Lack of Demonstrable Immunochemical Cross-Reactivities Between Alternaria and Cladosporium Extracts in Allergic Patients from Multiple U.S. Locations

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Abstract

Introduction: Alternaria and Cladosporium species are known to produce intracellular proteins with similar biological functions or enzymatic activities. These proteins display high sequence or structural homology and have been characterized as allergens, but their importance in IgE-mediated responses or cross-sensitivities between these fungi has not been determined.

Methods: Sera obtained from patients with positive skin tests to Alternaria alternata extracts were analyzed by ELISA and ImmunoCAP analyses. Direct ImmunoCAP assays were performed to determine IgE levels to a variety of fungi, including Alternaria (Al), Cladosporium herbarum (Ch) and Cladosporium sphaerospermum (Cs). Cross-inhibition analyses with Alternaria and Cladosporium extracts on both ImmunoCAP and ELISA solid phases were conducted to assess the degrees of shared or common allergens between these genera. Serum samples or pools with different fungal IgE specificity patterns were evaluated.

Results: A total of 85 patients from allergy clinics in 4 U.S. states (WI, AR, GA, FL) were studied. A majority of patients (57, 67%) displayed positive skin tests to Al and negative results to Ch. Positive skin reactions to both Al and Ch were found in 14 patients (16%), with no patients reacting exclusively to Ch. Approximately half of patient sera (42, 49%) possessed positive IgE ImmunoCAP reactions to Al and negative reactions to Ch. Positive IgE levels to both Al and Ch were detected in 10 sera (12%, including 4 sera from patients with positive skin tests to both extracts), with no sera reacting with Ch and not Al. ImmunoCAP inhibition analyses of Al and Ch allergens in sera exhibiting different IgE-binding patterns with Al and other fungi were highly specific for homologous allergens, with no measurable cross-reactivities observed between Al, Ch and Cs extracts. ELISA inhibition assays with Al, Ch and Cs extracts and 3 different in-house mold-positive serum pools also produced no evidence of shared or cross-reactive allergens.

Conclusions: Cytoplasmic proteins reported to be shared by Al, Ch and Cs were not recognized as prominent allergens. Immunochemical cross-reactivities between these fungi may be observed when individual proteins are investigated, but were not apparent among the multiple Al allergens characterized in this study.

Introduction/Overview

Fungal allergens are often present at moderate to high concentrations in both intracellular and extracellular fractions of laboratory cultures.

Most allergenic fungi studied to date produce a diverse repertoire of protein allergens possessing varied prevalence and IgE-binding activities among sensitive patients. In some cases, proteins sharing the same biological function or enzymatic activities have been identified in some phylogenetically-related and unrelated fungal organisms, supporting the notion that close sequence or structural homologies of these protein molecules could result in allergenic cross-reactivities between a wide range of fungi.

Alternaria alternata and *Cladosporium herbarum* are among the best studied allergenic fungi, and are included on skin test panels employed in most allergy clinics. A large percentage of the protein allergens identified and characterized from these organisms exhibit similar functions or catalytic activities, as illustrated below.

Alternaria alternata	Cladosporium herbarum	Protein function/activity
Alt a 1	None	Unknown
Alt a 3 Alt a 5 Alt a 6 Alt a 7 Alt a 8	Cla h HSP70 Cla h 5 Cla h 6 Cla h 7 Cla h 8	Heat shock protein 70 Acidic ribosomal protein P2 Enolase *S. cerevisiae* protein YCP4 Mannitol dehydrogenase
None	Cla h 9	Vacuolar serine protease
Alt a 10 Alt a 12 Alt a 13 Alt a NTF2	Cla h 10 Cla h 12 Cla h GST Cla h NTF2	Aldehyde dehydrogenase Acidic ribosomal protein P1 Glutathione-S-transferase Nuclear transport factor 2

Published studies have confirmed both sequence homology and IgE cross-reactivity for most of the above allergen pairs. However, it has also been noted that reactions to these allergens are not sufficient to explain the complex overall IgE reactivities to Al or Ch extracts, and their importance and clinical relevance to fungal IgE reactions in different areas of the United States have not been determined.

