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Placebo-Controlled Immunotherapy with *Cocos nucifera* Pollen Extract

Key Words

Immunotherapy
C. nucifera pollen
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Specific IgE
Specific IgG

Abstract

The effectiveness of *Cocos nucifera* pollen extract immunotherapy (CPE-IT) was studied in 96 patients allergic to *C. nucifera* pollen. A placebo-controlled study was performed at random for a period of 6–12 months. The clinical status of the patients measured by the symptom-medication scores demonstrated that *C. nucifera* pollen-allergic patients had significant ($p < 0.005$) clinical improvement after CPE-IT in comparison to placebo treatment. Serological study resulted a significant reduction ($p < 0.001$) of specific IgE and significant elevation ($p < 0.01$) of specific IgG in post-therapeutic patients' sera which were correlated significantly ($r = 0.45$, $p < 0.001$); the changes of the above immunoglobulin levels in the placebo-treated patients were nonsignificant. However, there was no correlation between symptom-medication scores and changes in specific serum IgE or IgG levels.

Introduction

Immunotherapy (IT) with allergens has been in clinical use since 1911 [1]. Well-controlled studies have convincingly demonstrated the beneficial effects of IT for the treatment of allergic rhinitis and asthma [2–6]; however, only a partial decrease in symptoms rather than 'cure' has been found. Immunological events that occur during IT include a rise in specific 'blocking' IgG antibodies, fall in specific IgE antibodies below pretreatment levels and finally, the induction of suppressor T cells. For the evaluation of IT the clinical status of the patients is considered [5]. However, the clinical relevance of the above findings is not certain for the individual patient since the beneficial effect of IT does not correlate precisely with concomitant reduction in specific IgE levels [7]. Gleich et al. [8] showed an abrupt increase in serum ragweed IgE in some patients

receiving initial ragweed IT at the same time there was a beneficial effect in vivo. Some investigators [9–11] found a positive correlation between rise in IgE and IgG levels during preseasonal hyposensitization with pollen extracts; on the other hand, a negative correlation between the rise in IgG and the fall in IgE was also reported [12].

The coconut (*Cocos nucifera*) tree grows throughout the world, especially in the lateral part of tropical belt. It flowers round the year and releases a large quantity of pollen [13], which is one of the sources of inhalant allergens responsible for immediate type I allergic reaction in humans [14]. However, IT with *C. nucifera* pollen extract has not been attempted so far.

The aim of the present investigation is to study the clinical response of *C. nucifera* pollen extract IT (CPE-IT) in patients allergic to *C. nucifera* pollen. The changes in symptom-medication score, specific IgE and IgG re-

sponses before and after IT have been longitudinally compared. The changes in the above Ig response with two allergenic fractions, C_nII and C_nVII have also been compared to those with CPE.

Materials and Methods

Patients

One hundred and five patients ranging in age from 6 to 56 (mean $\pm$ SD: 30.1 $\pm$ 9.86 years) were included in the study. They were selected on the basis of the following criteria: (1) all patients had symptoms of asthma and/or rhinitis throughout the year; (2) the existence of *C. nucifera* pollen allergen sensitivity in patients had been established by positive skin prick test (SPT) to CPE and the presence of CPE-specific serum IgE detected by Phadezym RAST (Pharmacia Diagnostics AB, Uppsala, Sweden). (3) None of the patients had multiple pollen allergy as assessed by SPT and Phadezym RAST to some common species viz., *Azadirachta indica*, *Cyanodon dactylon*, *Delonix regia*, *Caesalpinia pulcherrima* and *Areca catechu*, found in greater Calcutta. (4) None of the patients had satisfactory clinical improvement with medication and none had received any form of specific IT against any pollen.

Pollen

Floral materials collected from the matured intact flowers of *C. nucifera* were dried in an incubator at 37°C. Pollen was obtained from dried mass by successive sieving through 300-, 200- and 100- μ m mesh in a sieve-shaker. The purity of the pollen was examined [15] and found to be 98%, plant parts 1.6% foreign pollen 0.1% and foreign particles 0.3%.

