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***Fusarium solani*: Prevalence of skin reactivity and antigenic allergenic analysis**

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The prevalence of Fusarium solani reactivity in atopic individuals with symptoms of mold allergy was assessed with skin test and RAST. In addition, F. solani preparations were evaluated for antigenic/allergenic activity. Atopic individuals tested, 24.5% (n = 69), had positive skin reactions to a phosphate-buffered saline extract of F. solani, and these responses were statistically correlated with RAST results. Immunoelectrophoretic techniques demonstrated that this extract was antigenic in rabbits and allergenic in man. Gel filtration of F. solani extract on a Bio-Gel A 0.5 m column demonstrated three peaks of ultraviolet-absorbing material. The column eluate with the greatest RAST inhibition activity was associated with a protein peak having a molecular weight >341 kilodaltons; however, all peaks demonstrated inhibitory activity. These studies suggest that extracts of F. solani contain several allergens that differ in molecular weight and charge. (J ALLERGY CLIN IMMUNOL 77:842-9, 1986.)

It has long been recognized that inhalation of fungal fragments can produce allergic symptoms in susceptible individuals. Both upper and lower respiratory tract symptoms have been reported, but the actual prevalence of asthma or rhinitis resulting from inhalation of mold spores and/or mycelial fragments is not

Abbreviations used

PBS:	Phosphate-buffered saline
RT:	Room temperature
CIE:	Crossed immunoelectrophoresis
CRIE:	Crossed radioimmunolectrophoresis
Kd:	Kilodaltons
FRIE:	Fused-rocket immunoelectrophoresis
FRRIE:	Fused-rocket radioimmunolectrophoresis
UV:	Ultraviolet

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known.¹ Although present in the atmosphere in considerably higher concentrations than pollen grains, fungal spores have not been as widely studied as pollen aeroallergens.²

Recently, detailed studies of molds and their relationship to allergy have been initiated. The Fungi Imperfecti, which contains many of the species thought to induce IgE-mediated allergic respiratory disease in man, includes the Deuteromycetes as well as asexual stages of a variety of Ascomycetes and Basidiomycetes. Genera of Fungi Imperfecti, which have been studied in relation to allergy, include *Cladosporium*, *Aspergillus*, and *Alternaria*.³⁻¹¹ Our knowledge of antigenic/allergenic activity of these genera has increased, but other fungal preparations, including species of *Fusarium*, have not been well studied. *Fusarium* can be present in the air in levels up to 158/cu ft¹² and is reported to be among the genera that are most responsible for induction of respiratory allergic disease.¹³ Crude preparations of the species *F. solani*, in particular, are frequently used in clinical practice as a skin test reagent to determine the atopic status of patients and in immunoassays (RAST). In combination with other mold extracts, *F. solani* is also used for immunotherapy, although no controlled trials have been performed to determine therapeutic efficacy. Thus, it is important to characterize the allergens present in this organism.

The purpose of this study was to examine the prevalence of *F. solani* reactivity in patients with symptoms of mold allergy and to evaluate *F. solani* antigens/allergens by use of immunoelectrophoretic techniques.

MATERIAL AND METHODS

Extract preparation

Extract was prepared by sonicating (4° C for 10 minutes) 500 mg of powdered, defatted *F. solani* (obtained from Greer Laboratories, Lenoir, N. C., and verified by their consultant mycologist, Roger Crenshaw [material contained both spores and mycelia]) in 50 ml 0.01 mol/L PBS (pH 7.2). Light microscopy revealed that approximately 50% of the mycelia were disrupted. The sonicate was mixed overnight at 4° C and centrifuged at 48,000 × g for 30 minutes at 4° C, and then the pellet was discarded. The supernatant was concentrated to approximately 12 ml by Amicon ultrafiltration (Amicon Corp., Danvers, Mass.) (UM 2; molecular weight exclusion 1000) and dialyzed (molecular weight exclusion 3500) for 72 hours at 4° C against PBS. The retentate was centrifuged at 105,000 × g for 30 minutes at 4° C, the dry weight was determined, and the supernatant was frozen at -20° C until use. Multiple batches of extract were prepared and pooled. Final yield of the extract was 5.4% of the starting material.

