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Clinical and Laboratory Investigations

Atopy patch test reactions to *Malassezia* allergens differentiate subgroups of atopic dermatitis patients

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Summary

Background The yeast *Malassezia* is considered to be one of the factors that can contribute to atopic dermatitis (AD).

Objectives To investigate the reactivity to *Malassezia* allergens, measured as specific serum IgE, positive skin prick test and positive atopy patch test (APT), in adult patients with AD.

Methods In total, 132 adult patients with AD, 14 with seborrhoeic dermatitis (SD) and 33 healthy controls were investigated for their reactions to *M. sympodialis* extract and three recombinant *Malassezia* allergens (rMal s 1, rMal s 5 and rMal s 6).

Results Sixty-seven per cent of the AD patients, but only one of the SD patients and none of the healthy controls, showed a positive reaction to at least one of the *Malassezia* allergens (extract and/or recombinant allergens) in at least one of the tests. The levels of *M. sympodialis*-specific IgE in serum correlated with the total serum IgE levels. Elevated serum levels of *M. sympodialis*-specific IgE were found in 55% and positive APT reactions in 41% of the AD patients with head and neck dermatitis. A relatively high proportion of patients without head and neck dermatitis and patients with low total serum IgE levels had a positive APT for *M. sympodialis*, despite lower proportions of individuals with *M. sympodialis*-specific IgE among these groups of patients.

Conclusions These results support that *Malassezia* can play a role in eliciting and maintaining eczema in patients with AD. The addition of an APT to the test battery used in this study reveals a previously overlooked impact of *Malassezia* hypersensitivity in certain subgroups of AD patients.

Key words: atopic dermatitis, atopy patch test, *Malassezia*, *Pityrosporum*, recombinant allergens, skin prick test

Atopic dermatitis (AD) is a common inflammatory skin disease characterized by a chronically relapsing course, a distinctive clinical appearance and severe pruritus. Most patients with AD have elevated serum IgE and positive skin prick test (SPT) reactions to a wide variety of allergens. However, approximately

20% of patients with the clinical phenotype of AD have low serum IgE levels and a lack of detectable environmental allergen-specific serum IgE or positive SPT reactions.^{1,2}

IgE antibodies to the opportunistic yeast *Malassezia*, previously also denoted *Pityrosporum*, are often found in patients with AD,^{3–9} but only occasionally in patients with atopic respiratory diseases without AD,^{5,7} and are not reported in healthy controls^{4,6–8} or patients with seborrhoeic dermatitis (SD).^{4,8} Several

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IgE-binding components have been found in *Malassezia*.^{10,11} The genes for nine *Malassezia* allergens, Mal 1–9, have been identified and cloned.^{12–16} Elevated proliferation of peripheral blood mononuclear cells (PBMC) in response to *in vitro* stimulation with *Malassezia* extract has been found in AD patients with specific serum IgE to *Malassezia*,^{6,17–19} in combination with a T-helper (Th)2-like cytokine profile.^{18,19} However, this elevated proliferation of PBMC to *Malassezia* is not seen in patients with SD.²⁰ Treatment with ketoconazole has been shown to improve the eczema^{21–23} and to decrease the levels of *Malassezia*-specific IgE and total serum IgE in patients with AD.²³ Furthermore, patients with AD have been reported to react positively to the *Malassezia* atopy patch test (APT).^{4,6,8}

The APT reaction, an eczematous skin reaction induced by application of aeroallergens on non-lesional skin of patients with AD, was first described in 1982 by Mitchell *et al.*²⁴ and has thereafter been evaluated in many studies.^{25–27} Although several attempts at standardization have been made,^{28,29} there is still no consensus on how to perform the APT. The APT is frequently used for research purposes but is still not commonly used as a diagnostic tool despite clinical and microscopic similarities between the APT reaction and an acute AD lesion.

The aims of this study were to investigate further the occurrence of *Malassezia* allergy in adult patients with AD, the relationship between a positive APT reaction, specific IgE and clinical features in AD and to study the sensitization to different *Malassezia* allergens. We therefore measured specific serum IgE levels, and SPT and APT reactions to *M. sympodialis* extract and three recombinant *Malassezia* allergens (rMal s 1, rMal s 5 and rMal s 6). Our results support a role for *Malassezia* in eliciting and maintaining eczema in patients with AD. In addition, reactivity to *Malassezia* was found among patients with low total serum IgE levels and/or without head and neck eczema, indicating that *Malassezia* allergy is important also in these subgroups.

