Original Article

Phoenix sylvestris Roxb Pollen Allergy: A 2-Year Randomized Controlled Trial and Follow-up Study of Immunotherapy in Patients With Seasonal Allergy in an Agricultural Area of West Bengal, India

P Chakraborty,[1] I Roy,[2] S Chatterjee, [2] S Chanda,[3] and S Gupta-Bharracharya[4]

[1]Department of Botany, Sree Chaitanya College, Habra, West Bengal, India
[2]Department of Allergy, Institute of Child Health, Calcutta, India
[3]Centre for Study of Man and Environment, Salt Lake City, Calcutta, India
[4]Department of Botany, Bose Institute, Calcutta, India

Abstract. *Background.* Although the efficacy of allergen immunotherapy has been demonstrated in seasonal pollen allergy, there is no report of a double-blind placebo-controlled trial with standardized pollen extract in seasonal respiratory allergy from India. In the agricultural area of eastern India, *Phoenix sylvestris* Roxb or date sugar palm is grown or cultivated and seasonal allergic rhinitis is common during the pollen season.
Objective: The objective of the present study was to observe the clinical and immunological changes during a 2-year double-blind placebo-controlled trial of immunotherapy with standardized *P sylvestris* pollen extract in respiratory patients sensitive to pollen from this wild date palm. Thirty-five subjects with typical seasonal allergic rhinitis with or without bronchial asthma were selected. A symptom–medication score (based on a questionnaire and diary) was correlated with pollen counts as recorded in a Burkard sampler. Eighteen subjects were randomized to a specific immunotherapy (SIT) group receiving regular injections containing standardized allergen extract and 17 to a placebo control group. Changes in the level of specific immunoglobulin (Ig) E, IgG1, and IgG4 were recorded at 3-month intervals. Measurement of wheal diameter, total IgE level and forced expiratory volume in 1 second (FEV_1) were performed before starting and a month after finishing therapy.
Results: The SIT group showed decreases of 33.5% and 57% from the baseline symptom–medication scores during the first and second treatment season, respectively. This group showed significant decreases in skin-reactivity to *P sylvestris* pollen extract and in specific IgE levels, and significant increases in FEV_1, specific IgG1 (1.95–3.2 times higher) and IgG4 (21.24–30.83 times higher). There were no significant changes in total IgE levels. The control group showed no significant changes for any parameter except the development of new sensitization in 2 cases (to *Saccharum officinarum* pollen grain and *Alternaria* species spores). The rate of local adverse reactions was 0.024%.
Conclusion: After a 2-year study, allergen immunotherapy with standardized *P sylvestris* pollen extract was found to be effective in seasonal respiratory allergic subjects susceptible to *P sylvestris* pollen with a narrow range of sensitization.

Key Words: *Phoenix sylvestris* pollen. Seasonal rhinitis. Double-blind placebo-controlled trial. Immunotherapy.

Resumen. *Antecedentes*: Aunque se ha demostrado la eficacia de la inmunoterapia con alérgenos para el tratamiento de la alergia estacional al polen, no existen informes de estudios controlados con placebo de doble ciego con extracto de polen estandarizado para el tratamiento de la alergia respiratoria estacional en la India. En la zona agrícola del Este de la India se planta y cultiva la *Phoenix sylvestris* Roxb (datilera silvestre) y las rinitis alérgicas estacionales son comunes durante la época de polinización.
Objetivo: El objetivo de este estudio fue observar los cambios inmunológicos y clínicos durante un periodo de dos años. En este estudio doble ciego, controlado con placebo, la inmunoterapia consistió en la administración de extracto de polen de *P sylvestris* estandarizado a pacientes con síntomas respiratorios y sensibles al polen de esta palmera. Se seleccionaron 35 sujetos con rinitis alérgica estacional típica con o sin asma bronquial. Los índices de cantidad de síntomas y fármacos (basados en un cuestionario y en un diario) se correlacionaron con los recuentos de polen registrados en un recolector de pólenes tipo Burkard. Dieciocho sujetos se aleatorizaron al grupo de inmunoterapia específica (ITE) que recibió regularmente inyecciones de extracto de alérgeno estandarizado y 17, al grupo de referencia con placebo. Los cambios en los niveles de inmunoglobulina (Ig) E, IgG1 e IgG4 específicos se registraron en intervalos de tres meses. Se realizaron mediciones de diámetro de ronchas, nivel total de IgE y volumen espiratorio máximo en el primer segundo (FEV_1) antes de empezar el tratamiento y un mes después de finalizado éste.
Resultados: El grupo ITE presentó descensos de un 33,5% y un 57% con respecto a los valores de cantidad de síntomas y fármacos iniciales durante la primera y segunda temporada de tratamiento, respectivamente. Este grupo presentó descensos significativos en la reactividad cutánea al extracto de polen *P sylvestris* y en los niveles de IgE específica, y aumentos significativos en el FEV_1, IgG1 específica (1,95 – 3,2 veces) e IgG4 (21,24 – 30,83 veces). No hubo cambios significativos en los niveles IgE totales. El grupo de referencia no presentó cambios significativos en ninguno de los parámetros, excepto dos casos en que se produjeron nuevas sensibilizaciones (al polen de *Saccharum officinarum* y a las esporas de *Alternaria*). La tasa de reacciones adversas locales fue de un 0,024%.
Conclusión: Tras el estudio de dos años de duración, se concluyó que la inmunoterapia con alérgenos a base de extracto de polen de *P sylvestris* estandarizado es efectiva en sujetos con alergias respiratorias estacionales susceptibles al polen de *P sylvestris* con un rango limitado de sensibilización.

