

Original article

Molecular and immunological characterization of cytochrome c: a potential cross-reactive allergen in fungi and grasses

Background: Recombinant allergens are required for component-resolved diagnosis/therapy of allergic disorders. The study was aimed to express and characterize an allergenic protein from *Curvularia lunata* and study its cross-reactivity.

Methods: A clone encoding a 12-kDa protein screened from the cDNA library of *C. lunata* was sequenced and expressed in pET22b+ vector. The purified protein was characterized by biophysical and immunological methods.

Results: The sequence of gene encoding a 12-kDa protein showed homology to cytochrome c. It was expressed in *Escherichia coli* yielding 0.5 mg protein/l culture and designated as Cur 1 3. The absorption and circular dichroism spectrum of Cur 1 3 were similar to horse cytochrome c and the protein reacted with cytochrome c antibody. ELISA with different fungal-positive patients' sera showed ≥ 3 times specific IgE to Cur 1 3 compared with healthy controls. Mice anti-Cur 1 3 reacted with tropical and temperate grass extracts. Protein also reacted with grass-positive patients' sera. *In vitro* stimulation of peripheral blood mononuclear cells from *C. lunata*, fungi or grass-positive patients with it released significant levels of Th2 cytokines. *In vivo* testing of this protein in allergic patients showed marked positive skin reactivity in 60% fungal and 43% grass-positive cases. Cross inhibition assays (EC₅₀) demonstrated allergenic cross-reactivity of Cur 1 3 with fungi and grasses.

Conclusions: Cytochrome c, a major allergen from *C. lunata* was cloned, sequenced and expressed. It was identified as a cross-reactive allergen among fungi and grasses and has potential for clinical application.

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Fungi are a major source of aeroallergens responsible for allergic asthma and rhinitis (1). *Curvularia lunata* is an important fungi afflicting sensitization in 7–16% patients with respiratory allergy in India (2, 3) and 18–28% in other countries (4, 5).

Curvularia lunata shows cross-reactivity within *Curvularia* species and with fungi such as *Alternaria alternata*, *Aspergillus fumigatus*, *Cladosporium herbarum*, *Epicoccum purpurascens* and *Fusarium solani* (6, 7). Proteins of 12, 26, 31, 45 and 50 kDa are cross-reactive allergens among these fungi. Cross reactivity occurs because of similarity in sequence or structural fold between two or more proteins belonging to different genus or within species under the same genus. Because of this, hypersensitive patients show skin reactivity to other allergen sources that may not be prevalent in their environment. Cross-reactive allergens, viz. parvalbumin (8), profilins (9), tropomyosin (10), lipid transfer protein (11) and glutathione-S-transferase (12, 13), have been isolated and characterized from various sources. The purified cross-reactive allergens can minimize the extracts used for

diagnosis and show promise for component-based immunotherapy (14).

Cytochrome c (12 kDa) was demonstrated as an allergen from ragweed, Kentucky blue grass, bermuda grass, rye grass and dicotyledons, viz. *Plantago lanceolata* and *Echium plantagineum* (15–18). Earlier, a 12 kDa cross-reactive allergen was identified in fungi (7). Studies have emphasized the need of recombinant allergens for effective diagnosis/therapy and elucidating the mechanism of allergy. The present study was aimed to clone and express cytochrome c from the fungus *C. lunata* and assess its allergenic cross-reactivity with fungi and grasses.

Material and methods

Skin testing and patients' sample

Patients of asthma and rhinitis were selected based on history, skin tests, clinical and laboratory investigations at Out Patients' Department, V. P. Chest Institute, Delhi. Asthma was diagnosed as

per American Thoracic Society guidelines (19). The diagnosis of rhinitis was assigned to patient(s) having two or more of the symptoms, viz. post nasal drip/watery nasal discharge/sneezing/itching of nose and/or nasal congestion most of the times for a period of 2 years (20). Intradermal tests (IDT) were performed with *C. lunata* and other allergen extract(s) on patients during the year 2003–2004 to detect sensitization. Allergen extracts for IDT were procured from Antigen Lab, Institute of Genomics and Integrative Biology, Delhi, approved by drug controllers. Skin tests were graded after 20 min based on the wheal size of the positive control, i.e. histamine diphosphate (100 µg/ml) (21). Sera from fungal-positive patients ($n = 96$) were collected to assess IgE cross-reactivity of cytochrome c (Cur 1 3) among fungal allergens. Sera from healthy nonatopic individuals were collected for use as negative control. The institutional ethics committee approved the study protocol and informed consent of each patient was obtained for skin tests and blood collection.

Cloning and sequencing of allergen from *C. lunata*

cDNA library of *C. lunata* was constructed in Uni ZAP XR vector. *Escherichia coli* XL-1-Blue MRF' strain is RecA⁻ and contained F' episome, essential for Uni-ZAP XR vector. The F' episome present in *E. coli* XL-1-Blue MRF' strain is required for phage infection. It contains enzymatically inactive β-galactosidase gene for enzyme-based nonrecombinant selection, expresses the genes forming F' pili on the surface of the bacteria and has the lac repressor gene.

The cDNA library was screened using human serum pool (1 : 10 v/v in Tris buffered saline [TBS]) of *C. lunata*-hypersensitive patients, preabsorbed (1 : 1v/v) with *E. coli* XL-1 Blue lysate at a concentration of 1 mg/ml. Briefly, the phages containing cDNA library were used to infect *E. coli* XL-1 Blue cells, plated on NZY agar plate (density 300–400 plaques/plate) and incubated at 42°C for 4 h. The nitrocellulose disc soaked in 10 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was overlaid on the plate and incubated overnight at 37°C. Subsequently, membranes were washed with TBS, blocked with 3% defatted milk at 37°C for 1 h and incubated with preadsorbed pooled patients' sera overnight at 4°C. The overlaid membranes were washed with TBS-Tween 20 (0.05%) and incubated with anti-human IgE peroxidase, 1 : 1000 (Sigma, St Louis, MO, USA) at 37°C for 2 h. The positive clones were verified by secondary screening. The plaques for positive clones were excised using Ex-assist helper phage to get phagemid as DNA template for polymerase chain reaction (PCR) sequencing. The insert sizes of various clones were determined using PCR with T3/T7 primers. PCR product was purified using DNA isolation kit (Biological Industries, Beit Haemek, Israel) and sequenced on automated DNA sequencer (ABI-377; Applied Biosystems, Foster City, CA, USA).

