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The allergens of *Epicoccum nigrum* Link

I. Identification of the allergens by immunoblotting

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Two atmospheric isolates of Epicoccum nigrum (EN) were grown under sporulation conditions. Dialyzed extracts of spores, (>95% pure) and pure mycelia were used for skin testing, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and immunoblotting. By skin testing, 49 of the 126 atopic patients were found to be sensitive to EN in St. Louis, Mo., and Corpus Christi, Texas, combined. On immunoblotting, which was performed on 17 sera, 44 bands (12.3 to 119.0 kd) were detected; six were unique to spore, four were unique to mycelium, and 34 were common to both. No single band bound IgE from all sera. The most frequent band corresponding to 42 kd occurred in 11 sera. Five other bands were recognized by more than one half, whereas the remainder bound fewer sera. All skin test-positive patients had positive immunoblots; the number of bands recognized varied from three to 25. Spore or mycelium-specific, as well as common bands were recognized by 13 of 17 sera. Two sera recognized only spore and mycelium-specific bands. Only spore-specific bands were bound by two sera. No strain differences were detected. The binding patterns were comparable in the sera from both St. Louis, Mo., and Corpus Christi, Texas. These data suggest that EN is a significant allergen in urban communities. Allergenic proteins occur in both spore and mycelium, suggesting that both must be included in the reagents for skin testing and immunotherapy. (J ALLERGY CLIN IMMUNOL 1992;90:11-20.)

Key words: *Epicoccum nigrum*, atmospheric isolates, mold growth, spore, mycelium, skin testing, SDS-PAGE, immunoblotting, IgE binding, allergy

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Airborne mold spores are major components of aeroparticulates and a significant cause of seasonal and perennial disease. Mold spores often outnumber pollen by 1000:1 or more in the air.¹ The purpose of this ongoing study by our group is a systematic analysis of the mold aerobiology and immunology comprising two climatologically dissimilar regions.

Recent aerobiologic investigations^{2,3} by us have demonstrated the occurrence of *EN* Link spores in the St. Louis, Mo., and Corpus Christi, Texas, metropolitan regions. Volumetric aerosampling in Corpus Christi demonstrated that *EN* was one of the most frequent mold spores captured and made up 1.3% of the total aerospora.² Similarly, gravity sampling onto

culture plates in St. Louis demonstrated that *EN* was the third most common viable spore encountered.³

EN is a ubiquitous mold distributed widely throughout the United States.^{4,6} Although up to 60 species within the form genus *Epicoccum* have been described, more recent studies have suggested that most of these represent strain variations of *EN*.⁷ The only other currently recognized *Epicoccum* form species, *E. andropogonis* (Ces) Schol-Schwarz is a secondary parasite on *Claviceps* spp, the ergot-causing fungus observed on the members of the grass family (Poaceae). *EN* grows avidly as a saprophyte on the rotting vegetation, paper, or textile, or as a weak parasite on various plants.⁷ For this reason, a number of workers have suggested that this mold may be a significant problem in rural settings, particularly after harvesting.⁸ Indeed, an earlier study had demonstrated high levels of sensitivity to this mold in a rural population.⁶ *EN* has also been recovered from the dust and air of the air-conditioning ducts.⁹

Studies by Portnoy et al.¹⁰ in the rural setting demonstrated that large numbers of mold-sensitive individuals reacted to an "in-house"-prepared *EN* extract.¹⁰ In contrast, this same population had previously demonstrated only a limited skin reactivity to a commercial extract.⁶ Despite being a significant cause of allergic disease, little study has been done to identify allergens in *EN*. In the present study, we have isolated several atmospheric strains of *EN* and developed culture conditions that yield high levels of sporulation, allowing us to prepare pure extracts of spores and mycelia. These same extracts were used for immunoblotting and have allowed us to identify the allergens in *EN*.

MATERIAL AND METHODS

Atmospheric isolates of *EN* were collected by exposing culture plates with PDA, prepared as previously described,¹¹ to the air for 15 minutes and then allowed to grow at room temperature.³ Two strains were isolated and purified by growing single spores on PDA. These isolates, called strain 1 and 2, will be available from the American Type Culture Collection. The strains were identified by pigment formation when they were grown at 20° C under constant illumination with Gro-Lights (Sylvania [GTE Products Corp., Danvers, Mass.]). Under these conditions, strain 1 produced a brilliant yellow pigment and did not sporulate, whereas strain 2 produced a deep red pigment and limited numbers of spores. In both cases, pigment diffused into the medium. High levels of spore formation in both strains 1 and 2 were induced when the temperature was increased to 23° C, and the constant illumination was with a combination of Gro-Light and Cool-white fluorescent lamps. The selection of PDA as growth medium was based on the fact that our initial attempts to use a number of synthetic or defined media^{10, 12, 13} resulted in significant decrease in the growth