Skin testing

A total of 69 atopic individuals were referred to the Charity Hospital or Tulane University Hospital Allergy Clinics with perennial rhinitis symptoms with or without associated asthma (consistent with mold allergy) and were tested.

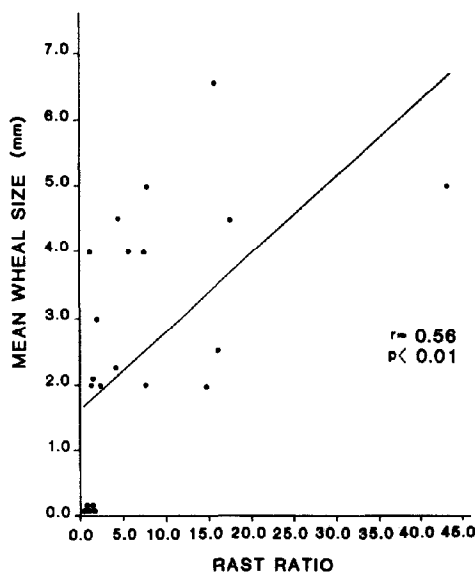


FIG. 1. Skin prick test results and RAST reactivity of atopic individuals tested with a 10 mg/ml of 0.01 m PBS *F. solani* extract.

These subjects had previously been skin prick tested with a panel of common inhalant allergens and were classed as atopic if they had a positive reaction to two or more of these allergens. In our clinics standard skin prick testing was performed with 10 mg/ml of *F. solani* extract in 50% glycerol-PBS; histamine diphosphate (1 mg/ml) was used as the positive control, and PBS diluent served as the negative control. Wheal size was determined by averaging the diameter in two dimensions. A reaction was considered positive if it was 2 mm greater than the negative control.

RAST

RAST with *F. solani* extract was performed on 33 of the 69 individuals who were skin prick tested and on whom sera were available. Filter paper discs were activated after the method of Ceska and Lundkvist¹⁴ by use of CNBr (Sigma Chemical Co., St. Louis, Mo.). Activated discs were coupled with 10 mg/ml of the *F. solani* extract or, as a control, human serum albumin (Miles Laboratories Inc., Elkhart, Ind.) in borate buffer (pH 8.0) overnight on a clinical rotator at RT washed three times in ethanolamine (1 mol/L, pH 8.0), then washed three times with assay buffer (500 ml 0.2 mol/L of Tris buffer, pH 7.5; 500 ml 1.8% NaCl [w/v]; 10 ml 5% NaN₃; 5 ml Tween 20; 2 gm of bovine serum albumin), and stored in assay buffer at 4° C until use.

For the test 100 µl of serum was added to tubes containing either an *F. solani*- or an human serum albumin-coupled disc, incubated overnight at RT on a rotator, and washed three times with 2.5 ml of physiologic saline to remove unreacted serum. One hundred microliters of ¹²⁵I-labeled anti-IgE (Pharmacia, Piscataway, N. J.) containing approx-

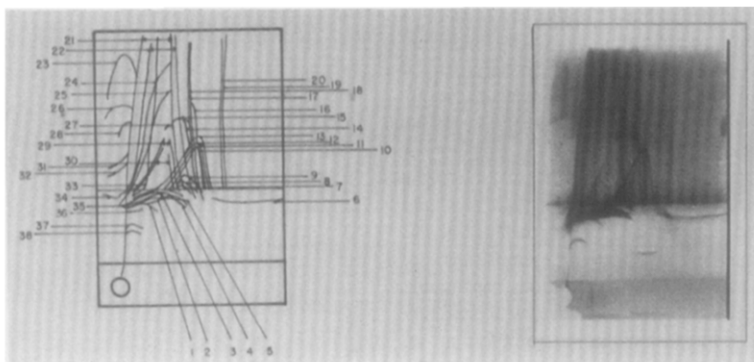


FIG. 2. CIE photo and line drawing of *F. solani* antigens. (Reproduced with permission from Lehrer SB, Aukrust L, Salvaggio JE: Respiratory allergy induced by fungi. Clin Chest Med 4:23, 1983.)