Homology search

The open reading frame (ORF) of the sequence was identified by homology search in NCBI database at both DNA and protein level. The sequence alignment was performed using alignment program CLUSTAL W.

Sub-cloning in pET22b+expression vector

The ORF showing homology to cytochrome c was amplified using the forward primer 5' GAT CCG AAT TCT ATG GGT TTC GAA CAG GGC GAT 3' and reverse primer 5' AAG CTT AAG CTT TTT GGT CTC GTG CTC CTG 3' having *Eco*R1 and *Hind*III

restriction sites, respectively. The PCR conditions included: 95°C, 5 min and then 25 cycles of 95°C, 60 s; 52°C, 90 s; 72°C, 60 s with a final extension at 72°C for 10 min. The digested PCR product was purified and ligated into pET22b+ vector. The correct insert size was confirmed by restriction digestion and PCR amplification. Plasmid containing cytochrome c was sequenced and designated as pCL2.

Expression and purification of recombinant protein

pCL2 was transformed into *E. coli* BL21 cells, grown till optical density (OD) 0.6, induced with 1 mM IPTG for 4 h and cells harvested. His-tagged protein was purified using Ni-NTA affinity purification (Qiagen, Hilden, Germany) under native conditions. The eluate was further purified on a Sephadex-G50 resin (GE Healthcare, Uppsala, Sweden) in phosphate-buffered saline (PBS). The purified protein was designated as Cur 1 3. Endotoxin levels were detected in the purified protein using LAL kit (Sigma, USA).

Characterization of Cur 1 3

Absorption spectra. Absorption spectrum of Cur 1 3 (1.5 mg/ml in 20 mM phosphate buffer) was measured in UV-visible range and compared with horse cytochrome c (SRL, Mumbai, India) used as reference.

Circular dichroism of Cur 1 3. To elucidate the secondary structure of Cur 1 3, CD spectra were recorded with 1 mg/ml protein in 20 mM phosphate buffer in the far-UV range (190–600 nm) on Jasco 715 (Jasco, Easton, MD, USA). Thermal scans in the range of 10–100°C were also carried out to determine the melting temperature (T_m) of Cur 1 3.

Immunoblotting. Cur 1 3 (10 µg) resolved on 15% SDS–PAGE (22) was transferred onto nitrocellulose membrane (NCM) (23). The NCM strips were blocked with bovine serum albumin, washed with PBS and incubated overnight at 4°C with 15 individual *C. lunata*-positive patients' sera (1 : 10) or sera samples from grass-positive patients. After washing, strips were further incubated with anti-human IgE peroxidase (1 : 1000 v/v; Sigma) for 3 h at 37°C and developed using diaminobenzidine.

In another experiment, grass pollen extracts, viz. *Lolium perenne*, *Phleum pratense*, *Poa pratensis*, *Imperata cylindrica*, *Zea mays*, *Pennisetum typhoides* and *Cenchrus ciliaris* (Allergon AB, Uppsala, Sweden) were transferred, probed with anti-cytochrome c (commercial or raised against Cur 1 3 in mice) by immunoblot. The strips were incubated with anti-mouse IgG peroxidase (1 : 1000) (Sigma) for 3 h at 37°C and developed using diaminobenzidine.

Skin testing with Cur 1 3. For clinical evaluation, Cur 1 3 (10 µg/ml) and spore-mycelial extract of *C. lunata* (1 : 500 w/v) were tested intradermally (21) on 77 patients with allergic rhinitis, asthma and/or both. Blood was collected from patients with marked positive skin test to

Cur 1 3 and either fungi or grasses or both for immunobiochemical assays. Cur 1 3 and *C. lunata* were also tested on 20 healthy individuals and blood collected for use as controls.

ELISA. Specific IgE levels against Cur 1 3 were determined by an enzyme-linked immunosorbent assay (ELISA) (24) using hypersensitive individual patients' sera from fungal-positive, grass-positive or from both groups of patients.

Histamine release assay. Blood samples of Cur 1 3-positive patients, those positive to fungi, grasses or both and controls were processed and challenged with Cur 1 3 in the concentration range of 5–50 ng (13). The histamine standards in the range of 0–100 nM and released histamine in the supernatant were acylated. The acylated histamine in all the samples, controls and standards was measured by ELISA as per the manufacturer's instructions (Immunotech, Marseille, France). As a negative control, spontaneous histamine release was measured in unchallenged sample and total histamine was estimated by cell lysis.

ELISA inhibition to assess cross-reactivity of Cur 1 3. Microtitre plate was coated with 40 ng of Cur 1 3 in carbonate buffer (pH 9.6) and incubated overnight at 4°C. The wells were washed with PBS, blocked and incubated with grass or fungal-positive sera preincubated with Cur 1 3 (0.01–1000 ng). The wells were washed and developed as in ELISA. Cur 1 3 was also used for self-inhibition and an insect allergen extract was used as negative control.

