

Original article

Clinical and immunologic evaluation of *Cedrus deodara* pollen: a new allergen from India

Background: Allergy to pollen from gymnosperms is well documented in the West. However, many allergenic species are native to the Himalayan region of India, and *Cedrus deodara* (Pinaceae) was selected for allergologic investigation. The objective was to define the allergologic and immunochemical aspects of *C. deodara* pollen.

Methods: Pollen antigen from *C. deodara* (CD) was prepared and characterized by biochemical and biologic assays. Specific IgE binding was determined by means of ELISA and immunoblotting.

Results: CD pollen antigen caused marked skin sensitivity in 7.5% of an atopic population. A significantly elevated level of CD-specific IgE antibodies was observed in 65.8% of the skin-positive patients. Immunoblotting showed protein fractions of 37, 44, 58, and 78 kDa with 100% binding with the patients' sera suspected to be due to carbohydrate moieties.

Conclusions: Patients from the Himalayan region, where CD occurs naturally, were sensitized more than patients from distant places. The immunochemical characterization revealed multiple protein fractions from low to very high molecular mass (14–126 kDa) mostly in the acidic pI range. CD pollen has been recognized as a new allergen from India for the first time.

A. Rawat, A. Singh, A. B. Singh

Aerobiology and Allergy Laboratory, Centre for Biochemical Technology, Delhi

S. N. Gaur

V.P. Chest Institute, Delhi

L. Kumar

Department of Pediatrics, PGI, Chandigarh

I. Roy

Institute of Child Health, Calcutta

P. Ravindran

Medical College, Trivandrum, India

Key words: allergen characterization; *Cedrus deodara*; Himalayan tree; sensitization.

Dr A. B. Singh
Aerobiology and Allergy Laboratory
Centre for Biochemical Technology
Delhi University Campus
Mall Road
Delhi-110 007
India

Accepted for publication 15 February 2000

The role of pollen as a causative agent of respiratory allergic disorders is very well established, as is evident from the recent increase of reports from across the world (1–4). India is blessed with the richest flora on the earth, from alpine tundra to Rajasthan desert. Consequently, it provides considerable variation in the quality and quantity of airborne pollen in different ecogeographic regions of the country (5–8). Although studies on the allergenic properties of airborne pollen from various species have been carried out by several workers in India (9–12), information on allergy to aerial pollen from Himalayan tree species has been completely

lacking, although a sizable population is exposed to various Himalayan airborne pollens, and many are likely to be sensitized.

Pine pollen, one of the important components of Himalayan air spora, is not considered to be highly allergenic although some reports suggest sensitization to its pollen as well (13–15). Beside natural pine forests in the Himalayan region, pine trees are also planted in parks and gardens in various parts of the country, and people are exposed to variable concentrations of its pollen. *Cedrus deodara* (CD), also belonging to the family Pinaceae, is a large evergreen tree and occurs as natural forest cover in the outer Himalayas from Kashmir to Garhwal at an altitude of 1300–3500 m above msl. It has been reported to be one of the important components of airborne pollen in the Himalayan region. Its pollen is present in the air from

Abbreviations. CD: *Cedrus deodara*; ELISA: enzyme-linked immunosorbent assay; IEF: isoelectric focusing; NC: nitrocellulose; OD: optical density; SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis.

August to December, with maximum concentration during October (16–19). The pollen of CD is even transmitted to the plains of India about 500 km from the source (20).

In view of the extensive distribution of CD in the Himalayas and the prevalence of its pollen in the atmosphere of the Himalayas and the distant plains, we decided to study the allergenic properties of its pollen with particular reference to respiratory allergic disorders in the population of different geographic regions of India, and partially to characterize its pollen antigen.

Material and methods

Source material

The pollen was collected from the Shivalik range of the Himalayan region during the pollination period in October–November. These pollen grains were then processed for >95% purity by sieving through different grades of sieves (100, 200, and 300 mesh/cm²). To remove lipids and irritants of low molecular mass, the pollen sample was defatted with diethyl ether by repeated changes, until the ether became colorless. The defatted pollen powder was then completely vacuum-dried and stored at 4°C in airtight containers until further use.