Palabras clave: Polen de *Phoenix sylvestris*. Rinitis estacional. Estudio doble ciego controlado con placebo. Inmunoterapia.

Introduction

Phoenix sylvestris Roxb, commonly known as date sugar palm or wild date palm, grows naturally in the tropics and subtropics of the Indian subcontinent and is also cultivated for its edible fruits and to provide ingredients for sweets and beverages. This tree is wind pollinated and the airborne pollen grains are known to cause respiratory allergy in susceptible individuals. During pollen season *P sylvestris* pollen contributes 14% to 16% of the aeropollen load [1] and a previous study showed that out of 540 respiratory allergic patients in the metropolitan area of Calcutta 44.07% had a positive skin reaction to *P sylvestris* pollen [2]. In eastern India, particularly in West Bengal, people residing in the agricultural area have a good chance of exposure to *P sylvestris* pollen due to the large number of date sugar palm plantations. A population was selected for the present study from the agricultural belt of the north 24-Parganas district situated in the northern suburbs of Calcutta, where pollinosis due to *P sylvestris* pollen is quite frequent.

Although many new and improved pharmacological drugs have been introduced to reduce the symptoms of allergic disease, only allergen immunotherapy targets the natural cause of allergic reaction [3]. The efficacy of such therapy has been proven in a number of studies involving patients allergic to airborne pollen grains [4-6]. There is very little information available on clinical and immunological changes during immunotherapy with pollen allergen extracts from the Indian subcontinent, however. In Indian diagnostic clinics, the crude extracts of common allergens are still being used, even though the application of standardized allergen is widely accepted [7].

We conducted a randomized double-blind placebo-controlled trial of *P sylvestris* pollen specific immunotherapy (SIT), enrolling respiratory allergic patients from an agricultural area of north suburban Calcutta. The subjects were sensitive to *P sylvestris* pollen and presented typical symptoms of rhinoconjunctivitis during the *P sylvestris* pollen season. The SIT was carried out for 2 years with subcutaneous injections of standardized *P sylvestris* pollen extract based on an important IgE reactive fraction. The clinical efficacy was assessed by recording symptom and medication scores and changes in levels of IgE, IgG1, IgG4 specific to *P sylvestris* pollen and also the total IgE level in both the SIT and placebo groups.

Materials and Methods

Pollen Counts

The airborne *P sylvestris* pollen count was continuously recorded with a 7-day volumetric spore trap (Burkard,

Hertfordshire, UK) placed on a half-meter high concrete base on a farm in the study area from August 1998 to July 2001 (3 years). The exposed tapes were microscopically examined following the guidelines of the British Aerobiology Federation [8]. All enrolled patients were living within 5 km of the sampling site.

