
Antigenic and allergenic cross-reactivity of *Epicoccum nigrum* with other fungi

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Background: Previous studies have identified *Epicoccum nigrum* (EN) as an important aeroallergen. Shared allergenicity among some fungi responsible for type I allergic disorders has been reported.

Objective: To study the cross-reactivity among different fungi and to identify immunoglobulin (Ig)G and IgE binding components shared between EN and 10 other fungi known to cause respiratory allergy.

Methods: Cross-reactivity studies were carried out by enzyme-linked immunosorbent assay (ELISA) and immunoblot inhibition using both rabbit antiserum raised against EN and pooled sera from patients' EN-positive skin test.

Results: A large number (82%) of EN-sensitive patients showed positive skin reactivity to other fungal extracts. ELISA inhibition revealed >50% inhibition in binding of EN-specific rabbit antibodies with *Alternaria alternata*, *Curvularia lunata*, *Cladosporium herbarum*, and *Penicillium citrinum* extract, whereas the other extracts showed only 20 to 40% inhibition. Rabbit antisera to *A. alternata*, *C. herbarum*, and *C. lunata* reacted with five to seven bands in EN, demonstrating the presence of shared antigens among these fungi. EN requires an amount of 100 ng for 50% IgE ELISA inhibition, whereas 175 ng of *A. alternata*, 160 ng of *C. lunata*, and 268 ng of *C. herbarum* and *P. citrinum* were required for the same. IgE immunoblot and immunoblot inhibition further revealed that 43-, 26-, and 17-kD allergenic bands were shared by EN and *A. alternata*, whereas the 80- and 37-kD bands were common to both EN and *C. lunata*. EN and *C. herbarum* shared 63- and 36-kD allergenic bands, whereas EN and *P. citrinum* shared the 34-kD band.

Conclusion: EN showed maximum cross-reactivity with *A. alternata* followed by *C. lunata*, *C. herbarum*, and *P. citrinum*. This information will be useful in treating EN-allergic patients.

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INTRODUCTION

Several fungi are known to induce type I allergic disorders in predisposed individuals. More than 80 genera of fungi are associated with respiratory allergy,¹ and among them, *Aspergillus fumigatus*, *Alternaria alternata*, *Cladosporium herbarum*, *Curvularia lunata*, *Fusarium solani*, *Rhizopus nigricans*, and *Epicoccum nigrum* (EN) have been recognized as important allergens throughout the world.^{1–5} Mold-allergic individuals often show sensitivity to more than one fungi. This may be because of the presence of cross-reactive proteins in different fungi. The knowledge of cross-reacting epitopes among allergens has important implications in reducing the panel of diagnostic and therapeutic allergens.⁶

EN is a common fungus found on rotting vegetation and damp places in both indoor and outdoor environments. It is known to cause respiratory allergic disorders, hypersensitivity pneumonitis,⁷ and allergic fungal sinusitis.⁸ In our earlier study, we standardized EN extract and identified the allergenic proteins.⁵ Portnoy et al⁹ have shown cross-reactivity between EN and *Alternaria* by enzyme-linked immunosorbent assay (ELISA) inhibition. Later, O'Neil et al¹⁰ demon-

strated shared allergenic determinants between EN and a Basidiomycete, *Armillaria tabescens*. However, neither of these studies identified the cross-reacting allergenic proteins. In the present study, cross-reacting allergenic proteins in EN and 10 other allergenic fungi have been identified using allergic patients' sera and rabbit anti-EN sera.