Materials and methods

Study design

Patients with AD or SD and healthy controls were recruited and investigated at three Swedish university hospitals between October 1999 and June 2000. The patients with AD and SD were referred to the hospital

or were selected from the diagnosis register. The healthy controls were mainly recruited among staff and medical students.

Inclusion criteria for the AD patients were diagnosis according to the U.K. working party criteria³⁰ and skin lesions not only restricted to the hands. Exclusion criteria for the participants in the study were skin diseases other than those being investigated, autoimmune diseases, immune deficiencies, malignant diseases, pregnancy or lactation, immunosuppressive treatment, and age below 18 or above 55 years. The healthy controls and patients with SD had no clinical symptoms or history of allergy and the healthy controls had no symptoms or history of skin diseases. Use of systemic glucocorticoids, systemic antifungal treatment or ultraviolet therapy was not allowed for 2 months before the investigation. Topical antifungal treatment was not permitted for 1 month before and topical corticosteroids were not allowed on the test sites for 1 week before the study. Antihistamines were withdrawn 5 days before the investigation. At the first visit subjects were interviewed about their medical history, the severity of their eczema was assessed using SCORAD (severity scoring of AD),³¹ and a blood sample was drawn.

Five of the originally recruited patients with AD were excluded from the study: three did not return for the second visit, and two could not be evaluated (one due to irritant reactions to the tape and one due to technical problems with the test). One healthy control was excluded because the SPT could not be evaluated due to dermographism. Two patients with AD from whom no blood sample could be obtained but who participated in the SPT and APT were included in the study. All participants gave their informed consent. The study was approved by the Regional Ethics Committee.

Subjects

In total, 132 patients with mild to severe AD, 14 with SD and 33 healthy controls were included in the study (Table 1). The 98 (74%) AD patients with head and neck dermatitis had significantly higher total serum IgE ($P < 0.01$) than the 33 AD patients without head and neck dermatitis. However, there were no statistically significant differences in SCORAD, number of patients with rhinoconjunctivitis, asthma or positive Phadia-top[®] (Pharmacia Diagnostics AB, Uppsala, Sweden) between the patients with and without head and neck dermatitis.

Table 1. Characterization of the subjects

	<i>n</i>	Gender F/M (<i>n</i>)	Age (years)	SCORAD ^a	Head and neck AD (%)	Rhino- conjunctivitis (%)	Asthma (%)	Rhino- conjunctivitis and/or asthma (%)	Total serum IgE ^b (kU L ⁻¹)	Total serum IgE ^b > 122 kU L ⁻¹ (%)	Phadiatop [®] positive ^c (%)
AD patients	132	83/49	31 ^d (18–55) ^e	37 ^d (8–86) ^e	74 ^f	72	50	79	310 ^{d,g} (2–23 800) ^e	69 ^g	78 ^g
SD patients	14	7/7	33 (18–55)	NA	NA	0	0	0	9 (2–45)	0	7
Healthy controls	33	29/4	38 (21–55)	NA	NA	0	0	0	25 (4–255)	18	24

AD, atopic dermatitis; SD, seborrhoeic dermatitis; NA, not applicable. ^aAssessed by SCORAD.³¹ ^bImmunoCAPTM (Pharmacia Diagnostics AB), reference range 1.6–122 kU L⁻¹. ^cPhadiatop[®] (Pharmacia Diagnostics AB), serum IgE to any of 11 common aeroallergens. ^dMedian. ^eRange. ^fRecorded in all but one AD patient. ^gInvestigated in 130 AD patients.

Malassezia sympodialis extract and recombinant allergens

Crude yeast extract was prepared from strain no. 42132, American Type Culture Collection (ATCC), as previously described.¹⁰ This strain was earlier denoted *P. orbiculare* or *M. furfur*, but recent retyping has shown it to be *M. sympodialis*.³² Recombinant *Malassezia* allergens rMal s 1, rMal s 5 and rMal s 6 (corresponding to allergens expressed naturally in ATCC strain no. 42132)^{12,15} and a recombinant control allergen (rAca s 13, a minor allergen from the dust mite *Acarus siro*)³³ were produced in the *Escherichia coli* system as described previously.^{12,15,33}