Abbreviations used

<i>EN</i> :	<i>Epicoccum nigrum</i>
PDA:	Potato/dextrose agar
EDTA:	Ethylenediaminetetraacetic acid
SDS-PAGE:	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
NC:	Nitrocellulose
PBS:	Phosphate-buffered saline, 0.154 mol/L of NaCl/20 mmol/L of phosphate, pH 7.35
HSA:	Human serum albumin
NFDM:	Nonfat dry milk
MW:	Molecular weight

rate and did not yield significant spore formation. Among the semidefined media tested, both PDA and potato/dextrose yeast agar elicited excellent results.

Spores were isolated from the actively sporulating 7- to 10-day-old colonies. The black/dark brown spores were gently lifted off the colonies with a scalpel blade. Pure mycelia were isolated by growing the mold in liquid potato/dextrose in 150 mm T flasks in the dark with constant gentle agitation. The mycelial balls were collected after 5 to 7 days of active growth and then washed briefly to remove traces of the growth medium. The purity of both the spores and mycelia was evaluated microscopically, based on the fragment analysis of the unstained preparations.

Extracts of spores and mycelia were prepared from the purified preparations. Briefly, the spores or the mycelia were rapidly frozen in liquid N₂ and then powdered in a mortar and pestle (chilled to the temperature of liquid N₂). The powder was rapidly added to 125 mmol/L of ammonium bicarbonate buffer, pH 8.1, containing 5 mmol/L of EDTA (1:5 Wt/Vol), and the mixture was further disrupted in a Polytron (Brinkman Instruments, Inc., Westbury, N.Y.) with 6- to 10-second bursts at 50% of the maximal setting. Debris were sedimented at 2200 rpm in a Beckman J-6M (Beckman Instruments, Inc., Irvine, Calif.) centrifuge, and the extract was further purified by centrifugation at 14,500 g in a Beckman J-21M centrifuge. The clarified extract was then dialyzed with Spectra/Por3 MWCO 3500 dialysis membrane (Spectrum Medical Industries, Inc., Los Angeles, Calif.) against ammonium bicarbonate buffer and stored at 4° C. The quantitative measurement of total proteins in the extracts was performed with trinitrobenzene sulfonate as described previously.¹⁴

During the initial experiments, freeze-dried, frozen, or fresh-powdered mold material was extracted in a variety of solutions: distilled water, PBS, pH 7.3, phosphate buffer, pH 7.7, Tris HCl, pH 8.0, and ammonium bicarbonate buffer, pH 8.1. In some experiments, EDTA, phenylmethyl sulfonyl fluoride, or insoluble polyvinyl pyrrolidone was included in the extraction medium.¹⁵ Both EDTA and phenylmethyl sulfonyl fluoride are proteolysis inhibitors, whereas polyvinyl pyrrolidone is known to bind with polyphenolic compounds, such as melanin, a pigment pres-

ent in the spores, and thus prevents binding and precipitation of melanin with the proteins. The extraction procedures involved disruption of the spore or hyphal walls by homogenization/sonication or by use of a Polytron, followed by constant agitation, stirring at 4° C for periods extending up to 48 hours. Of these procedures, the one noted above elicited the highest yield of protein with the broadest range of MWs.

Potato/dextrose control was prepared by clarifying the liquid growth medium by centrifugation, followed by dialysis against ammonium bicarbonate buffer and then lyophilization. For biochemical investigations, lyophilized medium was suspended 1:5 (wt/vol) in 125 mmol/L of ammonium bicarbonate buffer and stored at 4° C.

For skin testing, extracts diluted 1:100 in PBS containing 0.1% HSA (Hollister-Stier Laboratories, Spokane, Wash.) were first filtered through 0.44 and 0.22 µm cellulose acetate filters (Millipore Corp., Bedford, Mass.), and then equal volumes of sterile glycerol were added. Lyophilized potato/dextrose medium was reconstituted 1:100 in PBS/HSA and filtered as above. Skin testing was performed with spore and mycelial extracts of both strains 1 and 2 on adult volunteers with the skin prick test. Controls included PBS/HSA diluent and histamine along with the dilute potato/dextrose extract and were tested interdermally as well as by the prick test. Skin tests were graded by comparing reactions to the test reagents with diluent and histamine controls.¹⁶ All patients were informed of the nature of the study and signed an informed consent form. This study has been approved by the Human Experimentation Committee of Washington University School of Medicine, St. Louis, Mo.