MATERIALS AND METHODS

Preparation of Fungal Extract

EN (strain 4625, Indian Agricultural Research Institute, New Delhi, India) was grown in Sabouraud broth containing yeast extract for 13 days in stationary cultures at 25° C. Fungal material containing spore and mycelial components of *A. fumigatus*, *Aspergillus tamarii*, *A. alternata*, *Candida albicans*, *C. herbarum*, *C. lunata*, *F. solani*, *Mucor hiemalis*, *Penicillium citrinum*, and *Rhizopus oryzae* were obtained from the Antigen Production Division, Centre for Biochemical Technology, Delhi, India. The surface growth (spore-mycelial mass) was thoroughly washed with distilled water to remove the media proteins, if any, and then lyophilized. The freeze-dried spore-mycelial mass was ground to powdered form and then extracted in 1:20 wt/vol of 125 mmol/L ammonium bicarbonate buffer containing 5 mmol/L ethylene diamine tetraacetic acid and 1 mmol/L phenylmethylsulfonyl fluoride.⁵

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Skin Tests and Collection of Patient Sera

Informed consent of patients and normal subjects was obtained for skin tests and sera collection.¹¹ Intradermal tests were then carried out with EN and other fungal extracts (1:500 wt/vol) on patients with nasobronchial allergy at the Clinical Research Centre, V.P. Chest Institute, Delhi, India, according to the method of Shivpuri and Agarwal.¹² Phosphate-buffered saline was used as negative control and histamine diphosphate as positive control. Sera were collected from 50 patients showing marked positive skin reactions ($>3 \times$ negative control, 12 mm diameter) to EN and/or other fungal extracts. Sera were also collected from 17 nonallergic persons to be used as negative control.

Rabbit Antiserum

Antiserum was prepared by immunizing rabbits as described earlier.⁵ Animal use was in accordance with the Guidelines for Care and Use of Animals in Scientific Research.¹³

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Spore-mycelial extracts of EN and other fungi were resolved on 12% gel (Minigel electrophoresis apparatus, Bio-Rad, Hercules, CA) using Tris-glycine buffer.¹⁴ Approximately 150 μ g protein of each fungal extract was loaded in separate lanes. The resolved proteins were stained with Coomassie brilliant blue R-250 or used for immunoblot analysis.

Immunoblot

SDS-PAGE separated proteins of different fungi were electrophoretically transferred onto nitrocellulose membrane using transblot apparatus (Bio-Rad) as described by Towbin et al.¹⁵ The transferred proteins were checked by staining with Ponceau S (Sigma, St. Louis, MO) before incubation with pooled serum. The cross-reactive proteins in EN extract were

visualized using three separate serum pools (a, b, c as in Table 1). These serum pools were prepared with patients' sera skin tests positive to EN, but not to the fungal extract being tested on immunoblot and used diluted 1:5 vol/vol with phosphate-buffered saline. For immunoglobulin (Ig)G, rabbit anti-EN serum (1:1,000 vol/vol) was used. The cross-reactive bands were detected using anti-human IgE or anti-rabbit IgG conjugated with peroxidase (1:1,000 vol/vol; Sigma, St. Louis, MO) and substrate diaminobenzidine¹⁶ with hydrogen peroxide in sodium acetate buffer. The nitrocellulose strips containing transferred EN proteins were probed with normal human sera (NHS) and preimmune rabbit sera as controls to rule out nonspecific IgE/IgG binding.

ELISA Inhibition

IgG/IgE ELISA inhibition was carried out as described earlier.¹⁷ EN extract at a concentration of 1 μ g of protein per 100 μ L extract in carbonate-bicarbonate buffer (pH 9.6) was coated on the wells of microtiter plate (Maxisorp, Nunc Immuno module, Roskilde, Denmark) and incubated overnight at 4° C. Fifty microliters of different fungal extract (20 ng to 5 μ g) preincubated overnight at 4° C with 50 μ L of serum (1:1000 vol/vol rabbit antiserum or 1:5 vol/vol patient serum pool positive only to EN) was added to the coated ELISA plate. The bound IgG was detected by incubating the plate with anti-rabbit IgG peroxidase conjugate. For IgE, anti-human IgE peroxidase conjugate was used. The absorbance was read at 490 nm (Spectramax R 190, Molecular Devices Corporation, Sunnyvale, CA) and the amount of antigen required for 50% inhibition of binding to EN antibodies was calculated. Preimmune rabbit sera/NHS were used as negative controls for IgG/IgE ELISA inhibition.