Antigen preparation

The defatted dried pollen powder was extracted at 1:20 (w/v) in 0.05 M phosphate-buffered saline (pH 7.8) by continuous stirring for 20 h at 4°C. The supernatant was cleared by centrifugation at 10 000 rpm for 30 min and then dialyzed against distilled water with a Visking tube (cutoff 7 kDa) for 24 h, with repeated changes of water. Subsequently, centrifugation was repeated, and the supernatant was passed through a Millipore filter membrane (0.45 µm), lyophilized in small aliquots, and stored at –20°C until further use.

Protein content

The protein content of CD-pollen extract was estimated by the method of Lowry et al. (21) with a slight modification, as the protein was precipitated with phosphotungstic acid and then dissolved in 2% sodium hydroxide. The experiment was repeated five times within 10% variability, and the mean value was taken for protein concentration.

Skin tests

Selection of patients. To assess the allergenicity of the antigenic extract, the skin prick test was performed uniformly on 531 patients with nasobronchial allergy attending four hospitals (Delhi, Calcutta, Chandigarh, and Trivandrum) in India. The diagnosis and symptomatology of these patients are given in Table 1.

Table 1. Diagnosis and symptomatology of 531 patients (322 male, 209 female) selected for skin prick test with CD-pollen extract

Place	Total no. of patients tested	Diagnosis*			Symptomatology**		
		BA	AR	BA + AR	S	P	I
Chandigarh	86	40	15	31	40	17	29
Calcutta	182	28	45	109	73	24	85
Delhi	200	9	40	81	83	33	84
Trivandrum	63	11	14	38	25	19	19

* Diagnosis: BA – bronchial asthma; AR – allergic rhinitis.

** Symptomatology: S – seasonal; P – perennial; I – irregular.

Prick test. The lyophilized antigen was reconstituted for skin prick test at 1:10 w/v ratio in 50% glycerinated phosphate-buffered saline (pH 7.8). Along with the pollen extract, glycerinated buffered saline and histamine hydrochloride (1 mg/ml) were used to act as negative and positive controls, respectively. The wheal diameter and erythema were measured after 15–20 min of the test and graded according to the criteria laid down by Singh et al. (22). Beside CD extract, other common inhalant allergens were also tested on these patients to detect their offending allergens (Table 2) for diagnosis and immunotherapy, but this does not form a part of this study.

Serum samples. Venous blood was drawn from patients showing marked skin positivity. Blood was also drawn from patients showing negative skin reaction to act as control. Expressed sera were cleared of cell debris by centrifugation and stored at –70°C for subsequent immunologic studies.

Enzyme-linked immunosorbent assay (ELISA)

Specific IgE antibodies against CD antigen were measured by indirect ELISA by the method of Sepulveda et al. (23). Polystyrene 96-well, microtiter plates (Nunc, A/S Roskilde, Denmark) were coated with CD extract and incubated overnight at 4°C. The plates were then thoroughly washed with 0.05 M phosphate-buffered saline (PBS), pH 7.4. After thorough washing, free sites were blocked with 1% BSA in PBS at 37°C for 2 h.

Table 2. List of allergens tested at four medical centers in India

Pollen	
<i>Ailanthus excelsa</i>	<i>Alnus nitida</i>
<i>Amaranthus spinosus</i>	<i>Cedrus deodara</i>
<i>Cassia fistula</i>	<i>Cynodon dactylon</i>
<i>Delonix regia</i>	<i>Dodonaea viscosa</i>
<i>Holoptelea integrifolia</i>	<i>Mallotus philippensis</i>
<i>Prosopis juliflora</i>	<i>Ricinus communis</i>
<i>Sarcococca saligna</i>	<i>Syzygium cumuni</i>
Fungus	
<i>Alternaria alternata</i>	<i>Aspergillus fumigatus</i>
<i>A. versicolor</i>	<i>Cladosporium cladosporioides</i>
House dust	

Table 3. Results of skin prick test with CD extract (1:10 w/v) at different centers

Sites	Allergic subjects skin tested	Skin reactivity*			Positivity			
					1+ to 3+		2+ and 3+	
		1+	2+	3+	n	%	n	%
Chandigarh	86	3	7	1	11	12.8	8	9.3
Delhi	182	4	11	2	17	9.3	12	7.1
Calcutta	200	38	16	—	54	27.0	16	8.0
Trivandrum	63	5	3	—	8	12.7	3	4.8
Total	531	50	37	3	90	16.9	40**	7.5

* No 4+ skin reactivity was observed.