Patients and Design

After approval of the protocol by the ethics committee of the hospital of the Institute of Child Health, Calcutta, we recruited patients to randomize to 2 groups to receive SIT or placebo in a double-blind design. Originally 62 patients were invited from the outpatient clinic of the allergy department of the Institute of Child Health. Patient candidates were selected according to a history of severe seasonal rhinitis in the *P sylvestris* pollen season (January–March) and poor symptom control in previous seasons despite regular antiallergy treatment with sodium cromoglycate and antihistamines. The inclusion criteria were a doctor's diagnosis of seasonal rhinitis–rhinoconjunctivitis or allergic asthma or combination of both. The diagnosis was according to case history, clinical assessment of symptoms and pulmonary function tests. In all cases, sensitization towards *P sylvestris* pollen was measured by skin prick test against a panel of allergen extracts containing house dust mite, cat or dog dander, pollen grains of *Saccharum*, *Azadirachta*, *Cocos*, *Eucalyptus*, *Areca*, *Borassus*, *Delonix*, *Peltophorum*, *Carica*, *Catharanthus*, and *Phoenix* species and spores of *Alternaria alternata*, *Cladosporium herbarum*, *Aspergillus* species mix; specific IgE enzyme-linked immunosorbent assay (ELISA) was also performed. Patients with additional prick reactions to other important allergen groups (eg, house dust mites, all types of mold spores, animal dander) besides pollen grains were excluded, as were those with chronic or severe asthma symptoms, history of immunotherapy or any kind of systemic, autoimmune or joint diseases or malignancy.

After an interview concerning aspects of the patient's medical history that might reduce the ability to survive a systemic allergic reaction (as described above), 35 patients (age range, 20–59 years) were finally recruited. Their occupations were as follows: 37.14% were farmers, 20% students, 11.42% housewives, 11.42% casual farm workers, 8.57% landowners, 5.71% teachers, and 5.71% wild date sap collectors.

For comparison of antibody titers, 19 healthy controls were also studied.

After enrollment, signed informed consent forms were collected from the patients and baseline data were recorded for an entire year (July 1998 to June 1999). Patients were then randomized to receive SIT (n = 18) or sterile phosphate buffered saline (PBS) as the placebo (n = 17). The therapy schedule started in the period of September–December 1999, shortly before the pollen season, and continued for 2 years up to August–November 2001. Both the subjects and the administering personnel were blinded as to the composition of the injection vials. Prior to starting the SIT schedule, blood serum samples were collected; further samples were collected at 3-month intervals until the end of the second year.

Outcome Measure: Symptom–Medication Score

A symptom-medication score was compiled from the following measures taken during the course of the study:

– *Perception of symptoms, numerical score.* Once a month, the patients were asked about changes in their symptoms during that period. Possible answers were much worse, –3; worse, –2; bad, –1; no change, 0; good, 1; better, 2; much better, 3. Later, these values were converted to numbers between 0 and 3 (+3 = 0; 2 and 1 = 1; 0 and –1 = 2; and –2 and –3 = 3).

– *Symptoms and medication questionnaire and diary.* During the pollen season, every patient kept a diary to take down daily symptoms affecting lungs (breathlessness, tight chest, cough, wheeze), nose (sneezing, blocked, runny), eyes (itching, redness, streaming, swelling), mouth and throat (itching, dry). Numerical values placed on symptoms ranged from 0 (no symptoms) to 3 (very severe symptoms). Additionally, the duration of symptom scores were multiplied by the time complaints lasted (1, less than one week; 2, 1 to 4 weeks; 3, more than 4 weeks). Daily medication was also recorded as follows: 0, no medication; 1, sodium cromoglycate, locally if needed; 2, sodium cromoglycate regularly and antihistamine if needed; 3, antihistamine regularly and ß-antagonist or local or inhaled cortisone if needed.

– *Overall severity, visual analog scale.* Once a month patients recorded the degree of their complaints on an open scale ranging from "no symptoms" to "severe symptoms." Afterwards these values were converted to scores ranging from 0-3.

The mean of all scores for each patient served as the total symptom–medication score for every 3-month period.

Skin Prick Tests

Skin prick tests were carried out with *P sylvestris* pollen extracts (1:50 wt/vol). Histamine phosphate (1 mg/mL) and PBS were used as positive and negative controls. According to international guidelines, positivity was defined as a wheal diameter at least 3 mm larger than the negative control [9]. Tests were performed with 20 μL of allergen solution placed on the ventral side of the forearm and the site was pricked with a 26-G disposable hypodermic needle. The wheal was measured after 20 minutes. The reaction was graded from +1 to +4 as described by Stytis et al [10].

Pulmonary Function Tests

Pulmonary function tests were performed with a computer-assisted spirometer. Subjects performed 3 forced expiratory maneuvers starting from the maximum inspiratory position, or at least until 2 flow–volume curves were obtained with forced expiratory volume in 1 second (FEV_1) differing by no more than 5% [11]. The tests were performed during pollen season prior to starting the therapy schedule and after finishing the 2-year follow-up period. The data were analyzed for the subjects with allergic rhinitis along with asthma (n = 8 for the SIT group and n = 6 in the placebo group).