Allergen

C. nucifera pollens were defatted with peroxide-free diethyl ether, dried and extracted with 0.15 M phosphate-buffered saline (PBS, 1:50 w/v), pH 7.8, by overnight stirring at 4°. The extract was clarified by centrifugation at 10,000 rpm for 30 min and the clear supernatant after dialyzing for 24 h was filtered through 0.22- μ m Millipore filter (Millipore, Bedford, Mass., USA). The filtrate, *C. nucifera* pollen extract (CPE), was stored at 4°C in 5-ml aliquots in sterile vials. A portion of CPE was freeze-dried, desiccated and stored at -20°C.

CPE, after ammonium sulfate precipitation (0–90%), was fractionated by ion-exchange chromatography on a DEAE-Sephadex A-50 column (seven fractions, C_nI, C_nII ... and C_nVII). C_nII and C_nVII gave a high response on SPT. Direct IgE-ELISA and ELISA inhibition revealed that both the fractions had strong IgE-binding activity [16]. Both C_nII and C_nVII were found to be homogeneous by gel filtration and SDS-PAGE (12%) [17]. However, IEF produced two bands in C_nII (pI 3.1 and 3.5) and one band in C_nVII (pI 3.8). They have 39% common epitopes and both show highest IgE-specific reactivity at pH 7.5 [17].

For IT, CPE in 0.15 M PBS was standardized by its protein content [18] followed by addition of phenol (0.4% v/w). For placebo treatment, only carbol saline (0.15 M PBS containing 0.4% phenol) was used. Each solution was finally filtered through 0.22- μ m Millipore filter and the filtrate was stored at 4°C in 5-ml aliquots in sterile vials pending use for IT.

Table 1. Dose schedule for immunotherapy

Number of subcutaneous injections	Interval between two consecutive doses, days	Doses (μ l) $\times$ number of consecutive injections
1–16	7	50 $\times$ 4 100 $\times$ 4 150 $\times$ 2 200 $\times$ 2 250 $\times$ 2 300 $\times$ 2
17–28	15	350 $\times$ 2 400 $\times$ 2 450 $\times$ 2 500 $\times$ 2 550 $\times$ 2 600 $\times$ 2
29–36	20	650 $\times$ 2 700 $\times$ 2 750 $\times$ 2 800 $\times$ 2
37 up to cure	30	850 $\times$ 2 900 $\times$ 2 950 $\times$ 2 1000–up to cure

The dose schedule was the same for all patients irrespective of sex, age and specific IgE/IgG levels.

For 1+ SPT-sensitive patients, the concentrations of CPE in vaccines were 0.15 μ g/ μ l. For 2+, 3+ and 4+ patients, the concentrations were 0.075, 0.05 and 0.038 μ g/ μ l, respectively.

Immunotherapy Schedules

For CPE-IT, a series of subcutaneous injections of graded doses of CPE in carbol saline were given to patients at varying intervals (table 1) whereas for placebo the patients received only carbol saline of same volume. When a systemic reaction (respiratory tract symptoms, hypotension, etc.) or a large local reaction (wheal > 3 cm in cross-diameter) occurred, the dose increment was stopped and the next dose was the 2nd last dose given before this reaction. The next doses were continued according to the dose schedule (table 1).

Evaluation of the Patients

Patients attending the clinic, National Institute for Allergy and Asthma Research (NIFAAR), Calcutta, India, were made aware of the possible symptoms that could appear and the type of medication which they should take (table 2 and 3). A chart presenting all the treatments that might be required was available to the patients and their doctors. Patients complete daily forms in the preceding month before starting IT and during the last half of every 3rd month. Asthma and nasal symptoms were recorded on the forms along with all the medications used. The symptom and medication scores were made according to Bousquet et al. [5] and calculated for each patient.

Table 2. Symptom-medication score: scoring system for medications

No medication	0
Sodium cromoglycate (inhaler or nasal spray)	1
Antihistamine (terfenadine or dexchlorpheniramine maleate)	2
Salbutamol/terbutaline (inhaler/nasal spray)	3
Salbutamol/terbutaline (oral)	4
Steroid (beclomethasone or prednisolone)	5
Daily symptom scores were recorded.	

Skin Prick Test

An individual solution of CPE and seven fractions of *C. nucifera* (C_nI, C_nII ... C_nVII) and five other pollen allergens (*Azadirachta indica*, *Cydonodon dactylon*, *Delonix regia*, *Caesalpinia pulcherrima* and *Areca catechu*) extracts was prepared (1.5 mg protein/ml) in carbol saline and sterilized by Millipore filtration (0.22 µm). The subjects were requested not to take antihistaminics, steroids and ephedrine for 48 h before SPT. The test was performed by placing 10 µl of each solution on the volar surface of the forearm of the subjects approximately 5 cm apart followed by pricking each site with a disposable hypodermic needle (No. 26). The wheal responses were measured after 20 min and graded 1+ to 4+ according to Platts-Mills et al. [19] using carbol saline as control.