Skin testing in St. Louis was performed to the *Epicoccum* extracts and control as noted above. Each of these individuals were also skin tested, either simultaneously or previously, to a battery of 48 perennial and seasonal allergens. This battery contained eight molds: *Alternaria*, *Aspergillus*, *Cladosporium*, *Fusarium*, *Helminthosporium*, *Penicillium*, *Phoma*, and *Spondylocadium* (all molds obtained from Hollister-Stier Laboratories). Based on a clinical history of perennial symptoms or symptoms consistent with the peak of airborne mold spores in the St. Louis area and skin test reactivity to the eight molds tested, we divided our patients into three groups: (1) patients who were mold sensitive, (2) patients who were atopic but were not mold sensitive (based on history and three or more positive skin test reactions), and (3) nonatopic volunteers (negative history and no positive skin reactions). No exclusions for food intolerance to potatoes were made in the study population after questioning each patient and finding none. Blood was collected from patients with positive prick tests to *EN* spores, mycelia, or both, and sera were prepared within 120 minutes of collection.¹⁷ A pool of sera from nonatopic individuals served as control.

Blood was also collected from *EN*-sensitive individuals in Corpus Christi where atopic patients are routinely tested for sensitivity to *EN* with a commercial extract (Meridian Biomedical, Inc., Round Rock, Texas). Patients were also tested to the eight molds noted above as well as *Aureobasidium*, *Curvularia*, *Geotrichum*, *Monilia*, *Nigrospora*, and

Stemphylium. Frozen sera were then shipped to St. Louis by overnight carrier.

SDS-PAGE extracts of strains 1 and 2 mycelia and spores, as well as potato/dextrose control, were mixed with lysis buffer¹⁸ and boiled for 5 minutes. Extracts were separated on 0.75 mm thick 12% "minigels" as previously described.¹⁹ The gels were stained with Coomassie brilliant blue or were fixed according to a method described by Guevara et al.²⁰ and stained by silver impregnation with the Rapid-Ag stain kit (ICN Biochemicals, Irvine, Calif.) as described by the manufacturer.

For immunoblotting, separated proteins were electrophoretically transferred to NC sheets as previously described.¹⁹ Each gel contained individual lanes containing strains 1 and 2 spore and mycelial extracts. The NC sheets were dried and frozen at -20° C until use. Allergen detection was performed with sera of patients or control subjects diluted 1:4 with PBS containing 5% NFDM. After the overnight incubation with patients' sera, the NC sheets were washed and incubated with an affinity-purified rabbit anti-human IgE coupled to alkaline-phosphatase (K & P Immunochemicals, Gaithersburg, Md.) diluted 1:500 in PBS/NFDM, and incubated overnight at 4° C. The NC sheets were washed in PBS/NFDM and in distilled water, and the IgE-binding bands were detected with the 5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium kit (K & P Immunochemicals) as described by the manufacturer.

Specificity was confirmed by inhibition experiments. Immunoblotting was repeated with a patient's serum previously demonstrated to have maximum IgE binding to spores and mycelia of both strains to discern any nonspecific IgE binding. The serum was preincubated (1:1) with an equal mixture of the extracts of spore and mycelium of both strains. This procedure was performed at 4° C for 1 hour. The remainder of the procedure was similar to the preceding immunoblotting protocol.

RESULTS

EN strains 1 and 2 produced abundant morphologically similar spores and mycelia. Extracts were prepared from harvested spores that were >95% pure with minimal contamination by hyphal elements or agar and mycelial balls that were 100% pure (Fig. 1). On epicutaneous or intradermal skin testing in St. Louis, none of the patients reacted to the potato/dextrose control. No skin reactions to either mycelial or spore extracts were noted in nonatopic patients (three of the total 31 skin tested individuals), demonstrating the absence of nonspecific histamine-releasing activity in either preparation. Of the atopic patients tested in St. Louis, 13 of 28 patients were prick test positive to *EN*. The results of the positive individuals are presented in Table 1. There were variable differences in the skin reactivity between the mycelial and spore extracts from strain 1 or 2. Most patients reacted equally well to both the spore and mycelial extracts.