Allergen Extract and SIT

The active treatment was performed using *P sylvestris* pollen extract standardized with one of its principal allergenic fraction (fraction IIa), which gives rise to 2 IgE-reactive bands (33 and 66 kDa) in sodium dodecyl sulfate polyacrylamide gel electrophoresis after being purified by ammonium sulphate fractionation and gel filtration with Sephacryl S-200 column (Pharmacia, Sweden) [2]. The pollen grains were defatted with diethyl ether and extracted in PBS (0.1 M sodium phosphate, and 150 mM sodium chloride, pH 7.2) by continuous stirring for 16 hours at 4°C. After centrifugation at 12 500*g* for 45 min, the supernatant was dialyzed in PBS and passed through 0.22 μM Millipore filter (Millipore Corp, Bedford, Massachusetts, USA). The filtrate was then lyophilized and stored (–70°C) in sterile vials. We used a 1:5000 wt/vol dilution for the induction phase and a 1:2500 wt/vol dilution for the maintenance phase of immunotherapy. The solutions contained 1 μg and 2 μg of Fr IIa per mL, respectively. The placebo preparations included only sterile PBS. The weekly subcutaneous injection schedule included a 24 week induction phase with a starting dose of 0.05 μg and a final dose of 0.5 μg (injection volume 0.05–0.5 mL) of Fr IIa. This phase had been completed during February–April of the first treatment year. It was followed by an 18-month maintenance phase with injections at 2-week intervals of a dose of 0.5 to1.0 μg of Fr IIa (injection volume 0.25–0.5 mL). During pollen season, the dose was reduced 20% to 40% in symptomatic patients. The patients were observed for 30 minutes after each injection [12].

If an adverse local reaction was recorded, the subsequent doses were reduced to that of the last injection applied without any reaction and the interval between doses was increased (from 2 to 4 weeks, for example).

Changes in Total and Specific IgE Antibodies

Serum total IgE was measured with a Pathozyme total IgE quantification kit (Omega Diagnostics, Edinburgh, UK) following the manufacturer's instructions. Change in *P sylvestris* pollen specific IgE was determined by ELISA. Each ELISA well was coated with *P sylvestris* pollen extract (protein content 1 μg/well) and incubated for 3 hours at 25°C. After overnight blocking at 4°C with 1% bovine serum albumin in PBS, the plates were incubated with 50 μL of patients' sera followed by anti-IgE peroxidase conjugate (Sigma, St Louis, Missouri, USA) in proper dilution (1:1000 vol/vol in blocking solution). Each incubation step was followed by 3 washings of 5 minutes with PBS-Tween (10 mM PBS, pH 7.2 containing 0.05% Tween 20). O-phenylene diamine (OPD, 1 mg/mL in citrate-phosphate buffer, pH 5.0 with 0.01% H_2O_2) was used as the substrate for color development. The reaction was stopped by 4 M H_2SO_4 after 30 minutes at room temperature in the dark. The absorbance was measured with an ELISA reader at 492 nm. Specific IgE was expressed as the ratio of optical density of patient to pooled control sera (P/N value).

Changes in Specific IgG1 and IgG4 to *P sylvestris* pollen extract

Diluted patient serum or control serum (1:15 for specific IgG1 and 1:4 for specific IgG4 in diluent buffer; PBS-T containing 1% bovine serum albumin; 50 μL) was added to each well coated with *P sylvestris* pollen extract (protein content 1 μg/well). After incubation for 3 hours at 25°C, the wells were washed with PBS-T 3 times. Then 50 μL of 1:1000 (vol/vol) biotin-labeled goat antihuman IgG1 or IgG4 antibody (Sigma, St Louis, Missouri, USA) was added and incubated for 2 hours at 25°C. The wells were then washed three times with PBS-T and incubated with 1:1000 (vol/vol) streptavidin peroxidase (Sigma) for 30 minutes prior to another washing step, which was followed by another incubation with OPD substrate as described earlier. After the reaction was stopped, the optical density was measured at 492 nm. Specific IgG1 and IgG4 were also expressed as the P/N value for individual patients.

