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Cross-reactivity between antigens of fungal extracts studied by RAST inhibition and immunoblot technique

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We have used the RAST-inhibition technique in homologous and heterologous inhibitions of the Aspergillus fumigatus, Alternaria tenuis, and Cladosporium herbarum RAST assays followed by the immunoblot technique to assess the degree of shared allergenic determinants in these extracts. In the Aspergillus RAST, there was little or no inhibition with Cladosporium and Alternaria, but considerable cross-reactivity was found between Alternaria and Cladosporium. Inhibition by Alternaria of the Cladosporium RAST was found in a dose-dependent fashion in one serum, whereas all four sera in the Alternaria RAST were inhibited by Cladosporium in this fashion. Logit transformation of the Alternaria RAST-inhibition curves produced common slopes with Alternaria and Cladosporium in three of the four sera, indicating their immunologic identity. The immunoblot technique confirmed the degree of cross-reactivity found by RAST inhibition among the molds. This evidence of common allergenic determinants in these fungi could help to explain the observation that mold-allergic patients often have skin test reactions to several fungi. (J ALLERGY CLIN IMMUNOL 1987;79:627-33.)

Aspergillus fumigatus, *Alternaria tenuis*, and *Cladosporium herbarum* are among the most important allergenic fungi; all belong to the class Deuteromycetes (Fungi Imperfecti) that have an apparent lack of sexual reproduction and are only present as mycelia or spores. Because of lack of characterization, purification, and standardization of fungal antigens in the past and the innate tendency of fungi to mutate, skin test results have been highly variable.

RAST inhibition uses human IgE antibody specificity to investigate allergenic relationships between fungal genera; we have used this technique to examine the cross-reactivity among antigens in *A. fumigatus*,

Abbreviation used

SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

A. tenuis, and *C. herbarum* extracts. We have tried to obviate the controversy about the use of spore or mycelial extracts¹ by using fungal materials from one commercial supplier that contained both spores and mycelia. The immunoblot technique has then been applied to confirm the findings and provide an estimation of molecular weights of the allergens.

METHODS AND MATERIAL

Antigens

Cultures of *A. fumigatus*, *A. tenuis*, and *C. herbarum*, containing mycelia and spores, were prepared by Allergon AB, Engelholm, Sweden, and then extracted and lyophilized by Dome Hollister-Stier Miles Laboratories, U. K.

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TABLE I. *A. fumigatus*, *A. tenuis*, and *C. herbarum* Phadebas RAST classes of the 10 sera studied

Patient		Phadebas RAST class		
No.	Symbol	<i>A. fumigatus</i>	<i>A. tenuis</i>	<i>C. herbarum</i>
1	○	<u>4</u>	3	3
2	●	<u>4</u>	2	2
3	△	<u>4</u>	2	2
4	▽	<u>4</u>	3	2
5	▲	<u>3</u>	<u>4</u>	2
6	□	1	<u>4</u>	<u>4</u>
7	■	4	<u>4</u>	<u>4</u>
8	▼	3	<u>4</u>	3
9	◇	0	0	<u>4</u>
10	◆	0	2	<u>3</u>

Sera used in the respective RAST inhibition studies are underlined. Phadebas RAST class, level of allergen-specific IgE antibody: 4, very high; 3, high; 2, moderate; 1, low; and 0, absent or undetectable.

Patient sera

Ten Brompton Hospital outpatients who had both a positive skin prick test (wheal ≥ 3 mm in diameter) and a Phadebas RAST class 3 or 4 to one, two, or all three of the molds, *A. fumigatus*, *A. tenuis*, and *C. herbarum*, were chosen for study. None of the 10 patients had undergone immunotherapy, and all had, in addition, a positive skin prick test to extracts of one or more of the common allergens, grass pollen, *Dermatophagoides pteronyssinus*, and cat fur.

The patients are identified by different symbols (Table I) that are followed throughout this article.

RAST

The three mold extracts were each coupled to CNBr-activated paper discs (Schleicher & Schuell, Keene, N. H., No.589/3) with 10 mg of dry weight per 1 gm of discs according to the method of Axen et al.² In all RAST assays, 50 μ l of serum per disc was incubated for 16 hours at room temperature in duplicate. After washing, 50 μ l of ¹²⁵I-labeled anti-IgE (Pharmacia Diagnostics AB, Uppsala, Sweden) was added to each tube for a second 16-hour incubation. After further washing, the tubes were counted in a gamma counter. The Phadebas RAST birch reference system (Pharmacia Diagnostics AB) was included in the assays. This system is calibrated against the first British standard 75/502 for human serum immunoglobulin E and enables comparability in results obtained on different occasions and from different centers. The key to Phadebas RAST classes is presented in Table I.

