

Short communication

Allergenic cross-reactivity of *Curvularia lunata* with other airborne fungal species

Background: *Curvularia lunata* is an important fungus for respiratory allergic disorders. Previous studies indicated cross-reactivity of *Curvularia* with other fungi. However, the cross-reactive allergenic component (s) were not identified. The present work was carried out to study the shared allergenic components of *C. lunata* and others.

Methods: Cross-reactivity studies were performed using pooled hypersensitive patient sera to *C. lunata* by ELISA, immunoblot, immunoblot inhibition and ELISA inhibition.

Results: Many *C. lunata* sensitive patients showed positive skin test to five other fungi. *Alternaria alternata* exhibited maximum (68%) whereas *Cladosporium herbarum* showed the least (17%) skin reactivity. Immunoblots of fungal extracts with pooled sera showed common proteins. *Fusarium solani* and *C. herbarum* showed negligible IgE binding. IgE ELISA inhibition with *C. lunata* showed 92% inhibition whereas *A. alternata* and *E. nigrum* showed 84% and 63%, respectively. Immunoblot inhibition with self protein showed complete loss of IgE-binding activity. Proteins of 26, 31, 38, 45 and 50 kDa of *C. lunata* were inhibited by *A. alternata* and *E. nigrum*, whereas *A. fumigatus* inhibited 26, 45 and 50 kDa proteins.

Conclusions: Significant allergenic cross-reactivity exists among proteins of *C. lunata*, *A. alternata* and *E. nigrum*. Proteins of 26, 31, 38, 45 and 50 kDa are shared allergens in these fungi.

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Fungi are important triggers of respiratory allergies (1–4). Several fungi belonging to the class Deuteromycetes viz. *Alternaria*, *Aspergillus*, *Cladosporium*, *Curvularia*, *Epicoccum* and *Fusarium* are responsible for IgE-mediated allergic reactions in atopic individuals (5). Nasobronchial allergy patients usually show IgE-mediated response to multiple fungi, though some of these fungi may not be present in the vicinity of the patient. Therefore, sensitivity to multiple fungi might be the consequence of cross-reactive protein (s). Cross-reactivity can elicit positive allergy tests in subjects with no clinical symptoms. This is caused by IgE antibodies directed against common epitopes of a protein from different allergenic source.

Studies have revealed the presence of various cross-reactive proteins in fungi (6–10). Enolase, a major allergenic protein showed high degree of cross-reactivity between different classes of fungi, e.g., *Ascomycetes* and *Imperfecti* (6). This protein has recently been shown to share allergenicity with *Hevea* latex enolase (7). Alkaline serine proteinase is a cross-reactive protein present in *Aspergillus oryzae* and *Penicillium citrinum* (8). A peroxisomal membrane protein from *A. fumigatus* (*Aspf3*) showed shared IgE-binding epitopes with

P. citrinum and *Candida boidinii* (9, 10). Apart from fungi, proteins such as paralbumin, profilins, tropomyosin were cross-reactive from different allergenic sources (11, 12). Tropomyosin is present in almost all vertebrates and invertebrates. Besides, it may be the basis for cross-reactivity between food and allergen (s) of animal origin (13). *Bet v 4*, two-EF-hand calcium-binding birch pollen allergen (14) showed homology with similar protein from Bermuda grass (*Cyn d 7*), Timothy grass (*Phl p 7*), weed pollen (rape seedoil, *Bra r 1* and *Bra r 2*) and tree pollen (alder, *Aln g 4*, olive, *Ole e 3*). Utilization of cross-reactive proteins in clinical practice may reduce the number of fungal extracts used in allergy diagnosis and may be sufficient for immunotherapy. Johansson *et al.* (15) have proposed a revised nomenclature for allergic disorders to improve diagnosis.

Curvularia lunata is an important inhalant allergen and may cause respiratory mycoses similar to ABPA (1, 16, 17). *C. lunata* extract showed sensitivity in 7–26% of the atopic population (18–20). We have observed close antigenic and allergenic relationship among *Curvularia* species. Amongst them, *C. senegalensis* is most anti-

genically and allergenically related to *C. lunata*. Based on the data, *C. lunata* was selected as a representative species from this group (21).