Peripheral blood mononuclear cell (PBMC) proliferation and cytokine analysis on antigen challenge. A dose-dependent proliferation of PBMCs was carried out using different concentrations of Cur 1 3 and *Curvularia* extract (1, 5, 10 and 50 µg) to determine the optimal concentration of protein for positive stimulation. PBMCs were isolated by Ficoll-Hypaque centrifugation from heparinized blood of the same 20 patients used for histamine release assay (Sigma). For lymphoproliferation assays, 10⁵ cells/well were cultured in RPMI-1640 media and challenged with 10 µg of *Curvularia* extract and/or Cur 1 3. Phytohaemagglutinin 5 µg was used as positive control and negative control comprised unstimulated cells. Plates were incubated for 72 h and the culture supernatant collected for cytokine assays. To the wells 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 100 µg/ml) (Sigma) was added and incubated for 2 h. Cells were lysed in acidic isopropanol and read at 570 nm. Simultaneously, cells were stimulated with lipopolysaccharide (LPS) in the concentration range detected in three different preparations of Cur 1 3, i.e. < 4 EU/ml.

Interleukin (IL)-4, IL-5 and γ -interferon (IFN- γ) in PBMC culture supernatants from the above-mentioned

patients were measured using ELISA (BD Pharmingen Opt EIA kits; BD Pharmingen, San Diego, CA, USA). To detect the presence of T-regulatory IL-10, levels were determined in the supernatant of cultured PBMCs from 10 patients (four fungal, four grass and two sensitized to fungi and grasses both) and a control sample by ELISA (BD Pharmingen Opt EIA kits). Briefly, capture antibody (1 : 250) for each cytokine was coated on plates, incubated overnight and blocked with PBS containing 10% fetal bovine serum (Sigma) for 1 h. Standards (5–500 ng), 100 µl supernatant of sample (1 : 2 v/v) or controls were added to wells and incubated at 37°C for 3 h. The plates were washed, incubated with biotinylated detector antibody, colour developed using *o*-phenylene diamine and read at 490 nm in an ELISA reader.

Statistical analysis

Both parametric and nonparametric statistical analyses were performed to compare the means of specific IgE values, skin test reactivity and histamine release between healthy controls and allergic individuals using the Student's *t*-test. *P* < 0.05 was considered significant in all cases. All the results were analysed by box plots of medians with interquartile range.

Results

Cloning, expression and screening of cytochrome c from *C. lunata*

Immunoscreening of cDNA library with pooled *C. lunata*-hypersensitive patients' sera identified 12 positive clones and one sequence showed significant homology to cytochrome c (NCBI: AY034827). The homology ranged from 77% to 96% at the DNA level and from 80% to 98% at the protein level. Its ORF was 327 bp encoding 110 amino acids with a molecular mass of 12 kDa. Cur 1 3 has the conserved sequence C(A/S)QCHT present in the active site of cytochrome c. The sequence homology of Cur 1 3 with cytochrome c from various sources including allergenic fungi is presented in Table 1.

The yield of purified Cur 1 3 was 0.5 mg/l and was ~10–12% of the total protein. It resolved as a 12-kDa protein on SDS-PAGE with pI of 9.5 (data not shown).

Table 1. Sequence homology of Cur 1 3 with cytochrome c from various fungi

Homology with	Organism	Accession no. (NCBI)	% Similarity	
			Protein	DNA
Cytochrome c	<i>Neurospora crassa</i>	P00048	97	85
Cytochrome c	<i>Aspergillus niger</i>	P56025	84	81
Cytochrome c	<i>Saccharomyces cerevisiae</i>	P00045	80	77
Cytochrome c	<i>Fusarium oxysporum</i>	G1616892	83	81
Cytochrome c	<i>Candida albicans</i>	P53698	84	80

NCBI-BLAST (BLAST 2 sequences) and CLUSTALW multiple alignment programs were used to deduce the percentage sequence similarity at DNA and protein levels.

Commercial polyclonal anti-cytochrome c (Sigma) reacted with Cur 1 3 on immunoblot confirming it as cytochrome c. Endotoxin levels from three different batches of recombinant Cur 1 3 preparation were <4 EU/ml.

Characterization of Cur 1 3

Physicochemical properties. Absorption spectra of Cur 1 3 and horse cytochrome c showed peaks at 410 and 510 nm characteristic of cytochrome family of proteins (Fig. 1A). CD of Cur 1 3 showed negative peak in the far-UV range near 220 nm signifying the predominant α helix population similar to horse cytochrome c (Fig. 1B). It showed 47% α helix and 32% β sheets comparable to computationally predicted values. Its T_m was 57°C as determined by CD scans at different temperatures (10–100°C).