The sera chosen for RAST inhibition studies (underlined in Table I) were additionally RAST tested against the remaining molds, and the Phadebas RAST classes on undiluted sera are also presented in Table I. Cord blood sera were tested in all three RAST assays as measures of the background levels.

RAST inhibition

Four sera that were RAST positive to each of the three molds, *A. fumigatus*, *A. tenuis*, and *C. herbarum*, were titrated out, and the dilution at the top of each linear part of the curve was chosen for inhibition studies. Seven concentrations of inhibitors (100 μ l) from 0.98 μ g to 2 mg were preincubated with the chosen serum dilution (100 μ l) for 16 hours. The respective mold RAST was carried out, and percentage binding of the ¹²⁵I-anti-IgE was compared with that of the unabsorbed serum dilution. Results are illustrated in Figs. 1 to 3. Analysis by logit transformation of RAST-inhibition curves was made where it was possible. To assess any nonspecific inhibition in the RAST-inhibition tests, an unrelated allergen, mixed grass pollen (Bencard, U. K.), was used similarly (from 0.98 μ g to 2 mg concentration) as an inhibitor in each of the three mold RAST assays (Fig. 4) with a serum pool from the mold-hypersensitive patients in this study.

SDS-PAGE

A resolving 15% SDS-PAGE gel with a 3% acrylamide upper stacking gel was cast according to the method of Laemmli³ in a vertical Protean slab cell (Bio-Rad Laboratories, Watford, Herts, U. K.). Lyophilized samples of the three mold extracts were solubilized in 0.5 mol/L of Tris/HCl buffer, pH 6.8, containing 3% SDS at 60° C for 30 minutes. The prepared samples and a low-molecular-weight standard protein solution (Pharmacia), solubilized at 100° C for 5 minutes, were electrophoresed in duplicate at 18 mA for 1 hour and followed by 4 hours at 30 mA. The gel was fixed in 25% propan-2-ol/10% acetic acid for 1 hour, followed by staining in 0.1% Coomassie blue R250 for 16 hours, and destaining in 8% acetic acid: 5% methanol (Fig. 5).

An identical gel was cast as described above. The three-mold extract samples were electrophoresed in quadruplet with low- and high-molecular-weight standards (Pharmacia). After SDS-PAGE, the gel strip containing the standards sample was cut away, fixed, and stained, whereas the rest of the gel was processed by the immunoblot technique.

Immunoblot

Proteins from the SDS-PAGE gel were electrophoretically transferred onto a nitrocellulose sheet as described by Towbin et al.⁴ A Trans-Blot cell (Bio-Rad) was used, and the transfer was carried out at 0.1 A overnight in 25 mmol/L of Tris, 192 mmol/L of glycine buffer, pH 8.3, containing 20% w/v of methanol. Any additional protein-binding sites were blocked by immersing the nitrocellulose sheet in 2% bovine serum albumin in phosphate-buffered saline for 1 hour at 39° C. After washing, the sheet was cut into the 12 respective strips and processed for autoradiography.

Autoradiography

One serum per antigen was inhibited with the three molds, as for the RAST-inhibition assays, and detailed in Table II. The prepared sera were gently rotated over the 12 individual nitrocellulose strips in blocks with individual strip compartments for 16 hours. After washing, the strips were given

