Original Article

Cross-allergenicity between Aspergillus restrictus, Aspergillus fumigatus and Alternaria alternata determined by radioallergosorbent test inhibition

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ABSTRACT

Aspergillus restrictus, an osmophilic fungus, is abundant in house dust. We have shown previously that the incidence of immediate hypersensitivity to A. restrictus is comparable to that for Aspergillus fumigatus and Alternaria alternata in asthmatic children. Radioallergosorbent test (RAST) inhibition was used to determine whether A. restrictus shares similar allergenic components with A. fumigatus and A. alternata. Mycelial mats of the three species cultivated on completely synthetic media were used for extract preparation. IgE antibodies to each fungus were measured with RAST using a polyvinyl chloride microplate as a solid phase. Analysis of a serum pool obtained from nine asthmatic children with a positive RAST to A. restrictus showed that A. restrictus inhibited the RAST to A. restrictus, A. fumigatus and A. alternata by more than 80%. Similar results were observed with A. fumigatus and A. alternata. Additionally, when 13 serum samples with a positive RAST to A. restrictus were tested separately, A. restrictus substantially inhibited the A. restrictus RAST in all subjects tested. A. fumigatus and A. alternata inhibited the A. restrictus RAST in 10 and 8 of the samples studied, respectively. These findings indicate that A. restrictus shares allergenic components with A. fumigatus and A. alternata. The allergenic cross-reactivity between A. fumigatus and A. alternata was also demonstrated.

Key words: Alternaria alternata, Aspergillus fumigatus, Aspergillus restrictus, cross-allergenicity, osmophilic fungus, radioallergosorbent test inhibition

INTRODUCTION

Household fungi may be significant causal agents of childhood asthma, particularly because young children tend to spend much of their time indoors. Recent mycological studies1-4 using culture media with low water activity (e.g. MA64,1 DG185) have shown that house dust contains many species of osmophilic fungi, one of the most common being Aspergillus restrictus. There is evidence that these osmophilic fungi, especially A. restrictus, grow well in house dust containing dry organic substances at normal levels of humidity.6-9

We have shown previously that A. restrictus may act as an aeroallergen10 since IgE-mediated skin hypersensitivity to this fungus was demonstrated in asthmatic children by skin prick test and a radioallergosorbent test (RAST) using its mycelial extract. In addition, A. restrictus had a comparable incidence of positive skin prick tests and RAST to Aspergillus fumigatus and Alternaria alternata. A. restrictus, therefore, seems to play as important a role in childhood asthma as A. fumigatus and A. alternata. We observed significant correlations between the RAST values for A. restrictus and those for A. fumigatus and A. alternata in our previous study,10 providing circumstantial evidence for shared allergenic components in A. restrictus and the other species. Indeed, allergenic cross-reactivity was demonstrated between A. restrictus and A. fumigatus in our previous study.11 We have now investigated whether A. restrictus shares allergens with A. alternata using RAST inhibition.

We previously used an A. restrictus extract that was prepared from its mycelial mat cultivated in an organic medium, M40Y, to investigate the cross-allergenicity between A. restrictus and A. fumigatus.11 In the present study, a completely synthetic defined medium, yeast nitrogen base agar medium, was chosen for preparing the A. restrictus extract. A preliminary study using sodium dodecyl sulfate polyacrylamide gel electrophoresis showed a difference in protein composition between these two
A. restrictus extracts (unpubl. data). Therefore, the allergenic relationship between the two Aspergillus species was also examined using the newly prepared extract.

METHODS

Strains

Extracts were prepared from A. restrictus 002, A. fumigatus NUM11017, and A. alternata 001. A. restrictus 002 and A. alternata 001 were obtained from the Public Health Research Institute of Kobe City, Japan while A. fumigatus NUM11017 was provided by the Laboratory of Medical Mycology, Nagoya University School of Medicine, Nagoya, Japan.

