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## Identification of specific IgE binding proteins in Castor bean (*Ricinus communis*) pollen obtained from different source materials

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Allergenic components of *Ricinus communis* pollen obtained from different stages of inflorescence, different time intervals, different years and places were studied by immunoblot analysis. Proteins separated by SDS-PAGE and transferred to NC were identified using pooled sera from 15 skin and RAST positive patients. The IgE binding components in M.W. range of 14 to 70 kD were identified. The protein fractions of 70, 66, 64, 60, 50, 45, 36, 22 and 14 kD are the most prominent allergenic bands. Six samples collected during same pollination season from the same place showed similar allergenic profile. Of the samples collected from different stages of inflorescence, pollen of immature buds showed only three bands as compared to 18 from mature buds and flowers. Variability was seen in the IgE binding components of pollen stored for different years and obtained from different geographic regions of India. The IgE binding pattern of fifteen sera were heterogeneous. The number of bands identified by different sera varied from 3 to 18. Two protein components of 66 and 36 kD were recognised by 14 (93.3%) of the 15 sera studied. The result suggests that there exists variations in the specific IgE binding pattern in pollen samples of Castor Bean, obtained from different source materials.

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Pollen extracts are widely used, both for diagnosis and treatment of allergic diseases. It has been repeatedly demonstrated that commercial allergen extracts from the same material vary markedly, both between manufacturers and on batch to batch basis from the same manufacturer (Helm et al. 1984, Maasch et al. 1987). Thus there is an urgent need for improved standardized allergens in general and pollen in particular. However, much attention has been paid to the final product of an allergenic extract but not to the raw material. The quality of pollen determines the potency of the final extract. Factors that affect the quality of pollen include pollen authenticity, method and time of collection, processing and storage conditions (Anderson 1985). The allergenic extracts of pollen samples are often crude mixtures of proteins or glycoproteins and contain only few allergenic components in addition to irritants and toxic substances. Therefore, removal of these components and identification of clinically important allergens is essential for safe and effective diagnosis and therapeutic treatment. The subject of this study is the pollen of *Ricinus communis* (RC) growing wild and also cultivated in different parts of the world and well known for its allergenicity (Layton et al. 1962, Shivpuri et al. 1963, Singh et al. 1973). Beside pollen, the Castor Bean dust allergy is also reported as an occupational hazard for a long time (Panzani

1957, Thorpe et al. 1988). The present work is aimed at finding out the allergenic components of pollen samples collected from different source materials: (1) different stages of inflorescence, (2) from different places of India during the same pollination season, and (3) pollen stored for different years using immunoblot technique.

### MATERIALS AND METHODS

#### Pollen samples

We have described the detailed method of pollen collection in our earlier report (Singh et al. 1992). In brief six samples, from 1987–88 season (October–March), collected at monthly intervals from bloomed flowers, were used. Two more pollen samples collected from immature buds (>4 mm diam) and mature buds (<4 mm diam) on the same day (12.01.89) from the same community were also used to study the IgE binding pattern in two developmental stages of the flower. Pollen samples, collected during pollen seasons 1984–1987 and stored at 4°C after defatting, were used to study the storage effect. In addition to these samples from Delhi, pollen were also collected by us from different geographic regions of India (Bangalore, Bhopal, Nagpur, Tiruchirappalli, Trivandrum and Visakhapatnam) during 1988 pollination season.

Microscopic analysis of all the samples revealed pollen purity varying from 88% to 96%.

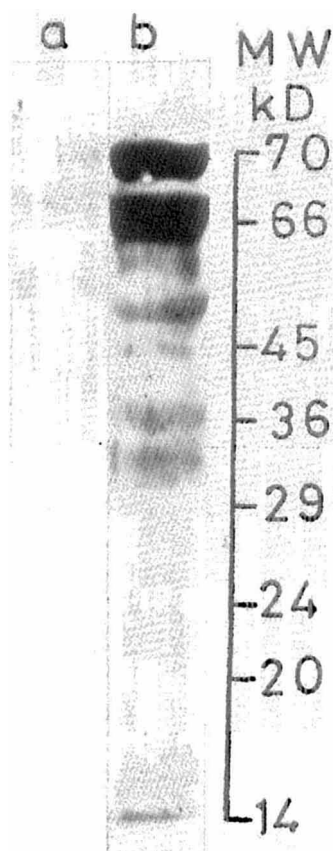


Fig. 1. Identification of IgE binding proteins by enzyme labelled anti-IgE to *Ricinus communis* pollen from immature (a) and mature buds (b), using pooled sera from 15 hypersensitive patients. Molecular wt markers (m.w.).