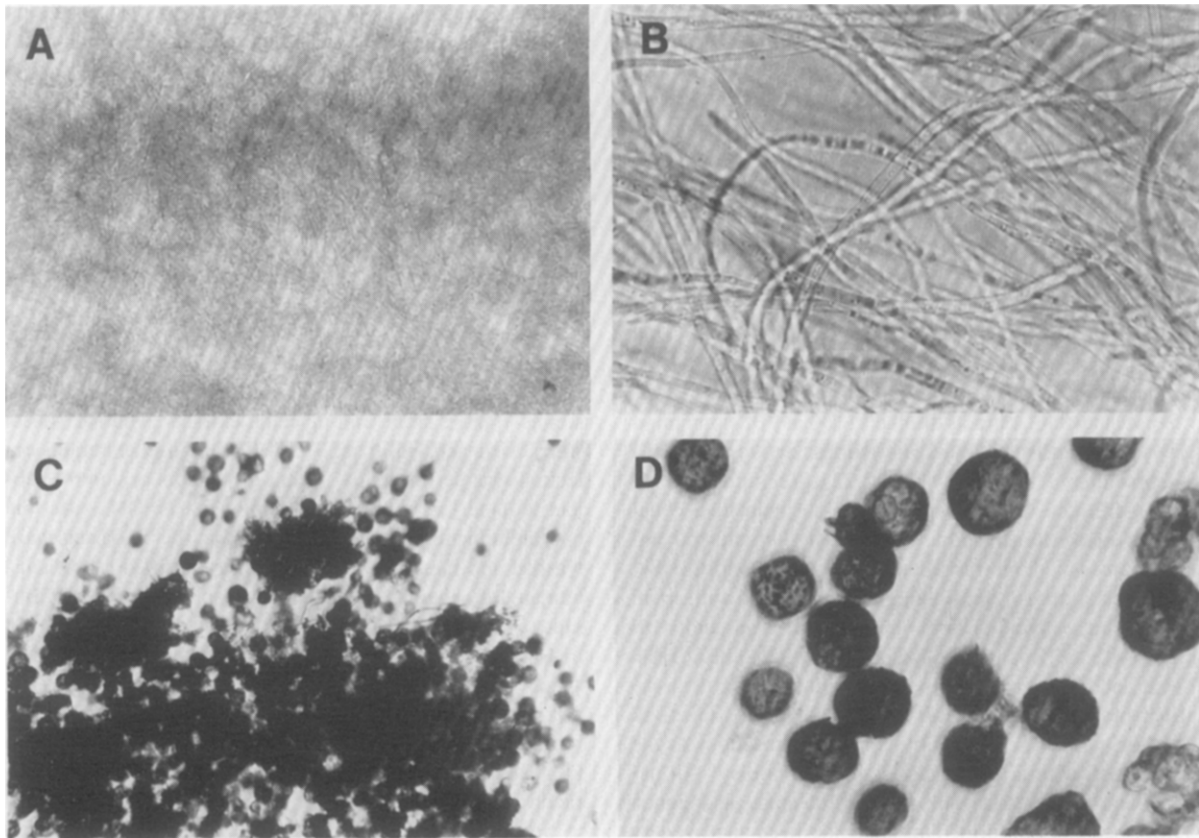


FIG. 1. Harvested mycelia and spores of *EN* used for extract preparation. **A** and **B**, Mycelia. **C** and **D**, Spores. (Original magnification: **A** and **C**, $\times 150$; **B** and **D**, $\times 860$.)

TABLE I. Skin reactivity to spore and mycelial extracts of *EN* in sensitive atopic patients in St. Louis

Patient No.	Spore extract		Mycelium extract	
	Strain 1	Strain 2	Strain 1	Strain 2
1	3+*	Neg	2+	3+
2	Neg	Neg	Neg	2+
3	4+	4+	4+	4+
4	3+	3+	3+	1+
5	4+	4+	4+	Neg
6	4+	Neg	4+	Neg
7	3+	Neg	Neg	Neg
8	Neg	1+	3+	2+
9	3+	Neg	Neg	Neg
10	3+	Neg	3+	Neg
11†	2+	2+	Neg	2+
12†	2+	Neg	1+	Neg
13†	2+	Neg	Neg	1+

Neg. Negative.

*Results are for prick testing with the Multitest (Lincoln Diagnostics, Decatur, Ill.) procedure.

