

An approach to fungal antigen relationships by radioallergosorbent test inhibition

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Antigenic relationships among three species of Aspergillus (A. fumigatus, A. glaucus, and A. flavus) were examined by paired cross-radioallergosorbent test (RAST) inhibition analysis, an in vitro technique based on human IgE antibody specificity. Alternaria tenuis was found to be antigenically unrelated to each of the three species of Aspergillus and was used as a negative control. A single test serum yielded uninhibited RAST indices of 6, 7.4, 8.1, and 7.8 for A. fumigatus, A. glaucus, A. flavus, and Alternaria tenuis, respectively. At a concentration of 10 mg/ml, A. fumigatus inhibited A. glaucus RAST by 63% and A. flavus RAST by 62%. A. glaucus inhibited A. fumigatus RAST by 36% and A. flavus RAST by 63%. A. flavus inhibited A. fumigatus RAST by 44% and A. glaucus RAST by 81%. Each species of Aspergillus produced significant but only partial inhibition of RAST to each of the other two species analyzed. Results indicate the existence of both shared and unique antigens among these three species of Aspergillus. Paired cross-RAST inhibition may be used as an approach to study species relationships among genera of several classes of clinically relevant fungi. Unless many strains are employed, data obtained do not represent a definitive analysis of species, because of possible different degrees of inhibition by various strains of a particular species. They do, however, allow for the antigenic comparison of two or more crude, poorly characterized preparations thought to be important in human allergic disease.

Species of *Aspergillus* are known etiologic agents in at least five distinct pulmonary disorders: allergic bronchopulmonary aspergillosis, allergic asthma, aspergilloma (fungus ball), hypersensitivity pneumonitis, and invasive aspergillosis.¹ Each disorder is characterized by unique immunologic features. Two of these disorders, allergic bronchopulmonary aspergillosis and allergic asthma, occur predominantly in atopic persons and are known to involve type I IgE-mediated hypersensitivity. Because it is based on human IgE antibody specificity, radioallergosorbent test (RAST) inhibition is an assay clinically relevant

for standardization of diagnostic reagents used to evaluate IgE-mediated diseases. Although our study does not represent a definitive analysis of species, because of the few strains employed, it was designed as a model for examination of antigenic relationships among species of *Aspergillus* or related *Deuteromycete* species.

MATERIALS AND METHODS

Fungal antigens

Cultures of *Aspergillus fumigatus* (Afu) (Duke University No. 5167), *A. glaucus* (Agl) (American Type Culture Collection No. 9643), *A. flavus* (Afl) (Carolina Biological No. LBA 275), and *Alternaria tenuis* (Alt) (ATCC No. 7431) were grown in Czapek-Dox broth (saccharose 30 gm, sodium nitrate 3 gm, dipotassium phosphate 1 gm, magnesium sulfate 0.5 gm, potassium chloride 0.5 gm, ferrous sulfate 0.01 gm) for either 10 or 25 days at 37° C on a shaker incubator (aerated). The medium was then decanted and the mat washed three times in acetone to remove medium components. After drying, the mat was homogenized in a ball mill for 1 hr. Extraction of the resulting powder with Coca's solution (1:20 w/v) at 4° C for 48 hr at

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TABLE I. RAST with fungal antigens

	<i>A. fumigatus</i>	<i>A. glaucus</i>	<i>A. flavus</i>	<i>Alternaria tenuis</i>
RAST index	6.0 $\pm$ 0.9*	7.4 $\pm$ 1.1	8.1 $\pm$ 1.2	7.8 $\pm$ 0.6
(mean $\pm$ SD)	6.3 $\pm$ 1.0†	7.1 $\pm$ 0.8†	8.4 $\pm$ 1.2†	—
Binding (percent	12.0*	13.6	15.1	14.1
total counts)‡	12.5†	13.0	15.9	—

*Twenty-one-day growth.

†Ten-day growth.

‡Nonatopic serum pool produced less than 2% binding for each antigen.

pH 8 and at 4° C for 5 days at pH 7.75 was followed by passage through a Millipore filter. The extract was then divided into two aliquots and dialyzed exhaustively against phosphate-buffered saline (PBS), pH 7.2, and borate buffer, pH 8. Extracts were concentrated to approximately 20 ml (Amicon UM10) and adjustments made to achieve a 10 mg/ml dry weight concentration of each preparation.