Serological Assays

Patients sera were collected before initiation of IT and after 6–12 months of its progress. For comparative serological study sera from 45 normal subjects were also collected. After collection sera were stored at –50°C prior to use.

One of the criteria for the selection of patients was to ascertain the presence of CPE-specific serum IgE antibody which was determined by Phadezym RAST. Solid-phase antigen was obtained by coupling the CPE solution (10 mg/ml) to CNBr-activated paper disks (6 mm dia) according to the method of Ceska and Lundqvist [20]. Phadezym RAST was performed according to Wide et al. [21] with Phadezym RAST reagent (Pharmacia Diagnostics AB, Sweden) according to the manufacturer's instructions.

CPE, C_nII and C_nVII specific serum IgE and IgG antibodies before and after IT were measured by ELISA [22]. 100 µl of each allergen (100 µg/ml in 10 mM carbonate buffer, pH 9.6) was added separately to each well of the 96-well PVC microtiter plate (Cat No. 77-176-05, activated, lot No. 44-41-N2; Flow Lab., UK) and incubated at 4°C for 24 h followed by washing (5 min × 4) with 10 mM PBS containing 0.05% Tween 20, pH 7.4 (PBS-T). The nonspecific sites were blocked with 1% BSA (100 µl) at 37°C for 1 h. 100 µl of diluted sera (1:10 for specific IgG) was added to each well after washing with PBS-T. Following incubation at 37°C for 3 h, the wells were again washed with PBS-T and incubated with 100 µl of diluted horseradish peroxidase-antihuman IgE/IgG conjugate (1:100 for specific IgE and 1:250 for specific IgG) for 1 h at 37°C. The enzyme-antibody conjugate was prepared according to Avrameas and Ternynck [23]. Finally, the wells after washing with PBS-T were incubated with 100 µl of *o*-phenylenediamine (1 mg/ml in 0.05 M citrate-phosphate buffer, pH 5.0 containing 0.01% H₂O₂) at room temperature for 30 min in the dark with gentle shaking. Absorbance of each well was measured at

492 nm in a Titertek Multiskan Plus MK II ELISA reader (Flow Lab., UK).

The serum IgE was assayed from a standard curve obtained from a serum pool of 10 highly *C. nucifera* pollen sensitive patients. The serum pool (dilution 1:10) was assigned to *C. nucifera* pollen-specific IgE concentration of 1,000 arbitrary units per milliliter (AU/ml) [24]. The assay of serum IgG was made from a standard curve obtained from a serum pool of 10 highly desensitized (≥ 2 years) patients. The serum pool (dilution 1:100) was assigned to *C. nucifera* pollen specific IgG concentration of 1,000 AU/ml.

The dilution effects on serum IgE in pooled sera of 4 CPE-treated, 4 placebo-treated and 4 normal subjects against CPE were compared. One part of each serum pool was depleted of IgG antibodies in a Protein A-Sepharose 4B column separately. Those IgG-depleted patient's serum pools were then used for specific IgE binding. Another part of the pooled sera was used as usual. The reactivities were compared by ELISA.

Design of the Study

Randomization of Patients. Patients were randomly assigned to one of two treatment groups: CPE-treated and placebo-treated. Eighty-six patients were treated with CPE and 19 patients received placebo. Nine patients, 8 from the CPE-treated and 1 from placebo group discontinued IT (for personal reasons) after 6 months and were considered dropouts. The study was performed blindly throughout. One physician was responsible for assigning the doses of allergen or placebo to be administered and another, who was unaware of which treatment each patient was receiving, was responsible for the clinical management of the patient during the progress of IT.

Parameters Studied. Before IT and after 6–12 months of its progress, CPE, C_nII and C_nVII specific serum IgE and IgG in patients were titrated. All patients had a follow-up based on a symptom-medication score diary that was filled in before the initiation of IT and in the last half of every 3rd month.

