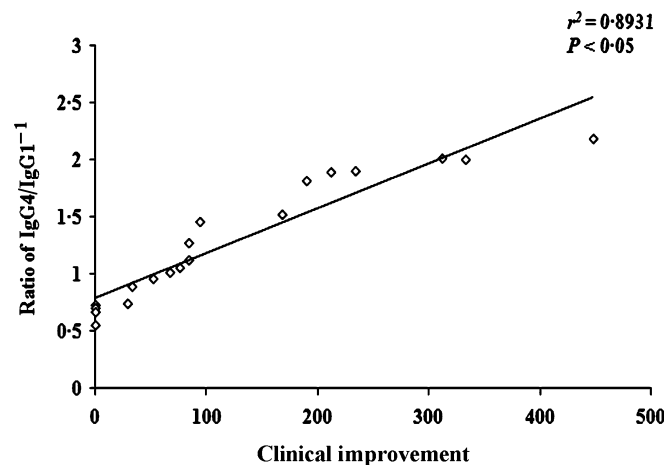


Figure 3 Correlation of IgG4/IgG1 ratio with clinical improvement (Change in symptom score T₀-T₁) in active immunotherapy group patients.

Table 4 Immunoblot results for specific IgE and IgG4 at T₀ and T₁ of active group patients

Protein bands (molecular weight in kDa)	IgE No. of patients		IgG4 No. of patients	
	T ₀	T ₁	T ₀	T ₁
97	7	7	5	10
72 and 78 (Per a 3)	13	8	9	13
43 (Per a 9)	11	10	3	6
37 (Per a 7)	10	8	5	8
~28 (Per a 10)	16	10	5	11
23	11	13	4	10

T₀, baseline [i.e. before immunotherapy (IT)]; T₁, after 1 year of IT.

IgE binding to other allergenic components also reduced in some patients post-IT. Five patients showed decreased intensity to 72- and 78-kDa components. Two patients showed decreased IgE binding to 37- and 23-kDa proteins. One patient showed decreased IgE binding to 43-kDa protein. Four patients did not show any change in IgE binding with protein components, while patient numbers 3 and 18 showed increased IgE bands post-IT (Table 4; Fig. 4).

Changes in IgG4 directed against cockroach major allergens. Unlike IgE blots, IgG4 immunoblotting demonstrated higher intensity and more number of bands after 1 year