†Serum not available for further screening.

indicating that either there were shared allergens within the two preparations or that most patients made IgE directed against allergens in both spores and hyphae. Five patients reacted only to strain 1, whereas one patient reacted only to strain 2. Two patients reacted only to the spore extract, and one patient reacted to only mycelial extract. All *EN*-positive patients were sensitive to other molds by skin testing. None of the nonmold-sensitive atopic patients reacted to any of the *EN* reagents. In Corpus Christi, 36 of the total 98 (92 mold sensitive and six nonmold sensitive) atopic patients with respiratory symptoms were positive on skin testing with a commercial preparation of *EN*. Similar to the patients in St. Louis, only mold-sensitive atopic patients reacted to *EN*. None of the six nonmold-sensitive atopic patients and two nonatopic individuals gave positive reactions. Those patients who were *EN* sensitive had symptoms of allergic rhinitis, conjunctivitis, or asthma that were perennial or, if these were seasonal, corresponded to the peaks of *EN* spores in the aeroparticulate in either St. Louis or Corpus Christi. There were no clinical characteristics that allowed us to differentiate patients who were sensitive to *EN* from patients who did not react to *EN* extracts.

The SDS-PAGE pattern for the spore and mycelial preparations is illustrated in Fig. 2. Although there were marked differences in the banding pattern between the spore and mycelial extracts, there were a number of bands that were common. Moreover, there was no variation in the banding pattern between either the mycelial or spore extracts of the two strains. The PDA control did not exhibit any band(s) (results not presented). Of the skin test-positive individuals tested, 10 patients from St. Louis and seven from Corpus Christi provided sera for immunoblot analysis. All 17 sera demonstrated positive immunoblots to the spore extracts, mycelial extracts, or both. Some of the representative blots revealing various patterns of IgE binding are illustrated in Fig. 3. All immunoblots demonstrated significant patient-to-patient variability. There were no differences in the IgE-binding bands for the individual patient between strains 1 and 2, even for those patients who had previously demonstrated strain differences with skin prick test. In all, 44 IgE-binding bands were detected in spore and mycelial extracts (Table II). IgE binding occurred to proteins with apparent MW varying from 12 to 119 kd. The total numbers of bands detected in spore and mycelial extracts were 40 and 38, respectively. Of these, 34 bands were common, whereas six were unique to spore and four unique to mycelium. The frequency of the binding of patient's sera to each spore

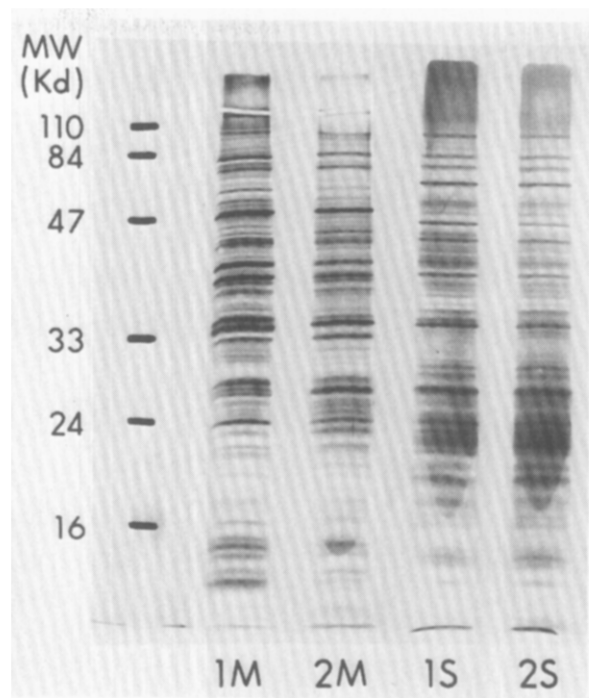


FIG. 2. SDS-PAGE patterns of mycelial and spore extracts of *EN* in 12% gel stained with silver (2.25 μ g of protein sample per lane); far left lane, MW markers; 1M, strain 1 mycelial extract; 2M, strain 2 mycelial extract; 1S, strain 1 spore extract; 2S, strain 2 spore extract.

or mycelial band is indicated in Table II. None of the bands was recognized by all 17 sera from *EN*-positive patients. The most frequent band was number 27 that bound to 11 sera, followed by bands 2 and 14 binding IgE in 10 sera, bands 11 and 22 binding in nine sera, band nine binding in eight sera, with the remaining bands binding in one to seven sera. For an individual band that appeared to have common epitope(s) and thus shared antigenic determinants between spore and mycelium, not every patient's sera bound to both spore and mycelial extracts. Thus, the number of sera reacting with a specific common band differed among spore and mycelial preparations. The total number of IgE-binding bands recognized by a single serum varied from three to 25 (Table III). The maximum number of bands recognized by any serum was 16 for the spore extract, 20 for the mycelial extract, and seven for the common bands. Two responders from St. Louis bound IgE exclusively to the spore extract, whereas another two responders had bands with both spore and mycelial extracts but no common bands. None of the sera bound IgE only to the mycelial extract. The overall band numbers, distribution, and binding were comparable in sensitive patients from St. Louis and Corpus