### Antigen

Antigen was extracted in 0.05 M Phosphate buffered saline (PBS), pH 7.8 in 1:10 (w/v) by continuous stirring at 4°C for 20 hrs. The samples were then centrifuged at  $27,000 \times g$  for 30 min and supernatant was dialysed (cut off 3500 daltons) against distilled water. After dialysis, samples were again centrifuged and passed through millipore filter (0.45  $\mu$ m). Filtered samples were lyophilised and stored at -70°C till further use.

### Sera

Sera were collected from blood samples of respiratory allergic patients showing markedly positive (2+ to 4+) prick test reactions to pollen extracts (1:10, w/v) of *Ricinus communis* in relation to histamine hydrochloride 1 mg/ml. Sera were also collected from five non-allergic persons showing no clinical symptoms and negative skin test. All the sera were tested for the presence of specific IgE antibodies to RC pollen antigen by RAST (class 2 and above). The RAST positive sera of fifteen such patients pooled together, as well as from individual patients, were studied. Sera from all the five non allergic persons were RAST negative.

### Gel electrophoresis

SDS-PAGE was performed in 10% polyacrylamide gel. 20  $\mu$ l of

each sample, containing 80  $\mu$ g of protein, heated with equal amount of sample buffer (0.01 M Tris HCl, pH 8.0, 0.001 M EDTA, 2% SDS, 10% glycerol, 5% B mercaptoethanol, 0.1% bromophenol blue dye) for 2 min at 100°C. Samples were loaded and gel was run at 4 mA/well using 0.01 M Tris glycine buffer containing 0.1% SDS. The gel was calibrated with marker protein obtained from Sigma chemical Co. USA.

### Electrophoretic transfer of proteins to nitrocellulose (NC) membrane

The proteins separated by SDS-PAGE were electrophoretically transferred to NC membrane at 20 mA for 5 hrs using Tris glycine buffer (25 mM Tris, 192 mM Glycine, 20% methanol, pH 8.3) (Towbin et al. 1979). After transfer, unbound sites were blocked with 3% BSA in phosphate buffer saline (PBS) for 3 hrs. After blocking membrane were repeatedly washed with PBS containing 0.5% Tween 20.

### Probing of NC strips

NC strips were incubated overnight (16 hrs) with patients sera diluted to 1:10 with PBS containing 0.03% Tween 20 and 0.1% sodium azide (TPBS). After extensive washing, strips were incubated with Anti human IgE peroxidase conjugate, obtained