** Only two patients out of 40 were monosensitized to *C. deodara*.

Again, after washings, sera diluted to 1:10 (0.05% BSA in PBS) were added to each well and incubated at 4°C. Alkaline phosphatase-labeled antihuman IgE antibodies diluted 1:1000 with 0.05% BSA in PBS were used as secondary antibody, and were incubated for 4 h at 37°C. For color development, *p*-nitrophenyl phosphate (pnpp) was used as substrate. After terminating the reaction with 5 N NaOH, optical density (OD) was measured at 410 nm with the Immuno-Reader (Dynatech Medical Products Ltd, Guernsey, UK).

Statistical analysis

OD values for different serum samples were analyzed according to the criteria of Kauffman et al. (24). Percent binding of CD-specific IgE was calculated for each sample in relation to control sera and pool of highly positive sera detected by ELISA. OD values depending on percent binding were categorized into four groups: 0–15%, 15–30%, 30–50%, and >50%. Sera showing more than 30% binding were considered to have significantly raised ($P < 0.01$) specific IgE against CD-pollen extract. The standard deviation of OD values at each center was also calculated.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE)

SDS–PAGE was carried out by the vertical discontinuous method with the buffer system of Laemmli (25) with Mini-Protein II slab gel apparatus (Bio-Rad, Hercules, CA, USA). The proteins were electrophoresed on 12% separating gel at constant current. Protein bands were detected by staining with Coomassie

brilliant blue R-250. The molecular mass of the fractions was calculated by calibrating with standard marker protein (Pharmacia, Uppsala, Sweden).

Isoelectric focusing (IEF)

IEF was performed, as described by Grafin (26), with precasted, broad-range (3.5–9.5) Ampholine PAG plates (Pharmacia, Uppsala, Sweden). Pollen extract was electrofocused on gel along with pI marker (Pharmacia, Uppsala, Sweden) to calibrate the pI of proteins in CD extract. IEF was run for 15 min at 100 V, followed by another 15 min at 200 V, and finally at 450 V for 2 h. The gel was stained with Coomassie brilliant blue R-250, as in SDS–PAGE.

Immunoblot

The proteins separated on SDS–PAGE were electrophoretically transferred to nitrocellulose (NC) membrane by the method of Towbin et al. (27) with Bio-Rad Mini-Transblot apparatus at 4°C. To block the free-binding sites, protein-loaded NC membrane was treated with 3% BSA in PBS (pH, 7.4) containing 0.05% Tween 20 (PBST) for 3 h at 37°C. After thorough washings with PBST, membranes were cut to the size of individual lanes of the gel and incubated with pooled sera as well as sera of individual patients (diluted 1:5 in 1% BSA in PBS). The strips were washed and incubated with peroxidase-labeled antihuman IgE (1:1000 with 1% BSA in PBS). The bound antibodies were detected by immunostaining with substrate solution (3'-3' diamino

Table 4. Percent binding of CD-specific IgE antibodies in sera of clinically sensitive patients as compared to control performed by ELISA

Places	No. of sera tested	% Binding OD values at 410 nm				% Correlation
		<15 0–0.072	15–30 0.073–0.155	30–50 0.156–0.265	>50% >0.265	
Chandigarh	6	1	—	3	2	83%
Delhi	13	—	4	5	4	69.2%
Calcutta	16	8	—	2	6	50%
Trivandrum	3	—	—	1	2	100%
Total/average	38	9	4	11	14	65.8%

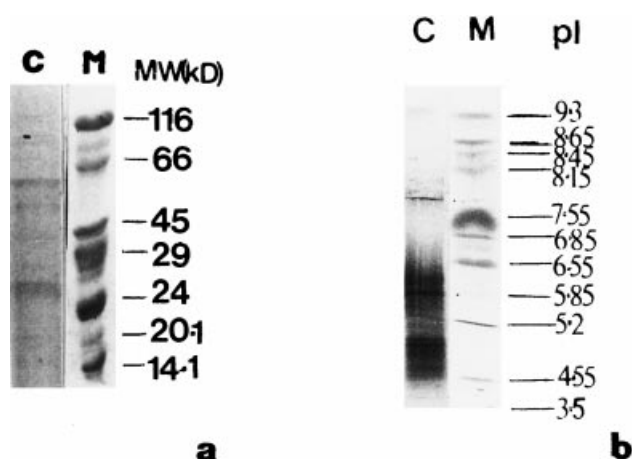


Figure 1. a) SDS-PAGE protein profile of antigenic extract of *C. deodara* (C) pollen in 12% discontinuous gel. M: molecular weight markers. b) IEF pattern of proteins of *C. deodara* pollen extract (C) pI range (3.5–9.5) PAG plates. M: pI markers.

benzidine in PBS, 0.15% H_2O_2). Developed strips were treated with distilled water containing 0.2 N HCl in traces for 2–3 min and stored.