Extract preparation

Aspergillus restrictus was grown on yeast nitrogen base (YNB) agar medium composed of 6.7g of YNB (Difco Laboratories, Detroit, MI, USA), 600g of sucrose and 15g of agar per liter at 25°C for 3 weeks. A. fumigatus was cultivated in YNB liquid medium consisting of 6.7g of YNB and 30g of sucrose per liter at 25°C for 3 weeks in a stationary flask according to the method described previously. A. alternata was cultivated on Czapek-Dox agar medium (Gibco Diagnostic, Grand Island, NY, USA) at 25°C for 11 days. Mycelial mats of A. restrictus and A. alternata were stripped from each medium using a scalpel, and a floating mycelial mat of A. fumigatus was harvested by filtration using cotton gauze. Washings from the mycelial mat of A. restrictus were planted on M40Y and Czapek-Dox agars. Three weeks later, the gross morphological characteristics and the microscopic morphology of the fungal colonies were evaluated to verify their identity and purity. Similarly, the identity and purity of those of A. fumigatus and A. alternata were confirmed using Czapek-Dox and potato dextrose agars. These mats, suspended 1:5 weight:volume in Coca's solution, were individually homogenized by a Polytron homogenizer (Kinematica, Basel, Switzerland) for 5 min at 4°C. The homogenates were incubated for 72 h at 4°C with gentle stirring and centrifuged at 10000g for 30 min at 4°C. The supernatants were dialyzed against several liters of distilled water overnight at 4°C and then lyophilized. Protein contents of the extracts were measured with a Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, CA, USA). Bovine serum albumin (BSA; Sigma Chemical, St Louis, MO, USA) was used as the protein standard.

Radioallergosorbent test

Sera from 94 asthmatic Japanese children (mean age ± s.d. 12.0 ± 3.6 years) and 35 non-atopic patients (8.7 ± 4.8 years) were tested by RAST to A. restrictus, A. fumigatus and A. alternata. Serum from patients with aspergillosis was not included. RAST was performed by our method. Briefly, each well of a polystyrene microplate (Sumitomo Bakelit, Tokyo, Japan) was filled with 50 µL of 0.1 mol/L sodium carbonate buffer (pH 9.8) containing A. restrictus (protein concentration: 50 µg/mL), A. fumigatus (20 µg/mL) and A. alternata (50 µg/mL), and incubated at 37°C for 3 h. After blocking the unoccupied sites of the wells with human serum albumin (3% w/v, Calbiochem, San Diego, CA, USA) in 0.1 mol/L sodium carbonate buffer (pH 9.8), 50 µL of the serum sample was diluted 1:4 with 0.01 mol/L phosphate-buffered saline (PBS; pH 7.2) and was incubated independently in the antigen-coated wells at room temperature. Three hours later, the non-reactive serum was removed, 125I radioactivity on the surface of each well was measured in a gamma counter (ARC-600, Aloka, Mitaka, Japan) 16 h after the application of 50 µL of 125I-labeled antihuman IgE (Pharmacia Diagnostics, Uppsala, Sweden) containing approximately 20 000 cpm. These assays were performed in duplicate. RAST values were expressed as standard deviation (s.d.) units of the 35 non-atopic subjects as follows:

\[ \text{s.d. units} = \frac{(125I \text{ uptake of a sample} - \text{mean 125I uptake of normal controls})}{\text{s.d. of 125I uptake of normal controls}} \]

Our previous study demonstrated the specificity of RAST for detecting IgE antibodies binding to allergenic determinants of A. restrictus, A. fumigatus and A. alternata using the methods of RAST inhibition and serum dilution tests.

RAST inhibition

Of the 94 serum samples examined by RAST, 13 samples (Table 1) showed over 5 s.d. units of RAST values for A. restrictus. A serum pool was prepared from equal volumes of nine (No. 1–9) of the 13 sera. The extracts of A. restrictus, A. fumigatus and A. alternata, and BSA, used as the inhibiting antigen, were

Table 1. RAST values for Aspergillus restrictus, Aspergillus fumigatus and Alternaria alternata in 13 serum samples