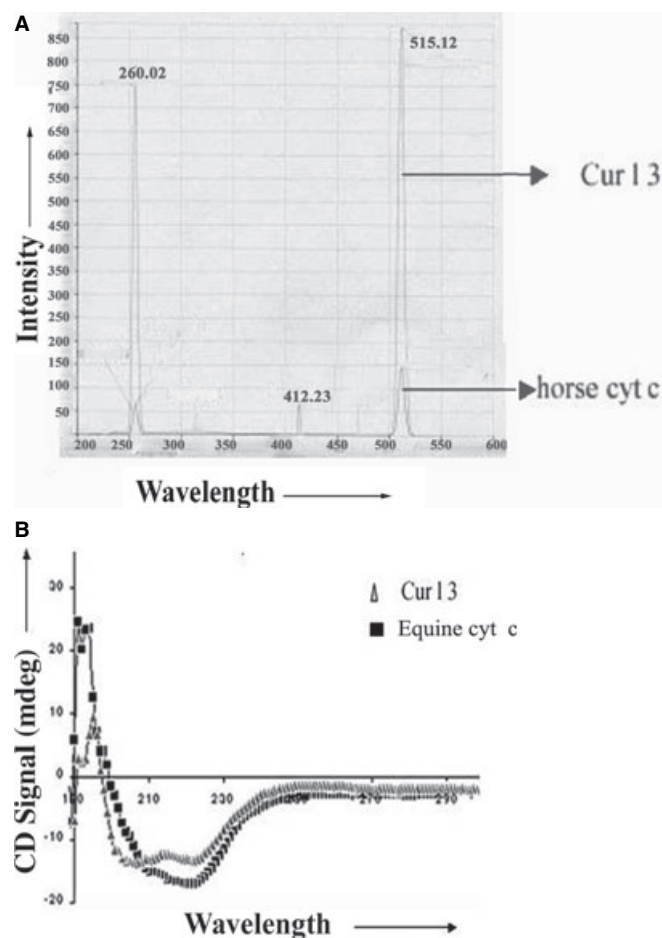


Figure 1. Absorption spectra and circular dichroism. (A) Absorption spectra of Cur 1 3 and the horse cytochrome c (standard). Proteins (1 mg/ml) were taken in 20 mM phosphate buffer and spectra recorded between 200 and 600 nm. (B) CD of Cur 1 3 (Δ) and horse heart cytochrome c ($\blacksquare$). Proteins (1 mg/ml) were taken in 20 mM phosphate buffer and spectra recorded in the far-UV range (190–300 nm).

Allergenic activity. Cur 1 3 reacted with all the 15 *Curvularia* hypersensitive patients' sera on immunoblot indicating it as a major allergen (Fig. 2, lanes 1–15). It did not react with control sera (lanes C1–C4). ELISA with Cur 1 3 and *C. lunata* extract using these individual patients' sera gave mean ODs of 0.472 ± 0.215 and 0.641 ± 0.291 respectively. Specific IgE values for 10 of the 15 patients' sera were ≥ 3 times of control. This demonstrates Cur 1 3 as a major allergen.

Cross-reactivity of Cur 1 3

Cur 1 3 binding with fungal-positive patients' sera. Cur 1 3 demonstrated IgE binding with all 96 fungal-sensitive patients' sera and the OD values were 0.5 or above suggesting the cross-reactivity of Cur 1 3 among fungi (Table 2). ELISA cut-off for positive value was taken as ≥ 3 times of control. It was observed that patients positive to *A. alternata*, *E. purpurascens*, *F. solani* and *A. fumigatus* showed higher IgE binding against Cur 1 3.

Cur 1 3 binding with grass-positive patients' sera. Cur 1 3 showed IgE binding on immunoblot with *L. perenne*, *P. pratense*, *P. pratensis*, *I. cylindrica*, *Z. mays*, *P. typhoides* and *C. ciliaris*-hypersensitive patients' sera (Fig. 3A). It shows that IgE antibodies present in the sera of temperate and tropical grass-sensitized patients cross-react with Cur 1 3. These pollen extracts also reacted with Cur 1 3 mice antibody and gave a discrete band at 12 kDa suggesting cross-reactivity (Fig. 3B).

Cur 1 3-inhibited IgE binding. Immunoglobulin-E ELISA inhibition with Cur 1 3 as inhibitor showed a dose-dependent inhibition of grass and fungal-hypersensitive pooled patients' sera. EC_{50} for inhibition of Cur 1 3 was 1 ng with grass and 1.1 ng with fungal-positive patients'

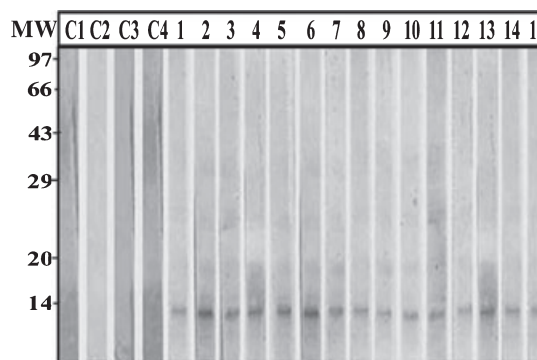


Figure 2. Immunoblot with *Curvularia*-hypersensitive patient's sera: Cur 1 3 resolved on 15% SDS-PAGE, transferred to nitrocellulose membrane, incubated with four control serum samples (1 : 10) (lanes C1–C4) or 15 *Curvularia* hypersensitive patients' sera (1 : 10) (lanes 1–15) and detected using anti-human IgE peroxidase (1 : 1000). MW, molecular weight marker.

Table 2. IgE binding to Cur 1 3 with fungal allergic patients' sera

S. no.	Fungus	Patients screened	Sp. IgE (OD ₄₉₀)
1	<i>Alternaria alternata</i>	20	0.7–1.1
2	<i>Cladosporium herbarum</i>	11	0.6
3	<i>Fusarium solani</i>	9	0.7
4	<i>Epicoecum purpurascens</i>	7	0.5–1.1
5	<i>Curvularia lunata</i>	20	0.8–1.5
6	<i>Aspergillus fumigatus</i>	15	0.7–0.9
7	<i>Rhizopus oryzae</i>	2	0.7
8	<i>Mucor hiemalis</i>	3	0.5
9	<i>Penicillium citrinum</i>	2	0.6
10	<i>Candida albicans</i>	7	0.6
11	Controls	20	0.1–0.2

Patients selected for the study showed clinical symptoms of bronchial asthma/allergic rhinitis/ both. Patients showing 2+ or more skin test reactivity with respective fungi were considered marked positive. The control serum samples were drawn from healthy individuals not having any history of sensitization/allergic symptoms. The specific IgE values represented were mean values from three experiments.

sera, respectively, confirming the cross-reactive nature of cytochrome c (Fig. 3C).

Lymphocyte proliferation and cytokine release. Dose-dependent lymphoproliferation assay with 10 and 50 µg of Cur 1 3 or *Curvularia* extract gave three times or more proliferation compared with the negative control. The 10 µg antigen concentration was chosen for further experiments (Fig. 4A). PBMCs of fungus and/or grass-sensitized patients challenged with 10 µg of Cur 1 3/ *Curvularia* showed three times or more proliferation when compared with the negative control (Fig. 4B–D). A parallel proliferation using LPS as stimulant (in the concentration range 0.04–4 EU/ml) showed background proliferation comparable to control values.