Results

Protein concentration

The mean soluble protein content of the pollen extract of CD was observed to be only 1.76 mg/ml of the 1:10 (w/v) concentration.

Skin test

The results of skin prick tests conducted on 531 patients with nasobronchial allergy at four different centers are presented in Table 3. The highest skin reactivity with CD antigen (1+ to 3+ grade) was observed in patients at Calcutta (27%), followed by Chandigarh (12.8%), Trivandrum (12.7%), and Delhi (9.3%). Subjects show-

Table 5. Mean OD values at 410 nm for specific IgE antibodies in sera of skin-prick-test-positive patients from different centers in India

Center	No. of sera tested	Mean OD value	SD
Chandigarh	6	0.214	± 0.1195
Delhi	13	0.251	± 0.1636
Calcutta	16	0.232	± 0.3086
Trivandrum	3	0.267	± 0.0774

ing marked positivity, i.e., 2+ and above degree of reaction, were considered clinically important, as they have been reported to correlate better with patient history and other diagnostic tests such as provocation test and ELISA (28, 29). The maximum 2+ and above positivity was observed in patients at Chandigarh (9.3%), followed by Calcutta (8%), Delhi (7.1%), and Trivandrum (4.8%). Monosensitivity to CD extract was found only in two patients from Chandigarh. An average of 7.5% of the patient tests showed 2+ and above skin positivity from all the centers, constituting the sensitization pattern of the Indian population. Most of the patients tested against common inhalant allergens showed multiple sensitivity in the range of 2.1–11% among the different centers.

Specific IgE (ELISA)

The specific IgE-binding (percent) pattern in the sera of 38 skin-positive patients (2+ and above) from different places in India, as compared to control sera showing negative skin test to CD extract, is analyzed in Table 4. Of the 38 sera tested, nine showed binding of 0–15% and were considered negative. CD-specific binding of 15–30% was observed in four serum samples, which were considered positive. Serum samples from 11 patients showed more than 30% binding, but less than 50%, while the remaining 14 sera showed even more than 50% binding with the antigen, and these results were

Table 6. Specific IgE-binding fractions of CD extract with pooled * and 12 individual sera of clinically sensitive patients

Sera no.	Molecular mass (kDa)																		Total
	126	124	116	87	86	78	67	66	62	58	53	44	42	39	37	32	28	25	
1.*	+	+	+	–	+	+	+	+	+	+	+	+	+	–	+	+	–	+	16
2.	+	+	+	–	–	+	+	+	–	+	+	+	–	–	+	+	–	+	13
3.	+	+	+	–	–	+	+	+	+	+	+	+	+	–	+	+	–	+	15
4.	+	+	+	–	–	+	+	+	+	+	+	+	+	–	+	+	–	+	15
5.	+	+	+	–	+	+	–	–	+	+	+	+	+	–	+	+	–	+	14
6.	+	+	–	–	–	+	+	–	+	+	+	+	+	–	+	+	–	+	13
7.	+	+	+	–	–	+	+	+	–	+	+	+	+	–	+	+	–	+	14
8.	+	+	+	–	–	+	+	+	–	+	+	+	+	–	+	+	–	+	14
9.	+	+	–	–	–	+	+	+	–	+	+	+	+	–	+	+	–	+	13
10.	+	+	–	–	–	+	–	+	+	+	+	+	+	–	+	+	–	+	13
11.	+	+	–	–	–	+	–	+	+	+	+	+	+	–	+	+	–	+	13
12.	+	+	–	–	–	+	–	+	+	+	+	+	+	–	+	+	–	+	13
13.	+	+	+	–	+	+	–	+	+	+	+	+	+	–	+	+	–	+	15

Protein fractions of 22, 25, 32, 37, 44, 53, 58, 78, 124, and 126 kDa showed 100% with IgE antibodies. Fractions of 14, 17, 28, 39, and 87 did not show binding with any serum sample.

considered to be statistically significant for CD-specific IgE in comparison to control sera.