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>A. restrictus</th>
<th>RAST (s.d. units)</th>
<th>A. fumigatus</th>
<th>A. alternata</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>19.9</td>
<td>27.6</td>
<td>48.6</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>18.4</td>
<td>39.0</td>
<td>11.1</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>26.3</td>
<td>89.1</td>
<td>28.7</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>20.9</td>
<td>16.8</td>
<td>33.7</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>14.5</td>
<td>11.9</td>
<td>28.3</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>8.9</td>
<td>12.0</td>
<td>9.7</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>51.9</td>
<td>0.3</td>
<td>87.1</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>31.4</td>
<td>-0.3</td>
<td>8.2</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>12.8</td>
<td>0.3</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>16.2</td>
<td>22.9</td>
<td>12.8</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>285.1</td>
<td>345.6</td>
<td>148.8</td>
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</tr>
<tr>
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<td>7.3</td>
<td>11.9</td>
<td>21.7</td>
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</tr>
<tr>
<td>13</td>
<td>10.0</td>
<td>9.7</td>
<td>184.6</td>
<td></td>
</tr>
</tbody>
</table>
dissolved in PBS (pH 7.2) at serial concentrations ranging from 0.4 to 200 µg/mL of protein. These were added to an equal volume of serum pool and then incubated overnight at 4°C. RAST to A. restrictus, A. fumigatus and A. alternata were carried out as described previously. In addition, the 13 serum samples were analyzed separately to detect IgE antibodies to allergenic components of A. restrictus that were shared with the other fungal species. The serum was diluted 1:2 in PBS (pH 7.2) and mixed with an equal volume of PBS (pH 7.2) containing each fungal extract at a concentration of 0, 4, 200 or 1000 µg/mL of protein. After incubation for 16 h at 4°C, the A. restrictus RAST was performed. To exclude the possibility that IgE antibodies bound to these fungal extracts without immunologic specificity for binding antigen, we evaluated the fungal extracts for inhibition of RAST to Dermatophagoides farinae and ovalbumin. The mite extract, donated by Torii Pharmaceuticals (Tokyo, Japan), and ovalbumin (Sigma Chemical) were dissolved at a protein concentration of 50 µg/mL with 0.1 mol/L sodium carbonate buffer (pH 9.8). Sera with high titters of IgE antibodies to these antigens, after being diluted 1:2 in PBS (pH 7.2), were incubated with equal volumes of each of the three fungal extracts dissolved at serial concentrations ranging from 0.4 to 200 µg/mL of protein. The procedures for RAST inhibition as described above were then carried out. Results were expressed in terms of percent RAST inhibition, which was defined as follows:

\[
100 \times \frac{\text{radioactive count without inhibition} - \text{radioactive count after inhibition}}{\text{radioactive count without inhibition} - \text{background}}.
\]

A level of percent RAST inhibition more than 20% was considered to be significant.

**Results**

The yield in mycelium of A. restrictus, A. fumigatus and A. alternata was approximately 36.5, 36.0 and 133.1 g of wet weight per liter of culture medium, respectively. Their mycelia were not misidentified or contaminated by other organisms. The protein content of the extracts of A. restrictus, A. fumigatus and A. alternata was 4.99, 8.30 and 2.73%, respectively. In the RAST, sera from 27 asthmatic patients showed values over 2 s.d. units in A. restrictus RAST, 22 in A. fumigatus RAST, and 33 in A. alternata. In the 35 non-atopic subjects, the mean (s.d.) of RAST values for A. restrictus, A. fumigatus and A. alternata was 4.99, 8.30 and 2.73%, respectively. In the RAST, sera from 27 asthmatic patients showed values over 2 s.d. units in A. restrictus RAST, 22 in A. fumigatus RAST, and 33 in A. alternata. In the 35 non-atopic subjects, the mean (s.d.) of RAST values for A. restrictus, A. fumigatus and A. alternata was 4.99, 8.30 and 2.73%, respectively. In the RAST, sera from 27 asthmatic patients showed values over 2 s.d. units in A. restrictus RAST, 22 in A. fumigatus RAST, and 33 in A. alternata. In the 35 non-atopic subjects, the mean (s.d.) of RAST values for A. restrictus, A. fumigatus and A. alternata was 4.99, 8.30 and 2.73%, respectively. In the RAST, sera from 27 asthmatic patients showed values over 2 s.d. units in A. restrictus RAST, 22 in A. fumigatus RAST, and 33 in A. alternata. In the 35 non-atopic subjects, the mean (s.d.) of RAST values for A. restrictus, A. fumigatus and A. alternata was 4.99, 8.30 and 2.73%, respectively. In the RAST, sera from 27 asthmatic patients showed values over 2 s.d. units in A. restrictus RAST, 22 in A. fumigatus RAST, and 33 in A. alternata. In the 35 non-atopic subjects, the mean (s.d.) of RAST values for A. restrictus, A. fumigatus and A. alternata was 4.99, 8.30 and 2.73%, respectively.