In the present study, cross-reactivity between *C. lunata* and five fungi was determined using pooled patient serum. Immunoblot and ELISA inhibitions were carried out in order to establish the allergenic relationship amongst them.

Material and methods

Strains and cultures

Curvularia lunata (*C. lunata*, strain no. 4624) and *Alternaria alternata* (*A. alternata*, strain no. 718) were obtained from National Chemical Laboratory, Pune, 4/1008, India. *Aspergillus fumigatus* (*A. fumigatus*, strain no. 902), *Cladosporium herbarum* (*C. herbarum*, strain no. 718), *Epicoccum nigrum* (*E. nigrum*, strain no. 4625) and *Fusarium solani* (*F. solani*, strain no. 3596) were procured from the Indian Agricultural Research Institute, Delhi. They were sub-cultured in 2% agar slants in potato dextrose medium and incubated at $25 \pm 2^\circ\text{C}$ until sufficient sporulation occurred.

Preparation of the extracts

Fungi were mass cultivated on Sabouraud's broth as stationary cultures for 13 days at $25 \pm 2^\circ\text{C}$. Spore-mycelial mass was harvested, freeze-dried and the antigens were extracted (22).

Protein profile of fungal extracts

An amount of 100 μg protein of each fungus was resolved on 10% reduced SDS-PAGE (21, 22), stained with Coomassie Brilliant blue and destained.

Skin tests and collection of serum

Intradermal tests were performed with fungal extracts on naso bronchial allergy patients attending the out-patient department at the V. P. Chest Institute, Delhi (22). Informed consent of subjects was obtained prior to the skin tests and blood collection. Serum from five nonallergic subjects was pooled and used as negative control.

IgE ELISA

ELISA was performed with pooled patient's sera (skin test positive to *C. lunata* and negative to other fungi) to evaluate the IgE-binding activity of different fungi. Microtitre plate (Nunc-MaxiSorpTM Surface, Roskilde, Denmark) was coated with *C. lunata* proteins and the plate was developed as described (21, 22).

Immunoblot with human antibodies

SDS-PAGE separated proteins were electrophoretically transferred, blocked and incubated overnight with pooled sera and immunoblot developed (22).

ELISA inhibition

C. lunata (1 $\mu\text{g}/100 \mu\text{l}$) was coated on the microtiter plate and incubated overnight at 4°C . The free sites were blocked and kept overnight with a preincubated mix of 50 μl pooled sera (1 : 5 v/v) and 50 μl of fungal extracts at 0.0125–6.4 μg protein concentrations (21).

Immunoblot inhibition

IgE binding to *C. lunata* proteins were inhibited with proteins of other fungal extracts. SDS-PAGE-resolved *C. lunata* proteins were transferred to nitrocellulose membrane (NCM). The free sites were blocked and incubated overnight at 4°C with a preincubated mix of 500 μl pooled sera (1 : 5 v/v) and a 500 μl fungal extract containing 50 or 500 μg protein (21).

Results

Skin sensitivity of different fungal extracts

Seven hundred and twenty-nine patients were tested with 13 common fungal extracts for allergy diagnosis during the year 2000. Fifty-three patients showed sensitivity to *C. lunata* extract and of these nine showed markedly positive skin reaction (2+ to 4+, Table 1) to *C. lunata* alone whereas 36 exhibited reactivity to *A. alternata* (68%), 24 to *E. nigrum* (45%), 20 to *A. fumigatus* (38%), 18 to *F. solani* (34%) and 9 to *C. herbarum* (17%).

Protein profile-SDS-PAGE

In order to determine the quality of fungal extracts, SDS-PAGE was performed. Different fungal extracts resolved into 10–21 CBB stained protein bands in the molecular weight range of 14–100 kDa (Table-1).

Cross-reactivity among different fungi

IgE-binding activity of fungal extracts was determined by ELISA (Table 1). *C. lunata*, *A. alternata*, *E. nigrum* showed maximum binding (Abs. 0.87–0.99) whereas *A. fumigatus*, *C. herbarum* and *F. solani* showed minimum binding (Abs. 0.29–0.36).