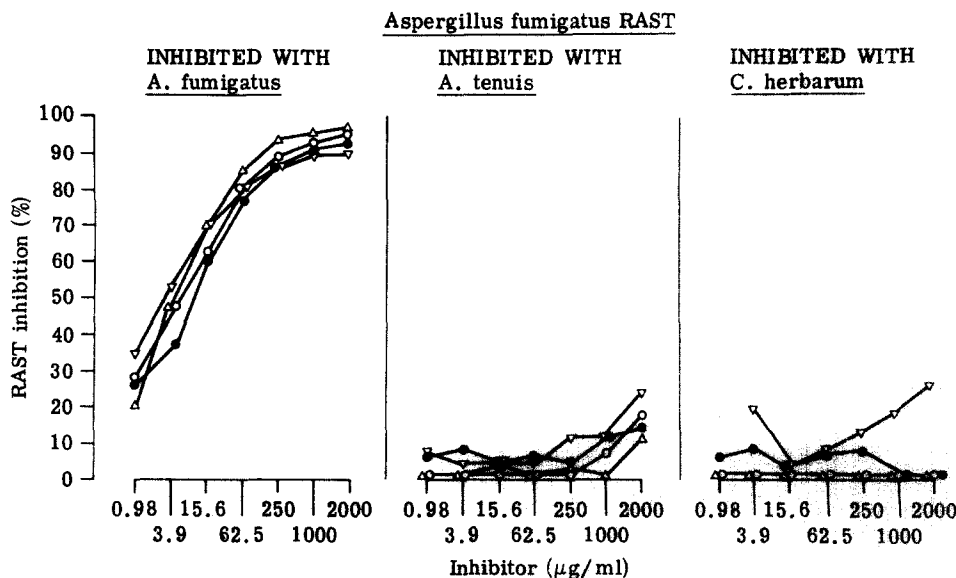


FIG. 1. Inhibition of *A. fumigatus* RAST with sera from four mold-hypersensitive patients. Phadebas RAST class and dilution of serum used: ($\circ$) class 3, 1:32; ($\bullet$) class 4, 1:16; (Δ) class 3, 1:16; and (∇) class 3, 1:8.

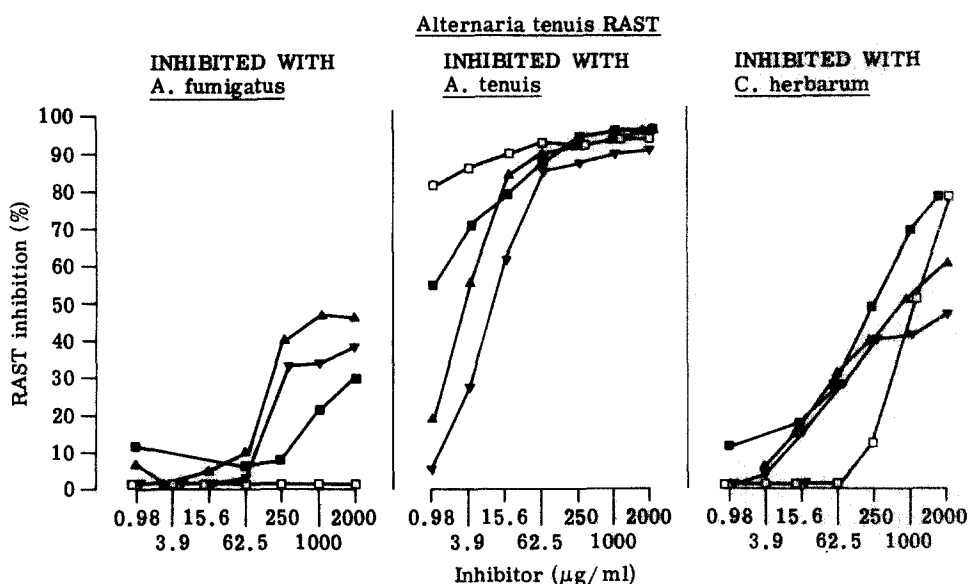


FIG. 2. Inhibition of *A. tenuis* RAST with sera from four mold-hypersensitive patients. Phadebas RAST class and dilution of serum used: ($\blacktriangle$) class 4, 1:2; ($\square$) class 3, 1:4; ($\blacksquare$) class 3, 1:4; and ($\blacktriangledown$) class 3, 1:2.

an additional 16-hour incubation with the tracer ^{125}I -anti-IgE (Pharmacia) (100 μl contained approximately 10,000 cpm). The washed and dried strips were affixed to a glass sheet with masking tape, exposed to X-Omat L Kodak film (Kodak Ltd., Hemel Hempstead, Herts, U. K.) with DuPont Quanta III (DuPont [U. K.] Ltd., Stevenage, Herts, U. K.) intensifying screens at -70°C for 100 hours, and were then developed.

RESULTS

RAST inhibition dose-response curves were obtained in each case with the homologous antigen (Figs.

1 to 3). The isotope percent binding of the serum dilutions used are presented in the legends to the figures.