imately 30,000 cpm was added, the incubation and washing were repeated, and tubes were counted on a gamma counter. Results were expressed as the ratio of test counts per minute divided by HSA counts per minute. A RAST ratio ≥ 2 was considered positive.¹³

Rabbit antiserum

Two new Zealand white rabbits were hyperimmunized as follows: 100 μ g of *F. solani* extract (diluted with PBS) emulsified with incomplete Freund's adjuvant (Difco Laboratories, Inc., Detroit, Mich.) injected into each popliteal lymph node. Four, 6, and 8 weeks later, rabbits were injected subcutaneously with 800 μ g of *F. solani* extract emulsified with 1.0 ml incomplete Freund's adjuvant every 2 weeks. Rabbits then received five biweekly injections of 5.0 mg of *F. solani* extract in complete Freund's adjuvant (Difco). A total of 28.4 mg of *F. solani* extract was injected per animal. Rabbits were bled via the marginal ear vein, and sera were tested for the presence of precipitating antibodies by use of double gel diffusion. For immunoelectrophoretic studies, the sera were pooled and concentrated to one fifth of original volume by ammonium sulfate precipitation (40%) and Amicon filtration (XM50). Sera collected before immunization were pooled and used as a negative control.

CIE

CIE was performed by a standard technique.⁵ Briefly, 20 μ l of *F. solani* extract (23 mg/ml) was electrophoresed into 1% agarose (Eastman, Rochester, N.Y.) (w/v) in Tris-barbital buffer (pH 8.6) at 10 V/cm for 30 minutes. Antigen-containing gel strips, 1 cm wide, were cut and transferred to the base of a 5 by 7 cm agar-coated glass slide, and a blank agar layer approximately 1.5 cm wide was poured adjacent to this strip. Agarose (2.1 ml) containing 400 μ l of normal rabbit sera or *F. solani* antisera was poured over the remaining area of the slide. Second dimension electrophoresis, performed at right angles to the first, was run at 2 V/cm for 18 hours. Slides were pressed, washed, dried,

and stained with Coomassie brilliant blue R-250 (Eastman). A reference pattern was drawn, and precipitin arcs were arbitrarily numbered.

CRIE

To perform CRIE, CIE plates were prepared with *F. solani* as above, except the final wash was in saline, and plates were dried under a stream of cool air. Six hundred microliters of sera from 12 *F. solani* RAST-positive individuals was diluted with 6.4 ml of phosphate buffer (70 mmol/L, pH 7.0) and incubated with a prepared CIE plate for 24 hours at RT. Controls, to assess nonspecific radio-staining, were a serum sample from an atopic individual who was RAST negative to *F. solani* and saline. Plates were washed four times for 10 minutes each with 10 ml of physiologic saline and incubated for 24 hours at RT with approximately 250,000 cpm of ¹²⁵I-labeled anti-IgE (Pharmacia) in 7 ml of incubation buffer. After washing four times with saline and once with distilled water, plates were dried, and autoradiography was performed.

CRIE classes were determined with the method of Aukrust and Aas.¹⁶ Discs were incubated with increasing dilutions of reference sera A (Pharmacia), washed with saline, incubated with 250,000 cpm of ¹²⁵I-labeled anti-IgE, re-washed, and autoradiography was performed. Radiostaining of test plates was compared with staining of discs. In general, CRIE class A represents the highest degree of radio-staining, and CRIE class H represents the lowest; however, in these experiments the highest staining was class B, and the lowest staining was class G.

