Allergology International (1996) **45**: 45–49

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

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Received 6 November 1995. Accepted for publication 26 January 1996.

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 studies[1–4] using culture media with low water activity (e.g. MA64,[1] DG18[5]) 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 aeroallergen[10] 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

Spergillus restrictus was grown on yeast nitrogen base (YNB) agar medium composed of 6.7 g of YNB (Difco Laboratories, Detroit, MI, USA), 600 g of sucrose and 15 g of agar per liter at 25°C for 3 weeks. *A. fumigatus* was cultivated in YNB liquid medium consisting of 6.7 g of YNB and 30 g of sucrose per liter at 25°C for 3 weeks in a stationary flask according to the method described previously.[12] *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.[7] Similarly, the identity and purity of those of *A. fumigatus* and *A. alternata* were confirmed using Czapek-Dox and potato dextrose agars.[7] 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 10 000 g 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. In a preliminary trial, we determined the optimal conditions for fungal culture including the duration of incubation and the culture medium. We found that in the conditions adopted here, the yield in mycelium of each fungus reached a plateau.

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.[10,12] Briefly, each well of a polyvinyl chloride microplate (Sumitomo Bakelite, 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, ^{125}I 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 ^{125}I-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:

s.d. units = (^{125}I uptake of a sample − mean ^{125}I uptake of normal controls)/s.d. of ^{125}I 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.[10]

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

Patient no.	RAST (S.D. units) *A. restrictus*	*A. fumigatus*	*A. alternata*
1	19.9	27.6	48.6
2	18.4	39.0	11.1
3	26.3	89.1	28.7
4	20.9	16.8	33.7
5	14.5	11.9	28.3
6	8.9	12.0	9.7
7	51.9	0.3	87.1
8	31.4	21.0	8.2
9	12.8	0.3	0.4
10	16.2	22.9	12.8
11	285.1	345.6	148.3
12	7.3	11.9	21.7
13	10.0	9.7	184.6

dissolved in PBS (pH 7.2) at serial concentrations ranging from 0.4 to 200 mg/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 mg/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 mg/mL with 0.1 mol/L sodium carbonate buffer (pH 9.8). Sera with high titers 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 mg/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 3 (radioactive count without inhibition 2 radioactive count after inhibition) / (radioactive count without inhibition 2 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 35.4 (6.78), 30.8 (5.52), and 33.0 (6.30) cpm/well, respectively. Total count was 17 873 cpm/well. For the RAST inhibition assays, we examined 13 of the 27 sera with over 2 s.d. units of RAST values for *A. restrictus* because the 13 serum samples showed values over 5 s.d. units in *A. restrictus* RAST (Table 1).

Dose-reponse 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

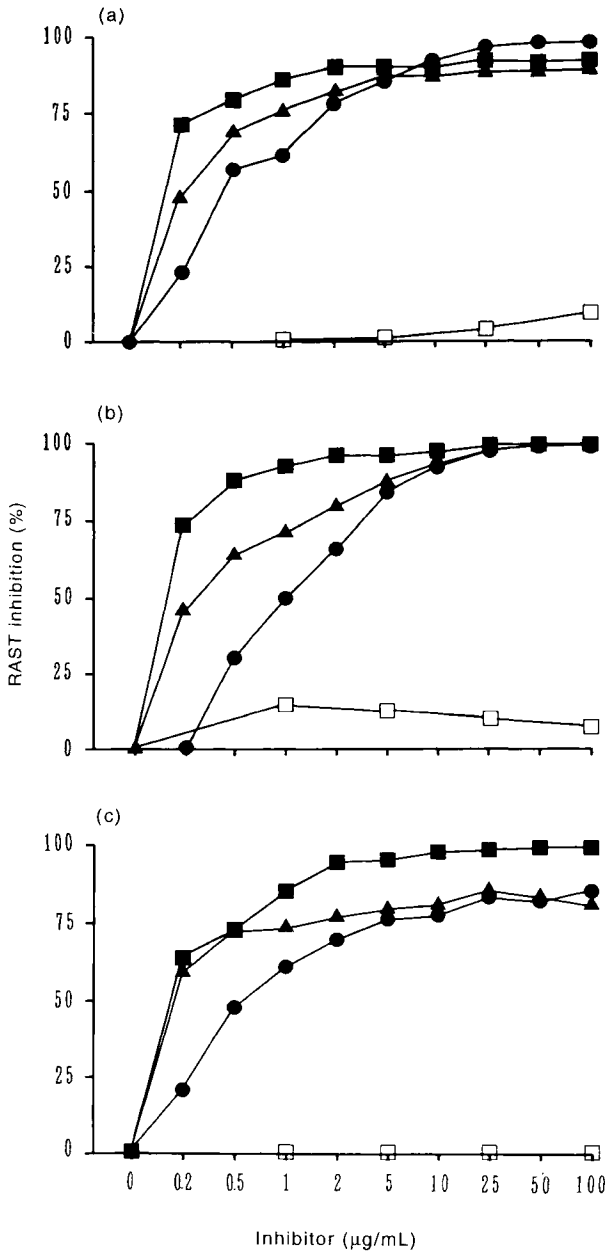

Fig. 1 Inhibition curves for RAST to (a) *Aspergillus restrictus*, (b) *Aspergillus fumigatus* and (c) *Alternaria alternata*. Mycelial extracts of *A. restrictus* (d), *A. fumigatus* (m) and *A. alternata* (j) and bovine serum albumin (u) was used as an inhibiting antigen. The total count of ^{125}I was 25 792 cpm/well in each RAST inhibition assay. Background counts for RAST to *A. restrictus*, *A. fumigatus* and *A. alternata* were 31, 35.5 and 39 cpm/well, respectively.