RAST

Cellulose disks were activated with CNBr according to the method of Ceska and Lundkvist² and coupled to each of the four fungal antigens at 10 mg/ml in borate buffer, pH 8, by incubating at 24° C for 6 hr on a rotator. A serum pool obtained from three nonatopic control subjects and a test serum obtained from an asthmatic subject demonstrating skin reactivity by prick test to each antigen were incubated independently for 6 hr with antigen-coated disks on a rotator at 24° C. After washing three times with PBS, ¹²⁵I anti-human IgE (Pharmacia, Piscataway, N. J.) was added to the disks and incubated overnight at 24° C. The disks were again washed and placed in vials, and radioactivity was determined for 5 min on a Beckman Biogamma Counter. Average counts per minute obtained with the test serum were divided by counts per minute obtained with the control serum pool to produce a RAST index. The percent binding of the total radioactivity added was also determined.

RAST inhibition

RAST inhibition was performed in two steps according to the method of Gleich et al.³ In the first step, antigen-coated disks, the RAST-positive serum, and the test antigen were incubated together for 6 hr on a rotator at 24° C. The second step was identical to that described for the RAST. Results were expressed in terms of percent inhibition by the test antigen beyond that obtained with PBS control. All tests were performed in triplicate.

Study designs

To examine the antigenic relationships among the three species of *Aspergillus*, each of three possible pair combinations were studied independently. These pair combinations were Afu and Agl, Afu and Afl, and Agl and Afl. For each pair, a cross-RAST inhibition study was performed as previously described.⁴ Briefly, each member of the pair was used as the test antigen for the inhibition of RAST to the

other member of the pair. Inhibition of Alt RAST was used as a negative control for each study. All assays were performed in triplicate.

Statistical tests

RAST inhibition results were evaluated by analysis of covariance.⁵

RESULTS RAST

The results of conventional RAST on the test serum are illustrated in Table I. IgE antibodies with specificity for each of the four fungal species were clearly demonstrated by this technique. Twelve percent to 15% binding to antigen-coated disks was obtained with the test serum, whereas less than 2% binding resulted with the nonatopic serum pool. There was little difference in RAST index employing antigen from either 10- or 21-day growth of *Aspergillus* species.

RAST inhibition

Cross-RAST inhibition was performed on each of the three pair combinations of *Aspergillus* species.

Afu and Agl. Maximal inhibition of Afu RAST was produced with Afu, but Afu produced no inhibition of RAST to Alt. The difference was significant at $p < 0.0005$. Afu produced a significant degree of inhibition to Agl RAST when compared with Alt RAST ($p < 0.005$) but somewhat less inhibition when compared with Afu RAST ($p < 0.005$) (Fig. 1, A). Conversely, maximal inhibition of Agl RAST was produced with Agl, but Agl produced no inhibition of Alt RAST. The difference was significant at $p < 0.005$, but less inhibition was produced when compared with Agl RAST ($p < 0.005$) (Fig. 1, B).

Afu and Afl. Maximal inhibition of Afu RAST was produced by Afu, but Afu produced no inhibition of Alt RAST. The difference was significant at $p < 0.0005$. Afu produced a significant degree of inhibition of Afl RAST when compared with inhibition of Alt RAST ($p < 0.0005$) but somewhat less inhibition

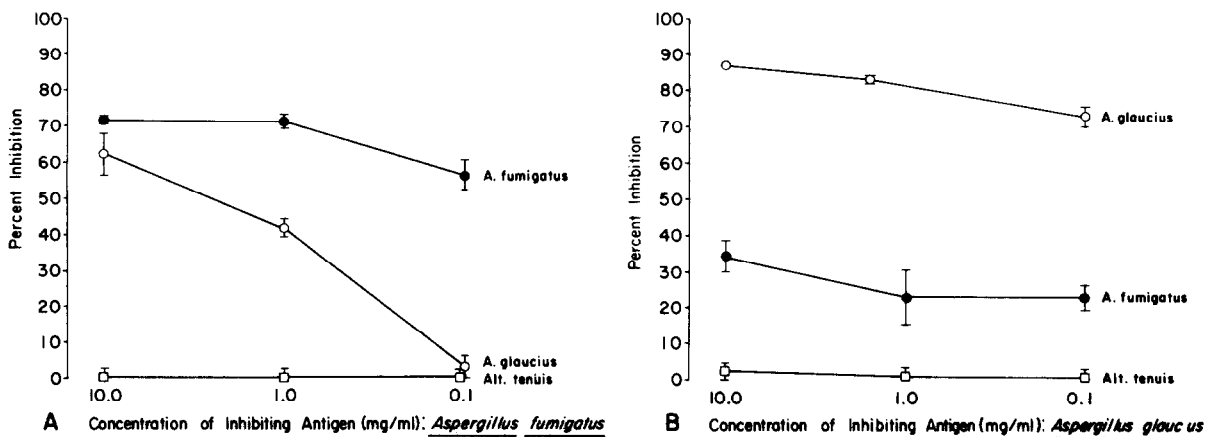


FIG. 1. A and B, Cross-RAST inhibition with *Aspergillus fumigatus* (Afu) and *A. glaucus* (Agl). A, Afu inhibited RAST to Agl significantly more than it inhibited RAST to Alt but less than it inhibited RAST to itself. B, Conversely, Agl inhibited RAST to Afu significantly more than it inhibited RAST to Alt but less than it inhibited RAST to itself.

