

Evaluation of the Levels of Specific IgE against *Cryptococcus diffluens* and *Cryptococcus liquefaciens* in Patients with Atopic Dermatitis

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Abstract: *Cryptococcus diffluens* and *Cryptococcus liquefaciens*, 2 basidiomycetous yeasts, frequently colonize the skin of patients with atopic dermatitis (AD). In this study, we investigated the presence of specific IgE antibodies against *C. diffluens* and *C. liquefaciens* in the sera of AD patients by using an enzyme immunoassay. Of the 122 AD serum samples tested, 43 (35.2%) and 50 (41.0%) were positive for specific IgE antibodies against *C. diffluens* and *C. liquefaciens*, respectively. The levels of specific IgE against the *C. diffluens* antigen and that against the *C. liquefaciens* antigen were strongly correlated ($r = 0.96$). In contrast, no remarkable correlation was observed between the levels of specific IgE against the 2 *Cryptococcus* species and that of specific IgE against *Malassezia restricta*. Competitive enzyme-linked immunosorbent assay (ELISA) inhibition tests revealed that *C. diffluens* and *C. liquefaciens* shared common antigens. This finding was consistent with the IgE immunoblotting data which demonstrated that several IgE-binding proteins with molecular masses of 77, 54, and 30 kDa were recognized in both *C. diffluens* and *C. liquefaciens* antigens. These results suggest that fungal components from *C. diffluens* and *C. liquefaciens* may act as allergens and play a role in the pathogenesis of AD.

Key words: Atopic dermatitis, *Cryptococcus* species, ELISA, Specific-IgE antibody

Atopic dermatitis (AD) is a chronic relapsing pruritic inflammation of the skin, affecting 10–20% children and 1–3% adults worldwide, and its prevalence is increasing in highly industrialized countries (13). During the last decade, there has been considerable interest in the mechanisms and triggering factors responsible for the increased microbial colonization of atopic skin. The microbial skin flora of AD patients differs from that of healthy people; *Staphylococcus aureus*, *Malassezia* species, and *Candida albicans* have been shown to frequently colonize the skin of AD patients (1, 2, 16). The sera of AD patients have elevated levels of specific IgE antibodies against these microbial antigens (8, 9, 12, 17, 18). Our recent study using a PCR-based non-culture method has shown that *Cryptococcus diffluens* and *Cryptococcus liquefaciens* as well as *Malassezia* species frequently colonize the skin of AD patients (15). The basidiomycetous yeasts *C. diffluens* and *C. liquefaciens* were previously classified as *C. albidus*. In 2000, *C. albidus* was reclassified into 12

distinct species (*C. aerius*, *C. arrabidensis*, *C. chernovii*, *C. cylindricus*, *C. diffluens*, *C. liquefaciens*, *C. oeirensis*, *C. phenolicus*, *C. saitoi*, *C. terricola*, *C. uzbekistanensis*, and *C. wieringae*) based on rDNA sequence analysis (3). *C. albidus* is considered to be one of the causative antigens of Japanese summer-type hypersensitivity pneumonitis (SHP) (5, 11). Previous studies have detected specific IgE antibodies against *C. albidus* in patients with AD and rhinitis (14). However, few studies have focused on detecting specific IgE antibodies against *C. diffluens* and *C. liquefaciens* in the serum samples of AD patients. In this study, we evaluated the levels of serum IgE against *C. diffluens* and *C. liquefaciens* in AD patients by using enzyme-linked immunosorbent assay (ELISA).

Materials and Methods

Subjects. Serum samples from 122 adult AD

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Abbreviations: AD, atopic dermatitis; ELISA, enzyme-linked immunosorbent assay; LNA, Leeming and Notman agar; PBS, phosphate buffered saline; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis; SHP, summer-type hypersensitivity pneumonitis; TMB, tetramethylbenzidine.

patients including 68 males, 52 females, and 2 patients of unknown genders whose ages ranged from 20 to 64 years (mean age, 36.2 ± 11.9 years) and 17 adult healthy subjects were obtained from Tokyo Medical University Hospital. AD was diagnosed according to the criteria of Williams et al. (19). The present study was approved by the regional ethics committee, and all the patients or their relatives provided informed consent.

