

CLINICAL REPORT

Serum IgE Reactivity to *Malassezia furfur* Extract and Recombinant *M. furfur* Allergens in Patients with Atopic Dermatitis

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IgE reactivity to the opportunistic yeast *Malassezia furfur* can be found in patients with atopic dermatitis (AD). We have previously cloned and expressed 6 recombinant allergens (rMal f 1, rMal f 5–9) from *M. furfur*. In the present study, we used ImmunoCAP to investigate whether these rMal f allergens can be useful in the diagnosis of *M. furfur*-associated AD compared with the *M. furfur* extract. A total of 156 adult patients with a clinical diagnosis of AD participated in the study. Sixty-four percent had increased total serum IgE levels, 79% had specific IgE antibodies to common inhalant allergens and 47% had IgE antibodies to *M. furfur* extract. IgE antibodies to any of the rMal f allergens were detected among 86 (55%) of the patients, 14 (16%) of whom did not react to the *M. furfur* extract. Any individual rMal f allergen detected between 32% and 89% of the patients ImmunoCAP-positive to the *M. furfur* extract, with the highest sensitivity for rMal f 9. Therefore, a couple of individual rMal f allergens can improve the diagnosis of *M. furfur*-associated IgE allergies in patients with AD. **Key words: head and neck dermatitis; ImmunoCAP; Phadiatop; recombinant allergen.**

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Atopic dermatitis (AD) is a chronic inflammatory skin disease with a wide variety of clinical manifestations. A subtype is combined with IgE-associated skin reactions to environmental allergens often together with elevated total serum IgE (1) and therefore deserving the term “atopic” (2). Atopy, as defined by Pepys (2), describes a familiar tendency to develop an IgE-sensitization to common inhalant allergens. This definition of atopy is useful for asthma and rhinoconjunctivitis, but for AD additional common allergens, such as food allergens and opportunistic yeasts, have to be considered. Therefore, the European Academy of Allergology and Clinical Immunology (EAACI), based on the proposal of its Nomenclature Task Force, with representatives of the EAACI Dermatology Section, has decided to recommend the term “Atopic Eczema/Dermatitis Syndrome (AEDS)” for AD (3).

In early infancy, AD starts on the scalp and face; however, head and neck dermatitis is mainly seen in adolescents and young adults (4). The lipophilic yeast *Malassezia*, previously denoted *Pityrosporum*, colonizes preferentially the sebum-rich

head and neck skin area of most healthy individuals. *Malassezia* is normally non-pathogenic, but under predisposing conditions it may act as a pathogen (5). Up to 65% of adult patients with AD have specific IgE reactivity to *Malassezia* yeasts (6–8). Furthermore, *M. furfur* extract induces higher T-cell responses in patients with AD than in healthy controls (9) and positive skin prick or patch-test reactions to *Malassezia* have been found in patients with AD (10–12). Treatment with ketoconazole can improve the eczema and decrease the specific IgE antibody and total serum IgE levels (13, 14).

Malassezia extract contains a wide range of IgE-binding proteins (15–18). However, the extracts vary in allergenic contents (19, 20) and allergens can denature during and after extraction. Moreover, some allergens exist in minute amounts in extracts, whereas others are hard to isolate. Many attempts have therefore been dedicated to the production of pure recombinant allergens to overcome this problem. So far, the cDNAs encoding 9 *M. furfur* allergens have been cloned (21–25), and recombinant allergens produced (24–26). We have previously found that rMal f 1 exhibits similar immunological properties as its natural counterpart and might be used for successful *in vitro* diagnosis of type I allergy in patients with AD along with other recombinant allergens from this yeast (26). Mal f 5–9 have been cloned from a *M. furfur* cDNA library (24, 25). Mal f 5 has sequence similarity to Mal f 2, Mal f 3 and to Asp f 3 allergen of *Aspergillus fumigatus* (24). Mal f 6 has significant homology to cyclophilin from *Schizosaccharomyces pombe* (24), whereas Mal f 1 and Mal f 7–9 are novel proteins that do not show homology with any known protein (21, 25).

In the present study, the specific serum IgE reactivity to *M. furfur* extract was compared with that of rMal f 1, and rMal f 5 to 9 in patients with AD. The aim was to evaluate the use of recombinant allergens for diagnostic purposes and possible identification of subgroups of AD.

MATERIAL AND METHODS

Patients

After informed consent, serum samples were obtained from 156 adult patients with AD diagnosed according to the clinical criteria of Hanifin & Rajka (27) (Table I). The patients had mild, moderate, or severe dermatitis as scored on the scale proposed by Rajka & Langeland (28). Sixty-three percent of the patients had head and neck distribution of their eczema, and 64% and 44% had past or present history of rhinoconjunctivitis and/or asthma, respectively (Table I). The study was approved by the Ethics Committees at the Karolinska Hospital and Lund University Hospital.