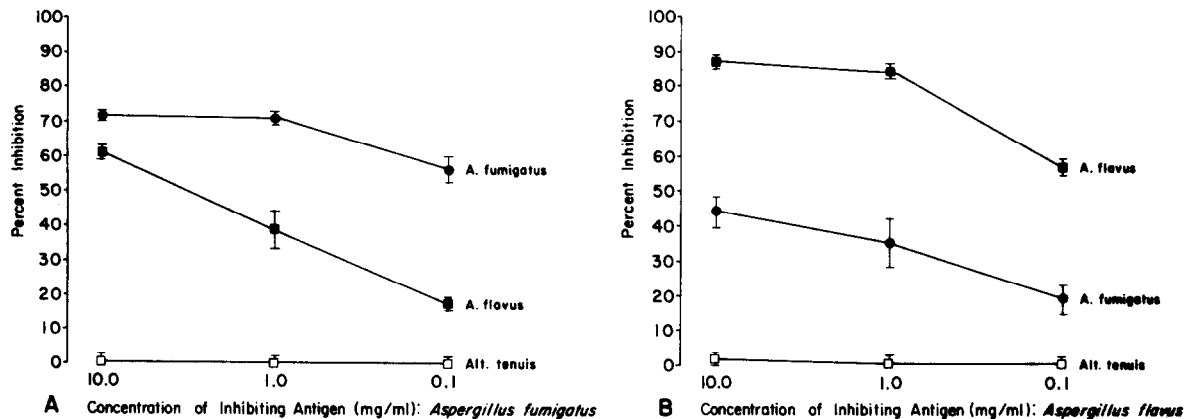


FIG. 2. A and B, Cross-RAST inhibition with *Aspergillus fumigatus* (Afu) and *A. flavus* (Afl). A, Afu inhibited RAST to Afl significantly more than it inhibited RAST to Alt but less than it inhibited RAST to itself. B, Conversely, Afl inhibited RAST to Afu significantly more than it inhibited RAST to Alt but less than it inhibited RAST to itself.

when compared with Afu RAST ($p < 0.0005$) (Fig. 2, A). Conversely, maximal inhibition of Afl RAST was produced with Afl, but Afl produced no inhibition of Alt RAST. The difference was significant at $p < 0.0005$. Afl produced a significant degree of inhibition of Afu RAST compared with Alt RAST ($p < 0.005$) but somewhat less inhibition when compared with Afl RAST ($p < 0.0005$) (Fig. 2, B).

Agl and Afl. Maximal inhibition of Agl RAST was produced with Agl, but Afl produced no inhibition of RAST to Alt. The difference was significant at $p < 0.0005$. Agl produced a significant degree of inhibition of Afl RAST when compared with Alt RAST ($p < 0.0005$) but somewhat less inhibition when compared with Agl RAST ($p < 0.005$) (Fig. 3, A). Conversely, maximal inhibition of Afl RAST was produced with Afl, but Afl produced no inhibition of Alt RAST. The

difference was significant at $p < 0.0005$. Afl produced a significant degree of inhibition of Agl RAST compared with Alt RAST ($p < 0.0005$) but somewhat less inhibition when compared with Afl RAST ($p < 0.005$) (Fig. 3, B).

DISCUSSION

Analyses with precipitating hyperimmune sera are most useful to demonstrate antigenic relationships of *Aspergillus* species; however, valuable information regarding allergenicity can be obtained by using specific IgE antibodies. Allergenic relationships among three species of *Aspergillus* were examined with the RAST inhibition assay. We found that comparable concentrations by dry weight of each species of *Aspergillus* (Afu, Agl, or Afl) produced significant but only partial inhibition of RAST-detecting IgE an-