Preparation of *Cryptococcus* and *Malassezia* antigens. *C. diffluens* (CBS160), *C. liquefaciens* (CBS968), *Malassezia restricta* (CBS7877), and *M. globosa* (CBS7966) were cultured at 32 C for 5 days on modified Leeming and Notman agar (LNA; 20 g glucose, 50 g malt extract, 1 g polypeptone, and 20 g bile salts) (Oxoid, Hampshire, U.K.) containing 1% Tween 40, 0.2% glycerol, and 50 µg/ml chloramphenicol (Sankyo, Tokyo). The cultures were harvested and then washed 5 times with phosphate-buffered saline (PBS). The cells were suspended in 3 volumes of PBS and then mechanically disrupted using a French press cell (Aminco, St. Louis, Mo., U.S.A.) at a pressure of 18,000 pounds per square inch. The cell suspension was centrifuged at 14,500 rpm for 1 hr. The protein concentration of the antigen extract was 3.0 mg/ml for *C. diffluens*, 3.0 mg/ml for *C. liquefaciens*, 0.4 mg/ml for *M. restricta*, and 2.9 mg/ml for *M. globosa*. The extract was stored at -20 C in the lyophilized state, and dissolved in PBS at an appropriate concentration before use.

Determination of specific IgE antibodies against *Cryptococcus* species. The levels of the IgE antibody against each of the *Cryptococcus* species and *M. restricta* were determined using the AlaSTAT Microplate method (Diagnostic Products Corporation, Los Angeles, Calif., U.S.A.) with slight modifications (7, 20). Briefly, antigens extracted from the yeasts were diluted using PBS (pH 7.2) to a protein concentration of 50 µg/ml. The wells of a microtiter plate (Corning, New York, N.Y., U.S.A.) were coated with 100 µl of each antigen for 18–20 hr at 4–8 C. Subsequently, 50 µl serum sample were added to the antigen-coated microplate wells, followed by peroxidase-labeled anti-IgE antibody. After adding the substrate solution, i.e., 3,3',5,5'-tetramethylbenzidine (TMB) dissolved in H₂O₂ the absorbance at 650 nm was measured for 5 min at 10-sec intervals. The IgE antibody level [units/ml] in the patients' samples was calculated using a standard curve based on a reference serum level in accordance with the 2nd IRP 75/502 of the World Health Organization. A specific IgE level greater than 0.35 IU/ml was considered to be positive.

Competitive ELISA inhibition test. Competitive

ELISA inhibition tests were performed to determine the specificity of the IgE binding to each antigen. Sera from 2 AD patients were used in this assay (Table 1). Starting at a concentration of 500 µg/ml, 10-fold serial dilutions of the extracts from the *Cryptococcus* and *Malassezia* species were prepared in PBS, and these were used as inhibiting fluid-phase antigens. A 25-µl aliquot of the patients' sera was mixed with an equal volume of the inhibiting fluid-phase antigens and incubated overnight at 4–8 C. This mixture was then added to the wells of an antigen-coated microtiter plate. The subsequent steps were identical to those followed for ELISA. Based on the results of the control samples, in which an equal volume of PBS was used instead of the inhibitors for preincubation, the inhibition of specific IgE binding was expressed in terms of % inhibition.

IgE immunoblotting test. Each fungal extract (100 µg) was subjected to sodium dodecyl sulfate-polyacrylamide (with a linear 10–20% gradient) slab gel electrophoresis (SDS-PAGE) and then transferred to a polyvinylidene fluoride membrane. The membranes with the blotted yeast components were incubated with a blocking buffer (PBS containing 5% skim milk powder) for 30 min at room temperature, cut into 4-mm wide strips, and immunoblotted. IgE-binding components were detected using the AlaBLOT kit (Diagnostic Products Corporation) according to the manufacturer's instructions (4). In brief, the membrane strips were incubated for 1 hr at room temperature with the individual serum samples diluted 1:10 in PBS; they were then washed 3 times with washing buffer and incubated with an anti-human IgE alkaline phosphatase conjugate for 1 hr. After washing, the IgE-binding components were visualized using a substrate solution containing 5-bromo-4-chloro-3-indolyl phosphate and nitroblue-tetrazolium (Wako, Tokyo).