Results

The 1999 *P sylvestris* pollen season (baseline pre-treatment season) had a mean count of 12.9 pollen grains/d/m^3 during the peak month, February. The following 2 seasons had mean counts of 10.8 and 11.7 pollen grains/d/m^3 during the peak month, respectively, representing decreases of 29% and 15%. The SIT group had a 33.5% ($P < .01$) and 57% ($P < .001$) decrease in the symptom–medication score during first and second treatment seasons of 2000 and 2001 when compared to the baseline peak month. There was no significant change in the control group (Figure 1).

The SIT and control groups were matched for mean age (SIT, 32.22 years; controls, 32.59 years), male-to-female ratio (SIT, 12:5; controls 12:6), and seasonal onset of respiratory allergic symptoms. The percentage of patients

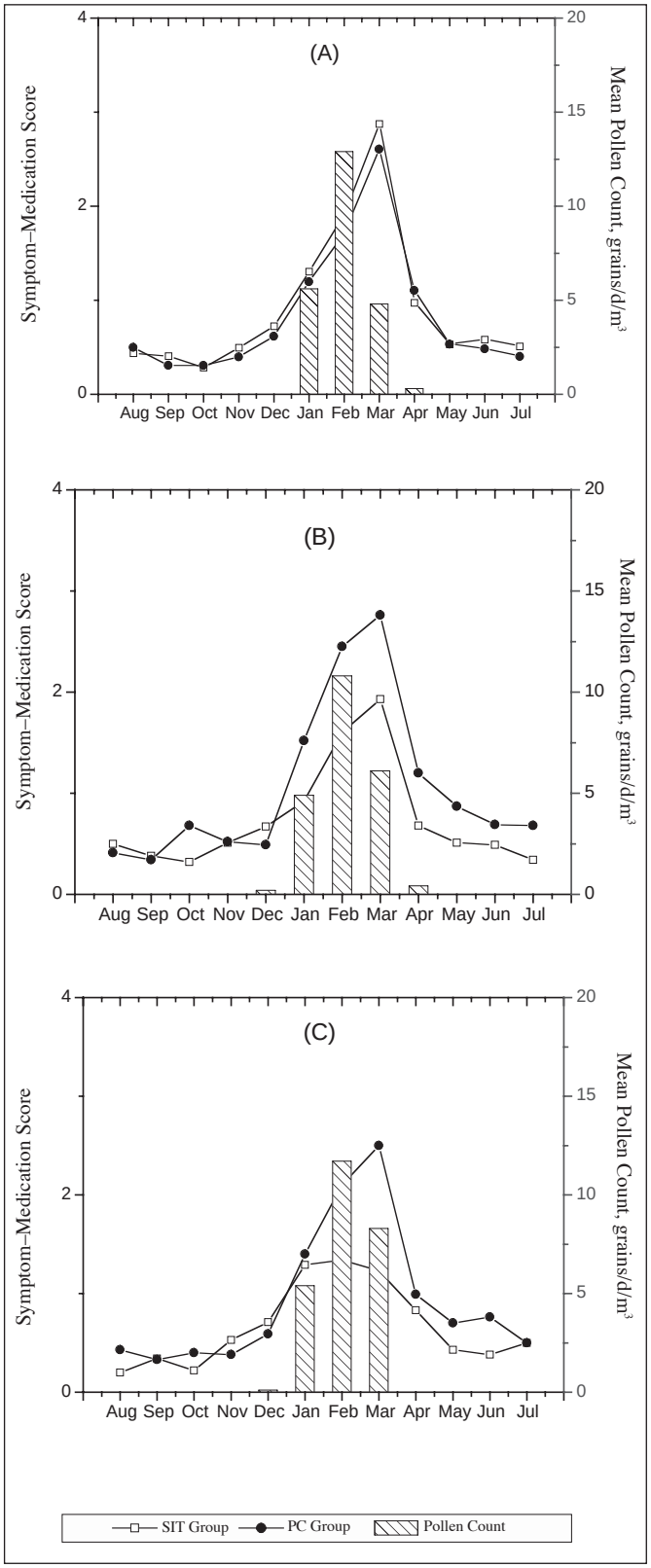

Figure 1. Changes in the symptom–medication score in the *Phoenix sylvestris* pollen season and at other times during the baseline study year (A), the first (B), and the second (C) treatment years in the allergen specific immunotherapy (SIT) and placebo control (PC) groups.

with seasonal allergy along with asthma was 44.44% (n = 8) in the SIT group and 41.17% (n = 6) in the control group. The mean wheal diameters in response to a SPT with *P sylvestris* pollen were similar in both groups upon patient enrollment; the ratio of the wheal diameter of the *P sylvestris* pollen extract to that of the positive control (histamine diphosphate) was 2.30 in the SIT group and 2.32 in the control group. All of the patients had seasonal rhinitis during the December–April period and 40% of them had bronchial asthma in addition to rhinitis. Five patients (2 in the SIT group and 3 in the control group) were monosensitive, whereas 45.71% were additionally sensitive to other related palm pollen types (*Areca, Borassus,* and *Cocos* species). Oligosensitive patients were sensitive to maximum of 3 pollen types altogether.