Higher secretion of IL-4 and IL-5 was observed on stimulation with 10 µg of protein. IL-4 levels were 100–325 pg/ml on stimulation with 10 µg of Cur 1 3 in fungal-positive patients whereas grass-positive patients released 89–250 pg/ml (Fig. 5A,B). IL-5 levels were comparable both for fungal-positive (110–250 pg/ml) and grass-positive samples (91–220 pg/ml) (Fig. 5A,B). Patients showing positive skin test to grasses and fungi yielded higher secretion of IL-4 (145–320 pg/ml) and IL-5 (150–270 pg/ml) on challenge with Cur 1 3 (Fig. 5A,B). The control subjects showed nondetectable levels of IL-4 and IL-5. IFN-γ (Fig. 5C) and IL-10 levels were in the range of 40–60 pg/ml on stimulation with 10 µg protein (Fig. 6) comparable to control samples. Analysis of cytokine secretion in both groups of patient samples on challenge with Cur 1 3 further established the cross-reactive nature of cytochrome c.

Skin reactivity of Cur 1 3. Of the 77 patients skin tested with Cur 1 3 and *C. lunata* extract, 36 were fungal

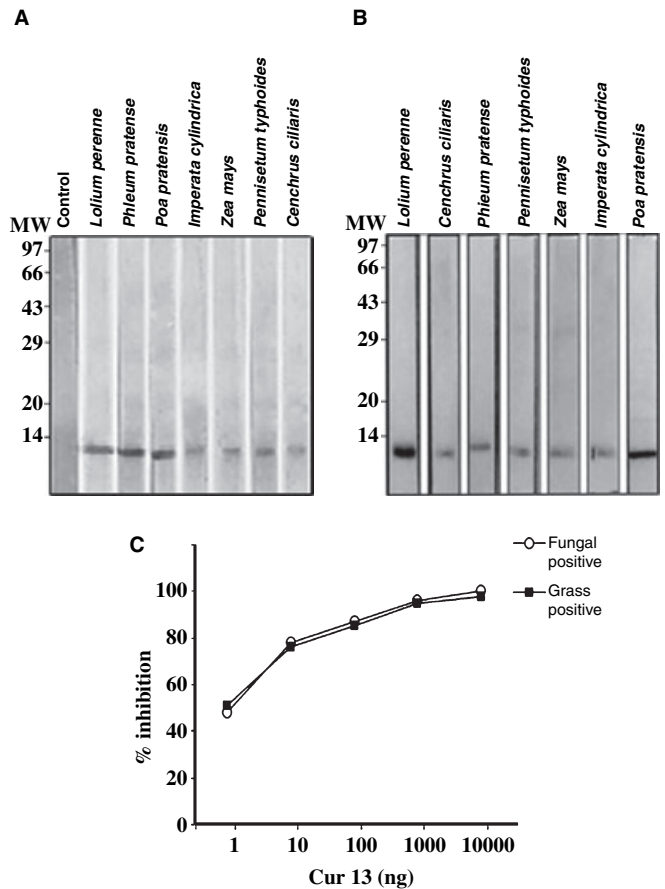


Figure 3. Cross-reactivity using immunoblot and ELISA inhibition: (A) Cur 1 3 resolved on 15% SDS-PAGE and transferred onto nitrocellulose. Specific IgE against Cur 1 3 in control and different pollen-positive sera was detected using anti-human IgE peroxidase. MW, molecular weight marker. (B) Different grass extracts resolved on SDS-PAGE, transferred onto nitrocellulose membrane, probed with anti-Cur 1 3 (1 : 1000) raised in mice and detected using anti-mouse IgG peroxidase. (C) ELISA inhibition was performed using Cur 1 3 as inhibitor. Cur 1 3 (0.01–1000 ng) was preincubated with fungal- and grass-positive patients' sera, added to Cur 1 3-coated plate and detected using anti-human IgE peroxidase (1 : 1000).

positive (11/36 were *C. lunata* positive), 32 grass pollen positive and nine insect/tree pollen positive. Cur 1 3 was positive in 21 of 36 fungal pollen sensitized cases, 14 of 32 grass-positive cases and in five cases positive to both fungi and grasses (Fig. 7). This *in vivo* cross-reactivity may be caused by common epitopes of Cur 1 3 in fungi and grass pollens.

Histamine release by Cur 1 3. *In vitro* basophil challenge was performed with Cur 1 3 and *Curvularia* extract on 20 of 40 Cur 1 3-positive patients. The skin test reactivity profile of these patients showed that 10 were fungal positive, five grass positive and five positive to both. Cur 1 3 released histamine in the range of