About 66% of the patients eliciting (2+ and above) marked positivity had shown elevated specific IgE titer in their sera. When OD values were analyzed by calculating standard deviation (Table 5), the least SD (+0.0774) was recorded in the binding pattern of sera from Trivendrum, while the maximum was recorded (+0.0386) in samples from Calcutta.

SDS-PAGE

The antigenic extract of CD pollen resolved into 17 distinct bands in the range 14–116 kDa (Fig. 1a). Pro0-4tein fractions of 37, 53, 87, and 116 kDa were most prominent and were stained strongly by Coomassie brilliant blue. However, protein fractions of 22, 25, and 67 kDa were less distinct and sharp. Bands at 14, 17, 28, 32, 39, 42, 44, 62, 66, 78, and 86 kDa were weak and poorly stained, but were clearly visible and distinguishable on gel.

IEF

Altogether, 20 bands were detected by IEF, of which 18 were in the acidic region (4.5–6.5), and only two bands at pI 7.1 and 9.3 were observed in the basic region (Fig. 1b). Protein bands at pI 4.3, 4.9, 5.0, 6.0, 6.1, and 9.4 were prominent and intensely stained, while bands at pI 4.5 and 4.8 were distinct but fine. However, other fractions were weak and poorly stained.

Immunoblot

The specific IgE-binding fractions of CD extract detected in the sera of nasobronchial allergic patients are analyzed in Table 6. Of the 17 protein fractions of CD antigen that resolved on SDS-PAGE, 13 were identified as specific IgE-binding fractions in the pooled

sera from 12 patients (Fig. 2). Apart from these, two high-molecular-mass protein fractions of 124 and 126 kDa and two of low mass (62 and 58 kDa) were also detected by immunoblot, but were not visible in SDS-PAGE fractionation. Protein fractions when probed with sera of individual patients showed more than 14 fractions binding with the sera of six patients. Sera from another six patients showed binding with 13 protein fractions of CD extract varying from 22 to 126 kDa. Interestingly, 10 IgE-binding fractions with molecular masses of 22, 25, 32, 37, 44, 43, 58, 78, 124, and 126 kDa were detected in the sera of all the 12 patients, thus showing 100% binding. Of these, fractions at 37, 44, 58, and 78 kDa were more prominent. It was observed that protein fractions of 14, 17, 28, 39, and 87 kDa did not show binding with either pooled or individual sera tested.

Discussion

The pollen of CD constitutes an important component of the Himalayan air spora in India and has also been reported to be present in the air of distant regions of the country due to sporadic plantations and the fact that the pollen is anemophilous (8, 16–19). Although the size of the pollen grains is large (65–70 μm), either whole grains or small fragments may penetrate the upper airways and induce type I hypersensitivity (30). Human sensitization to CD pollen in the Indian population has been assessed in the present study by skin prick test and CD-specific IgE antibodies in the sera of nasobronchial allergy patients.

The skin prick tests were conducted with 1:10 (w/v) concentration of CD extract, as other inhalant allergens being tested routinely by allergologists and supplied by manufacturers in India are in the same (w/v) concentration. The results with 1:10 concentration were found not to produce nonspecific positivity in healthy volunteers.