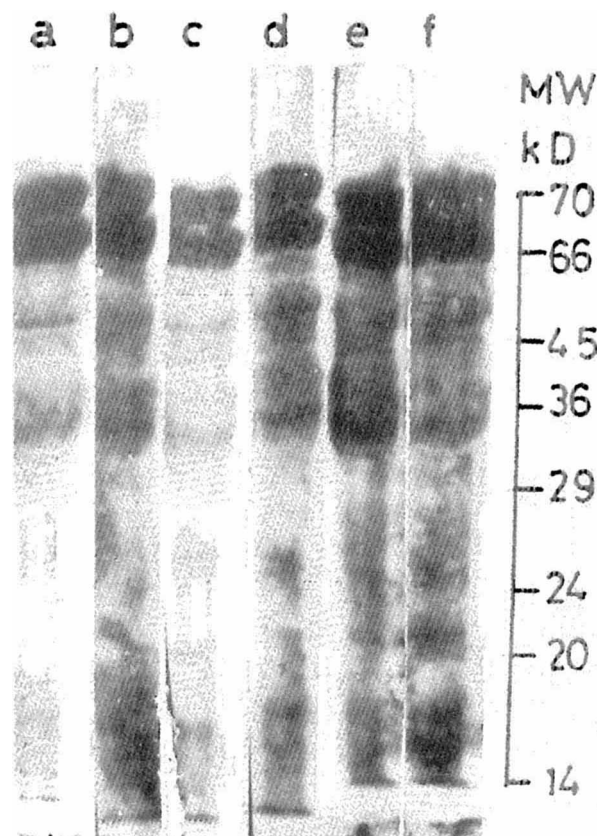


Fig. 2. Identification of IgE binding fractions by enzyme labelled anti human IgE following SDS-PAGE of *Ricinus communis* extract from pollen samples collected at monthly intervals during same pollination season using pooled sera from 15 allergic patients. - a. 25.10.87; b. 26.11.87; c. 24.12.87; d. 23.1.88; e. 20.2.88; f. 24.3.88 M.W. = molecular wt markers.

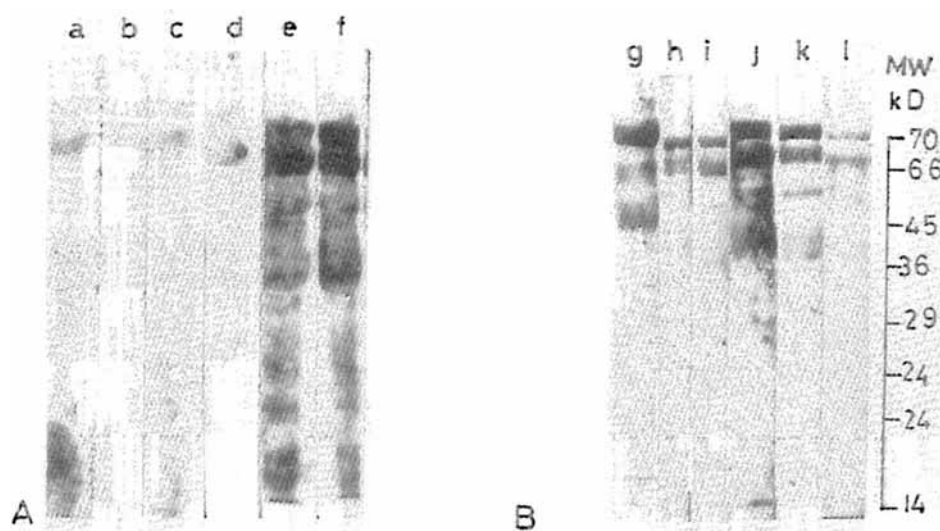


Fig. 3. IgE binding proteins of *Ricinus communis* antigens extracted from pollen of:

A. Different years (a-f: 1984-1989) using pooled sera.  
B. Six geographical regions of India collected during same pollination season.  
g. Bangalore; h. Bhopal; i. Nagpur; j. Tiruchirapally; k. Trivandrum; l. Visakhapatnam. - M.W. = molecular wt markers.

from Sigma Chemical Co., USA (1:500 in 0.03% TPBS) for 3 hrs. After several washings, strips were placed in DAB solution (10 mg of Diamino benzidine in 20 ml PBS) for 10 min in dark. After colour development strips were washed with distilled water and stored in dark.

## RESULTS

The SDS-PAGE protein profiles of different pollen samples used for immunoblot analysis in the present investigation are already reported in one of the earlier issues of this journal (Singh et al. 1992). In brief, protein profiles of pollen from different time intervals showed presence of similar protein bands from 14 kD to 70 kD. The 66 kD and 70 kD bands were the major bands present in all the samples. In sample from immature buds only few bands were present as compared to sample from mature buds or flowers. In stored samples from 1984 to 1987 from Delhi only 4 to 5 bands were present compared to more than twenty from fresh pollen samples of 1988 and 1989 seasons. In samples from different geographical regions the banding pattern was almost similar from Bangalore, Nagpur, Tiruchirapally and Visakhapatnam except for the absence of 70 kD protein in Bangalore and Nagpur samples and two protein bands around 15 kD in Bangalore sample. In extract of Bhopal bands of low molecular weights were not distinct.