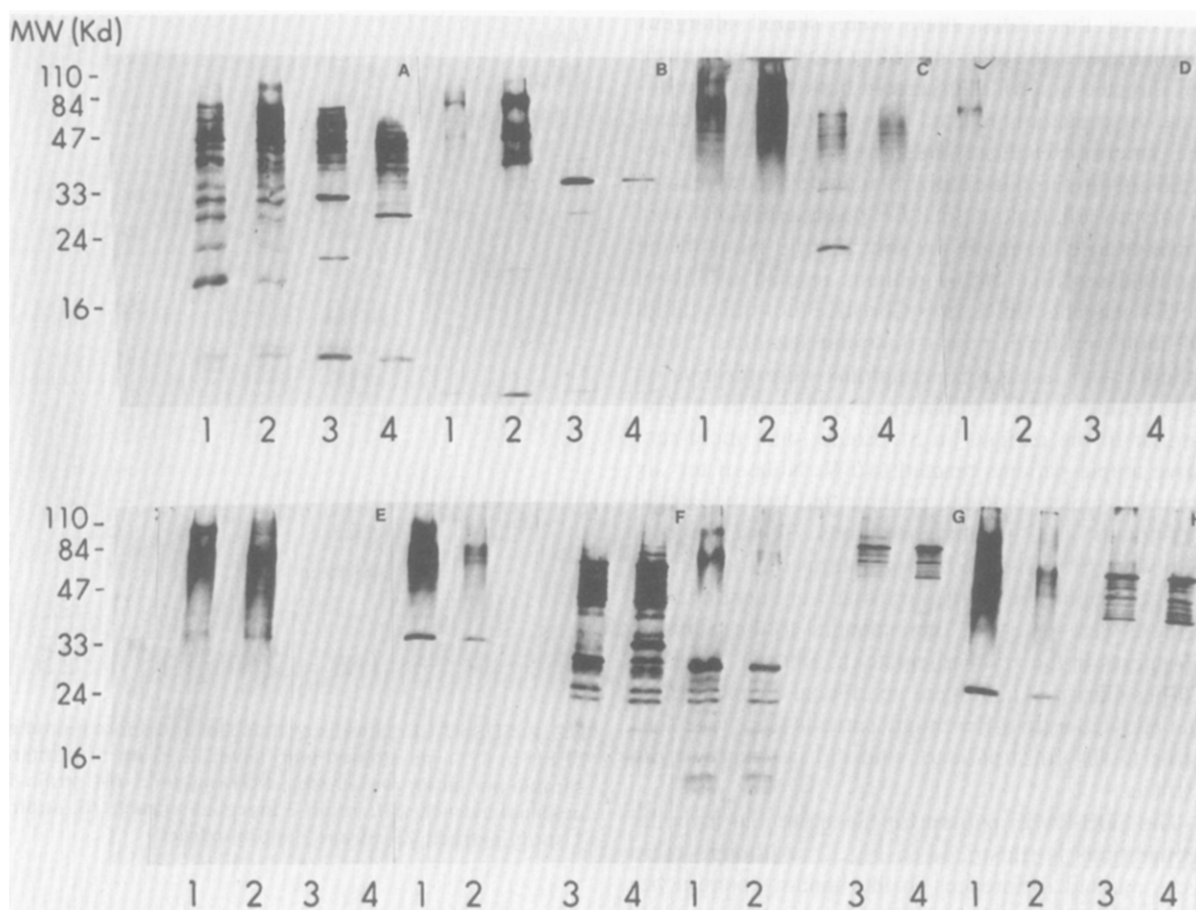


FIG. 3. *EN*-positive immunoblots revealing varying IgE-binding band patterns in different patients. Each lettered set (*A* through *H*) represents band pattern for an individual patient. Immunoblot *B*, patient numbered 4; *D*, patient 2; *H*, patient 1 of Table I. Rest of immunoblots are of patients from Corpus Christi who were skin tested with commercial extract. Far left lane, MW markers; lanes 1 and 2, spore extracts and lanes 3 and 4, mycelial extracts of strains 1 and 2, respectively.