Table I. Characterization of patients with atopic dermatitis (AD)

	<i>n</i>	Age years median (range)	Gender F/M	Head and neck distribution <i>n</i>	Past or present R and/or A <i>n</i>	Total serum IgE (kU/l)‡ median (range)	Elevated serum IgE* <i>n</i>	<i>M. furfur</i> specific IgE‡ (> 0.35 kU/l) <i>n</i>
AD patients	156	26 (18–81)	102/54	98	100/68	343 (2–22100)	100	74
Phadiatop‡ positive	123	26 (18–81)	76/47	88	95/64	516 (17–22100)	93	72
Phadiatop‡ negative	33	23 (18–56)	26/7	10	5/4	34 (2–1314)	7	2

‡Pharmacia CAP System™, Pharmacia Diagnostics AB, Uppsala, Sweden.

*Reference range: 1.3–263 kU/l for 5–20 year old individuals and 1.6–122 kU/l for adults.

‡Serum IgE reactivity to common aeroallergens.

F: Female; M: Male; R: rhinoconjunctivitis; A: asthma.

Preparation of *M. furfur* extract and recombinant Mal f allergens

Whole extract was prepared from a 4-day-old culture of the yeast *M. furfur* (strain no. 42132, American Type Culture Collection) on glycerol monostearate/olive oil medium at 37°C, as previously described (16). The cells were freeze-dried, re-suspended in phosphate-buffered saline, sonicated and centrifuged (16). The supernatant was filtered through a 0.8 µm filter (Advantec MFS, Inc., Pleasanton, CA). The protein concentration of the extract was measured by BCA Protein assay Reagent (Pierce Chemical Company, Rockford, IL) in accordance with the manufacturer's instructions.

Recombinant *M. furfur* allergens, rMal f 1 and rMal f 5–9) were expressed as His6-tagged proteins in *E. coli* as described previously (24–26). The rMal f 5 was recovered mainly from soluble fractions of the *E. coli* lysates, whereas rMal f 1 and rMal f 6 to 9 were located in inclusion bodies, which had to be solubilized before purification. The recombinant proteins were purified by Talon metal affinity resin (Clontech, Palo Alto, CA) (24–26). Purity of the recombinants was confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

IgE reactivity

Serum samples were analyzed for total serum IgE levels and IgE antibodies specific to 11 common aeroallergens (Phadiatop: Cladosporium, *Dermatophagoides farinae*, *D. pteronyssinus*, cat, dog, horse, birch, timothy grass, mugwort, olive and *Parietaria judaica*) by the CAP System™ IgE FEIA and Phadiatop®, respectively (Pharmacia Diagnostics AB, Uppsala, Sweden). Extract of *M. furfur* (strain no. 42132 from ATCC) and the rMal f allergens were covalently coupled to the cellulose solid phase (ImmunoCAP) (Pharmacia Diagnostics AB) and IgE-reactivity measured in the Pharmacia CAP System in accordance with the manufacturer's instructions. A positive ImmunoCAP result was defined as a value of at least 0.35 kU/l. Serum samples with IgE antibody reactivity to the recombinants, but not to the *M. furfur* extract, were retested with ImmunoCAP and checked in immunoblotting. Briefly, proteins of *M. furfur* were separated on gradient 7–20% SDS gels in reduced condition by electrophoresis and blotted onto polyvinylidene fluoride membranes (Millipore, Bedford, MA). The membranes were cut into strips and incubated with sera. Blotting and detection of IgE-binding proteins were performed as previously described (16, 26). Two sera with IgE antibodies specific to *M. furfur* extract and ImmunoCAP-positive to all the rMal f allergens were used as positive controls. For negative control, a strip of membrane was incubated in sodium phosphate buffer containing 0.2% bovine serum albumin and 0.1% Tween 20.

Statistical analysis

Differences between groups were evaluated with the Mann-Whitney U-test. Correlations were calculated with Spearman rank correlation analysis. The results are considered statistically significant at $p < 0.05$.

RESULTS

Among the 156 individuals with a clinical diagnosis of AD, 64% had an elevated total serum IgE, 79% had a positive Phadiatop test and 47% had IgE antibodies to the *M. furfur* extract (Table I). The total serum IgE levels were significantly higher ($p < 0.001$) in the Phadiatop-positive patients ($n = 123$) compared to the Phadiatop negative ones. Patients with head and neck dermatitis ($n = 98$) had higher levels of total serum IgE ($p < 0.001$) than those without head and neck dermatitis. A significant correlation ($r_s = 0.82$, $p < 0.001$, $n = 156$) was found between the levels of total serum IgE and IgE specific to the *M. furfur* extract.