Table 1. Skin Test Reactivity and Specific IgE to EN in Fungi-Sensitive Patients

Patients	Skin test positivity	Specific IgE to EN (OD at 490 nm)
1	EN(++), Can(+), Fus(++), Hel(+), Muc(++), Pho(+), Tri(+), Rhi(++)	0.799
2	EN(++), Muc(+), Fus(++), Pho(+)	0.542
3	EN(+), Rhi(+), Fus(+)	0.711
4	EN(++), Hel(++), Muc(++), Rhi(++), Fus(++)	0.454
5	EN(++)	1.208
6	EN(++), Tri(+), Fus(++)	0.578
7	EN(+), Fus(++), Trich(+)	0.440
8	EN(++), Fus(++), Pho(++)	1.020
9	EN(++), Rhi(+), Tri(+)	0.636
10	EN(++), Alt(++), Asp(+++), Can(+++), Cla(++), Cur(++), Hel(++)	0.751

Serum pools were made using equal quantities of serum from EN-allergic patients.

Pool a: The serum from patients #1, 2, 3, 4, 5, and 6 were pooled and used for immunoblots of EN, *A. fumigatus*, *A. tamarii*, *A. alternata*, *C. herbarum*, *C. lunata*, and *P. citrinum*.

Pool b: The serum from patients #5, 6, 7, and 8 were pooled and used for blots of *R. oryzae*, *M. hiemalis*, and *C. albicans*.

Pool c: The serum from patients #9 and 10 were pooled and used for blot of *F. solani*.

EN – *Epicoccum nigrum*, Rhi – *Rhizopus oryzae*, Tri – *Trichoderma lignorum*, Muc – *Mucor hiemalis*, Fus – *Fusarium solani*, Pho – *Phoma hybernica*, Hel – *Helminthosporium annamalum*, Alt – *Alternaria alternata*, Asp – *Aspergillus fumigatus*, Can – *Candida albicans*, Cla – *Cladosporium herbarum*, Cur – *Curvularia lunata*.

Immunoblot Inhibition

Immunoblot inhibition was performed to confirm the presence of IgE cross-reactivity among the antigens of EN, *C. lunata*, *A. alternata*, *C. herbarum*, and *P. citrinum*. SDS-PAGE-separated proteins of these five fungal extracts were transferred onto nitrocellulose membrane. The membrane was blocked, washed, and incubated overnight at 4° C with 10 mL of 1:5 vol/vol patients' serum pool (positive to multiple molds including EN) inhibited with 1 mg/mL of EN protein. Blots incubated with uninhibited serum pool served as positive controls. The blots were developed with enzyme substrate as described above.

RESULTS

Skin Tests

In all, 1,092 nasobronchial allergy patients were screened and skin tested with a battery of allergens including EN extract. Sixty-two (5.6%) showed markedly positive skin reactivity to EN. Of these, only 11 (18%) patients showed positive intradermal tests to EN extract alone, whereas the rest (51, or 82%) were also positive to other fungal extracts. Among these 51 EN-sensitive patients, 37% showed skin reactivity to *C. herbarum*, 32% to *A. alternata*, and 31% to *C. lunata* extract (Fig 1). Sera could be collected from 50 patients. This was analyzed by ELISA for specific IgE against different fungal allergens. Sera with high titers of IgE (absorbance or optical density [OD] value 10 times greater than NHS) were used for immunobiochemical experiments (Table 1).

ELISA Inhibition with Rabbit Antisera

Rabbit anti-EN antibodies (IgG) were used to quantitate the shared antigenic components in different fungi. Sixty nanograms of EN extract was required to produce 50% binding inhibition, whereas *C. lunata*, *A. alternata*, *C. herbarum*, and *P. citrinum* extracts required an amount of 100, 200, 350, and 1,700 ng, respectively. The other fungal extracts showed only 20 to 40% inhibition at the maximum concentrations tested (Fig 2a).