Results

Levels of Specific IgE Antibodies against Cryptococcus and Malassezia Species

The serum level of the specific IgE antibody in the 122 AD patients was 1.14 ± 2.52 U/ml (mean $\pm$ standard deviation) for *C. diffluens*, 0.95 ± 2.39 U/ml for *C.*

Table 1. Profiles of the serum samples from 2 AD patients used in the competitive ELISA inhibition tests

No.	Serum	Specific IgE antibody titer (U/ml)		
		<i>C. diffluens</i>	<i>C. liquefaciens</i>	<i>M. restricta</i>
1	#101	13.0	10.1	30.0
2	#104	22.2	23.0	225.0

Table 2. Specific IgE antibody levels against *Cryptococcus* and *Malassezia* species in sera from AD patients

	Number of patients (Sex M:F)	Age	Total-IgE (IU/ml)	<i>C. diffluens</i>		<i>C. liquefaciens</i>		<i>M. restricta</i>	
				Specific IgE antibody (U/ml)	Number of positive patients (%)	Specific IgE antibody (U/ml)	Number of positive patients (%)	Specific IgE antibody (U/ml)	Number of positive patients (%)
AD patients	122 (68:52)	34.5±10.8	5,975±9,677	1.14±2.52	43 (35.2%)	0.95±2.39	50 (41.0%)	32.2±72.6	95 (77.9%)
Healthy subjects	17 (6:11)	39.2±10.7	125±137	0.07±0.04	0 (0.0%)	0.08±0.03	0 (0.0%)	0.25±1.02	1 (5.9%)

Data represent mean ± standard deviation.

Table 3. The correlation coefficients between the specific IgE antibody levels against the two *Cryptococcus* species and *M. restricta* in sera from AD patients

		<i>C. liquefaciens</i>		<i>M. restricta</i>	
		neg	pos	neg	pos
<i>C. diffluens</i>	pos	0	43	0	43
	neg	72	7	27	52
	corr	$r = 0.96$		$r = 0.28$	
<i>C. liquefaciens</i>	pos			1	49
	neg			26	46
	corr			$r = 0.32$	

pos: positive, neg: negative, corr: correlation coefficient.

liquefaciens, and 32.2 ± 72.6 U/ml for *M. restricta* (Table 2). A positive increase (>0.35 IU/ml) in the serum levels of specific IgE against *C. diffluens*, *C. liquefaciens*, and *M. restricta* was detected in 35.2% (43/122), 41.0% (50/122), and 77.9% (95/122) AD patients, respectively. None of the healthy subjects showed remarkable levels of IgE antibodies to these *Cryptococcus* species. Table 3 presents the correlation coefficients between each of the specific IgE values against the two *Cryptococcus* species and *M. restricta* in sera from AD patients. The level of specific IgE against the *C. diffluens* antigen and that against *C. liquefaciens* antigen showed a strong correlation ($r = 0.96$). In contrast, the level of specific IgE against the *M. restricta* antigen showed a poor correlation with those of specific IgE against the *C. diffluens* and *C. liquefaciens* antigens ($r = 0.28$ and 0.32 , respectively).

Competitive ELISA Inhibition Test

To investigate the antigen specificity of each *Cryptococcus* species, competitive ELISA inhibition tests were performed using sera from 2 different AD patients. Figure 1A shows the inhibition of IgE binding to the *C. diffluens* antigen at different concentrations of the fluid-phase antigens from *C. liquefaciens*, *M. restricta* and *M. globosa*. The fluid-phase antigens from *C. liquefaciens* markedly inhibited IgE binding to solid-phase *C. diffluens* antigens in a concentration-dependent manner (95% inhibition was observed at the highest concentra-

tion). In contrast, the IgE binding to the *C. diffluens* antigen was hardly inhibited by the fluid-phase antigens from *M. restricta* and *M. globosa* (20–40% inhibition was observed at the highest concentration). Almost identical results were obtained with the *C. liquefaciens* antigen (Fig. 1B)—the fluid-phase antigen of *C. diffluens* remarkably inhibited IgE binding to the solid-phase antigen of *C. liquefaciens*, whereas the fluid-phase *M. restricta* and *M. globosa* antigens did not. These results demonstrated that *C. diffluens* and *C. liquefaciens* shared common antigens, but the *Cryptococcus* species did not share antigens with *Malassezia* species.

IgE Immunoblotting Patterns

To identify IgE-binding proteins, crude extracts from *C. diffluens*, *C. liquefaciens*, and *M. restricta* were analyzed using SDS-PAGE and Western blotting (Fig. 2). The patterns of the IgE-binding bands for the *C. diffluens* and *C. liquefaciens* extracts exhibited certain similarities in that IgE-binding bands with apparent molecular masses of 77, 54, and 30 kDa were recognized for both extracts (Fig. 2A and B). In contrast, the pattern of the IgE-binding bands obtained for the *M. restricta* extract was different from those of the IgE-binding bands obtained for the *Cryptococcus* species (Fig. 2C).