In the *A. fumigatus* RAST (Fig. 1), approximately 5 μg of *Aspergillus* antigen produced 50% inhibition. There was low ($\leq 20\%$) consistent inhibition with 2 mg of *A. tenuis* but no inhibition with *C. herbarum* at this concentration, with the exception of serum 4 that elicited 25% inhibition. Pollen inhibited the *Aspergillus* RAST by 6% at this top 2 mg concentration.

With the *Alternaria* RAST (Fig. 2), <4 μg of the homologous antigen elicited $>50\%$ inhibition with

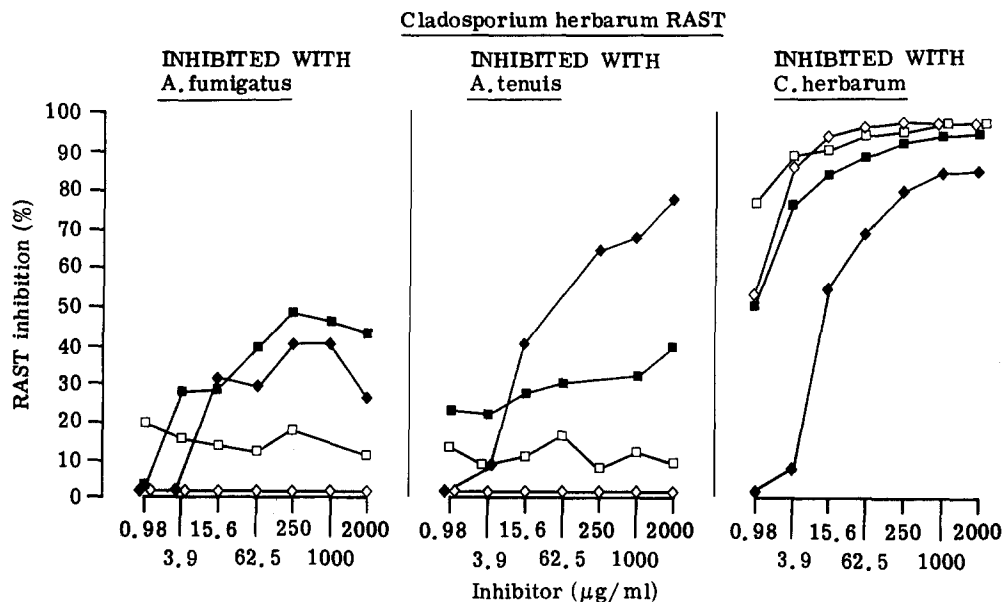


FIG. 3. Inhibition of *C. herbarum* RAST with sera from four mold-hypersensitive patients. Phadebas RAST class and dilution of serum used: (□) class 3, 1:16; (■) class 3, 1:4; (◇) class 4, 1:2; and (◆) class 3, 1:1.

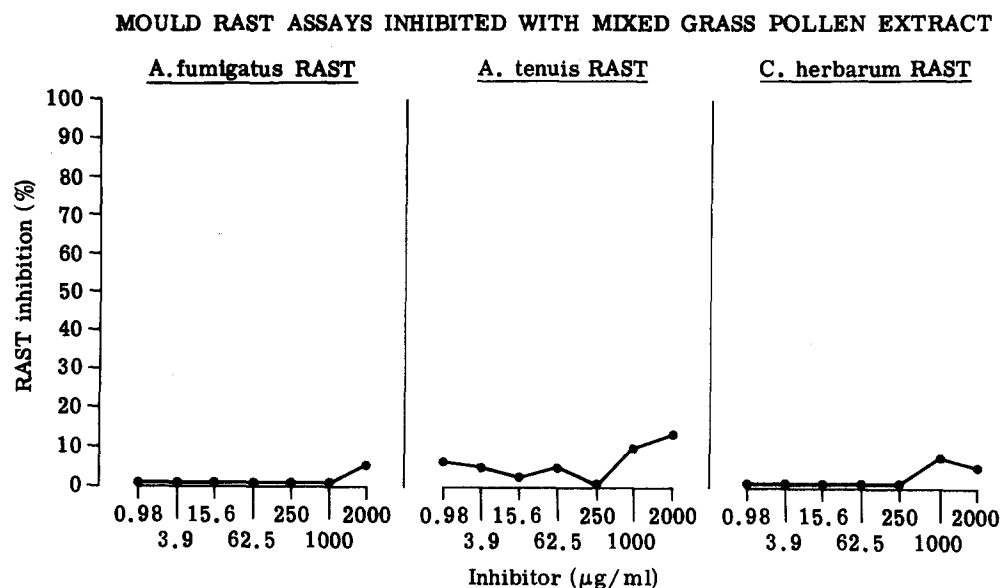


FIG. 4. Inhibition of *A. fumigatus*, *A. tenuis*, and *C. herbarum* RAST assays with mixed grass-pollen antigen.