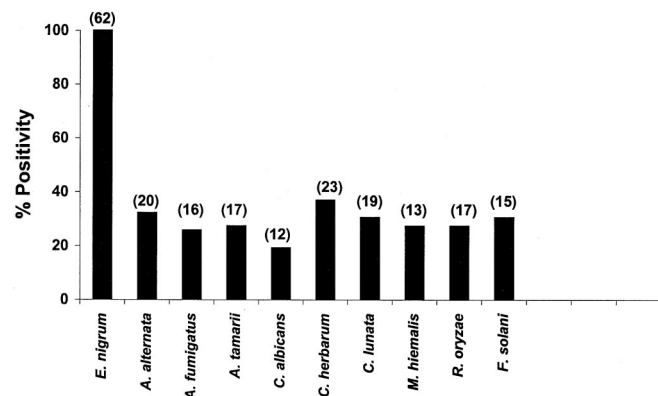


Figure 1. Intradermal test (ID) results (percent positivity, y-axis) with 10 different fungal extracts in 62 EN-sensitive allergy patients. The number of patients showing ID positivity to other fungi is given in parentheses.

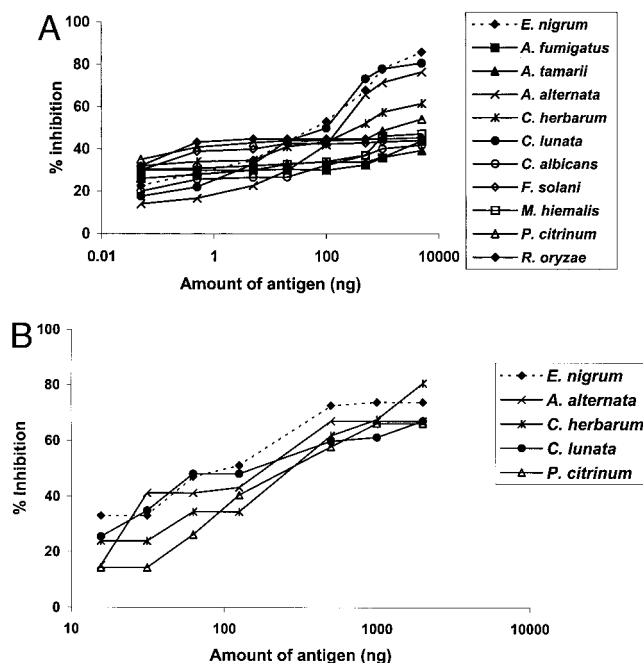


Figure 2. ELISA inhibition demonstrating the extent of cross-reactivity in different fungi. Different fungal extracts of varying concentration (20 ng to 5 μ g) were used as inhibitors. (a) IgG ELISA inhibition using rabbit anti-EN antibodies. (b) IgE ELISA inhibition using pooled sera from EN-hypersensitive patients.

ELISA Inhibition Using Patient's Serum Pool (IgE)

To check for the presence of cross-reactive proteins in fungal extracts including EN, inhibition studies were further carried out with serum pool of patients hypersensitive to EN using *A. alternata*, *C. lunata*, *C. herbarum*, and *P. citrinum* extracts as inhibitor. The other six fungal extracts were not included as they failed to inhibit 50% of EN-specific rabbit antibodies (Fig 2a).

EN extract required 100 ng of self-protein for 50% inhibition, whereas *A. alternata*, *C. herbarum*, *C. lunata*, and *P. citrinum* showed the same inhibition at 175, 268, 160, and 268 ng, respectively (Fig 2b). These extracts inhibited EN IgE antibodies in the same order as in ELISA inhibition with rabbit antisera (Fig 2a).

Immunoblot with Rabbit Antisera

The fungal extracts resolved into 11 to 25 protein bands in the molecular weight range of 14 to 100 kD (Fig 3). The transferred proteins on nitrocellulose membrane when probed with rabbit anti EN sera showed four to 11 IgG-reactive bands in different fungi (Fig 4a). *A. alternata* extract showed 11 immunoreactive bands with EN antisera. Eight bands were detected each in *A. tamarit*, *C. albicans*, *M. hiemalis*, and *P. citrinum* extracts, whereas *C. lunata*, *C. herbarum*, and *R. oryzae* extract showed only four IgG bands. The 38-kD band was present in EN and seven other fungi, whereas the 55-kD was detected in six fungi tested. The 77-, 67-, and 30-kD bands were also detected in EN and five other fungi (Table 2).