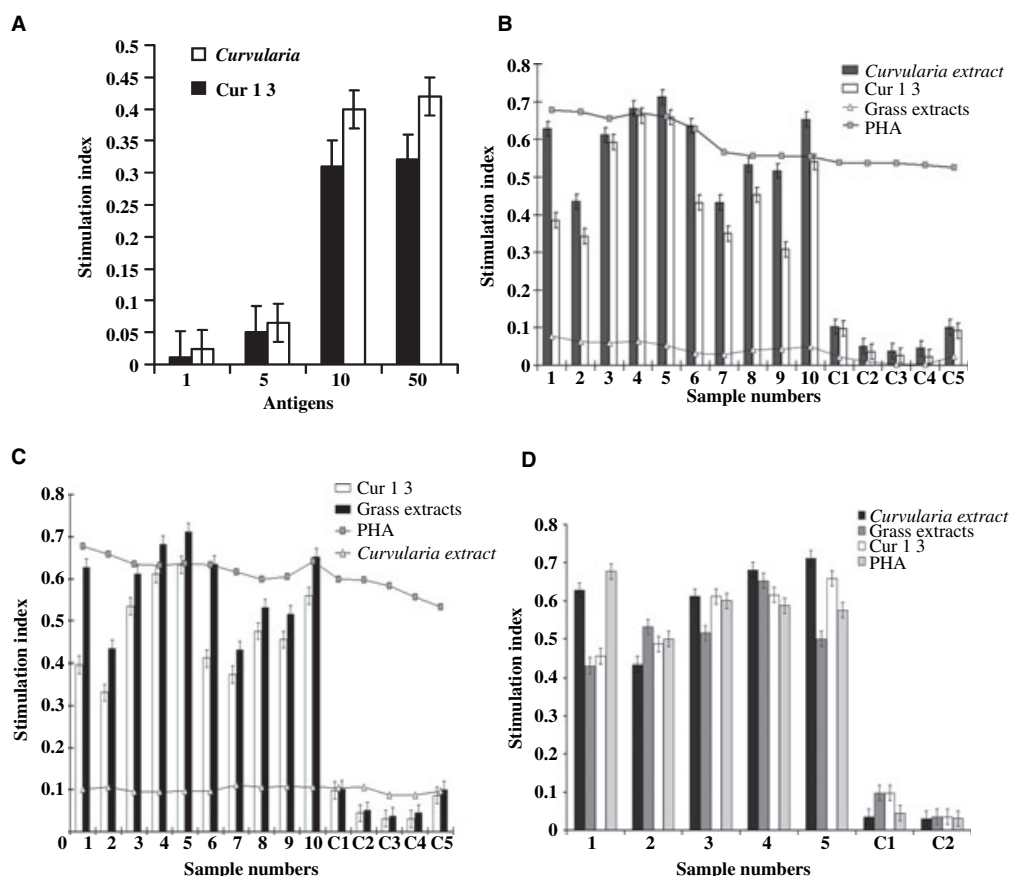


Figure 4. Lymphoproliferation assay using patients' PBMCs: (A) Dose-dependent assay with different concentrations of *Curvularia* extract and Cur 1 3 (1, 5, 10 and 50 µg). PBMCs isolated from the patients positive to (B) fungi, (C) grasses or (D) both were stimulated with 10 µg of Cur 1 3 and cultured for 72 h and proliferation measured by the MTT assay. Phytohaemagglutinin (PHA)-stimulated cells were taken as positive control while respective grass (B) or fungal (C) extracts were used to find basal proliferation in each case. C1–C5 in (B) and (C) correspond to stimulation of PBMCs isolated from healthy controls. Similarly, C1 and C2 were the control samples in (D).

13–50% in all the fungal- and grass-hypersensitive individual samples tested, demonstrating its role as a potent inducer of chemical mediators involved in allergic disease (Table 3). The controls showed histamine release in the range of 5–8% on challenge with *C. lunata* extract or Cur 1 3.

Discussion

Natural allergen extracts contain many IgE-binding proteins. Most of these proteins are not stable for sufficient duration and are degraded by inherent proteases. Therefore, the extracts lose potency and give false-negative results. Purified proteins offer improved and selective diagnosis/therapy of allergy but are difficult to generate native proteins. Recombinant allergens are homogeneous preparation and prove better reagents than whole mass extracts in clinical trials (14, 25). In recent years, recombinant allergens

from diverse sources have been synthesized and analysed for their biological functions. These allergens show activity like glycolytic enzymes (24, 26), lipid transfer proteins (11), proteases (27, 28), transferases (13), etc. This knowledge has helped in elucidating the common proteins among multiple sources. In the present study, cytochrome c allergen from *C. lunata* was cloned and expressed to investigate its role in fungus and grass pollen sensitization.

Studies show cross-reactivity among fungal allergens because of common antigenic determinants (6, 7). Both intrageneric or inter species cross-reactivity have been revealed using recombinant allergens such as vacuolar serine protease (*P. oxalicum* and *P. citrinum*) (28), enolase (*A. alternata* and *C. herbarum*) (26), glutathione-S-transferase (12) and nuclear transport factor 2 (*A. alternata* and *C. herbarum*) (29). The cross-reactive allergens have clinical utility as they can replace the large number of extracts used for diagnosis and therapy. Recently, cross-reactive food allergens have been impli-