Christi. *EN*-negative atopic control and nonatopic control subjects gave negative immunoblots. Nonspecific binding was not detected in the inhibition experiments (data not presented).

DISCUSSION

The study presented here, in conjunction with our aerosampling surveys,^{2, 3} demonstrates that *EN* is a significant allergen in both St. Louis, and Corpus Christi. Previous studies with skin testing and ELISA/ELISA-inhibition techniques have demonstrated sensitivity to *EN* in rural communities such as southeast Missouri^{6, 8, 10} and in a semirural university setting in Michigan.¹⁰

Atmospheric isolates of *EN* grew easily in the laboratory under well-defined culture conditions. The ability of the variation in culture conditions to induce

or prevent spore formation allowed us to evaluate allergens in both spore and hyphal elements. This finding is important since the results presented here demonstrate that unique allergens are present in both spores and mycelia and therefore extracts of *EN* for skin or in vitro testing must contain both the spore and mycelial allergens. However, the possibility of the introduction of variation between spore and mycelial extracts caused by the differences in the physical conditions that were used to obtain sporulating or nonsporulating colonies, along with the brief washing of the mycelial balls, cannot be completely disregarded at this stage. Although evidence exists for the presence of fungal allergens of metabolic origin in the culture broth,²¹⁻²³ spent culture medium was not examined in this study because it is usually considered less suitable for the preparation of standardized extracts.^{24, 25}

TABLE II. Distribution of IgE-binding bands in *EN*-positive immunoblots

Band No.	Apparent MW (kd)	No. of sera bound			Total No. of sera bound*
		Only to spore extract	Only to mycelial extract	To both extracts	
1	119	—	1	—	1
2	114	10	—	—	10
3	108	5	—	—	5
4	99	2	1	—	3
5	96	2	—	—	2
6	94	4	2	1	7
7	90	2	1	—	3
8	88	3	—	1	4
9	86	2	3	3	8
10	84	—	3	—	3
11	82	6	2	1	9
12	80	1	3	1	5
13	79	1	1	—	2
14	77	5	1	4	10
15	74	1	3	2	6
16	72	—	—	2	2
17	69	1	3	1	5
18	66	1	2	3	6
19	63	2	4	—	6
20	60	2	2	2	6
21	57	1	3	2	6
22	54	3	2	3	9
23	51	1	2	1	4
24	49	1	4	1	6
25	46	—	3	—	3
26	45	2	3	1	6
27	42	4	7	—	11
28	38	3	3	1	7
29	36	—	3	—	3
30	35	—	1	2	3
31	33	1	3	3	7
32	32	—	1	3	4
33	30	1	1	3	5
34	29	1	3	1	5
35	27	2	—	—	2
36	26	2	1	2	5
37	24	—	3	1	4
38	22	3	—	—	3
39	20	1	—	1	2
40	17	2	—	1	3
41	15	2	1	—	3
42	14	1	2	—	3
43	13	1	—	—	1
44	12	2	1	1	4

Determined by linear regression analysis of multiple immunoblots.

*N = 17.

We also noted that proper procedures were necessary for the preparation of *EN* extracts. Attempts to prepare extracts of either spores or mycelia with lyophilized material did not prove fruitful, yielding ex-

tremely low amounts of protein with limited spectra as determined by SDS-PAGE. Similarly, neither direct disruption of the material by the Polytron nor prolonged extractions (48 hours at 4° C) of either spore

TABLE III. IgE-binding band distribution in immunoblots of *EN*-positive individuals from St. Louis and Corpus Christi

<i>EN</i> -positive serum No.	No. of IgE-binding bands			Total No. of bands
	With spore extract	With mycelium extract	Common to spore and mycelium extracts	
1 SL*	16	14	5	25
2 SL	3	—	—	3
3 SL	5	14	5	14
4 SL	7	6	2	11
5 SL	4	11	3	11
6 SL	10	2	—	12
7 SL	2	5	—	7
8 SL	5	—	—	5
9 SL	4	4	3	5
10 SL	4	5	1	8
11 CC†	16	16	7	25
12 CC	5	4	2	7
13 CC	12	12	6	18
14 CC	9	2	2	9
15 CC	11	4	3	12
16 CC	11	20	6	25
17 CC	3	5	1	7

*SL, Serum from skin test-positive patients from St. Louis.