All patients completed the 2-year schedule. Figure 1 depicts the variation of average monthly symptom–

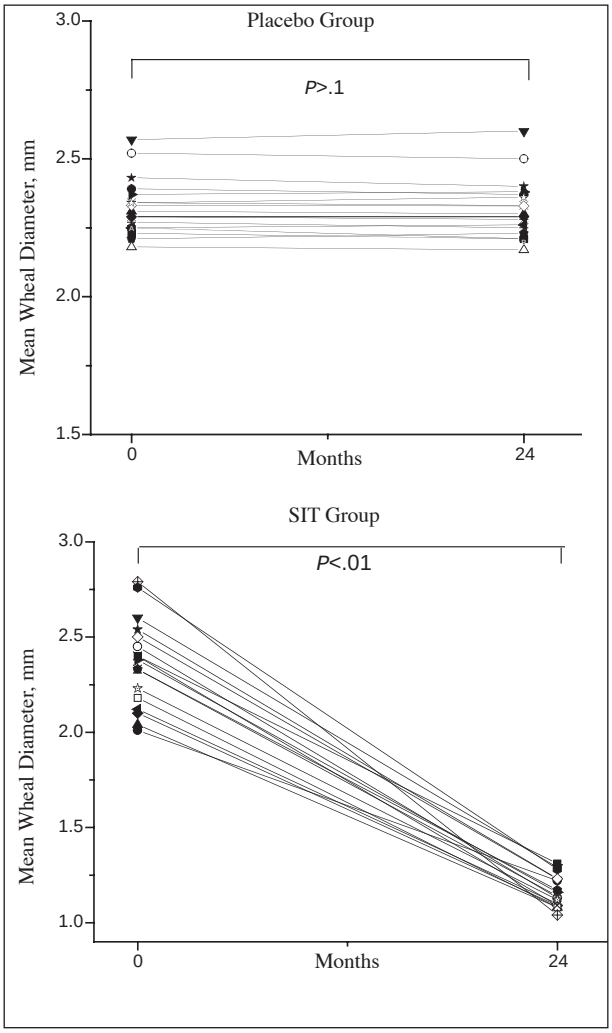

Figure 2. Changes in the mean wheal diameters (ratio of the wheal diameter against *Phoenix sylvestris* pollen extract to that of histamine) before starting and after finishing the therapy schedule in the allergen specific immunotherapy (SIT) and placebo control groups.