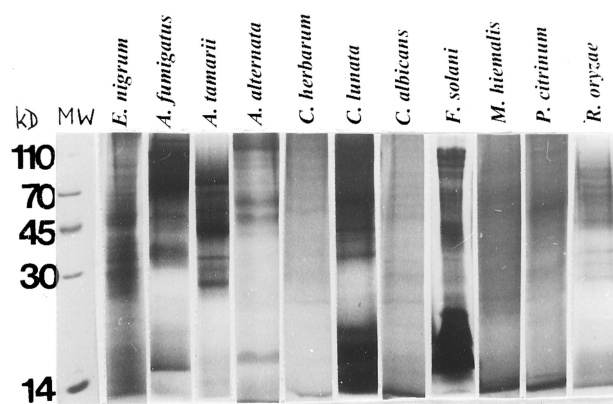


Figure 3. SDS-PAGE profile (12% resolving) depicting Coomassie brilliant blue R-250-stained protein bands in different fungal extracts. MW, molecular weight markers.

IgG cross-reactivity among EN, *C. lunata*, *A. alternata*, and *C. herbarum* was further evaluated by probing the EN blots with rabbit antibodies raised against these fungi. Each of the rabbit anti-*Alternaria* and anti-*Cladosporium* antibodies detected seven antigenic bands on an EN blot, whereas five bands were observed with rabbit anti-*Curvularia* antibodies (Fig 4b). Both, rabbit anti-*Alternaria* and anti-EN antibodies recognized 77- and 30-kD bands in EN and *A. alternata*. The 36-kD band in *C. herbarum* and EN was observed using rabbit anti-EN and anti-*Cladosporium* antibodies.

Immunoblot with Pooled Patients' Sera

IgE-binding proteins common to EN and other fungal extracts were studied using three separate serum pools described in Table 1. IgE-probed immunoblots of different fungal extracts showed 4 to 11 allergenic bands (Fig 5). *A. alternata* shared eight allergenic proteins with EN, followed by six with *C. lunata* and *A. fumigatus* (Table 3).

Immunoblot Inhibition Using Patients' Serum Pool

To confirm the allergenic cross-reactivity among EN, *A. alternata*, *C. lunata*, *C. herbarum*, and *P. citrinum*, immunoblot inhibition was carried out using pooled patients' sera positive to multiple molds. The serum pool was incubated with 1 mg/mL of EN protein. The bands that are inhibited in the blot are cross-reactive proteins present both in EN and the fungi tested (Fig 6).

EN extract inhibited IgE binding to all EN proteins on the immunoblot except for the 43- and 24-kD bands. In the case of *A. alternata*, EN inhibited IgE-reacting bands of molecular weight 88, 77, 74, 64, 59, 43, 26, and 17 kD. Of these, the 43-, 26-, and 17-kD bands were also observed in the IgE immunoblot described earlier (Fig 5). Similarly, among the four IgE recognized bands inhibited in *C. lunata* blot (viz 80, 67, 37, and 26 kD), the 80- and 37-kD bands were also present in the EN blot (Fig 5). EN inhibited IgE binding to six bands on the *C. herbarum* blot and four bands on *P. citrinum*