Discussion

C. albidus has been reclassified into 12 species, including *C. diffluens* and *C. liquefaciens*, based on the current taxonomic criteria (3). Our recent study has demonstrated that the skin of AD patients is highly colonized by *C. diffluens* and *C. liquefaciens* (15). This finding suggests that *C. diffluens* and *C. liquefaciens* in AD lesions may play a role in the pathogenesis of AD. Although the level of specific IgE against *C. albidus* in AD patients has previously been investigated, the specific IgE responses to *C. diffluens* and *C. liquefaciens* in AD patients remain uncertain. In the present study, we used ELISA to evaluate the levels of serum IgE against *C. diffluens* and *C. liquefaciens* in AD patients. We observed that 35% of the AD patients had specific IgE against *C. diffluens* and 40%, against *C. liquefaciens*.

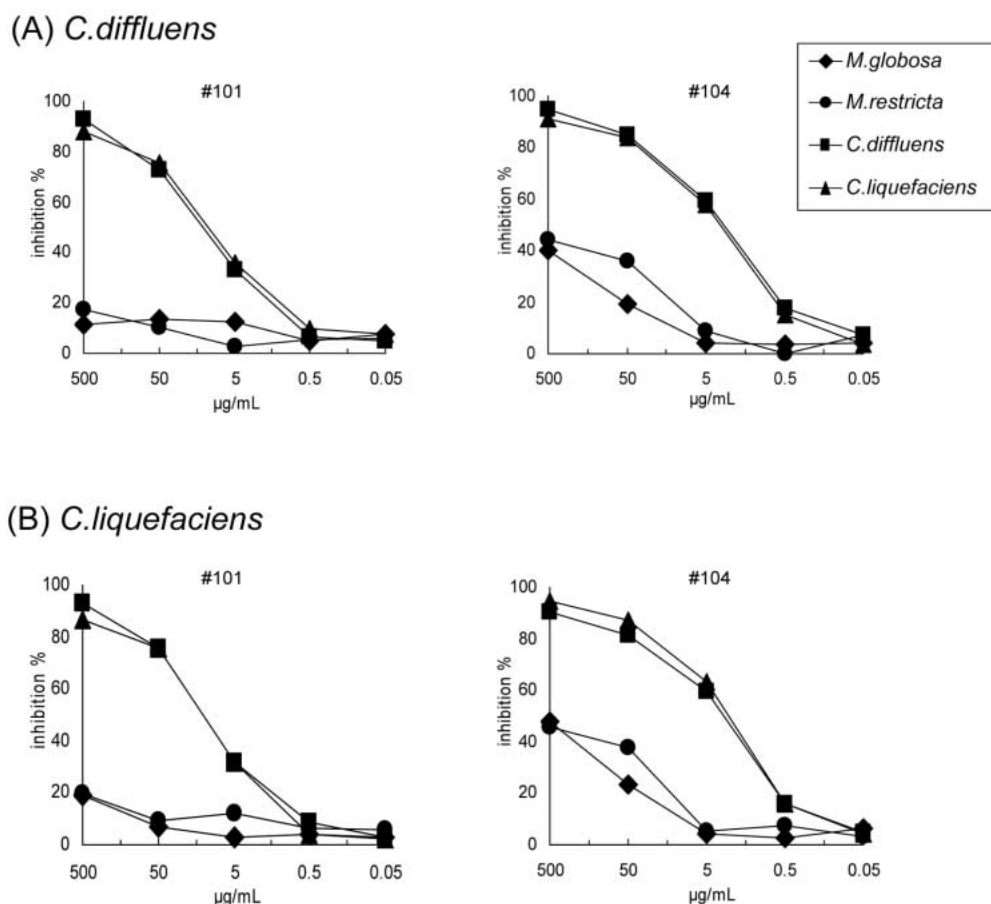


Fig. 1. Inhibition of IgE binding to solid-phase antigens from (A) *C. diffluens* and (B) *C. liquefaciens* by using fluid-phase inhibitory antigens of *Cryptococcus* species and *M. restricta*.

Further, none of the healthy subjects showed remarkable levels of IgE antibodies to these *Cryptococcus* species. These results indicate that components from *C. diffluens* and *C. liquefaciens* could act as allergens and provoke immediate-type hypersensitivity responses.