### Samples from different stages of inflorescence

The results of immunoblot with pollen samples collected on the same day from immature and mature buds using pooled sera are illustrated in Fig. 1. In sample collected from immature buds, only 3 bands of 70, 66 and 50 kD were detected as compared to mature buds which showed 18 bands in MW range of 14 to 70 kD.

### Samples from different time intervals

The immunoblot studies with extracts of pollen samples using pooled sera showed specific IgE antibodies against 18 proteins in M.W range of 14 to 70 kD (Fig. 2). Although, these samples showed similar allergenic profile, but bands of low M.W became more prominent in samples (d-f) collected from December onwards. The most prominent bands from different pollen samples are of 70, 66, 64, 60, 50, 45, 36, 22 and 14 kD.

### Samples from different years

Immunoblot analysis of extracts prepared from pollen stored for different years showed that sample of 1984, 1986 and 1987 had two faint bands of 70 and 66 kD (Fig. 3A). The sample from 1985 showed binding to 60 kD component in addition to above bands, but were very weak. The number of specific IgE binding proteins detected in samples of 1988 and 1989 (e, f) were 18 as mentioned earlier.

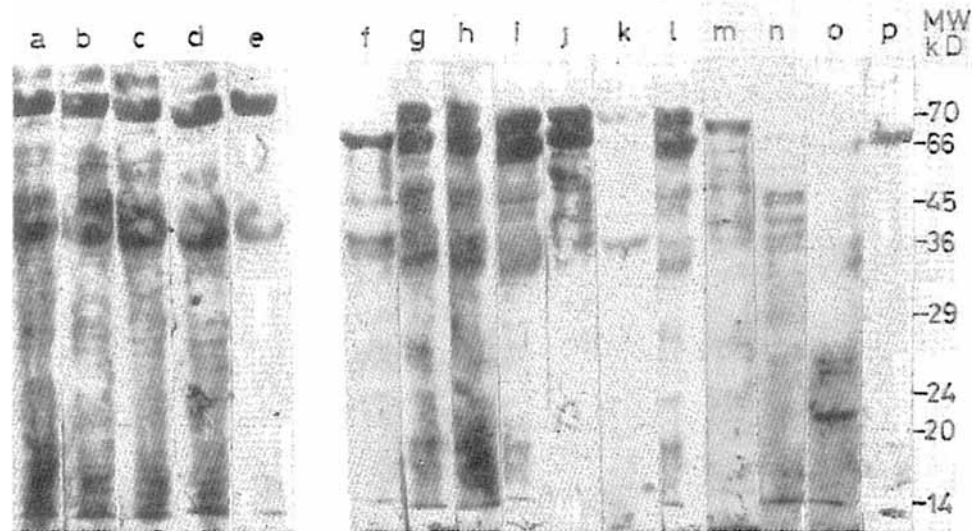
### Samples from different places

When IgE binding pattern of pollen samples from different places of India, beside Delhi, were compared (Fig. 3B), using pooled sera, Bangalore (g) and Tiruchirapally (j) samples showed six bands. Of these, 70, 66, 64 and 50 kD components were common to both. Sample from Trivandrum (k) showed five bands of 70, 66, 50, 42 and 40 kD. Samples from Bhopal (h) and Visakhapatnam (l) had four bands each while Nagpur sample showed only three bands of 70, 66 and 50 kD.

### Heterogeneity in binding of specific IgE antibodies

Heterogeneity was seen in the binding of specific IgE from sera of fifteen individual patients to various components of

Fig. 4. Heterogeneity in the IgE binding proteins recognised by the serum of 15 patients (a-o) sensitive to *Ricinus communis* pollen and control subjects (p).



RC pollen (Fig. 4). The number of bands recognised by sera of individual patients varied from 3 (k) to 18 (b-e) in M.W range of 14 to 70 kD. Four protein components of 64, 60, 50 and 14 kD were recognised by 86.6% of the cases where as 66 and 36 kD components were recognised by 93.3% of sera. Sera of four patients (b-e) showed similar binding pattern. When pooled sera from five control subjects was studied, a very faint band of 66 kD was observed.