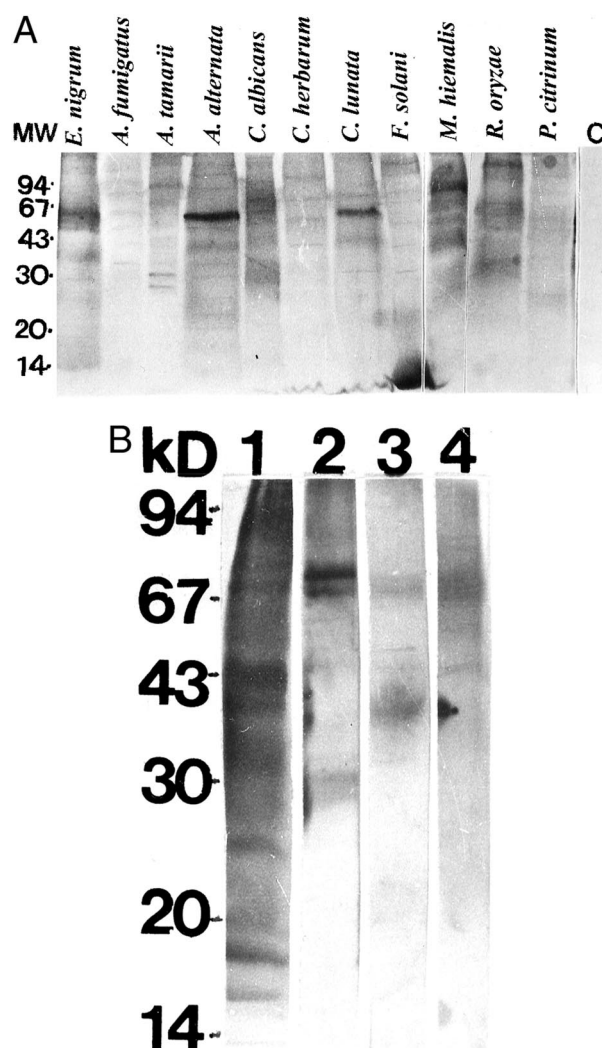


Figure 4. Immunoblot showing shared IgG bands (a) Different fungal extracts were separated on SDS-PAGE, transferred onto nitrocellulose membrane and probed with rabbit anti-EN antibodies. C, EN strip probed with preimmune rabbit sera. (b) EN extract was separated on SDS-PAGE and probed with antibodies raised against 1) EN, 2) *A. alternata*, 3) *C. herbarum*, and 4) *C. lunata*, raised in rabbits.

blot. The 63- and 37-kD IgE-recognized protein bands on the *C. herbarum* blot and the 34-kD IgE-recognized protein of the *P. citrinum* blot were also seen in the EN immunoblot probed with pooled patient sera hypersensitive to EN.

DISCUSSION

The degree of cross-reactivity between two species depends on the number and relative importance of the individual allergens that cross-react and the patient sera used. Studies have revealed that patients sensitive to one fungus show positive skin reactions and raised serum IgE to several other fungal allergens.^{1,6,18} The allergens of EN have recently been characterized^{5,19}; however, the cross-reactivity among EN

Table 2. Antigenic Bands Recognized by Rabbit EN Antibodies in Different Fungi

Fungi	Number of bands in immunoblot	Molecular weight of IgG bands (kD)
<i>Aspergillus fumigatus</i>	5	92, 77, 63, 50, 38
<i>Aspergillus tamarii</i>	8	92, 67, 55, 50, 43, 35, 32, 27
<i>Alternaria alternata</i>	11	94, 77, 57, 45, 38, 35, 32, 30, 25, 23, 19
<i>Candida albicans</i>	8	77, 67, 63, 55, 38, 34, 32, 30
<i>Cladosporium herbarum</i>	4	92, 74, 55, 36
<i>Curvularia lunata</i>	4	85, 57, 38, 30
<i>Fusarium solani</i>	5	85, 67, 55, 38, 30
<i>Mucor hiemalis</i>	8	85, 77, 67, 57, 45, 38, 34, 30
<i>Rhizopus oryzae</i>	4	67, 55, 45, 34
<i>Penicillium citrinum</i>	8	94, 77, 67, 55, 38, 32, 27, 25
<i>Epicoccum nigrum</i> (EN)	25	92, 85, 77, 74, 67, 63, 57, 55, 50, 45, 43, 38, 36, 35, 34, 32, 30, 27, 26, 25, 23, 20, 19, 18, 16