Different fungal extracts were immunoblotted with *C. lunata* sensitized pooled sera. *C. lunata* showed 14 IgE-binding proteins followed by *A. alternata* (9), *E. nigrum* (7) and *A. fumigatus* (5). Proteins of 12, 26, 31, 45 and 50 kDa from these fungal extracts reacted with anti-*Curvularia lunata* (CL) pooled sera indicating that these may be the cross-reacting proteins (data not shown). Proteins of *C. herbarum* and *F. solani* showed minimal reactivity suggesting little cross reactivity with *C. lunata* components (Table 1).

ELISA inhibition was performed to demonstrate cross-reactivity in quantitative terms (Fig. 1). IgE-binding inhibition to *C. lunata* allergens by self protein showed a reduction of 92% whereas 84% and 63% was obtained with *A. alternata* and *E. nigrum*, respectively, at 6.4 μg protein. *C. lunata* required 0.15 μg self protein for 50% inhibition whereas *A. alternata* and *E. nigrum* required 0.24 μg , 1.1 μg protein, respectively. *A. fumigatus* did not reach 50% inhibition at the highest concentration (Fig. 1).

Table 1. Allergenic cross-reactivity of different fungal extracts using intradermal test, ELISA and immunoblot

Fungal extracts	SDS-PAGE bands	No. of ID positive* patient (% reactivity)	ELISA†Abs. (nm)	Cross-reactivity	
				No. of bands	Immunoblot‡
					MW (kDa)
<i>A. alternata</i>	13	36 (68)	0.865	9	12 26 31 38 45 48 50 78 85
<i>A. fumigatus</i>	17	24 (45)	0.295	5	12 26 31 45 50
<i>C. herbarum</i>	10	20 (37)	0.324	1	12
<i>E. nigrum</i>	14	18 (34)	0.871	7	12 26 31 38 45 48 50
<i>F. solani</i>	10	9 (17)	0.356	1	65
<i>C. lunata</i>	21	53 (100)	0.985	14	12 23 26 31 38 41 45 50 53 58 65 78 80 88

*Intradermal tests were performed on 729 nasobronchial allergy patients. Fifty-three patients showed markedly (2+ to 4+) skin test reactivity to *C. lunata* extract. Sensitivity to other fungi was assessed in these patients. †Specific IgE ELISA values of different fungi using pooled hypersensitive sera. Normal human serum was used as negative control and subtracted from the absorbance of test samples. ‡Immunoblot identifying IgE-binding proteins using *Curvularia lunata* hypersensitive patient sera. Proteins in bold are cross-reactive allergens as determined by immunoblot inhibition.

Shared allergens in different fungi

In order to investigate IgE-binding cross-reactive proteins, immunoblot inhibition was performed using CL sensitive pooled sera (Fig. 2). *C. lunata* showed complete inhibition at 500 µg protein. *A. alternata* and *E. nigrum* showed inhibition of 26, 31, 38, 45, 50 kDa allergens, *A. fumigatus* showed inhibition of 26, 45 and 50 kDa proteins while *C. herbarum* and *F. solani* proteins showed negligible inhibition. Positive control showed 14 allergenic proteins and NHS showed no IgE-binding activity (Fig. 2). These results demonstrated close allergenic relationship between *C. lunata*, *A. alternata* and *E. nigrum*.

Discussion

Cross-reactivity among fungal species belonging to a genus or different genera have been described earlier (23–26). However, the cross-reactive proteins in fungi have not been characterized. The present study demonstrated multiple fungus sensitivity in *Curvularia*

positive subjects. Along with *C. lunata*, several patients showed sensitivity to *A. alternata*, *E. nigrum*, *A. fumigatus*, *F. solani* and *C. herbarum*.

In order to identify common protein components in these fungi, direct binding and inhibition experiments were performed using pooled sera hypersensitive to *C. lunata*. The results obtained showed cross-reactivity among *C. lunata*, *A. alternata* and *E. nigrum* proteins (Figs 1,2).