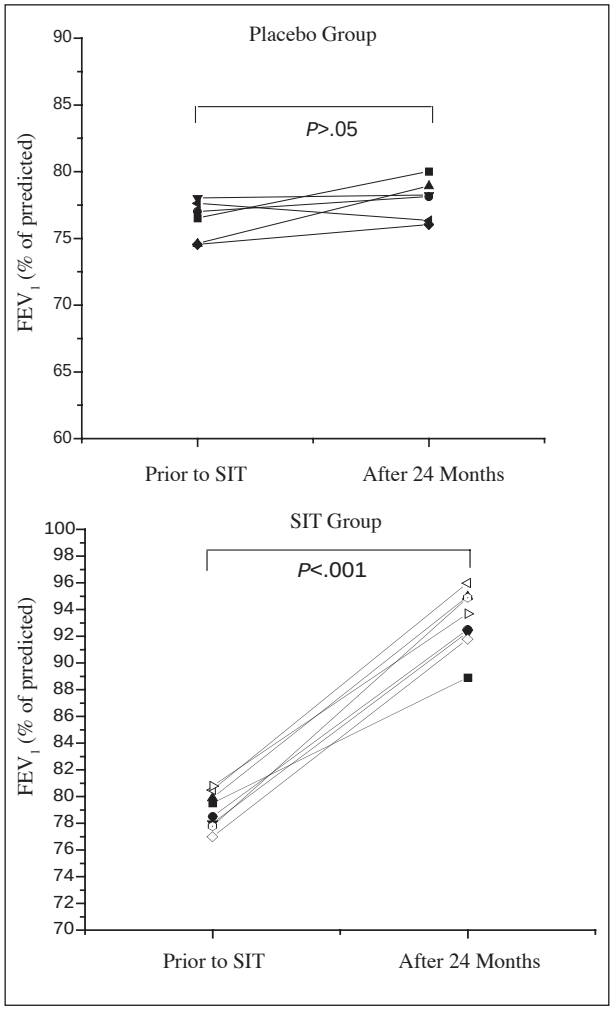

Figure 3. Changes in the forced expiratory volume in 1 second (FEV_1) (in the pollen season) before starting and after finishing 2 years of therapy in subjects with allergic rhinitis and asthma randomized to an allergen specific immunotherapy (SIT, n=8) or a placebo control (n=6) group.

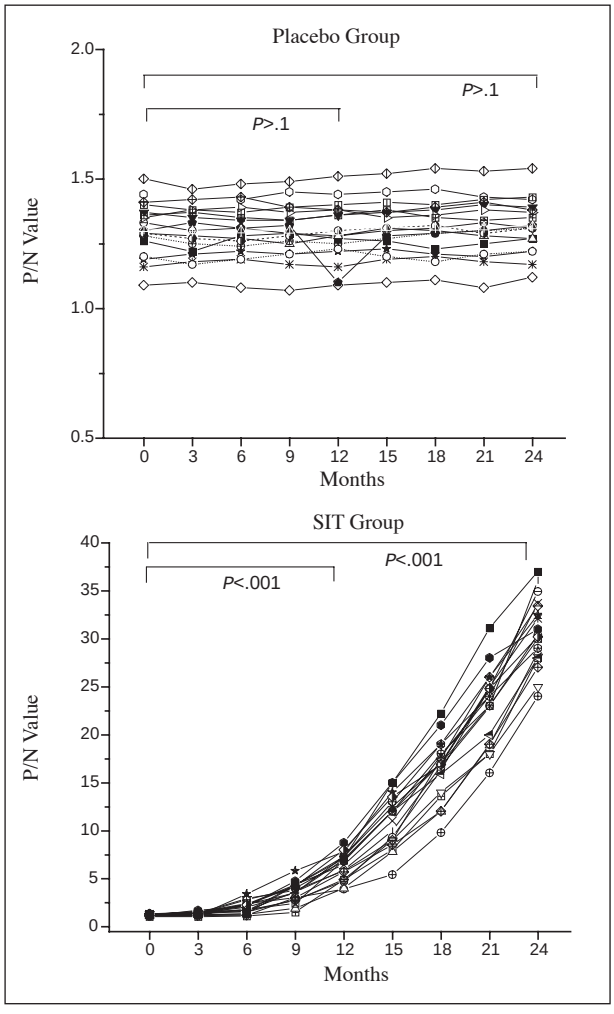

Figure 4. Changes in the level of specific IgG4 in the allergen specific immunotherapy (SIT) and placebo control groups during a 2-year therapy schedule. P/N indicates the ratio of optical density of patient to pooled control sera.

medication scores of both groups with as well as changes in airborne *P sylvestris* pollen levels. The mean symptom–medication scores were significantly correlated ($P < .001$) with the airborne pollen count. In the pretreatment season, the baseline score reached its peak in March (Figure 1A) following the pollen peak in February. During the first treatment season (Figure 1B), the symptom–medication score had less than 30% reduction during the peak period. In the second treatment season (Figure 1C), there was more than 50% reduction in the score in the same period. In both seasons, there was no significant change in the scores in the control group.

After 24 months of allergen immunotherapy, there was a significant decrease of skin reactivity to *P sylvestris* pollen extract in the SIT group ($P < .01$) but no significant change in the control group (Figure 2). In the control group 2 patients developed new sensitivities, 1 to grass (*Saccharum officinarum*) and 1 to *Alternaria alternata* spores.

In pulmonary function tests, we recorded a comparatively better performance for asthmatics in the SIT group than in the control group after completion of the 2-year schedule. FEV_1 also improved significantly between the baseline pollen season and after the SIT schedule (Figure 3).