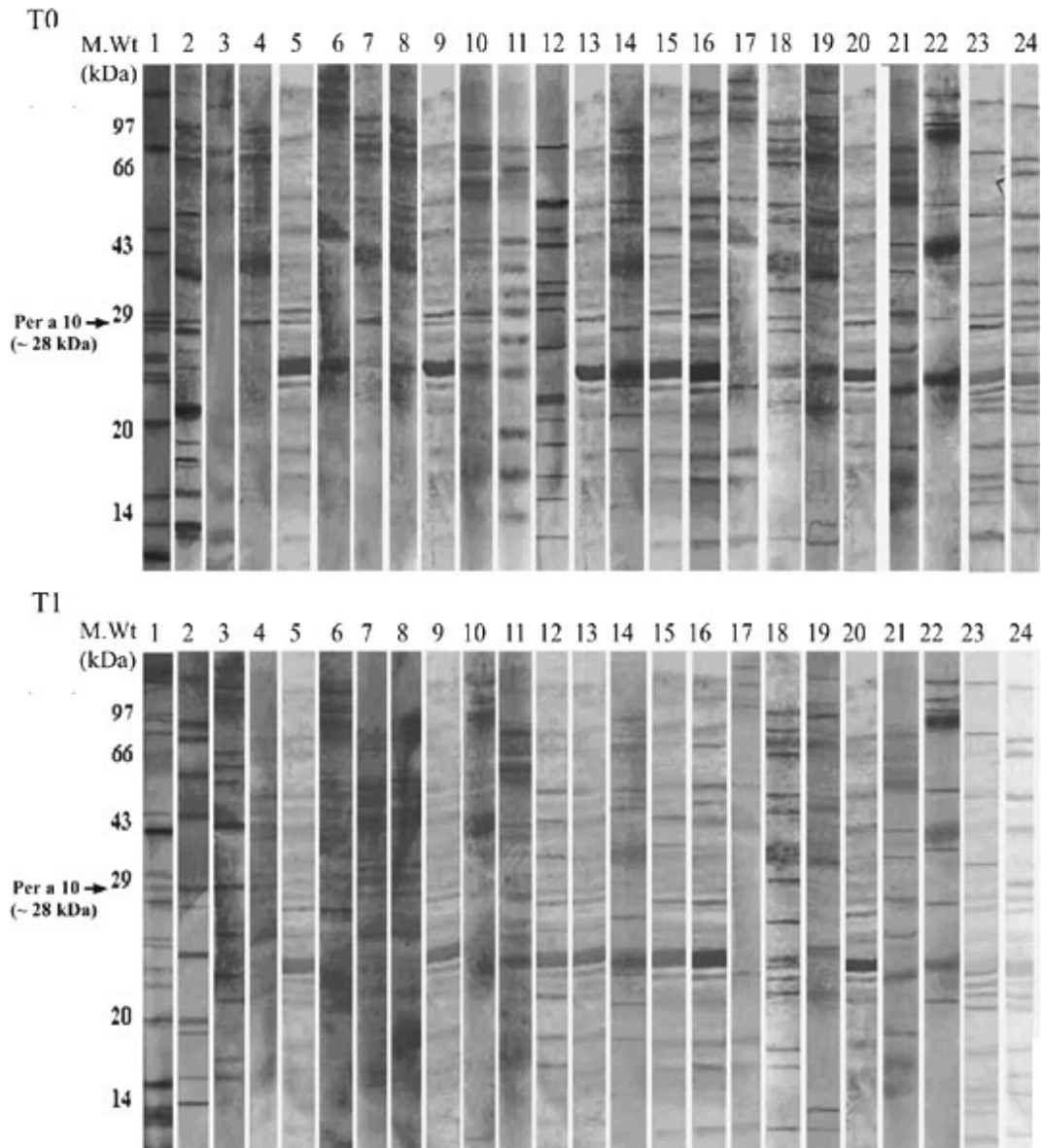


Figure 4 Protein profile of *Periplaneta americana* extract probed with individual cockroach hypersensitive patients' sera ($n = 24$) for IgE binding before (T_0) and after 1 year (T_1) of immunotherapy.

of cockroach IT than at baseline. Post-IT, 11 of 24 patients (46%) from active IT group showed IgG4 binding with 28-kDa major allergen of cockroach. The proteins of 72 and 78 kDa were detected in 13 of 24 patients (54%), whereas 10 of 24 patients (42%) demonstrated IgG4 binding with 97-kDa protein; 43-, 37- and 23-kDa proteins of MW showed IgG4 binding with six, eight and 10 patients' sera, respectively. Five of 24 patients did not show any change in IgG4 binding compared with baseline (Table 4; Fig. 5).

Discussion

The consensus reports and meta-analyses of placebo-controlled studies show that allergen IT is effective in reducing allergic symptoms and drug consumption in cases of respiratory allergy caused by pollens, mites, animal danders and moulds [22]. However, the merit of IT in asthma is questionable [12]. The present study was carried out to assess the effect of IT with cockroach extract in patients with

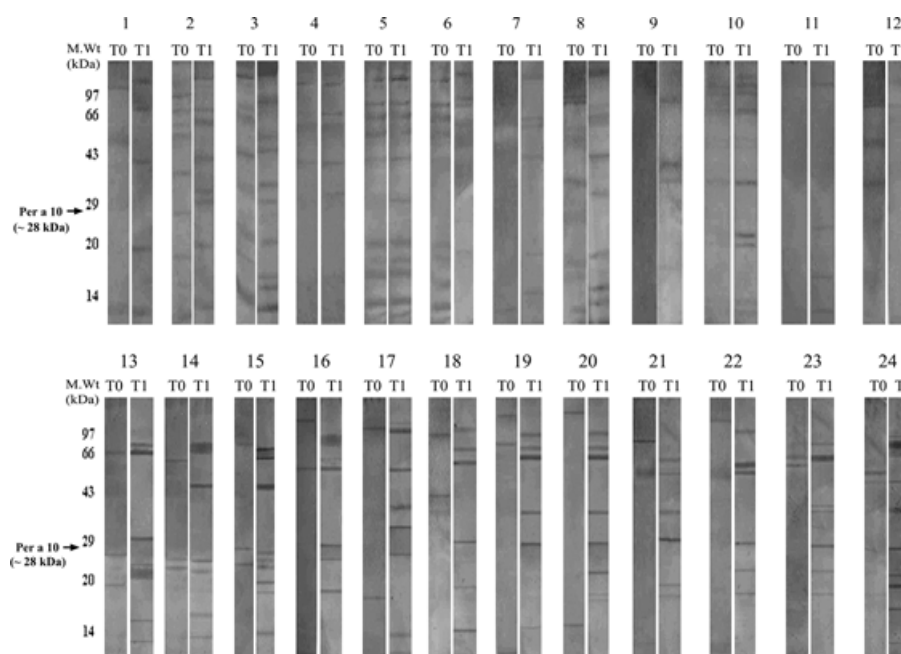


Figure 5 Protein profile of *Periplaneta americana* extract probed with individual cockroach hypersensitive patients sera ($n = 24$) for IgG4 binding before (T_0) and after 1 year (T_1) of immunotherapy.

allergic asthma and rhinitis based on clinico-immunological parameters.