three of the sera, whereas the fourth serum exhibited 61% inhibition at 15.6 μg . With the sera from patients 5, 7, and 8, *Aspergillus* at the highest concentration of 2 mg produced significant (30%, 46%, and 38%) inhibition. There was more consistently low inhibition by the pollen, reaching a maximum of 13% at 2 mg. *C. herbarum*, however, caused considerable inhibition of the *Alternaria* RAST, nearly 80% with the sera from patients 6 and 7 at 2 mg without evidence of any

flattening of the dose-response curve with addition of increasing inhibitor in any of the four sera. When inhibition lines of the *Alternaria* RAST with *Alternaria* and *Cladosporium* were compared by logit transformation, a common slope was found in three of the sera, indicating that *Alternaria* and *Cladosporium* were exhibiting immunologic identity.

In the *Cladosporium* RAST (Fig. 3) there was >50% inhibition by <1 μg of *Cladosporium* antigen

with sera from patients 6, 7, and 9. There was inhibition with *Aspergillus* in three of the four sera tested, but only with the sera of patients 7 and 10 was there any evidence of a dose-response relationship. Inhibition was produced with *Alternaria* in three sera, but only patient 10 demonstrated a dose-response relationship with 76% inhibition at 2 mg. Pollen inhibited the *Cladosporium* RAST (mean 7%) at the two top concentrations, and therefore, it would appear that certainly at 2 mg concentration in all three mold RAST assays, the unrelated pollen antigen is either producing nonspecific inhibition or does in fact have a low concentration of common allergens with the molds. Relating the pollen-inhibition results to the mold inhibitions, the degree that *Alternaria* and *Cladosporium* antigens inhibit the *Aspergillus* RAST cannot be taken as definitely significant, but in the other assays, any inhibition produced by the mold antigens was much greater than that produced by pollen and can therefore be taken as authentic.

All patients had a positive RAST to the remaining molds (Table I) except for patient 10 who had a Phadebas RAST class 0 *Aspergillus* RAST and patient 9 who appeared to be exclusively positive to *Cladosporium*. Neither *Aspergillus* nor *Alternaria* extract inhibited the latter patient's *Cladosporium* RAST, but this result was not consistent with patient 10 whose serum was considerably inhibited in the *Cladosporium* RAST by *Aspergillus*, despite the patient having a low *Aspergillus* RAST. The background level in the RAST assays was 0.4% isotope binding for *A. fumigatus* and *A. tenuis*, and 0.5% binding for *C. herbarum* (Phadebas RAST class 0).

The SDS-PAGE gel stained with Coomassie blue (Fig. 5) illustrates multiple protein bands in all three molds, the main common band occurring at 68K. Strong protein bands occur with *A. fumigatus* (channel 1) at 94K, 68K, 39K, and 17.5K, with *A. tenuis* (channel 2) at 94K, 68K, and 18K, and with *C. herbarum* (channel 3) at 68K, 28K, and 17K.

The immunoblot strips 1a, 2a, and 3a of Fig. 6 illustrate the autoradiographs produced by overlays of uninhibited individual sera from three mold-hypersensitive patients on *A. fumigatus*, *A. tenuis*, and *C. herbarum*, respectively. Self-inhibitions (1b, 2c, and 3d) left no visible lines. With the *Aspergillus* strips, there was negligible inhibition with *Alternaria* (1c) and *Cladosporium* (1d) with all allergen lines still visible. RAST inhibition had been only 11% with *Aspergillus* and 16% with *Cladosporium*. With the *Alternaria* strips, both *Aspergillus* (2b) and *Cladosporium* (2d) considerably reduced the density of the allergen bands of >100K, 80K, 68K, 39K, and 32K but not the allergens at 26K and 23K daltons compared