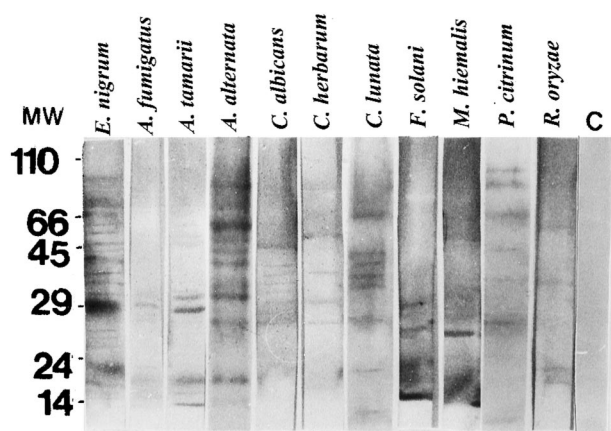


Figure 5. Immunoblot showing shared IgE bands. Fungal extracts were probed with serum pools from EN-sensitive patients as described in Table 1. C, EN strip probed with NHS.

allergens and those of commonly encountered fungi has not been established.

EN-hypersensitive patients may develop antibodies to other fungi during the natural course of exposure. Cross-reactive antigens between different species can be best shown using species-specific polyclonal antibodies.^{20–22} In this study, EN showed four to 11 shared antigenic bands with different fungal extracts tested. Several allergens have been reported to be enzymes/proteases. The 38-kD band was present in EN and seven other fungi. An aspartic proteinase having a similar molecular weight has been reported in *A. fumigatus*.²³ The proteins of molecular weight 30 to 34 kD were detected in EN and three to five other fungi. Many proteases of similar molecular weight range have also been reported in other fungi.^{24–26}

A dose-dependent inhibition of rabbit antibodies specific to EN was observed with *A. alternata*, *C. lunata*, *C. herbarum*, and *P. citrinum* extracts. However, the other fungal extracts tested did not yield 50% inhibition. Immunoblot analysis using IgG antibodies raised against *A. alternata*, *C. herba-*

rum, *C. lunata*, and *P. citrinum* identified several proteins in common between these extracts and EN (Fig 4a). The number of common bands in the immunoblot did not correlate with the amount of fungal extract needed for 50% inhibition in ELISA. This could be either attributable to the different binding affinities of the allergenic proteins as polyclonal antibodies have been used in this study, or the inherent differences in the methods used. ELISA evaluates the protein in one conformation, whereas SDS-PAGE and immunoblotting evaluates the protein in dissociated forms, which can have altered antibody-binding sites.

Earlier studies have detected cross-reacting IgE antibodies specific to *Candida* and *Pityrosporum* in the serum of patients with atopic dermatitis and asthma,²⁷ whereas *Aspergillus*-, *Alternaria*-, and *Cladosporium*-reactive antibodies were observed in the serum of patients allergic to multiple molds.²⁸ In the present study, 11 of 62 patients were specifically positive to EN, whereas the remaining were sensitive to multiple molds. As the immunologic responses vary between individuals, pooled patients' serum was used in the study.

Evaluation of immunoblots of different fungal extracts probed with different patient pools showed many shared allergenic bands. *A. alternata* and EN had the highest number of bands in common in this study (Fig 5, Table 3). Earlier studies had also reported cross-reactivity between EN and *Alternaria*.⁹ Extracts of *A. alternata*, *C. herbarum*, *C. lunata*, and *P. citrinum* demonstrated a dose-dependent inhibition of IgE binding to EN in solid phase. Portnoy et al⁹ reported that 100 µg/mL of *Alternaria* extract inhibited EN-specific IgE antibodies. In the present study, 1.75 µg/mL was required to achieve the same inhibition. These differences may be attributable to the quality of the extract, the strains of fungi used, or the different serum pools.