## DISCUSSION

Immunoblot has been developed as an efficient technique to study the specificity of the IgE antibodies to different components of allergenic extracts. In this method, first the antigens are separated by SDS-PAGE by their M.W. and then transferred and immobilised on a NC membrane. Proteins are detected by using specific antibodies followed by autoradiography or enzyme substrate method. We have used the latter technique. Since IgE antibodies are mainly responsible for immediate (Type I) hypersensitivity, the use of specific serum IgE antibodies provide definite answer about proteins to which patients are susceptible.

Of the pollen samples collected from different stages of inflorescence, binding pattern from mature buds and flowers were almost similar but immature buds showed binding to only 3 protein bands. When six samples collected at monthly intervals during the same pollination season were analysed, they showed similar allergenic profile with 18 bands, but the bands of low M.W. became more distinct and sharp in samples collected from December onwards.

When allergenic proteins from pollen stored for different years were compared, samples from 1984-1987 showed only 2-3 bands to which IgE antibodies showed binding. Poor protein profiles with 8 to 10 bands were detected in Coomassie Brilliant Blue R 250 stained gel in samples from 1984-1987, where as in 1988 and 1989 samples 27

bands were present (Singh et al. 1992). Samples from 1988 and 1989 were broadly in agreement with each other and showed better allergenic profile. Since the allergenic components of pollen samples from 1984-1987, as present in that particular year, are not available it is difficult to say that loss of allergenic determinants in these samples are due to storage conditions or were absent from the very beginning. It is possible that the pollen samples of these years were mainly from immature buds and not from flowers as pollen from immature buds contained few allergenic proteins. Variation in antigenic and allergenic components of *Ambrosia* pollen from different years and suppliers is also reported (Anderson et al. 1985, Maasch et al. 1987). It is also possible that moisture content in these pollen samples would have lead to the loss of allergenic components. Contrary to our observations, no significant variation in different samples of *Phleum pratense* from different seasons and suppliers was recorded by Maasch et al. (1986). When two lots of Russian Thistle extracts from five different commercial firms were compared, uniform allergenic components were observed (Mansfield et al. 1989).

The pollen samples of different places collected during 1988 pollen season showed variation in the allergenic determinants. The number of bands detected varied from 3 (Nagpur) to 18 (Delhi). The difference in the allergenic components of these samples could be due to variable climatic conditions prevailing in these areas, soil conditions and also stage of maturation of inflorescence from which pollen were collected. It is also possible that some of these patients might not have developed sensitivity to some of the protein fractions present in different samples. Variable amount of IgE in pollen samples collected from *Ambrosia artemisifolia* population growing in the same geographical location has been demonstrated (Lee et al. 1979). Considerable variability in biochemical and immunochemical properties in six pollen batches of *Olea* from different pollen seasons and geographic areas have been reported

(Barber et al. 1990). We also found similar variation in the allergenic profile of pollen samples of *Holoptelea integrifolia* stored for different years and procured from different geographical locations in India (Malik et al. 1991). Thus our studies confirm the geographical variation in allergenic components of different pollen samples. However, specific allergenic components from which pollen samples remain constant regardless of geographical origin but the response of allergic patients differ (Hemmens et al. 1988a, 1988b).

When binding capacity of 15 patients sera were compared, marked variations were seen. One of the patient's sera showed binding to only three proteins, where as ten sera showed very heterogenous response showing specific IgE binding from 6 to 14 proteins. Other patients showed almost similar allergenic profile identifying 18 bands. This could probably be due to patients not exposed to all the components of pollen. However, this variation could also be due to differences in the intensity of disease. Heterogeneity in the specific IgE binding of individual sera to different components of various pollen has been shown (Peltre et al. 1982, Ford et al. 1985, Shen et al. 1988). Majority of the patients showed binding to 66, 64, 50, 45, 40, 36 and 14 kD proteins suggesting them to be important allergenic determinants of RC pollen. In pooled sera from five non allergic volunteers, one faint band of 66 kD was seen. This could be due to the presence of antibodies against some cross reactive substance in some individuals.

## CONCLUSION

Thus we conclude from our studies that there exists (1) significant variation in the allergenic component of pollen samples from immature and mature buds, (2) insignificant variation in samples collected at different time intervals, (3) marked variation among samples from different years and from different geographical places, and (4) heterogeneity in the specific IgE binding patterns of individual patients sera.

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