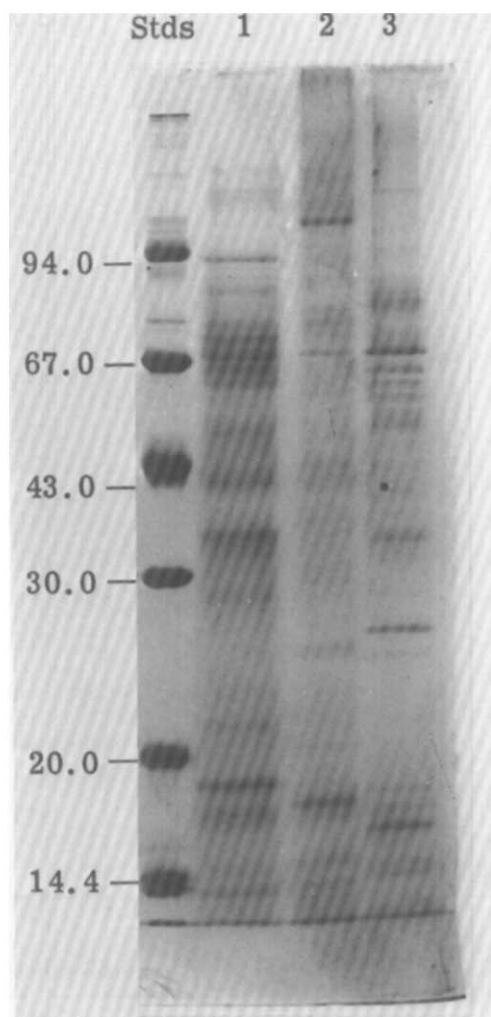


FIG. 5. Fifteen percent acrylamide SDS-PAGE stained with Coomassie blue. 1, *A. fumigatus*; 2, *A. tenuis*, and 3, *C. herbarum*.

with the unabsorbed sera strip (2a). This result was in keeping with the RAST inhibition of 31% found with *Aspergillus* and 40% with *Cladosporium*. With the *Cladosporium* strips, both *Aspergillus* (3b) and *Alternaria* (3c) inhibited the allergen response, but *Alternaria* inhibited to a greater degree with both the 39K and 35K allergens and to an even greater degree with the 23K dalton allergen. RAST inhibition had been 41% with *Aspergillus* and 68% with *Alternaria*.

DISCUSSION

The degree of IgE cross-reactivity between two species depends on the number and relative importance of the individual allergens that cross-react and the patient sera used to study the cross-reactivity because of the marked heterogeneity among mold-allergic patients with respect to allergy to single mold extract

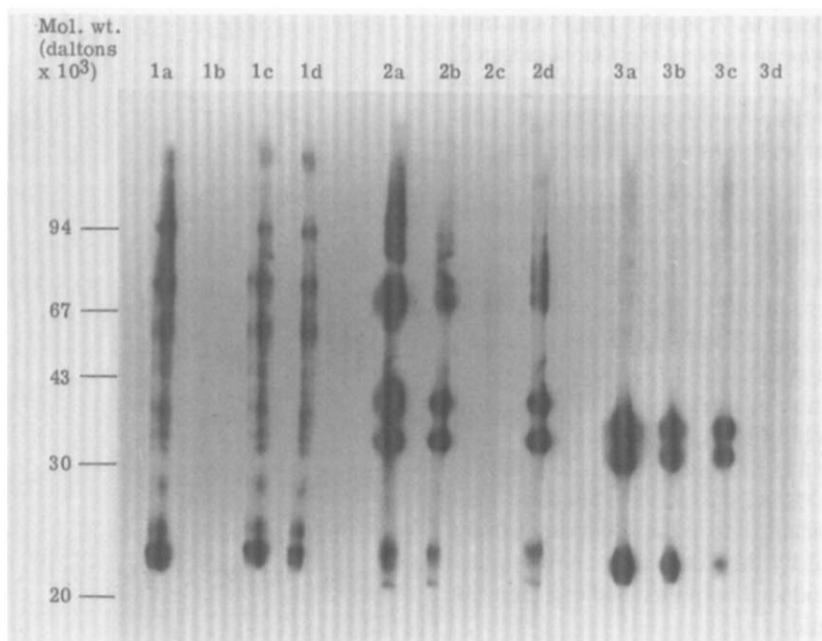


FIG. 6. Autoradiograph of immunoblot from 15% acrylamide SDS-PAGE gel. 1, *A. fumigatus* antigen; 2, *A. tenuis* antigen; 3, *C. herbarum* antigen; a, uninhibited serum overlay; b, serum inhibited with *A. fumigatus* overlay; c, serum inhibited with *A. tenuis* overlay; and d, serum inhibited with *C. herbarum* overlay.