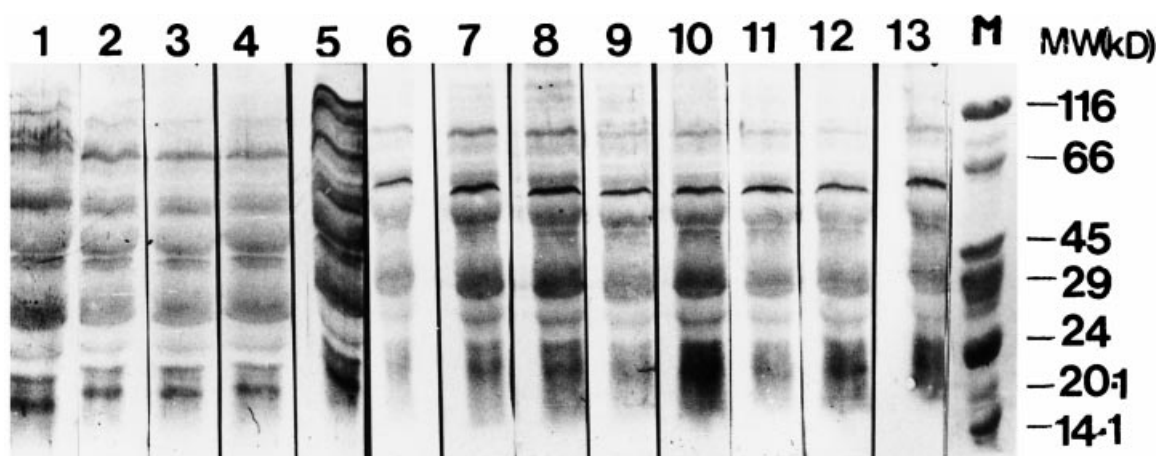


Figure 2. Specific IgE-binding protein fractions of pollen extract of *C. deodara* when transferred to NC membrane and probed with 12 individual and pooled serum samples from ELISA-positive patients. Lane 1) pooled sera; 2–13) individual patient serum; M) molecular weight markers.

Furthermore, no correlation was found between total soluble protein content and skin reactivity (31). To assess the biologic potency of the pollen antigens tested, only 2+ and higher skin reactions were taken into consideration, since they are likely to be clinically and immunologically significant, unlike 1+ skin reactions, which are also reported in a certain percentage of normal nonallergic individuals. Similar observations have also been made in our practice (28, 29).

The results of skin prick tests conducted at four different allergy centers in India with the extract of CD pollen revealed a variable degree of sensitization. No other pollen antigens from the Pinaceae family were tested along with CD. CD was tested on 531 patients and produced average skin positivity in 7.5% of the nasobronchial allergy patients. Only two patients were found to be monosensitized to CD. The skin positivity (2+ and above) in atopic patients at Chandigarh was highest, with 9.3% as compared to 8% at Calcutta and only 7.1% at Delhi. Patients showed the highest skin positivity at Chandigarh probably because of frequent exposure to CD pollen there. This city is located at the foothills of the Himalayas, and the patients reporting to this clinic are mostly from the northern Himalayan region. Although the CD tree does not grow naturally in the Calcutta and Delhi regions, CD pollen has been reported to drift in from the Himalayan region (20). Long-distance transport of allergenic pollen has also been reported for birch (*Betula* sp.) and *Juniperus ashi* (32, 33). Patients from Trivandrum, which is located in the extreme south of the country, far from the Himalayas, have shown the least sensitization (4.3%).

CD positivity among patients at places distant from the Himalayas, such as Trivandrum, could be due to cross-reactivity with pollen of other members of the same family (Pinaceae), as trees of *Pinus* spp. are planted in parks and gardens outside the natural habitat of the species, and release pollen into the local atmosphere with low frequency and concentration. Cross-reactivity among members of the same family has already been reported by several workers (34, 35). This could also be due to cross-reactivity of pollen antigens from even unrelated species, as reported by some workers (36). Skin sensitivity among patients who are exposed to low levels of allergens or even among unexposed patients may also occur, as reported by Lacovacci et al. (37), partly confirming our observation of low-level sensitization to CD pollen from Trivendrum due to cross-reactivity.

Significantly ($P < 0.01$) elevated levels of CD-specific IgE antibodies in the sera of patients showing 2+ or above positivity has been found. Of all 38 sera tested for CD-specific IgE, 65.8% showed greater than 30%

binding, as compared to control sera. The relatively weak sensitization pattern in the patients from Calcutta and Trivandrum was reflected by a low specific IgE titer in the sera of patients from these places. This could again be due to low pollen exposure to other members of the Pinaceae family.

The low concentration of protein in the CD extract is in conformity with the general observation of low protein content in the pollen of gymnosperms (37–39). Protein fractions of CD crude extract are present in the wide range of 14–116 kDa. High-molecular-mass protein has also been reported in the pollen antigen of *Pinus radiata* by Fountain & Cornford (15). Multiple protein fractions of 10–100 kDa in other gymnosperm species have been reported from Europe (37–39). By IEF, we were able to detect 20 protein bands mostly in the acidic range (4.5–6.5), except for two fractions observed in the basic region (pI 7.05 and 9.4).