Regarding immunoglobulin levels, there was no significant change in total serum IgE (data not shown) in either group ($P > .05$) between the pre- and posttreatment measurements. The *P sylvestris* pollen specific IgE level was significantly lower in the SIT group after a year ($P < .01$) and 2 years ($P < .01$), except for a certain amount of seasonal rise during the pollen season. In the control group, there was no overall significant change in specific

IgE level. The rise in specific IgG1 was remarkably significant ($P < .001$) after both 1 and 2 years of treatment in the SIT group. The mean specific IgG1 increased between 1.95 and 3.2 times after the 2-year period. In the control group there was a significant rise in specific IgG1 after a year ($P < .02$), but finally no significant change ($P > .1$) was found after 2 years. For specific IgG4 (Figure 4), there was a highly significant rise ($P < .001$) after both first and second years of treatment. The mean IgG1 value increased 21.24 to 30.83 times within this 2-year therapy period. For the control group, there was no significant difference ($P > .1$) after either 1 or 2 years.

Regarding the adverse reactions, we observed no systemic reaction in any of the 35 patients in the 2-year period. Four patients in the SIT group had episodes of local urticaria at the injection site and 1 had a single episode of local inflammation, giving a rate of local adverse events of 0.024% for a total of 2095 injections administered. All these reactions occurred in the maintenance phase. After dose reduction and an increase in the next interval for all patients experiencing such events, no more adverse effects occurred.

Discussion

The success of allergen immunotherapy depends on the use of consistently quality controlled and properly standardized extracts [13]. To date, no results from double-blind placebo-controlled randomized trials on SIT with any standardized pollen extract for seasonal respiratory allergy have been available for the Indian subcontinent. Our aim was to ascertain the safety and efficacy of SIT with *P sylvestris* pollen extracts.

There was no significant variability in seasonal *P sylvestris* pollen counts during the peak month of the 3 seasons. In addition, we found no significant difference between the baseline data of symptom–medication scores in the 2 groups. The overall score in the SIT group showed a 57% decline after 24 months of treatment, a difference which is remarkably significant ($P < .001$). Another report from Calcutta on placebo-controlled immunotherapy with *Cocos nucifera* pollen extract demonstrated significant ($P < .005$) clinical improvement after 6 to 12 months [14]. In immunotherapy, clinical improvements are usually observed during the first year [15], and the benefits remain for several years even after discontinuation [16], indicating a persistent immunological change during treatment.

We found a significant decrease in wheal diameter in the SIT group and no additional development of sensitization to other allergens. Lung function tests also demonstrated a significant improvement in the respiratory function of asthmatics in the SIT group. Such results support the notion that immunotherapy may have a protective role against new sensitivity [17] as well as the development of asthma in patients suffering from rhinoconjunctivitis [6].

Regarding the mechanism of SIT, earlier studies reported a decrease in specific IgE and an increase in specific IgG subclass levels [18]. There is a demonstrated immunomodulation from type 2 helper T cells to type 1 response [19], cytokine regulation, specific inhibition or activation of immunotolerance, which have reinforced the importance of allergen immunotherapy [20]. We found no significant change in total IgE level before and after SIT in either group of patients. The specific IgE level showed a significant decline after 12 months and 24 months of treatment in both groups.

There was a significant increase in specific IgG1 and an approximately 30-fold increase in allergen specific IgG4 levels in the SIT group. It is frequently observed that during immunotherapy, increased levels of specific IgG1 and IgG4 are related to clinical improvement [21, 22]. Among the proposed theories on the mechanism of action in immunotherapy, induction of antibodies antagonizing the activity of allergen specific IgE supports such observations in many cases [23,34], although there are controversies [25]. When a substantial increase in specific IgG4 level has been induced, SIT may be considered to be immunologically effective [26].

The probable cause of the relatively low rate of adverse reactions (0.024% of all injections) and no systemic reactions in the study period is the use of a higher dilution for the maintenance dose (1:2500 wt/vol instead of the recommended 1:30–1:100 wt/vol for pollen extracts) in the trial [27]. Though it has been reported that very low maintenance doses (eg, $1:10^6$) are not effective [28], our dilution seems to be sufficient to be effective and resulted in a significant change in clinical and immunological parameters.

Our results suggest that subcutaneous immunotherapy with standardized *P sylvestris* pollen allergen may be helpful for the treatment of susceptible seasonal respiratory allergic subjects with improved clinical and immunologic outcome.

Acknowledgements

Thanks are due to the Council of Scientific and Industrial Research, New Delhi, for providing a grant to the first author as research associate and senior research associate. We also thank the Director of the Institute of Child Health, Calcutta, for his kind approval of the project.

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Swati Gupta-Bharracharya

Department of Botany, Bose Institute
93/1 Acharya Prafulla Chandra Road
Calcutta, India
E-mail: swati@bosemain.boseinst.ac.in; swatigb29@yahoo.com