Statistical Analysis

Statistical analysis was performed with nonparametric tests. The Wilcoxon W test for paired data and the Mann-Whitney U test for unpaired comparison were used for the analysis, and *p* < 0.05 was considered significant. The correlation coefficient, *r*, between the changes of specific IgE and IgG levels was computed from deviation of scores from respective means.

Results

Homogeneity among Groups of Patients

The characteristics of the 96 patients continued IT all through the study and placed in the CPE-treated or placebo-treated groups are presented in table 4. The homogeneity between two groups was analysed by using Mann-Whitney U test. All parameters studied were comparable between the two groups.

Allergenic sensitivities of the Patients

CPE sensitivity of the patients, by SPT, is presented in table 4. SPT results of different fractions isolated from

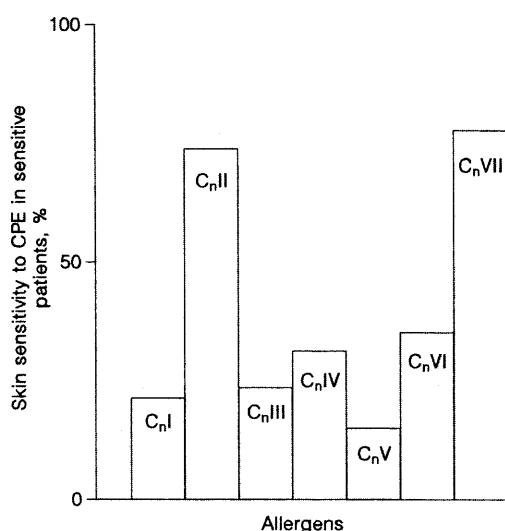


Fig. 1. SPT of different fractions isolated from CPE by DEAE-Sephadex A-50.

Table 3. Symptom-medication score: diary charts provided to the patients for asthma and rhinitis

<i>Asthma</i>	
No symptom	0
Mild occasional bronchospasm	1
Seasonal bronchospasm without/with congestion	1 or 2
Perennial bronchospasm without/with emphysema	2 or 3
<i>Rhinitis</i>	
No symptom	0
Episodes of sneezing (> 5)	1
Nasal blockade	1
Rhinorrhea	1 or 2
Pruritus of the nose	1

Daily symptom scores were recorded.

CPE by DEAE-Sephadex A-50 are shown in figure 1 which demonstrated that C_nII (76%) and C_nVII (80%) are much more skin sensitive than the other fractions. CPE-specific IgE in the patients' sera determined by Phadezym RAST is shown in figure 2, which demonstrated the antibody classes among the patients; 23 have class 1, 47 have class 2 and 8 have class 3 antibody among the CPE-treated patients, while in placebo-treated patients 5 belong to class 1, 11 belong to class 2 and 2 belong to class 3 antibody. The difference in CPE-specific IgE between CPE-treated patients (mean±SD, 1.29±1.2) and placebo-treated pa-