TABLE II

Antigen on nitrocellulose strip	Serum	Concentration	Serum overlay inhibitor (1 mg/ml)			
			None	<i>A. fumigatus</i>	<i>A. tenuis</i>	<i>C. herbarum</i>
<i>A. fumigatus</i>	▽	1:8	1a	1b	1c	1d
<i>A. tenuis</i>	▼	1:2	2a	2b	2c	2d
<i>C. herbarum</i>	◆	1:1	3a	3b	3c	3d

components.⁵ In our present study we have therefore tested four different patients' sera with each mold extract to achieve reasonable representation.

Although much work has been done on individual fungi, e.g., *Alternaria*⁶ and *Cladosporium*,⁷ there have been relatively few studies of cross-reactivity. Different species of the same genus may demonstrate significant differences as Kim and Chaparas⁸ found, studying several *Aspergilli*, and by Vijay et al.,⁹ studying *A. tenuis* and *A. solani*. Karr et al.,¹⁰ using only one serum, demonstrated that some cross-reactivity exists between the different species, *A. fumigatus*, *A. glaucus*, and *A. flavus*, but not between the different genera of *Aspergillus* and *Alternaria*. On the other hand, significant IgE cross-reactivity has been demonstrated between some different genera. Hoffman and Kozak¹¹ demonstrated cross-reactivity be-

tween *Alternaria*, *Helminthosporium*, *Spondylocadium*, *Stemphylium*, and *Curvularia* species. Similarly, Agarwal et al.¹² demonstrated that *Alternaria* and *Stemphylium* extracts contain multiple shared allergenic determinants including Alt-I, which was the major allergenic component in both extracts. Additionally, in 1983, Gravesen and Nyhølm¹³ demonstrated by crossed radioimmuno-electrophoresis that *A. alternata* and *Ulocladium chartarum* have multiple antigens in common, including Alt-I and other allergens.

With the four sera we tested in the *Aspergillus* RAST in this study, there was inhibition in only one of four sera with *Cladosporium* and only low inhibition was found with *Alternaria*. However, the *Aspergillus* extract was able to inhibit both the *Alternaria* and *Cladosporium* RAST assays to a greater extent, implying that some allergenic determinants were com-

mon among all three fungi. *Alternaria* inhibited the *Cladosporium* RAST, especially with serum 10, whereas *Cladosporium* extensively inhibited the *Alternaria* RAST in all four sera tested. Logit transformation of inhibition curves demonstrated that *Alternaria* and *Cladosporium* were exhibiting immunologic identity in their manner of inhibition. The considerable cross-reactivity between *Alternaria* and *Cladosporium* makes it difficult to distinguish between them in fungal RAST results in an individual patient. Some patients will be reacting to the same antigen present in both fungi. Individuals behave differently to the same mold, some exhibiting cross-reactivity (e.g., serum 10), whereas others do not (e.g., serum 9), identifying a biologic as well as a standardization problem.

The immunoblot confirmed that there was far less cross-reactivity between *Aspergillus* and each of the other two molds than between *Alternaria* and *Cladosporium*. However, *Aspergillus* was capable of partially inhibiting the allergens of >100K, 80K, 39K, and 32K daltons of *Alternaria* and those of 39K, 35K, and 23K of *Cladosporium*, demonstrating the presence of some cross-reacting allergens. *Cladosporium* inhibited the density of the allergen bands of *Alternaria* and to a greater degree *Alternaria* inhibited those of *Cladosporium* (39K, 35K, and 23K), confirming a more extensive cross-reactivity between these two molds.

The observation that patients allergic to one mold frequently have skin test reactions elicited by extracts of fungi of other species has two alternative explanations: the presence of shared antigenic determinants¹⁴ or separate immunologic responses to specific antigens of the different fungal species. Our evidence is more in keeping with common antigens shared by different fungal genera.

Cross-reactivity between different fungi is important. Knowledge of which molds have common antigens is relevant for diagnosis, particularly when immunotherapy is contemplated.

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