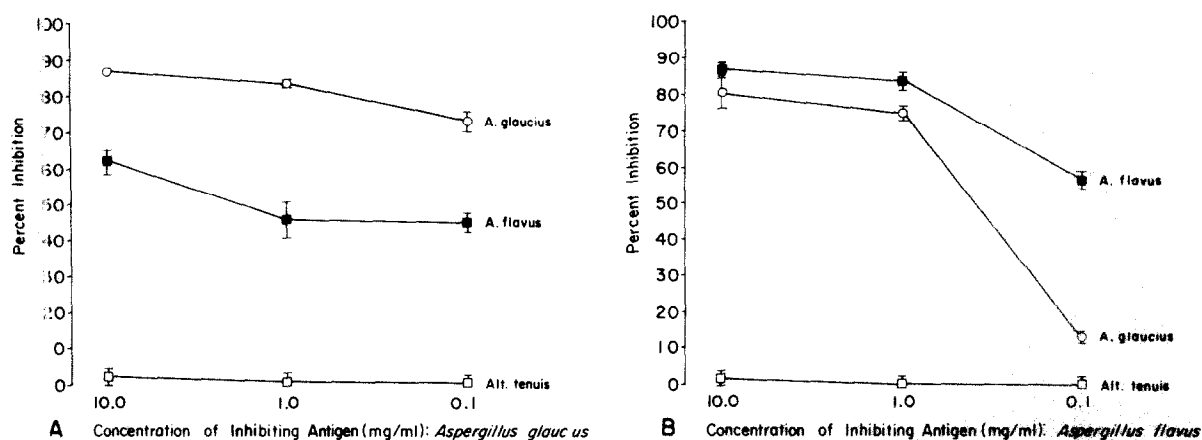


FIG. 3. *A* and *B*, Cross-RAST inhibition with *Aspergillus glaucus* (Agl) and *A. flavus* (Afl). *A*, Agl inhibited RAST to Afl significantly more than it inhibited RAST to Alt but less than it inhibited RAST to itself. *B*, Conversely, Afl inhibited RAST to Agl significantly more than it inhibited RAST to Alt but less than it inhibited RAST to itself.

tibodies to the other two species. To our knowledge there are no previously reported comparisons of antigens among species of *Aspergillus* using in vitro techniques based on human IgE antibody specificity to evaluate allergenicity.

Our recognition of common antigenic determinants among species of *Aspergillus* is in agreement with the findings of Bardana,⁶ who, using double-immunodiffusion analysis, found that a serum that reacted strongly with multiple bands to any one of a variety of species of *Aspergillus* almost always reacted in some degree to all. Perhaps of greater bearing on the analysis based on human IgE specificity is the work of Pepys⁷ and of Longbottom and Pepys,⁸ who, in studies of patients with allergic asthma and bronchopulmonary aspergillosis, also concluded that certain species of *Aspergillus* possess both shared and unique antigenic characteristics. In other related studies, Hoffman and Kozak,⁹ using RAST inhibition, found cross-reactivity between *Alternaria* and species of several other genera such as *Helminthosporium*, *Spondylioladium*, *Stemphyllium*, and *Curvularia*. They did not report a comparison with *Aspergillus*.

An important consideration in comparing studies is the antigenic variability in preparation of microorganisms. Using diffusion and electrophoresis techniques, Kim and Chaparas^{10, 11} found a maximum number of mycelial antigens in 4-day-old shaker-incubated cultures. Although a large number of precipitating antigens were still noted at 2, 4, and 6 wk, the number of these mycelial antigens diminished with length of culture. They also found mycelial extracts to be the most antigenically potent fungal component as assessed by direct RAST.¹⁰ Other investigators, however, employing cellular, mycelial, or

culture filtrate antigens of *Aspergillus* and related *Deuteromycete* species prepared from stationary culture growth, have often used 3-wk, or at times up to 3-mo, cultures for antigenic analysis.^{8, 12-14} In our study we noted little difference between antigen from 10- and 21-day shaker-incubated growth of these *Aspergillus* species in ability to detect specific antibody by RAST. A comparison of different analyses of mold antigens is, however, difficult without standardization of age of culture and extraction technique.

The adaptation of conventional RAST inhibition to paired cross-RAST inhibition provides several advantages for comparing antigenic relationships among poorly characterized, crude preparations. First, the cross-over aspect of this approach allows for discrimination between qualitative and quantitative antigenic differences among preparations. Second, the pairing process provides a systematic way to analyze antigenic relationships among many preparations. And third, relatively few determinations are required. Gleich et al.³ and Yunginger et al.¹⁵ have assessed antigenic potency by comparing the concentration of antigen producing 50% inhibition of RAST to a known standardized preparation. Differences in slopes of the dose-response curves for each preparation indicate qualitative differences among antigens. This approach requires an adequate number of determinations for each preparation to construct a dose-response curve and the existence of a well-characterized antigen standard. It is best suited for comparison and standardization of certain commercial antigenic preparations. Alternatively, the paired cross-RAST inhibition adaptation is particularly well suited for the analysis of antigenic relationships among antigen preparations where a well-characterized standard for com-

parison is lacking. Antigens of microbial origin fall in this category, and it was with this purpose that we developed our approach. It is hoped that such an approach will facilitate analysis of the clinically relevant species of *Aspergillus* and related species.

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