Decrease in skin reactivity, symptoms and improvement in lung function are important parameters for evaluating clinical efficacy of IT [23–25]. IT trial with *D. pteronyssinus* showed significant improvement in skin reactivity of asthmatic children [21], while IT with *Alternaria* extract found no significant changes in skin reactivity but an increase in the placebo group. In the present study, decrease in skin reactivity to cockroach allergen was more than 50% compared with baseline value in active IT group patients, whereas skin reactivity to cockroach extract in the placebo group showed only 10–20% change on either side. Also a significant increase in $PC_{20}FEV_1$ values, i.e. decrease in airway reactivity, was observed in active IT group patients (18/24) than that in placebo. The 15 of 18 patients in placebo group showed rise in airway reactivity but reduction in their symptom score from 157 to 96 may be attributed to drug therapy that controls symptoms but does not modulate the underlying cause of disease.

Cockroach IT trials showed significant reduction in symptom score, i.e. sneezing attacks, nose blowing and nasal obstruction at the third and fifth year of IT [13,14]. IT with *Cocos nucifera* and grass pollen has also demonstrated reduction in symptom and drug score [26,27]. In the present study, active IT group (21 of 24 patients) showed significant improvement than baseline in symptom score and nearly 25% had no symptoms post-IT for

1 year. Further, a significant reduction in the active group ($P < 0.0001$) was observed post 2 years of IT signifying its success in treating patients with asthma/rhinitis. Only 30% of the patients in the placebo group showed reduction in symptom score that may be attributed to pharmacotherapy [28]. Further, 70% of patients in active IT group showed reduction (10–15%) in drug score, compared with 20% of patients in placebo group after 1 year. Besides, many patients in the placebo group showed exacerbations in both symptoms and drug score. After 1 year, active IT group required less amount of short-acting β -2 agonists/oral antihistamines; however, after 2 years, highly significant reduction in drug score was observed ($P < 0.0001$).

Immunotherapy studies showed modest reduction in allergen-specific IgE levels and induction of allergen-specific IgG subclasses leading to reduction in the recruitment and activation of proinflammatory cells in target mucosa [29–31]. However, some of the studies showed no change in IgE levels associated with significant increase in IgG subclasses post-IT [32]. In the present study, a modest reduction in IgE levels (0.435 ± 0.135 – 0.402 ± 0.144) and significant increase in IgG4 were found in active group patients after 1 year of IT. Whereas after 2 years of IT, reduction in IgE level became significant (0.435 ± 0.135 – 0.315 ± 0.099) than baseline. It appears that tendency observed in the first year became more pronounced in the second year. Studies suggest that allergen IT for more than 1 year is necessary to determine statistically significant

therapeutic effects [33]. The samples collected after 2 years of IT ($n = 12$ patients) showed significant immunological changes in the present study. Eleven patients showed downregulation in specific IgE level, and 12 patients showed upregulation in IgG4 level. However, we could not obtain samples from all patients after 2 years of IT.

There are studies that show clinical improvement but not much change in immunological status of the patients [34]. In the present study, we observed a significant correlation between increase in IgG4/IgG1 ratio and clinical improvement. Previously, a study on grass pollen IT also showed association between rise in IgG4/IgG1 ratio and improvement in clinical symptoms [32]. It is probable that IT modulates and effects different immunological as well as nonimmunological phenomena and the clinical improvement is a consequence of different working mechanisms with time.

IgE immunoblotting was used to assess the effects of IT, as it demonstrates specific antibody affinity against distinct allergen components [35,36]. Also, IgG4 immunoblotting in different studies has provided an improved level of discrimination for the assessment of correlation between clinical efficacy and immunological response. IT with honey bee and birch pollen showed changes in intensity and number of IgE and IgG4 binding bands [36,37]. In the present study, immunoblotting demonstrated (T_1) disappearance or reduction in IgE binding to 28-kDa major allergen and other important allergens of cockroach than baseline (T_0). Whereas IgG4 immunoblotting showed distinct protein bands and *de novo* appearance of a 28-kDa band with most of the patients' sera post 1 year of IT with cockroach extract. The rise in IgG4 may be a parallel epiphenomenon reflective of other immune changes like generation of T regulatory cells that may be directly responsible for improvement in airway hyper-responsiveness and systemic allergic responses. In conclusion, cockroach IT for 1–2 years is potentially effective treatment for cockroach allergy in patients with asthma and rhinitis.

Acknowledgements

Authors thank Mr Ashok Kumar Sinha and Mr Dinesh Chand, V. P. Chest Institute, Delhi, for technical support in *in vivo* testing of patients. Authors acknowledge Dr Amit Diwakar, Delhi, for co-operation in patients' selection. Thanks are due to Department of Science and Technology and Council of Scientific and Industrial Research (Network project 05), New Delhi, for financial assistance to this study.

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Received 28 April 2010; accepted 4 January 2011

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