In this study, the allergenic relationships between Al, Ch and Cs have been assessed using commercial extracts, individual serum samples and pools from skin test-positive individuals, and ELISA and ImmunoCAP analyses in direct and competition (inhibition) formats.

Materials and Methods

Prick skin testing was performed at allergy clinics in 4 locations in the midwestern and southeastern United States (Madison WI, Little Rock AR, Atlanta GA and Fort Lauderdale FL). Skin test devices, extracts, controls and criteria for positive reactions were identical to those used in each clinical practice. Both wheal and erythema reactions were measured and recorded as diameters (mm) or class scores.

Blood samples were obtained from patients displaying positive skin prick tests to Alternaria. Following centrifugation, serum samples were frozen (-20°C) and shipped to Greer for immunochemical analyses. Proper informed consent and HIPAA guidelines were followed by each clinic, and all study activities were approved by investigational review boards.

Extracts of *Alternaria alternata* (Al), *Cladosporium herbarum* (Ch) and *Cladosporium sphaerospermum* (Cs) used for the laboratory analyses were commercial 1:20 w/v glycerinated preparations manufactured at Greer from cellular (mycelial) source materials.

ImmunoCAP 100 analyses (Phadia, Portage, MI) were conducted at Greer using Phadia reagents and assay conditions. Direct measurements of the IgE-binding activities of patient sera employed the following ImmunoCAP fungal solid phases: *Alternaria alternata* (m6), *Penicillium chrysogenum* (m1), *Cladosporium herbarum* (m2), *Aspergillus fumigatus* (m3) and *Fusarium moniliforme* (m9). Inhibitions of human IgE reactions with Alternaria ImmunoCAP allergens were performed by pre-incubation (2 hours, 20-25°C) of serum with 5-fold serial dilutions of Alternaria extracts. Percent inhibition values for extract dilutions were calculated relative to the reactivities of uninhibited (positive control) samples. IgE-binding potencies from % inhibition vs. log dilution dose-response curves were determined by parallel line bioassay analysis.

IgE ELISA inhibition experiments were conducted using Immulon 4 microtiter plates (Thermo Electron Corp., Milford, MA) and 3-fold serial dilutions of reference and test extracts co-incubated with an in-house human serum pool containing mold-positive IgE (lots HAM1-1, ZM-P1 or ZE-P4). IgE binding was detected after incubations with biotinylated anti-human IgE, avidin-alkaline phosphatase and chromogenic substrate p-nitrophenyl phosphate, with absorbances read at 405 nm. Percent inhibitions and relative IgE-binding potencies were calculated using a parallel line bioassay spreadsheet macro.

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Prick Skin Test (PST) and Direct IgE ImmunoCAP Results

A total of 85 subjects at 4 allergy clinics were evaluated by prick skin testing to multiple fungal extracts. Two-thirds of the study subjects displayed positive skin reactions (2+ or higher) to Al and negative reactions to all other fungi tested. No subjects reacted only to Ch or Cs. Serum from half of the study subjects exhibited positive ImmunoCAP reactions (1 kU/L or higher) only to Al allergens. A small percentage of patients showed positive skin or IgE reactions to both Al and Ch/Cs allergens.

Analysis	Number of subjects with positive reactions to ...			
	Al + Ch/Cs	Al only	Ch/Cs only	Al or Ch or Cs + other fungi
PST	14 (16%)	57 (67%)	0 (0%)	14 (16%)
IgE ImmunoCAP	10 * (12%) * 4 w/ Pos PSTs to both	42 (49%)	0 (0%)	33 (39%)

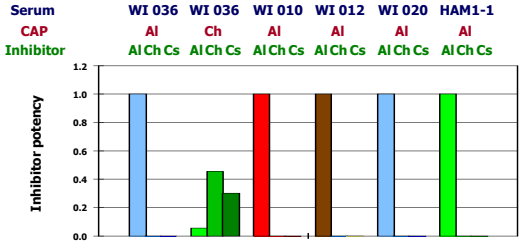
The Al and Ch/Cs reactivities of the 20 subjects positive by PST or ImmunoCAP to both allergens are summarized below. For most subjects, noticeable differences in the magnitude of the reactions to the 2 fungi were observed.