†CC, Serum from skin test-positive patients from Corpus Christi.

or mycelium yielded adequate extraction. It was for this reason that we used frozen powdered spores or mycelia for our extraction procedure. This provided an extract with high protein content and the broad spectrum of protein bands noted on SDS-PAGE. Since some commercial *EN* extracts are prepared from lyophilized material, this may account for the low frequency of skin test reactions to *EN* noted when such extracts were used in previous studies.⁶ Other commercial extracts prepared from material that has not been lyophilized may not have this problem, evidenced by the results from Corpus Christi in which an *EN* extract prepared only from nonlyophilized mold material was used (J. Brandewie, Meridian Biomedical Inc. Personal communication).

In the present study, 49 of the 126 atopic individuals were found to be sensitive to *EN* with comparable levels in both St. Louis and Corpus Christi. Skin testing results ranged from 1+ to 4+ among sensitive individuals who demonstrated varying response to the two strains, whereas additional testing of the patient's sera by immunoblotting did not reveal any such difference. Six of the 10 *EN*-sensitive patients from St. Louis (skin tested to both spore and mycelial extracts) reacted equally well to spore and mycelium with concordant results between skin testing and immunoblotting. These results are in contrast to the previously reported greater sensitivity to *EN* mycelium by Port-

noy et al.¹⁰ Of the remainder, the skin-reactivity patterns did not necessarily correlate with the IgE-binding patterns. This finding suggests that skin reactivity alone cannot be used to discern strain variation in the allergenicity for a particular mold.

EN-sensitive patients exhibited great diversity in the IgE binding by immunoblotting. No two patients demonstrated identical patterns, although relative similarities were present. Multiple, unique as well as common, allergenic proteins occurred in both spore and mycelium. The numbers and patterns of IgE binding observed in the sera from patients in the St. Louis area were found to be comparable to those from Corpus Christi. This finding demonstrates that there are no significant antigenic differences among strains of *EN* found in these climatologically dissimilar regions of the country.

Both spore and mycelial *EN* extracts contained a large number of proteins as determined by silver staining. Of these proteins, 44 bands bound IgE in sera from *EN*-sensitive patients. However, this may be an underestimate of the total number of allergens, since separation of the proteins occurred under dissociating conditions that may reduce subsequent immunologic reactivity of many proteins, as reported by Hoffman^{26, 27} for Hymenoptera venom. In contrast, use of nondissociating conditions to separate and blot proteins of various complex extracts can result in the

occurrence of fewer clinically significant IgE-binding bands.²⁸

When individual immunoblot patterns are analyzed, the common bands may be detected in spore, mycelial, or both extracts. This finding indicates that many of the bands contain multiple allergenic components. The occurrence of shared antigenic components among spore and mycelial extracts for a particular band was either due to the presence of comigrating proteins or to the antigenic similarity of epitopes between the two extracts. Further separation by two-dimensional techniques, such as isoelectric focusing of both extracts followed by SDS-PAGE and immunoblotting, as previously described,²⁹ are currently underway in our laboratory to determine the true number of the allergens.

The analysis of the allergens in *EN* is also compromised by the known proteolytic activity of the fungal extracts.¹⁵ Thus, many of the IgE-binding bands, particularly bands of lower MW, may represent proteolytic fragments bearing identical allergenic epitopes to those bands with higher MW. In contrast, experiments with a variety of extraction methods, including inhibitors of proteolysis, did not alter the Coomassie blue or silver-impregnation banding patterns nor the number of bands detected by immunoblotting. Similarly, there was no change in banding pattern or immunoblotting when extracts were stored at 4° C, indicating again low or absent proteolytic activity. This suggests that *EN*, in contrast to other fungi, such as *Alternaria* or *Cladosporium*, contains limited proteolytic activity.

Although *EN* has been demonstrated to possess significant antigenic similarity with *Alternaria* by Portnoy et al.,¹⁰ Hoffman and Kozak³⁰ have found limited cross-reactivity between *EN* and *Fusarium*. Since extensive intergeneric cross-reactivity of *Alternaria*, especially with the dark-spored Fungi Imperfecti, has already been demonstrated,³⁰⁻³² efforts are underway by our group to examine any such relationship(s) of *EN* with some of these fungi.

Our study demonstrates the presence of large numbers of allergenic proteins in *EN*. These large numbers, coupled with significant levels of *EN* spores in the ambient air,^{2,3} may account for the high frequency of responders to *EN* in metropolitan St. Louis and Corpus Christi area. This study also indicates that the reagents for the diagnosis of sensitivity to *EN* in atopic individuals and for immunotherapy must be prepared with appropriate techniques that will ensure the least degradation of the allergens during extract preparation and must contain both spore and mycelial components to be of value.

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