The occurrence of specific IgE to the rMal f allergens is given in Table II. Out of the 156 patients, 86 (55%) had positive ImmunoCAP to either of the rMal f allergens. Fourteen patients (17%) negative in ImmunoCAP to *M. furfur* extract were positive to either of the rMal f allergens and 8 of

Table II. Results of ImmunoCAP to *M. furfur* whole extract and rMal f allergens for 156 patients with atopic dermatitis

No. of patients ImmunoCAP +/– to <i>M. furfur</i> extract (class)	No. and (%) of patients ImmunoCAP-positive to									
	rMal f 1	rMal f 5	rMal f 6	rMal f 7	rMal f 8	rMal f 9	rMal f 1, 5–9			
23 (≥4)	20 (87)	13 (57)	21 (91)	13 (57)	21 (91)	23 (100)	23 (100)			
34 (3)	20 (59)	9 (26)	25 (74)	11 (32)	23 (68)	29 (85)	33 (97)			
17 (1&2)	3 (18)	2 (12)	5 (29)	0 (0)	5 (29)	14 (82)	16 (94)			
Total 74 (1–6)	43 (58)	24 (32)	51 (69)	24 (32)	49 (66)	66 (89)	72 (97)			
82 (0)	1 (1)	0 (0)	4 (5)	0 (0)	8 (10)	2 (2)	14 (17)			
Totals 156	44 (28)	24 (15)	55 (35)	24 (15)	57 (37)	68 (44)	86 (55)			

*The cut-offs for each ImmunoCAP class ≥4, 3, 2 and 1 are ≥17.5, 3.5, 0.7 and 0.35 kU/l, respectively.

these had IgE specific to rMal f 8 (Table II). Only 9 sera, all with an ImmunoCAP class 3 or above (≥3.5 kU/l) to the *M. furfur* extract and total serum IgE above 844 kU/l, were positive to all rMal f allergens. Two sera with IgE specific to the *M. furfur* extract (ImmunoCAP value 0.8 and 8.8 kU/l) did not have detectable IgE antibodies to any of the recombinant Mal f allergens (data not shown).

The number of patients with positive Phadiatop and IgE specific to different Mal f allergens is given in Table III. Among the 33 patients in the Phadiatop negative group, 4 patients had IgE specific to either rMal f 7 (ImmunoCAP class 4), rMal f 8 (ImmunoCAP class 3), rMal f 9 (ImmunoCAP class 1 and 2), or *M. furfur* extract (ImmunoCAP class 2 and 3) (Table III). Ninety-eight (63%) patients out of the total 156 patients with AD had head and neck dermatitis (Tables I and III), and 66 (67%) of those had a positive Phadiatop and IgE antibody to *M. furfur*. Fifteen patients (20%) with IgE specific to *M. furfur* extract had no eczema in their head and neck area.

All 14 sera ImmunoCAP-negative to *M. furfur* extract, but ImmunoCAP-positive to any of the rMal f allergens, were tested in immunoblotting with *M. furfur* extract. Ten sera (Fig. 1, nos. 1–6, 8, 10, 11 and 14) all with an ImmunoCAP value above 0.7 kU/l showed bands in immunoblotting, indicating that for some patients insufficient amounts of the allergens are present in the *M. furfur* extract-ImmunoCAP. Four sera (Fig. 1, nos. 7, 9, 12, 13) with ImmunoCAP values less than 0.7 kU/l showed no bands in immunoblotting, suggesting that the immunoblotting did not detect low levels of IgE antibodies to *M. furfur*.

DISCUSSION

M. furfur extracts contain a wide range of IgE-binding proteins (15–18), but variations in allergenic content and difficulties in standardization of the extract (20) made us produce recombinant allergens. In this study, we have coupled the rMal f allergens to ImmunoCAP to screen for specific IgE antibodies in adult patients with AD. Out of 156 patients, 74 (47%) had IgE antibodies specific to *M. furfur* extract and 86 (55%) to either of 6 rMal f allergens.

Mal f 1 has previously proved to be a major allergen using a large panel of sera (26). In the present study, Mal f 1, Mal f 6, Mal f 8 and Mal f 9 all fulfill the criteria of major allergens, as each recombinant allergen was recognized by more than 50% of the sera ImmunoCAP-positive to *M. furfur* whole extract. Our results further show that the sensitivity of rMal f 9 to distinguish the patients with IgE to *M. furfur* is 89% of that of the whole extract. Recombinant Mal f 9 is able to distinguish 53% of the patients with a positive Phadiatop, and 61% of the patients with head and neck dermatitis. These results could suggest that rMal f 9 is the most important allergen of those studied and a good candidate to be used in diagnosis.