Immunoblotting has been found to be an important laboratory tool to confirm the acceptability of source material for clinical studies. By immunoblot, we were able to detect 16 IgE-binding fractions from the sera of allergic patients, of which four fractions of 58, 62, 124, and 126 kDa were identified only by immunostaining, as these fractions were not observed in SDS-PAGE. A distinct heterogeneity was not observed in the binding pattern of 12 sera probed individually. The large number of bands (22, 25, 32, 37, 44, 53, 58, 78, 124, and 126 kDa) recognized by most of the 12 sera tested could be due to carbohydrate cross-reacting moieties in the CD-pollen extract. Cross-reactivity of carbohydrate moieties with specific IgE has also been reported by Afferni et al. (40).

In conclusion, more than 7% of nasobronchial allergy patients from India showed sensitization to CD-pollen extract. A significantly high level of CD-specific IgE antibodies was observed in the sera of patients. The crude pollen antigen of CD has been characterized, yielding 20 protein fractions in the range of 14–126 kDa. Both low- and high-molecular-mass protein fractions have been observed to bind to CD-specific IgE antibodies. A considerable degree of allergen cross-reactivity with carbohydrate moieties is suspected in the CD-pollen allergen. Allergen cross-reactivity is increasingly important for molecular characterization of allergens, and must be taken into account in the diagnosis of allergic patients.

Acknowledgments

We thank the Ministry of Environment and Forests, India, for financial assistance, and Mrs Charu Lal for providing technical help.