Gel filtration of *F. solani*

A 100 mg sample of the *F. solani* extract was fractionated by passage through a Bio-Gel A 0.5 m (100 to 200 mesh) (Bio-Rad Laboratories, Richmond, Calif.) column previously standardized with blue dextran (Pharmacia Fine Chemicals), human fibrinogen, human gamma globulin, ovalbumin, and lysozyme (all other column markers from

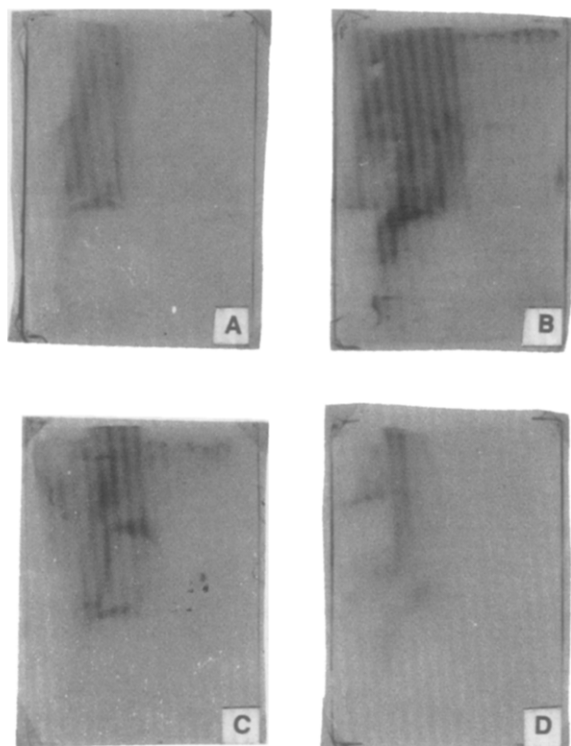


FIG. 3. CRIE photographic film from experiments with sera from selected *F. solani*-sensitive individuals. (Reproduced with permission from Lehrer SB, Aukrust L, Salvaggio JE: Respiratory allergy induced by fungi. Clin Chest Med 4:23, 1983.)

Sigma Chemical Co.). Material was eluted with PBS (pH 7.2, 0.01 mol/L) at 60 ml/hr, 5 ml fractions were collected, and UV analyses of fractions (optical density 230 and 280 nm) were performed.

RAST inhibition

RAST inhibition was performed by technique established in our laboratories¹⁷ with a serum pool from RAST-positive individuals. Pooled aliquots of five fractions, diluted 1:10 with PBS, were used as the inhibiting antigen. For the test, 100 μ l of test sera and 100 μ l of pooled column fractions were incubated for 1½ hours on a clinical rotator at RT, centrifuged at 1050 $\times$ g for 15 minutes, and a 100 μ l aliquot of each supernatant was assayed by RAST with an *F. solani* disc, as described above. Assays were performed in duplicate. A positive control consisted of a 100 μ l of serum sample and 100 μ l of PBS. Percent RAST inhibition was calculated as follows:

$$\frac{\text{Average test sample cpm}}{\text{Average positive control cpm}} \times 100 = \% \text{ RAST inhibition}$$

FRIE

FRIE was performed by a standard technique.¹⁸ Briefly, 8.3 ml of 1% agarose in Tris-barbital buffer (pH 8.6) was poured over a 2.5 cm wide strip of an 11 by 20.5 cm glass slide. Twenty-three milliliters of agarose, mixed with 2 ml concentrated hyperimmune rabbit *F. solani* antisera, was poured over the remaining area of the plate and allowed to congeal. Two millimeter wells were punched in alternating fashion across the bottom agar layer, and 5 μ l of each column fraction was applied to the appropriate well. Fractions were allowed to diffuse for 30 minutes at RT, and electrophoresis was performed at 3 V/cm for 18 hours. The plate was washed and stained as for CIE.

FRRIE

FRRIE was performed as follows: a FRIE plate was prepared as above except that the final wash was in saline, and the plate was dried with cold air. The plate was incubated overnight with 4.8 ml of pooled RAST-positive serum diluted to a total volume of 84 ml with phosphate buffer. The plate was washed four times with physiologic saline, incubated overnight with 3 $\times$ 10⁶ cpm of ¹²⁵I-anti-IgE, and

ing at peak 1 and more diffuse staining at peak 2. In addition, there was a wide area of diffuse staining on FRRIE that was poorly visible on FRIE. There was minimal staining at peak 3, but the major precipitin arc, as demonstrated by FRIE, did not stain.