In IgE immunoblot inhibition, *A. alternata*, *C. herbarum*, *C. lunata*, and *P. citrinum* showed four to eight cross-reacting allergens. The 43- and 24-kD bands in EN extract were not completely inhibited by 1 mg/mL of self-antigen, although the 43-kD band in *A. alternata* and *C. herbarum* extract was completely inhibited by EN. This may be attributable to

Table 3. IgE-Binding Bands Recognized in Different Fungi Using EN-Hypersensitive Pooled Patients' Sera

Fungi	Number of bands in immunoblot	Molecular weight of shared IgE bands
<i>Aspergillus fumigatus</i>	8	31, 29, 26, 20, 17, 14
<i>Aspergillus tamarii</i>	8	45, 30, 17, 14
<i>Alternaria alternata</i>	11	100, 66, 55, 45, 43, 34, 26, 17
<i>Candida albicans</i>	8	45, 37, 34, 30
<i>Cladosporium herbarum</i>	6	100, 63, 50, 37, 30
<i>Curvularia lunata</i>	8	80, 63, 43, 37, 34, 20
<i>Fusarium solani</i>	5	29, 20, 17, 14
<i>Mucor hiemalis</i>	6	45, 31, 29, 26
<i>Rhizopus oryzae</i>	4	34, 20, 17
<i>Penicillium citrinum</i>	7	100, 34, 20
<i>Epicoccum nigrum</i> (EN)	23	103, 100, 96, 80, 66, 63, 55, 50, 45, 43, 40, 37, 34, 31, 30, 29, 26, 25, 24, 20, 17, 15, 14

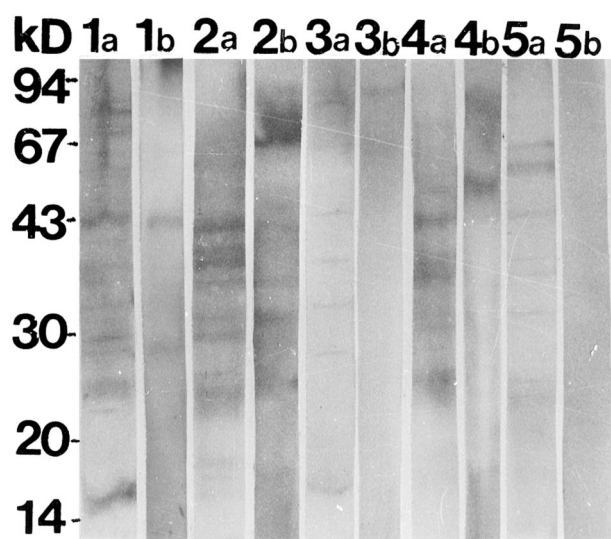


Figure 6. Immunoblot inhibition: fungal extracts. 1) EN, 2) *A. alternata*, 3) *C. herbarum*, 4) *C. lunata*, and 5) *P. citrinum* were separated on SDS-PAGE, transferred onto NC and probed with "a," EN-positive serum pool or "b," EN-positive serum pool incubated overnight with 1 mg/mL of EN.

higher content of the antibodies in the serum pool or lesser amount of these proteins in *A. alternata* and *C. herbarum*. Similarly, Shen et al²⁹ have also observed that *Penicillium notatum* extract could not completely inhibit IgE antibodies against its 34-kD allergen. They also found significant IgE cross-reactivity and N-terminal sequence homology among 34-kD major allergens in different *Penicillium* species. In another study by Shen and Hans,²⁶ N-terminal amino acid sequence analysis has shown that the cross-reactive 33/34-kD proteins of different *Penicillium* species and the 34 kD major allergen of *Aspergillus oryzae* may be an alkaline serine protease. In this study, the cross-reactive 34-kD band was found to be inhibited both in EN and *Penicillium*. The nature of this cross-reactive allergen can be determined after it is purified and sequenced.

CONCLUSION

The different serum pools used in IgE immunoblot and IgE immunoblot inhibition revealed three to eight cross-reacting allergens in EN, *A. alternata*, *C. herbarum*, *C. lunata*, and *P. citrinum*. Nowadays, purified/recombinant allergens are preferred for cross-reactivity studies as they are well defined.³⁰⁻³² However, such a study with EN is possible only when one of the major allergen is purified and sequenced. Until now, the cross-reactive allergens in EN have not been identified. The data presented here would thus form a useful basis for future studies.

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