These results were confirmed by cross inhibition. Inhibition of IgE-binding proteins with different fungi revealed cross reactivity among 26, 31, 38, 45 and 50 kDa proteins of *C. lunata*, *A. alternata* and *E. nigrum*. The 31 and 38 kDa protein of *C. lunata* showed incomplete inhibition with *A. alternata* and *E. nigrum* extracts. Though the serum used in IgE immunoblot inhibition is primarily against *C. lunata*, the possibility of patient's exposure to other allergens could not be ruled out. Moreover, incomplete inhibition of allergens by *A. alternata* and *E. nigrum* may be owing to the low content of these proteins in extract and/or the inherent difference in allergen binding. Shen

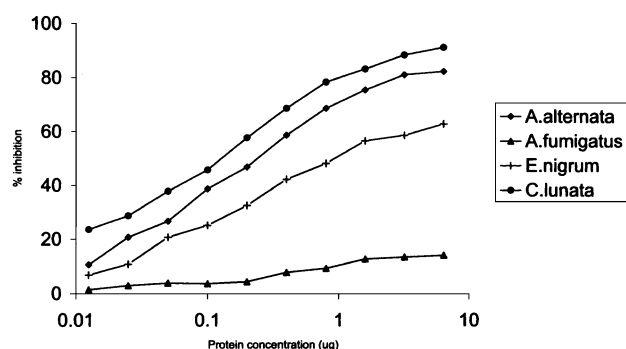


Figure 1. IgE ELISA inhibition with fungi. Microtitre plates were coated with *C. lunata* antigen, blocked and incubated with a mix containing 50 µl pooled sera and 50 µl of fungal extract from 0.0125 to 6.4 µg. Wells probed with pooled sera (1 : 10 v/v) for positive control and normal human serum were used as negative control.

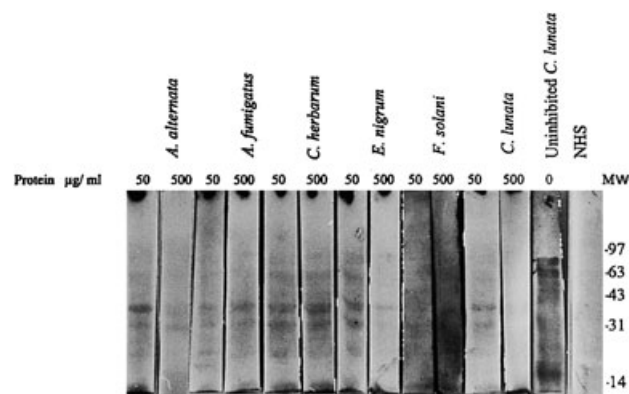


Figure 2. IgE immunoblot inhibition with fungi. *C. lunata* protein strips were blocked and incubated with mix containing 500 µl of pooled sera and 50 or 500 µg fungal extract. *C. lunata* proteins were probed with the pooled sera for positive control and normal human serum for negative control.

et al. (1999) have shown that the *P. notatum* extract could not completely inhibit IgE antibodies against its 34 kDa allergen. Furthermore, significant IgE cross-reactivity and N-terminal sequence homology amongst the 34 kDa major allergen in the two *Penicillium* species was observed (27). *C. herbarum* and *F. solani* showed no shared allergenicity with *C. lunata* by IgE immunoblot and ELISA inhibition.

Amongst the fungal allergens reported so far, proteases, enolases and dehydrogenases are the major cross-reacting allergens. The 31 kDa cross-reacting protein identified in the present study may be a protease as our preliminary study with *C. lunata* extract shows protease activity (unpublished observations). The 12 kDa cross-reactive protein could be ribosomal P₂ protein or profilin as these two proteins fall in the range of 11–14 kDa. Enolase may be the cross-reactive 45 kDa protein. However, the definite nature of these cross-reactive allergens can be determined after purification and sequencing. A 31-kDa glycoprotein from *A. alternata* has been purified, however, its biological

function is not known (28). Purified or recombinant allergens are preferable for cross-reactivity studies (29). But such a study with *C. lunata* proteins is feasible only when its allergen (s) are available in purified/recombinant form.

In summary, extensive allergenic cross-reactivity among proteins of *C. lunata*, *A. alternata* and *E. nigrum* has been demonstrated. This may be as a result of the presence of identical epitopes in proteins from different allergenic sources. The present study may form the basis for developing a cross-reactive protein (s) for multiple fungal sensitivity.

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