DISCUSSION

These studies demonstrated that a significant percentage (24.5%) of atopic individuals of the South in the area of the Gulf of Mexico with perennial rhinitis and asthma had positive immediate wheal-and-flare skin prick test reactions and demonstrable serum IgE antibody to our *F. solani* extract. Skin test reactions were statistically correlated with RAST ratios. CIE demonstrated that *F. solani* was antigenic in the rabbit; furthermore, 21 of the antigenic components of *F. solani* demonstrated positive radiostaining by CRIE. The column eluate with greatest RAST inhibition activity was associated with a peak having a molecular weight >341 Kd.

It is of note that many of the atopic individuals in this study reacted with a positive skin prick test not only to *F. solani* but also to at least one other member of the Fungi Imperfecti. It is not known if these fungi are cross-reactive such that exposure to one mold can elicit an immune response to another or if multiple reactions merely reflect an allergic diathesis (e.g., atopy). The relationship between cross-reactivity of fungal allergens among different genera or higher order classifications is not clear and has not been well studied. By use of RAST inhibition, species of *Aspergillus* (*A. fumigatus*, *A. glaucus*, and *A. flavus*) were demonstrated to cross-react extensively, although they were unrelated to *Alternaria tenuis*.¹⁷ Recently, however, partially purified *Cladosporium cladosporioides* fractions have been demonstrated to resemble fractions of *A. tenuis*.¹⁹ It is noteworthy that other fungal species, including *Coprinus micaceus* and *Alternaria alternata* may cross-react with ragweed pollen.²⁰ These studies suggest that, as more highly purified materials become available for testing, at least some of the multiple fungal reactions observed in atopic individuals could be as a result of shared allergenic components.

In direct RAST testing, nonspecific binding of total IgE to solid-phase carriers can alter the results. Use of a RAST ratio in which each person serves as his own control may minimize this effect.²¹ Results from RAST were statistically correlated with skin prick test reactions, although the correlation coefficient of 0.56 indicates that other factors influence the regression equation. The nature of these influencing factors is not immediately apparent. Among the possibilities are atopic state and age. In addition, since fungal allergens

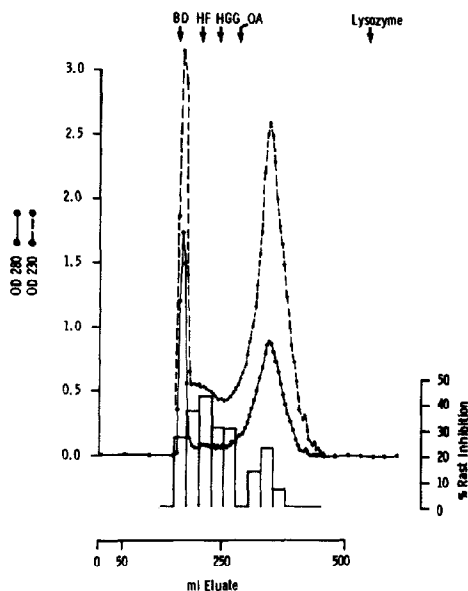


FIG. 5. Fractionation of *F. solani* on Bio-Gel A 0.5 m. Gel filtration of *F. solani* and percent RAST inhibition by use of column fractions.

could be polysaccharide or glycoprotein in nature, they may not readily couple to cellulose discs.²² Thus, it may be that nonprotein allergens are responsible for some of the positive skin reactions (not detected by RAST). Use of other techniques, such as the enzyme-linked immunosorbent assay and/or analytical isoelectric focusing and immunoprinting may prove of added benefit in analysis of mold allergens.²³⁻²⁵

The number of antigens detected by CIE (38) is low in comparison with other fungal preparations. For example, *Cladosporium herbarum* demonstrates at least 60 antigenic components⁵ and *Aspergillus fumigatus*, at least 52.²⁶ These differences may reflect differences in the organism itself or in extraction techniques. Disruption of *F. solani* mycelia and spores by sonication was demonstrated to be about 50%. Although other methods of extraction may have yielded a more potent extract, sonication did produce a highly active preparation.