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

Table 2. Inhibition of *Aspergillus restrictus* RAST with extracts of *A. restrictus*, *Aspergillus fumigatus* and *Alternaria alternata* in 13 serum samples with a highly positive RAST to *A. restrictus*.

Patient no.	Inhibiting antigen (μg/mL of protein)								
	A. restrictus			*A. fumigatus*			*A. alternata*		
	2	100	500	2	100	500	2	100	500
1	2.4	75.9	92.6	6.3	90.4	91.6	5.3	63.2	70.3
2	23.0	83.7	90.3	51.3	66.6	63.2	72.5	70.4	68.4
3	0	88.2	92.5	78.7	90.8	94.4	93.0	94.7	90.0
4	44.2	91.3	90.6	28.5	36.5	50.9	51.0	70.0	76.5
5	31.0	66.1	83.8	51.5	64.9	72.2	79.4	82.8	95.9
6	61.2	78.1	92.5	74.7	78.9	70.8	88.1	79.4	80.2
7	84.2	95.7	91.9	ND	ND	3.0	0	10.4	11.6
8	60.0	94.6	93.6	0	4.9	6.2	10.1	1.0	9.0
9	81.4	84.7	89.4	ND	ND	1.2	2.1	0	1.1
10	88.7	89.4	93.9	95.6	82.5	94.6	97.7	91.8	94.4
11	95.3	98.7	94.3	98.4	98.9	90.4	98.6	98.5	83.8
12	38.5	64.4	86.0	81.5	83.0	93.7	ND	ND	ND
13	42.5	62.8	75.9	44.4	78.9	72.1	ND	ND	ND

ND, Not done. Results are expressed as percent RAST inhibition.

the RAST to *D. farinae* or ovalbumin (<7%). BSA had no inhibitory effect on the RAST to any of the fungal species (<7%).

When sera with a highly positive RAST to *A. restrictus* were examined separately (Table 2), *A. restrictus* in amounts that exceeded 100 μg/mL of protein greatly inhibited its homologous RAST (75.9–94.3% in all 13 subjects). *A. fumigatus* and *A. alternata* also inhibited the RAST to *A. restrictus* to a similar extent in 10 and 8 of the 11 subjects studied, respectively. In the three serum samples without a positive RAST to *A. fumigatus* (No. 7–9), *A. fumigatus* did not significantly inhibit the *A. restrictus* RAST. A similar result was observed with *A. alternata* in patient No. 9. The sera from patients No. 7 and 8 with a positive RAST to *A. alternata*, *A. alternata* did not show any significant inhibition of the *A. restrictus* RAST.

Discussion

We have shown that *A. fumigatus* and *A. alternata* induced a significant inhibition of *A. restrictus* RAST in most of the sera obtained from asthmatic children who showed a highly positive RAST to *A. restrictus*. When a serum pool containing IgE antibodies to *A. restrictus* was examined, *A. fumigatus* and *A. alternata* also substantially inhibited *A. restrictus* RAST. These findings indicate that *A. restrictus* shares allergenic components with *A. fumigatus* and *A. alternata*, and that the cross-reacting components may include parts of key allergens of *A. restrictus*. Also, *A. restrictus* was a potent inhibitor of RAST to *A. fumigatus* and *A. alternata* in the RAST inhibition tests performed on the serum pool. The likelihood that *A. restrictus* contains parts of major allergenic components of *A. fumigatus* and *A. alternata* is supported by the results of our previous study[10] that demonstrated highly significant correlations between RAST values for *A. restrictus* and those for *A. fumigatus* and *A. alternata*.

The present study confirmed the allergenic cross-reactivity between *A. restrictus* and *A. fumigatus* as shown in our previous study,[11] 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.*[13] showed that the amino acid sequence of *Asp f* I, a major 18 kDa *A. fumigatus* allergen,[14] 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.*[15] 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.[16,17] 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.[18–22] 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.[1–4] *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.[7,23]

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.

ACKNOWLEDGEMENTS

We thank Professor Kenji Tanaka of the Laboratory of Medical Mycology, Nagoya University School of Medicine, and Dr Yoshio Matsuda of the Public Health Research Institute of Kobe City for their help. This study was supported in part by a grant for Health and Welfare Programs of the Ministry of Health and Welfare, Japan.

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