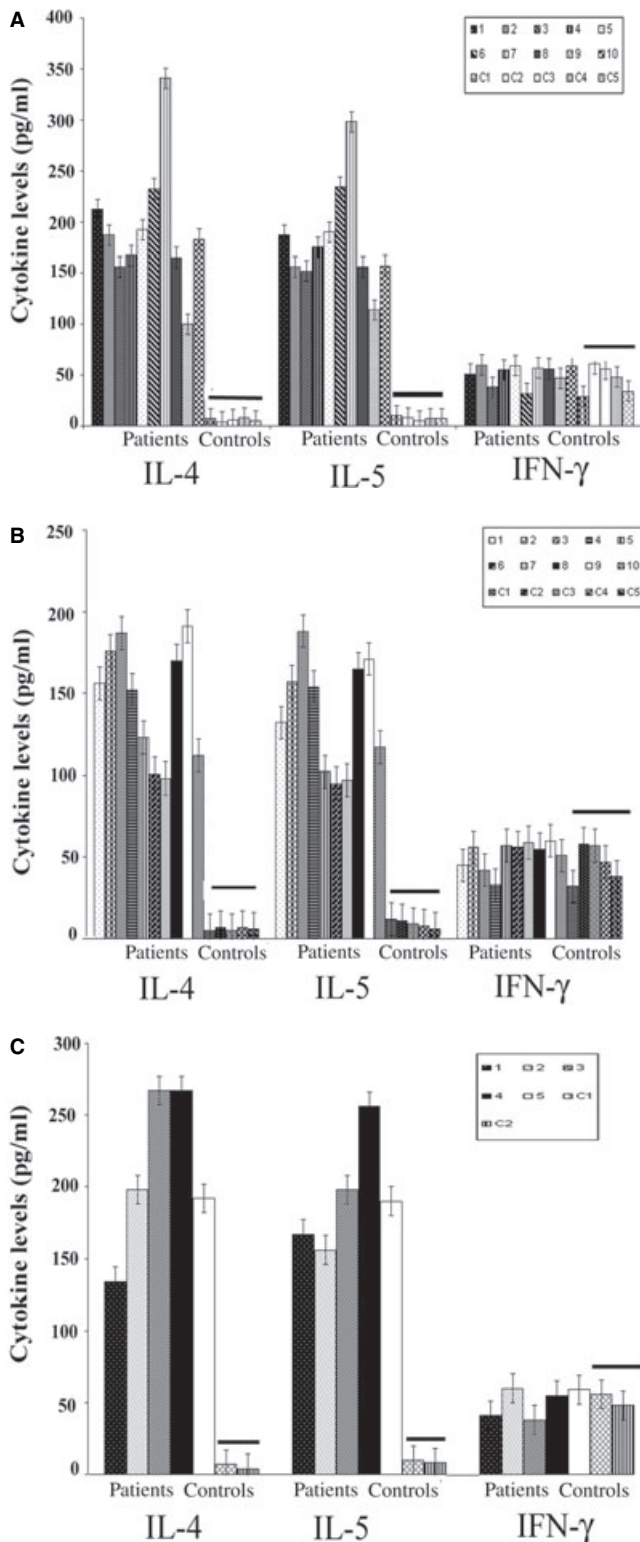


Figure 5. Cytokine levels on allergen stimulation in PBMC culture supernatant. IL-4, IL-5 and IFN- γ were measured in PBMC culture supernatants of (A) fungal-positive, (B) grass-positive and (C) grass- and fungal-positive patients. The values represent mean $\pm$ SD. Control samples are highlighted with lines above bars.

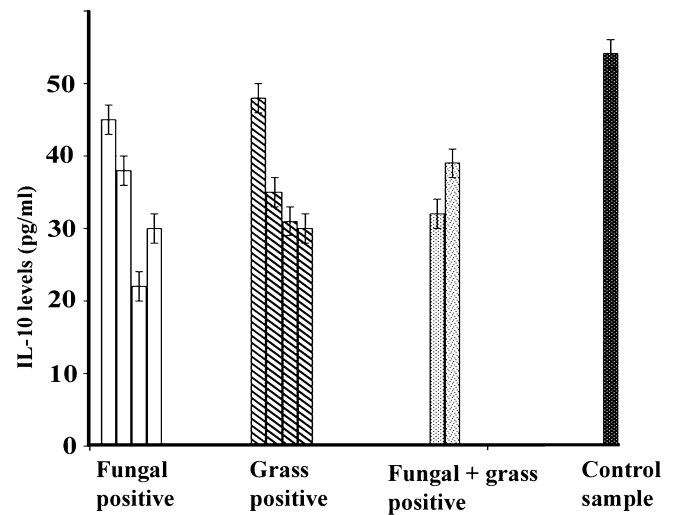


Figure 6. IL-10 levels on allergen stimulation in PBMC culture supernatant: IL-10 was measured in PBMC culture supernatants of patients and a control sample using ELISA. The results shown are mean $\pm$ SD.

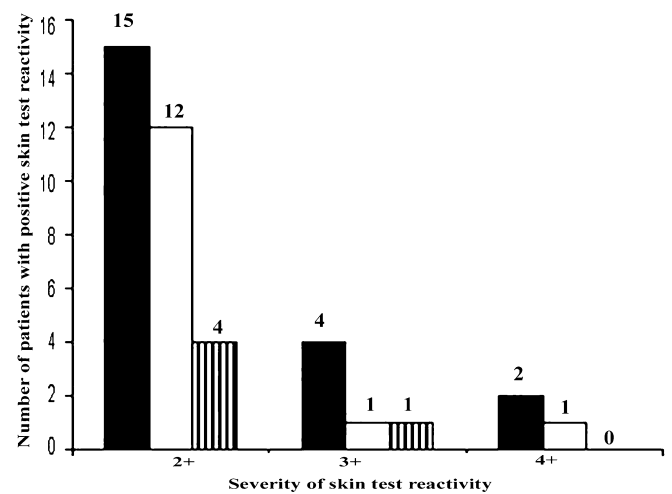


Figure 7. Skin reactivity with Cur 1 3: Bar graph representing skin reactivity and severity of Cur 1 3 on respiratory allergy patients [■] corresponds to fungal-positive patients, Cur 1 3 reacted with 21 of 36 patients; [□] corresponds to grass-positive patients, Cur 1 3 reacted with 14 of 32 patients and [▨] represents the group sensitized both to fungi and grasses. The number of patients with severity of skin reactivity, i.e. 2+, 3+ or 4+ is marked above the bars.

cated for clinical use (30). In the present study, fungal allergic patients' sera showed three times or more specific IgE against Cur 1 3 (cytochrome c) of *C. lunata*. Further, Cur 1 3 released significant amount of histamine and Th-2 type of cytokines from PBMCs of *C. lunata* and other fungal-positive patients. These data suggest

Table 3. Skin reactivity, specific IgE ELISA and % histamine release against *Curvularia* extract and Cur I 3