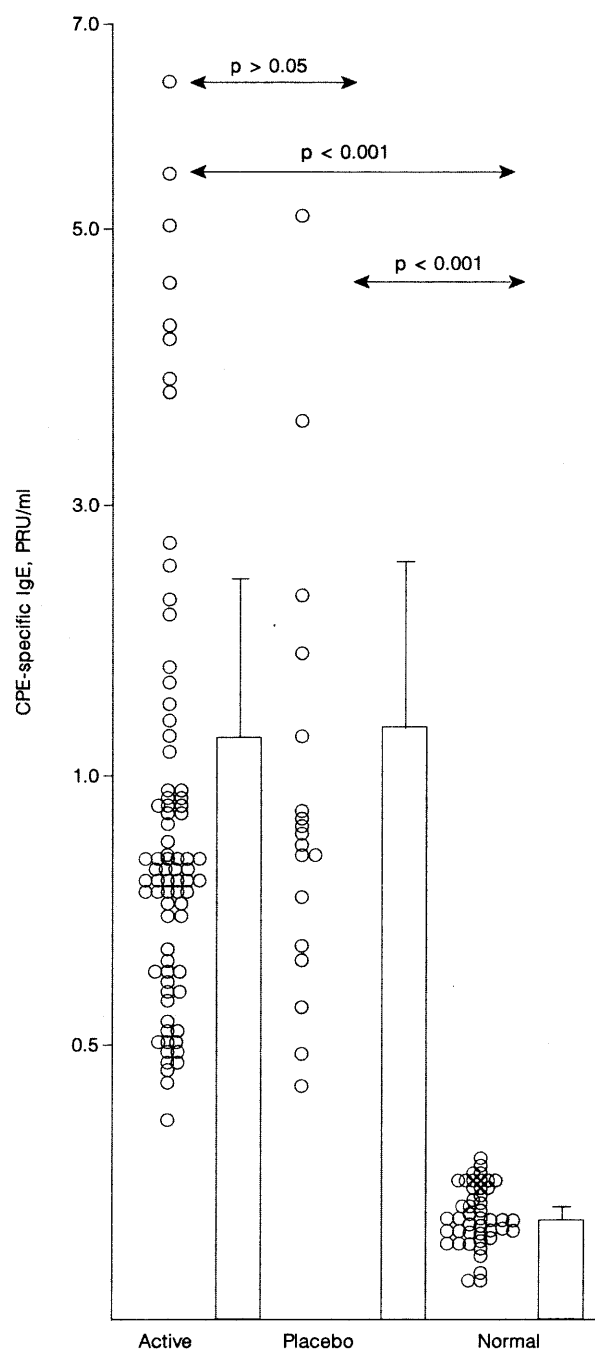


Fig. 2. Scattered diagram of the CPE-specific IgE levels of the selected patients against normal subjects. Phadezym RAST unit per milliliter (PRU/ml) and class antibody in patient serum was determined using reference birch pollen sera and allergen disks (Pharmacia Diagnostics, Sweden) according to the manufacturer's directions; class 1 antibody score (0.35–0.70 PRU/ml), class 2 (0.70–3.50 PRU/ml) and class 3 (3.50–17.50 PRU/ml).

tients (1.35 ± 1.24) was found to be nonsignificant ($p > 0.05$, Mann-Whitney U test); whereas CPE-specific IgE levels in both groups were found to be significantly higher ($p < 0.001$) than in the normal subjects.

Protocol for IT and Side Effects

In CPE-treated patients six local reactions and three systemic reactions occurred. Placebo-treated patients had two local reactions and no systemic reactions. In spite of the side effects, good blinding was achieved as the patients did not know which treatment they were receiving and the treatment was entirely random.

Symptom-Medication Scores

Symptom and medication scores were analyzed for asthma and allergic rhinitis (fig. 3). Before IT, it was observed that patients in both CPE- and placebo-treated groups had a similar mean symptom-medication score. Whereas after IT only CPE-treated patients had a significant clinical improvement compared to placebo treatment ($p < 0.005$, Mann-Whitney U test).

Effect of IT Regimens

From figure 4, it is evident that the binding of CPE to specific IgE in IgG-depleted (fig. 4a) or nondepleted (fig. 4b) patients' sera both pretherapy (PrT) and posttherapy (PoT) had nearly the same except that the specific IgE level in PoT placebo-treated patients was much higher than the PrT level. In normal sera, the dilution effect was almost nil.

Serum IgE and IgG antibody levels (mean $\pm$ SD) before and after IT are presented in table 5. The specific IgE and IgG levels of both groups of patients before IT were found to be comparable, and their differences were nonsignificant. In the CPE-treated patients, CPE-specific as well as C_nII- and C_nVII-specific IgE levels were found to have declined significantly ($p < 0.001$) after 6–12 months of IT as compared to the respective PrT or placebo groups. In contrast, the specific IgG levels were found to be significantly elevated for anti-CPE ($p < 0.01$ and $p < 0.025$), anti-C_nII ($p < 0.01$ and $p < 0.025$) and anti-C_nVII ($p < 0.01$ and $p < 0.01$) as compared to the corresponding PrT or placebo groups. In the placebo-treated patients both IgE and IgG levels for anti-CPE, anti-C_nII and anti-C_nVII were found to have elevated nonsignificantly. The serum IgE and IgG levels against CPE, C_nII and C_nVII in normal subjects were found to be significantly lower ($p < 0.001$) than the CPE- or placebo-treated patients.