Dose-response curves for RAST inhibition are presented in Fig. 1. When the single serum pool was examined, A. restrictus, A. fumigatus and A. alternata inhibited their homologous RAST by approximately 98% at the maximum concentration of 100 µg/mL of protein. Aspergillus restrictus at 100 µg/mL inhibited the RAST to A. fumigatus and A. alternata by 88.8% and 91.6%, respectively. A. fumigatus and A. alternata at the same concentration also inhibited their heterologous RAST by more than 80%. In contrast, none of these fungal extracts, even at the maximum concentration of 100 µg/mL, significantly inhibited
The present study confirmed the allergenic cross-reactivity between A. restrictus and A. fumigatus as shown in our previous study, although the protein composition of the A. restrictus extracts used in each study differed. We excluded the possibility that part of the medium constituents contributed to the allergenic relationship, because completely synthetic culture media were used in this study. Arruda et al. demonstrated that the amino acid sequence of Asp f I, a major 18 kDa A. fumigatus allergen, is 99% homologous to that of mitogillin, a cytotoxin produced by A. restrictus. This suggests cross-allergenicity between A. fumigatus and A. restrictus. Thus, our results are compatible with this sequence data, although there is no evidence that the extracts of A. fumigatus and A. restrictus that we used included Asp f I or mitogillin, respectively.

Karr et al. demonstrated cross-reactivity among the species of A. fumigatus, A. glaucus and A. flavus, but not between the genera Aspergillus and Alternaria (i.e. A. alternata). However, we have shown that A. alternata shares common allergens with A. fumigatus and A. restrictus. The discrepancy between these results may be due to strain differences in allergenic activity of the fungi. Indeed, the allergenic variability of different strains of A. alternata and A. fumigatus has been previously observed. The polyvinyl chloride microplate used in the present study may bind a larger amount of polysaccharide allergens than the polysaccharide-based solid phases, which may be one explanation for the discrepancy. Several recent studies have shown allergenic cross-reactivity among fungal genera. Our observations are in accord with those results.

The present study demonstrated that the sera of patients No. 7-9 with a positive RAST to A. restrictus did not contain IgE antibodies to cross-antigens between A. restrictus and the other fungi A. fumigatus and A. alternata, because when these sera were tested A. fumigatus and A. alternata did not inhibit A. restrictus RAST to any extent. Our results suggest that hypersensitivity to A. restrictus, in general, may be induced by A. fumigatus and A. alternata as well as by A. restrictus. However, occasional cases of hypersensitivity to A. restrictus may be induced by A. restrictus alone but not by A. fumigatus or A. alternata.

There is no firm evidence that A. restrictus is really a cause of clinical hypersensitivity. However, its frequent recovery from the environment suggests a possibility of the clinical relevance of this species in allergic disease. We assume that A. restrictus may initiate the development of fungal allergies in young childhood because it is commonly present in the home. A. fumigatus and A. alternata presumably are less significant contributors to allergic symptoms that occur indoors because their spores do not germinate at the relative humidity found in the typical domestic environment.

In conclusion, we demonstrated a cross-allergenicity among A. restrictus, A. fumigatus and A. alternata. This finding will be useful in identifying the fungi involved in fungal allergies.
ACKNOWLEDGMENTS

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CORRIGENDUM

This article originally appeared in Allergology International Volume 1, Issue 1, 1996. Unfortunately, it included a number of typographical errors and so the corrected version has been reprinted in full in this issue.