In a previous study, the sensitivity of rMal f 9 was 36% in the ImmunoCAP assay. However, only 25 sera RAST positive to *M. furfur* were investigated (25). Other studies using the ImmunoCAP assay to compare rLep d 2 and rTyr p 2 (recombinant allergens from the mites, *Lepidoglyphus destructor* and *Tyrophagus putrescentiae*), or rAsp f I (recombinant allergen from *Aspergillus fumigatus*) with corresponding whole extracts obtained a sensitivity of the recombinant allergen in the range 60–73% (29, 30). For diagnosis of allergy to

Table III. Number of patients positive in ImmunoCAP to *M. furfur* whole extract and rMal f allergens in relation to Phadiatop reactivity and presence of head and neck (H&N) dermatitis

Patient characteristics	No. of patients	No. and (%) of the AD patients with specific IgE to									
		<i>M. furfur</i> extract	rMal f 1	rMal f 5	rMal f 6	rMal f 7	rMal f 8	rMal f 9	rMal f 1, 5-9		
Phadiatop* positive	123	72 (59)	44 (36)	24 (20)	55 (45)	23 (19)	55 (45)	65 (53)	82 (67)		
Phadiatop* negative	33	2 (6)	0 (0)	0 (0)	0 (0)	1 (3)	2 (6)	3 (9)	4 (12)		
H&N dermatitis	98	68 (69)	41 (42)	24 (24)	51 (52)	23 (23)	51 (52)	60 (61)	71 (72)		

*IgE reactivity to common aeroallergens detected by Pharmacia CAP System™, Pharmacia Diagnostics AB, Uppsala, Sweden.

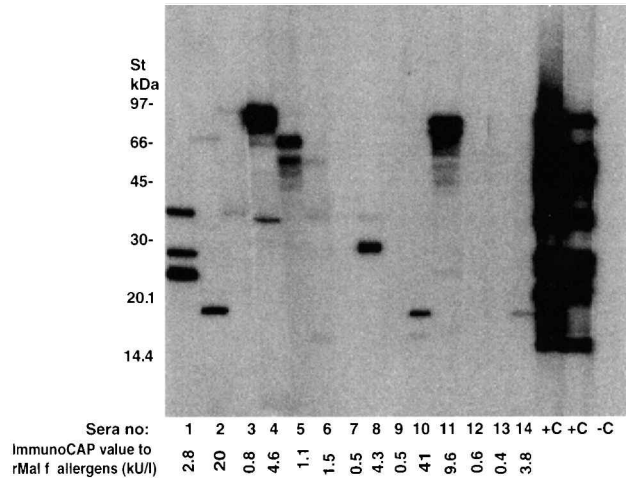


Fig. 1. Immunoblot analysis of *Malassezia furfur* extract using sera from patients with atopic dermatitis positive in ImmunoCAP to the rMal f allergens but negative to *M. furfur* extract. ImmunoCAP values to the rMal f allergens for each of 14 sera negative in ImmunoCAP to the *M. furfur* extract are shown. Sera from 2 patients ImmunoCAP-positive to the *M. furfur* extract were used as positive controls (+C). For negative control (-C) a strip of the membrane incubated with phosphate buffer instead of serum was used. Molecular mass markers are indicated on the left.

A. fumigatus (30), several recombinant allergens must be used, as this fungi can produce more than 40 allergens (30, 31). In contrast, only a few recombinant birch pollen allergens (rBet v 1, rBet v 2) seem to be sufficient to diagnose birch pollen allergy (32, 33). *M. furfur* contains many allergens and one could suspect that a high number of recombinant allergens is needed to achieve high diagnostic sensitivity. In this study, we obtained 97% sensitivity by using 6 recombinant allergens (Table II).

Fourteen sera, which were ImmunoCAP-negative to *M. furfur* extract, were ImmunoCAP-positive to rMal f allergens (Table II). Ten of these sera with ImmunoCAP values above 0.7 kU/l showed bands with *M. furfur* extract in immunoblotting (Fig. 1). It makes the pure rMal f allergens a useful tool for diagnosis of IgE-associated AD to *M. furfur*. Four Phadiatop-negative patients, two of them with normal serum IgE levels, were found to have IgE reactivity to *Malassezia* allergens (data not shown). Thus, by using Mal f allergens, additional patients could be identified as having IgE-associated AD.

In conclusion, a couple of individual or a mixture of rMal f allergens can serve to diagnose IgE reactivity to *M. furfur*, giving a better discrimination of patients with an IgE-associated allergy than the *M. furfur* extracts used so far. It remains to be seen if the recombinant allergens are effective in *in vivo* testing.

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