The finding that a high percentage of the precipitin arcs (21) (38% to 55%) in the *F. solani* extract demonstrated positive radiostaining in CRIE is similar to other crude fungal preparations.²⁷ For example, *C. herbarum* has been demonstrated to contain at least 36 allergens.²⁸ Eleven of the 21 arcs from *F. solani* that demonstrated radiostaining were only positive with serum from one individual, suggesting that they

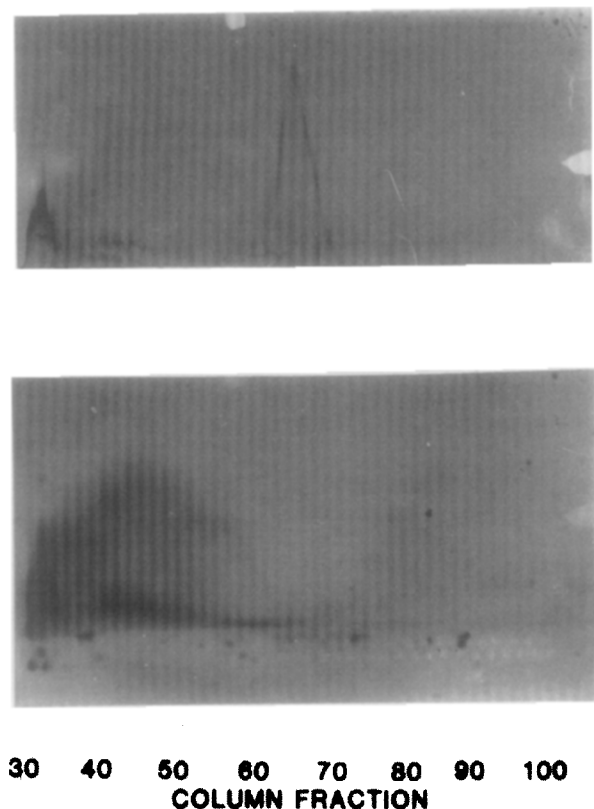


FIG. 6. FRIE (top) FRRIE (bottom) of *F. solani* Bio-Gel A 0.5 m column fractions.

may be of minor importance as allergens. One precipitin arc (No. 18) demonstrated staining at only CRIE class G. Although controls in our system indicated that staining at CRIE class G retained its specificity, other workers have suggested a lack of specificity at this class.²⁹ Important allergens in this extract appear to occur at precipitin arc Nos. 3, 21, and 22. Four of the RAST-positive individuals (ratios ranging from 4.1 to 14.6) did not demonstrate specific radiostaining. In addition, the serum with a RAST ratio of 42.9 had only four lines of radiostaining, and these are all CRIE class D. This may illustrate two of the limitations of CIE/CRIE, i.e., (1) that these tests are dependent on uniform rabbit antisera and (2) that separate components, which are immunogenic in rabbits, may not be allergenic in man.

As evidenced by the CIE/CRIE, *F. solani* is complex and contains components that are allergenic in man. Ideally, extracts used in clinical testing or treatment should contain all important allergens but should

be relatively free from nonallergenic protein materials. Some studies have indicated that repeated injections of allergen extracts in nonallergic individuals can induce specific IgE synthesis.³⁰ Since extracts of *Fusarium* are frequently included in mold mixtures for immunotherapy, it is important that they contain only pertinent allergens.

Previous investigators demonstrated improved correlation of RAST and/or skin test results by use of fungal antigens prepared by partial purification with chromatography or other procedures.^{9, 31} The demonstration of high-molecular-weight antigenic components (>341 Kd and <42 Kd) is not unusual in crude fungal preparations¹ and may be due to aggregation of components in the extract. That these antigenic components are allergenic is less common. The allergenic nature of the peaks of UV absorbing was, however, confirmed by both RAST inhibition and FRRIE studies.

These studies demonstrated that extracts of *F. so-*

lani contain several relevant allergens that differ in molecular weight and charge. They also suggest immunologic tests other than the RAST may be appropriate.

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