Patient no.	Sex/age/CH	Skin test reactivity			Specific IgE		% Histamine	
		Fungi	Grasses	Cur I 3	Cur I 3	<i>Curvularia</i>	Cur I 3	<i>Curvularia</i>
1	M/60/BA	-ve	<i>Cen3+</i> , <i>Cyn3+</i> , <i>Imp2+</i> , <i>Pen 2+</i> ,	3+	0.462	0.021	18	4
2	F/28/BA	-ve	<i>Cen3+</i> , <i>Cyn3+</i> , <i>Imp2+</i>	2+	0.318	0.031	12	6
3	M/27/AR	-ve	<i>Cen2+</i> , <i>Imp2+</i>	3+	0.482	0.012	32	9
4	F/31/AR	-ve	<i>Cen2+</i> , <i>Imp2+</i> , <i>Pen3+</i>	2+	0.381	0.084	19	6
5	M/29/Both	-ve	<i>Cen3+</i> , <i>Cyn3+</i> , <i>Imp2+</i> , <i>Pen 2+</i>	2+	0.410	0.061	13	7
6	F/24/BA	<i>Cur2+</i>	-ve	2+	0.452	0.294	46	12
7	M/25/Both	<i>Alt2+</i>	-ve	2+	0.391	0.025	21	18
8	F/48/BA	<i>Alt2+</i> , <i>Cur2+</i>	-ve	2+	0.317	0.123	16	16
9	M/38/both	<i>Alt2+</i> , <i>Cur2+</i> , <i>Epi2+</i>	-ve	2+	0.300	0.214	31	17
10	F/42/BA	<i>Alt2+</i> , <i>Asp2+</i> , <i>Cla2+</i>	-ve	2+	0.299	0.195	26	18
11	F/23/BA	<i>Alt2+</i> , <i>Can2+</i>	-ve	2+	0.471	N.D	13	11
12	F/39/BA	<i>Cla2+</i> , <i>Cur2+</i>	-ve	3+	0.672	0.567	21	25
13	M/29/AR	<i>Cur2+</i>	-ve	3+	0.576	0.432	23	22
14	M/23/BA	<i>Epi2+</i>	-ve	2+	0.515	0.654	25	27
15	M/14/Both	<i>Alt2+</i>	-ve	2+	0.532	0.543	16	18
16	M/28/BA	<i>Cla2+</i> , <i>Epi 2+</i>	<i>Cen3+</i> , <i>Pen2+</i>	2+	0.398	0.478	19	21
17	M/23/AR	<i>Alt2+</i> , <i>Asp2+</i> , <i>Cur2+</i>	<i>Cyn2+</i> , <i>Pen2+</i>	2+	0.528	0.612	28	31
18	F/18/BA	<i>Asp2+</i> , <i>Can2+</i> , <i>Cur2+</i>	<i>Cen2+</i> , <i>Cyn2+</i> , <i>Imp2+</i> , <i>Pen3+</i>	2+	0.376	0.634	31	33
19	M/27/BA	<i>Cla2+</i> , <i>Cur2+</i>	<i>Cen3+</i> , <i>Imp2+</i> , <i>Pen2+</i>	2+	0.310	0.423	15	16
20	M/29/AR	<i>Can2+</i> , <i>Cla2+</i> , <i>Cur2+</i>	<i>Cen2+</i> , <i>Cyn2+</i> , <i>Pen2+</i>	2+	0.384	0.712	39	41

Control value for IgE-specific ELISA against Cur I 3 = 0.050–0.128. Control value for histamine release was in the range of 4–6%. CH, clinical history; BA, bronchial asthma; AR, allergic rhinitis; Both: AR + BA; Alt, *Alternaria alternata*; Cur, *Curvularia lunata*; Cla, *Cladosporium herbarum*; Can, *Candida albicans*; Cen, *Cenchrus ciliaris*; Cyn, *Cynodon dactylon*; Imp, *Imperata cylindrica*; Pen, *Pennisetum typhoides*; % histamine release for control subjects was in the range of 3–8%; ND, not done. Skin reaction of 2+ and above was considered marked positive.

that Cur I 3 is a cross-reactive allergen among multiple fungi.

Cross-reactivity between distantly related sources is less commonly known but it has been established for enolase from fungi and latex (26), foods and tree pollens (31), etc. It could be the result of similarity in primary protein sequence, structural motifs or common carbohydrate determinants. The proteins with conserved sequence have higher chance of being cross-reactive. Cytochrome c is a conserved protein across diverse sources. It belongs to group X cross-reactive allergens. Earlier studies have shown the presence of cross-reactive cytochrome c allergen in Kentucky blue grass and perennial ryegrass (16). In our study, fungal cytochrome c (Cur I 3) recognized grass pollen-positive patients and released histamine/Th2 cytokines on challenge. It shows the diverse cross-reactivity of cytochrome c allergen. Intradermal testing on grass- and fungal-positive patients suggests the presence of shared allergenic determinants on Cur I 3.

The evidence of IgE reactivity against Cur I 3 in varied fungal and pollen-sensitized patients indicates its potential for clinical use. Both fungal and grass pollen allergy are prevalent throughout the world. The cross-reactive allergens aggravate the symptoms even in the

absence of actual allergen. Therefore, the knowledge of such cross-reactive allergens or epitopes can be exploited for better diagnostics/therapeutic reagents and studies at the molecular level (30). The primary structure of a protein can help map its epitopic regions using bioinformatic approaches. The residues involved in IgE binding can be detected by incorporating mutations in predicted B cell regions and synthesizing hypoallergenic variants having negligible IgE reactivity but intact T cell activity as suggested earlier (32). As Cur I 3 protein has a highly conserved sequence, cytochrome c from other sources can also be assessed for its allergenic potential.

In conclusion, recombinant Cur I 3 (cytochrome c, 12 kDa) from *C. lunata* is a major cross-reactive allergen among fungi and grasses. The protein shows promise for component-based diagnosis/therapy of allergic disorders.

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