Competitive ELISA inhibition tests revealed that *C. diffluens* shared common antigens with *C. liquefaciens*. Further, we demonstrated that *M. restricta* did not share common antigens with *C. diffluens* and *C. liquefaciens*. Consistent with this, Lintu et al. have previously demonstrated that the IgE binding to crude extracts of *C. albidus* is not inhibited by mannan from *Pityrosporum ovale* (*Malassezia furfur*) (10). In this study, IgE immunoblotting demonstrated that several IgE-binding proteins with molecular masses of 77, 54, and 30 kDa were detected in both *C. diffluens* and *C. liquefaciens* antigens. Ikeda et al. have identified one of the *C. albidus* antigens as laccase (6), an enzymatic protein with a molecular mass of 75–83 kDa (21). Further, Savolainen et al. have demonstrated weak IgE binding to a 46-kDa band of *C. albidus* antigen (14). However,

in our assay system, we were unable to detect this 46-kDa band. This discrepancy may be due to the difference in the classification of *C. albidus*.

In conclusion, we demonstrated that fungal components from *C. diffluens* and *C. liquefaciens* could evoke IgE responses in AD patients and that *C. diffluens* and *C. liquefaciens* contained common antigens. This suggests that AD patients primarily sensitized by either *C. diffluens* or *C. liquefaciens* may develop allergic symptoms on exposure to the other fungus due to cross-reacting IgE antibodies. Further studies are required to characterize the nature of these cryptococcal allergens to enable their diagnostic usage. To elucidate the role of cryptococci in AD, it is also required that further study be carried out, including the localization of fungal antigens in involved but not uninvolved sites, the measurement of specific serum IgG, and interleukin 4 induction by fungal antigens. Moreover, this is necessary for the effectiveness of antifungal therapy in patients with AD.

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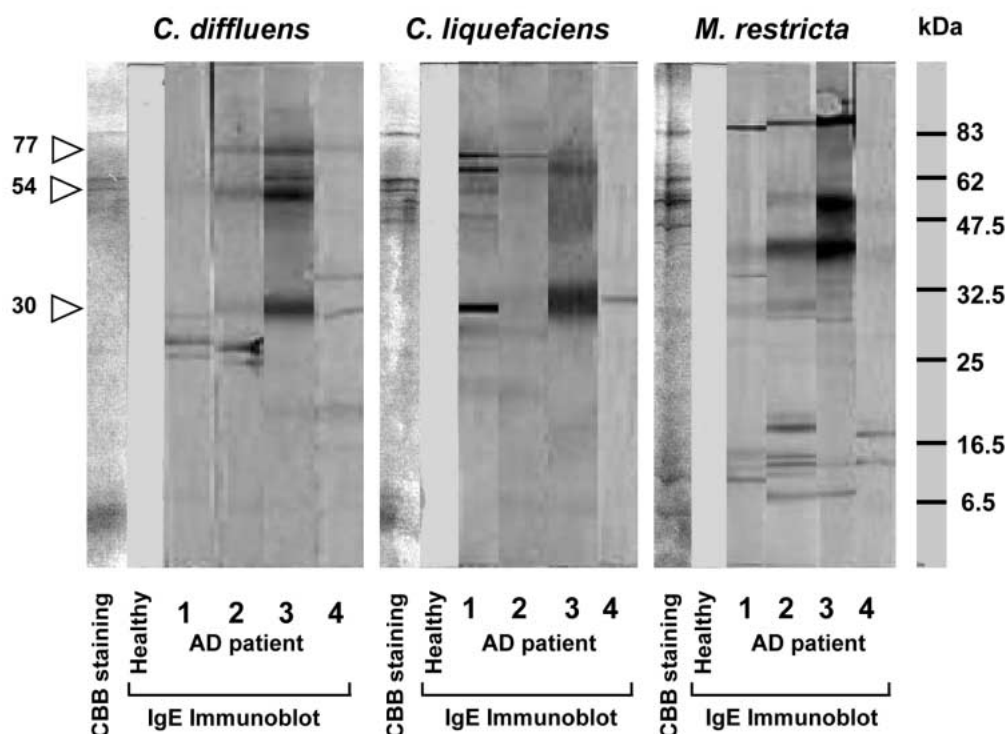


Fig. 2. IgE immunoblotting analysis of *Cryptococcus* and *Malassezia* extracts. Binding of serum IgE (4 serum samples from AD patients) to crude extracts of (A) *C. diffluens*, (B) *C. liquefaciens*, and (C) *M. restricta* are shown. The molecular mass markers are indicated.

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