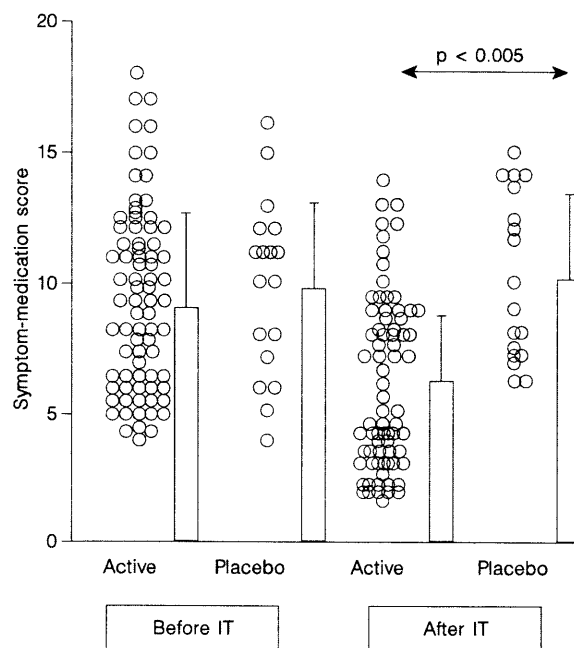


Fig. 3. Symptom-medication scores for asthma and allergic rhinitis.

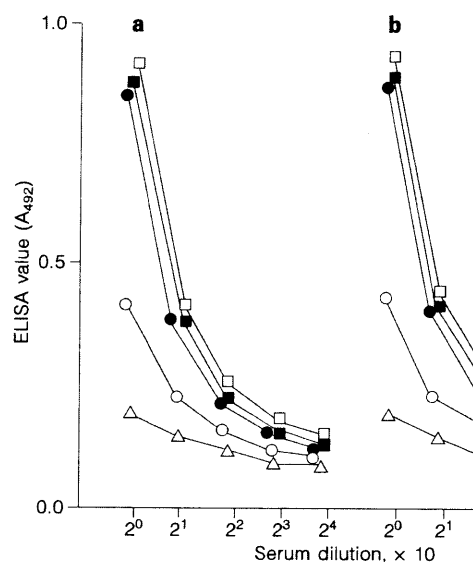


Fig. 4. IgE-binding activity (mean OD $\pm$ SD of each triplicate) of CPE of 4 CPE-treated patients, 4 placebo-treated patients and 4 normal subjects. **a** Specific IgE from IgG-depleted sera. **b** Specific IgE from non-IgG-depleted sera. Reactivities of CPE with specific serum IgE in CPE-treated patients before IT ($\bullet$) and after IT ($\circ$), in placebo-treated patients before IT ($\blacksquare$) and after IT ($\square$), and in normal subjects ($\triangle$).

Table 4. Comparison of the patient groups

	CPE-treated	Placebo-treated
Patients	78	18
Age, years		
Range	6–56	12–48
Mean	30.58±9.53	29.05±10.32
Male:female	43:35	10:8
SPT to CPE		
1+	13	16
2+	60	2
3+	4	–
4+	1	–
CPE-specific IgE, PRU/ml	1.29±1.2	1.35±1.2
Asthma, %	59 (75.64)	14 (77.78)
Duration, years		
Range	1–16	0.5–13
Mean	8.23±2.15	9.19±1.94
Severity score ¹	2.08±0.87	2.28±0.76
Rhinitis, %	57 (73.08)	13 (72.22)
Duration, years		
Range	0.5–13	0.5–12
Mean	7.54±2.08	6.86±1.87
Severity score ¹	2.57±0.97	2.95±0.86
Urticaria, %	21 (26.92)	4 (22.22)
Eczema, %	2 (2.56)	–
Itching of eyes, %	14 (17.95)	3 (16.67)

¹ Asthma and rhinitis were graded 0–4 according to the severity of symptoms and the medications required by the patients the month before starting IT.

The correlation coefficients, *r*, between the changes in specific IgE and IgG levels for CPE, C_nII and C_nVII were –0.452 (*p* < 0.001), –0.318 (*p* < 0.01) and –0.331 (*p* < 0.001), respectively. There was no significant correlation between symptom-medication scores for asthma and allergic rhinitis and specific IgE and IgG.

Discussion

The present study demonstrates the allergenic sensitivity to the CPE as evidenced by SPT and Phadezym RAST. The sensitization to *C. nucifera* pollen was comparable to that of earlier investigation [14] though conducted in different locations on different groups of patients suffering from asthma and allergic rhinitis.