References

- ORTEGA G, MORTNEZ PJ, MARTINIZ H, PALACIOS R, BELMONTE J, RICHART C. *Mercurialis annua* pollen: a new source of allergy sensitization and respiratory disease. *J Allergy Clin Immunol* 1992;**89**:987–993.
- SINGH AB, SINGH A. Pollen allergy – a global scenario. In: AGASHE SN, editor. *Recent trends in aerobiology and immunology*. Oxford: IBH, 1994:143–173.
- AMATO D, SPIEKSMAN FThM, LICCARDI G, et al. Pollen-related allergy in Europe. *Allergy* 1998;**53**:567–578.
- ARNON G, COHEN RC, YOAV W. Allergic response to pollen of ornamental plants: high incidence in general atopic population and especially among flower growers. *J Allergy Clin Immunol* 1998;**102**:210–214.
- SINGH AB, BABU CR. Studies on pollen allergy in Delhi: diurnal periodicity of common allergenic pollen. *Allergy* 1980;**35**:311–317.
- SINGH AB, BABU CR. Studies of atmospheric pollen allergens in Delhi: seasonal periodicity. *Ann Allergy* 1982;**48**:115–122.
- GUPTA S, CHANDA S. Aeropalynological survey in subtropical eastern Himalayas, Kurseong. *Grana* 1984;**28**:219–221.
- NAIR PKK, JOSHI AP, GANGAL SV. Airborne pollen spores and other plant materials of India – a survey. Lucknow: CSIR Centre for Biochemicals, Delhi and NBRI, 1986.
- SHIVPURI DN, SINGH AB, BABU CR. New allergenic pollens of Delhi state, India and their clinical significance. *Ann Allergy* 1979;**42**:49–52.
- SINGH AB. Airborne pollen types of allergenic significance in India. *Adv Aerobiol (Experientia Suppl)* 1987;**51**:61–64.
- SINGH AB, MALIK P, PARKASH D, GANGAL SV. Identification of specific IgE binding proteins in castor bean (*Ricinus communis*) pollen obtained from different source materials. *Grana* 1993;**31**:376–380.
- MONDAL AK, PARUI S, BISWAS SR, MONDAL S. Identification of the allergenic proteins of *Ipomea fistulosa* pollen – partial characterization and sensitivity test. *Grana* 1997;**36**:301–305.
- NEWMARK FM, ITKIN IH. Asthma due to pine pollen. *J Allergy* 1967;**25**:251–252.
- HARRIS RM, GERMAN DF. The incidence of pine pollen reactivity in an atopic population. *Ann Allergy* 1985;**55**:678–679.
- FOUNTAIN DW, CORNFORD CA. Aerobiology and allergenicity to *Pinus radiata* pollen in New Zealand. *Grana* 1991;**30**:71–75.
- LAKHANPAL RN, NAIR PKK. Atmospheric pollen survey at Almorah. *J Sci Ind Res* 1960;**17c**:80–87.
- KUMAR R. Aerospora in pine forests in India. *Grana* 1982;**21**:179–181.
- GAUR RD, KALA SP. Studies on the aerobiology of a Himalayan alpine zone, Rudranath, India. *Arctic Alpine Res* 1984;**16**:173–183.
- GAUR RD, KALA SP. Studies on aerobiology of Gopeshwar – spring periodicity of airborne pollen grains. *J Indian Bot Soc* 1984;**63**:266–271.
- MALIK P, SINGH AB, BABU CR, GANGAL SV. Atmospheric concentration of pollen grains at human height. *Grana* 1991;**30**:129–139.
- LOWRY O, ROSENBOROUGH N, FARR A, RANDALL R. Protein measurement with Folin phenol reagent. *J Biol Chem* 1951;**193**:263–275.
- SINGH AB, MALIK P, PARKASH D, GUPTA CK. Biological standardization of pollen allergens from India. *Asia Pac J Allergy Immunol* 1992;**10**:103–109.
- SEPULVEDA HJ, LONGBOTTOM J, PEPYS J. Enzyme linked immunosorbent assay (ELISA) for IgG and IgE antibodies to protein and polysaccharide antigens of *Aspergillus fumigatus*. *Clin Allergy* 1979;**9**:359–357.
- KAUFFMAN HF, BEAUMONT F, MEURS H, VAN DER HEIDE S, DE VRIES K. Antibody measurements against *Aspergillus fumigatus* by means of double diffusion and enzyme-linked immunosorbent assay (ELISA). *J Allergy Clin Immunol* 1983;**72**:255–261.
- LAEMMLI UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970;**227**:680–685.
- GRAFIN DE. Isoelectric focussing. *Methods Enzymol* 1990;**182**:459–477.
- TOWBIN H, STAEGHALIN T, GORDON J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci U S A* 1979;**76**:4350–4354.
- RACKEMAN FM, SIMON FA. Technique of intracutaneous test and result of routine test in normal persons. *J Allergy* 1935;**5**:204–207.
- SHIVPURI DN, SINGH K. Studies in yet unknown allergenic pollens of Delhi state metropolitan (clinical aspects). *Indian J Med Res* 1971;**50**:1411–1419.
- MICHEL FB, MARTY JP, QUET L, COUR P. Penetration of inhaled pollen in the respiratory tract. *Am Rev of Respir Dis* 1977;**115**:609–615.
- SINGH AB. Studies on atmospheric pollen of Delhi with reference to respiratory allergy. PhD thesis, Delhi University, 1977.
- WALLIN JE, SEGERSTRÖM U, ROSENHALL L, BERGMANN E, HJELMROOS M. Allergic symptoms caused by long-distance transport of birch pollen. *Grana* 1991;**30**:265–268.
- ROGERS C, LEVETIN E. A two year investigation of long distance transport of mountain cedar pollen into Tulsa, OK. Abstracts, International Congress of Aerobiology, Perugia, Italy, 1998, p. 40.
- BARLETTA B, AFFERNI C, TINGHINO R, MARI A, DI FALICE G, PINI C. Cross-reactivity between *Cupressus arizonica* and *Cupressus sempervirens* pollen extract. *J Allergy Clin Immunol* 1996;**98**:797–804.
- FERNANDEZ C, ESTEBAN MM, FIANDOR A, et al. Analysis of crossreactivity between Sunflower pollen and other pollen of Compositae family. *J Allergy Clin Immunol* 1993;**92**:660–667.
- PHAM NH, BALDO BA. Allergenic relationship between taxonomically diverse pollen. *Clin Exp Allergy* 1995;**25**:599–606.
- LACOVACCI P, AFFERNI C, BARLETTA B, et al. *Juniperus oxycedrus*: a new allergenic pollen from Cupressaceae family. *J Allergy Clin Immunol* 1998;**101**:755–761.
- CIMIGNOLI E, BROCCUCCI L, CERNETTI C, GERLI R, SPINOZZI F. Isolation and partial characterization of *Cupressus sempervirens* allergens. *Aerobiologia* 1992;**8**:465–470.
- PETTYJOHN ME, LEVETIN E. A comparative biochemical study of conifer pollen allergens. *Aerobiologia* 1997;**13**:259–267.
- AFFERNI C, IACOVACCI B, BARLETTA B, DI FELICE G, TINGHINO R, MARI A, PINI C. Role of carbohydrate moieties in IgE binding to allergenic components of *Cupressus arizonica* pollen extract. *Clin Exp Allergy* 1999;**29**:1087–1094.