Clinic/ Subject	Al PST w/e	Ch/Cs PST w/e	Al IgE CAP kU/L	Ch IgE CAP kU/L
WI 009	10/40	0/0	11.10	3.50
WI 036	18/46	0/0	17.60	1.69
WI 038	10/51	6/51	3.75	< 0.35
WI 039	13/46	6/30	15.30	1.40
WI 040	16/42	0/0	17.00	2.15
WI 041	10/36	11/40	1.81	2.42
AR 007	3+	2+	80.30	Not tested
AR 012	4+	3+	1.27	< 0.35
AR 014	2+	3+	0.45	Not tested
AR 015	2+	2+	< 0.35	Not tested
AR 017	2+	Negative	13.50	2.22
AR 019	3+	2+	< 0.35	Not tested
AR 026	3+	3+	5.28	7.93
AR 027	4+	2+	3,95	< 0.35
AR 028	4+	2+	2.00	< 0.35
AR 029	3+	2+	3.88	< 0.35
AR 030	3+	3+	3.97	0.45
FL 001	9/28	14/28	36.80	1.85
FL 002	6/11	Negative	7.53	1.49
FL 005	6/23	Negative	26.80	1.96

MPN 091609HD160

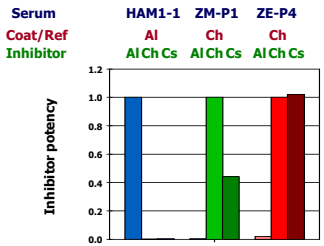
ImmunoCAP Inhibition Results

Inhibition of IgE ImmunoCAP reactivities by Al, Ch or Cs was examined for one serum displaying positive reactions to both Al and Ch (WI 036) and for 3 Al-specific sera. All 4 sera displayed complete self-inhibition with Al but little or no cross-inhibition between Al and Ch or Cs allergens present in the commercial Greer extracts. Similar results were obtained for an Al-specific in-house human serum pool (HAM1-1).



ELISA Inhibition Results

IgE ELISA inhibition analyses with microplates coated with Al or Ch extracts were performed using 3 different in-house human serum pools with varying fungal allergen specificities. Dose-response curves for homologous allergens (reference) ranged from 11-92% inhibition. Test extracts produced inhibition levels ranging from 0-33% for unrelated species (Al–Ch/Cs) and 10-77% for related species (Ch–Cs). Al and Ch/Cs extracts displayed virtually no IgE cross-reactivity with any of the serum pools included in these analyses, consistent with the ImmunoCAP inhibition data described above.



Conclusions

A large number of intracellular proteins possessing similar biological functions or enzymatic activities have been isolated from Al and Ch/Cs cultures and characterized as allergens based on IgE-binding properties. *In vivo* and *in vitro* cross-reactivities between these fungi would be expected in patients that recognize homologous regions of shared proteins as prominent allergens. Conversely, patients reacting to different epitopes or to unrelated allergenic proteins would likely exhibit minimal cross-reactivities between Al and Ch/Cs.

Skin test reactivities in patients tested with Al and Ch/Cs extracts at 4 U.S. allergy clinics did not reveal any dominant or consistent relationships between these fungi. A majority of patients displayed strong positive reactions to Al and negative reactions to Ch/Cs. Similar results were found when sera obtained from these patients were analyzed by direct IgE ImmunoCAP assays to these and other fungi. No patients displayed positive skin test or IgE reactions exclusively to Ch/Cs in this study.

IgE ImmunoCAP and ELISA reactivities of 4 individual patient sera and 3 in-house serum pools to immobilized Al allergens were unaffected by pre-incubation or co-incubation with Ch/Cs extracts. Al extracts produced near-complete inhibition of IgE binding to Al solid phase allergens under the identical extract dilution and reagent incubation conditions. Binding to Ch allergens was not influenced by inhibition with Al extract. Cs produced partial inhibition of Ch reactivity in some cases and near-complete inhibition in others.

In summary, the Al and Ch/Cs extracts examined in this study exhibited virtually no allergenic cross-reactivities when tested directly on patients (PST) or analyzed using patient sera and validated quantitative laboratory assays for IgE-binding activities (ImmunoCAP inhibition, ELISA inhibition).

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