From the above study, a small but clear differences were observed clinically and serologically between patients treated with CPE and placebo. Clinical benefit, as evaluated by symptom-medication scores, showed that these were

significantly reduced in the CPE-treated group in comparison to placebo (*p* < 0.005) which confirmed the value of IT with an appropriate dose schedule. Creticos et al. [25] similarly observed that both symptoms and release of mediators were significantly reduced by ragweed pollen-IT. The lack of efficacy in the placebo-treated patients might be due to the absence of CPE during the placebo treatment.

From the serological study, both specific IgE and IgG levels in the patients' sera were found to be much higher than in normals. Obviously, the rise in specific IgE is due to a type I allergic reaction, but the reason for the rise in IgG is obscure. One of the several factors may be the exposure of the patients to airborne *C. nucifera* pollen even under medication, thereby raising some amount of specific antibody of the IgG1 type [26].

Most of the patients receiving CPE-IT responded with significant regression of specific IgE (*p* < 0.001) and significant elevation of specific IgG (*p* < 0.01) antibodies although the specific IgE levels did not fall to normal levels even after 6–12 months of IT, while clinically the patients showed good recovery. In contrast, the specific IgE and IgG levels were found to increase nonsignificantly in the placebo treatment. These findings suggest the rise in specific IgG may play an important role in relieving patients who suffer from allergic illness and is likely to be related to the lowering of specific IgE. Several studies on hyposensitization demonstrated a selective rise in serum IgG [4, 27–30]. Some of them showed a quantitative increase in IgG which is weakly associated with clinical efficacy [29, 30]. Decrease in allergen specific IgE levels due to IT has also been observed in several studies in pollen-treated patients [4, 9, 31, 32].

Although the clinical efficacy was significant by symptom-medication score and a good correlation was found between lowering of specific IgE and elevation of specific IgG, no significant correlation between changes in symptom-medication score and specific IgE or IgG was noted. Thus, the differential contribution of CPE-IT to alleviating allergic disorders by suppression of specific IgE and stimulation of specific IgG is suggested, but could not be associated with clinical benefit based on our data. This would imply that so called 'blocking antibodies' are not the sole explanation of clinical improvement with IT. Other investigators have found a similar lack of correlation between levels of specific IgG antibodies and clinical benefit [32–34]. Further studies are required to explore the full potential of CPE-IT in the immunoregulation of *C. nucifera*-sensitive patients, and to determine whether these clinical and serological effects can be amplified by high-dose treatment for considerably longer periods of time.

Table 5. Specific serum IgE and IgG levels (mean $\pm$ SD of each triplicate) in CPE-treated and placebo-treated patients and normal subjects to CPE, C_nII and C_nVII

Allergen	CPE-treated (n = 78)		Placebo-treated (n = 18)		Normal (n = 45)
	BT ^a	AT ^b	BT ^c	AT ^d	
<i>Specific IgE, AU/ml</i>					
CPE	698±54 (NS)	291±32***	724±43 (NS)	783±51***	85±13***
C _n II	686±65 (NS)	319±28***	695±52 (NS)	746±38***	130±18***
C _n VII	691±59 (NS)	332±27***	708±61 (NS)	765±46***	142±20***
<i>Specific IgG, AU/ml</i>					
CPE	478±42 (NS)	582±26**	453±48 (NS)	469±37*	91±16***
C _n II	463±53 (NS)	591±45**	487±39 (NS)	506±41*	162±14***
C _n VII	456±57 (NS)	607±42**	429±45 (NS)	461±35**	157±18***

BT = Before therapy; AT = after therapy. AU/ml, given in Materials and Methods, *p < 0.025; **p < 0.01; ***p < 0.001; NS, not significant.

^a p values compared to BT placebo values.

^b p values compared to BT CPE-treated values.

^c p values compared to AT placebo values.

^d p values compared to AT CPE-treated values.

The changes in C_nII- and C_nVII-specific serum IgE and IgG levels before and after CPE-IT were almost same as those of CPE and thus were not significant.

It is evident that changes in specific IgE values by subsequent dilutions (fig. 4) were of same order of magnitude in the presence or absence of specific IgG antibody in both PrT and PoT sera. It seems that the interfering factors, if any, in the binding capacity of specific IgE were constant in both PrT and PoT patients' sera. The above study also showed that the specific IgG did not inhibit the specific IgE determination which could be explained by the fact that the different epitopes of the same allergen might be responsible for specific IgE and specific IgG binding.

In conclusion, the results presented in this study confirm that CPE is an effective immunotherapeutic agent for use in the management of CPE-sensitized atopic patients.

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