For each allergen (extract and various recombinants), one single batch was prepared and used for the SPT and APT in the three test centres throughout the whole study. The 'rMal s mix' preparation consisted of one-third each of rMal s 1, rMal s 5 and rMal s 6, giving a final total protein concentration of 100 µg mL⁻¹ (for SPT) and 4 mg mL⁻¹ (for APT), equal to the protein concentrations used for the single recombinant allergen tests. In the SPT concentration (100 µg mL⁻¹ protein), the test batches had a nucleic acid content of less than 0.6 ng µL⁻¹ (DNA dipstickTM, Invitrogen, San Diego, CA, U.S.A.) and an endotoxin content of less than 62.5 EU mL⁻¹ (Limulus test, performed by Apoteket AB, Stockholm, Sweden). BCA Protein Assay Reagent (Pierce Chemical Company, Rockford, IL, U.S.A.) was used for determining the protein concentration of the extract, whereas the protein concentrations in the recombinant allergen preparations were estimated by spectrophotometric absorbance at 280 nm. Protein purity was checked with sodium dodecyl sulphate–polyacrylamide gel electrophoresis. No significant changes, for extract or recombinant

proteins, were found in repeated measurements of concentration and purity after the study.

Skin prick test and atopy patch test

The SPT was performed in duplicate on the forearms³⁴ by an experienced nurse. The protein concentration of the allergens was 100 µg mL⁻¹. Histamine dihydrochloride (10 mg mL⁻¹; ALK, Hørsholm, Denmark) was used as a positive control and phosphate-buffered saline (PBS) as a negative control. The tests were evaluated after 15 min and the reaction was graded as mean diameter (mm) of the weal. A reaction with a mean diameter of 3 mm or more was considered as positive.

The APT was performed on non-lesional skin of the back, as previously described.⁸ The skin was tape stripped 15 times with TransporeTM tape (3M, Sollen-tuna, Sweden). The allergens (20 µL) were applied on paper discs in Finn chambers (8 mm; Epitest Ltd, Tuusula, Finland) under coded conditions (individually randomized for each subject). The recombinant allergens were tested at a concentration of 4 mg mL⁻¹ and the *M. sympodialis* extract in a twofold serial dilution from 5 to 0.6 mg mL⁻¹. PBS was used as a negative control. The patch tests were removed after 48 h and a physician evaluated the skin reactions, still under coded conditions. The test results were scored from 0 to 3+, where 0 = negative reaction; 1+ = erythema, infiltration, possibly papules; 2+ = erythema, infiltration, papules and small vesicles; and 3+ = erythema, infiltration, papules and large vesicles.^{8,35}

Specific serum IgE analysis

Specific serum IgE for *M. sympodialis* was analysed with ImmunoCAPTM (m70, Pharmacia Diagnostics AB;

prepared from yeast extract according to Zargari *et al.*;¹⁰ ATCC strain no. 42132). In this paper, the short commercial designation 'm70' is used. For analysis of specific serum IgE to the recombinant *Malassezia* allergens rMal s 1, rMal s 5 and rMal s 6, the allergens were immobilized on to cellulose solid phase (ImmunoCAPTM) by covalent binding (MIAB Uppsala, Sweden). The recombinant ImmunoCAPs were tested in the Pharmacia CAPTM system according to the manufacturer's instructions. A value of 0.35 kU L⁻¹ or more was considered as positive.

Statistical analysis

Differences between two groups were analysed with the Mann–Whitney *U*-test. Differences between more than two groups were first analysed with Kruskal–Wallis ANOVA by ranks, whereafter *post hoc* comparisons were made with the Mann–Whitney *U*-test. Correction for multiple comparisons was made using the Bonferroni method. The significance tests followed a two-sided alternative hypothesis. Correlations were calculated using Spearman rank correlation analysis. $P < 0.05$ was considered as statistically significant.

Results

Reactions to *Malassezia sympodialis* extract

Positive SPT reactions to *M. sympodialis* extract were found in 51% of the 132 patients with AD. Forty-five per cent of the 130 AD patients tested for m70 had specific serum IgE to *M. sympodialis* extract and 40% were m70+/SPT+ (Fig. 1a). A weak but statistically significant correlation was found between SPT reactions (mm) and m70 (kU L⁻¹) in the AD patients positive in one or both of those tests ($r_s = 0.27$, $P < 0.05$, $n = 74$). No positive SPT reactions or m70+ were found in the SD patients or healthy controls.

Positive APT reactions to *M. sympodialis* were found in 38% of the patients with AD (Fig. 1a), in one (7%) of the SD patients and in none of the healthy controls. Twenty-four per cent of the AD patients were m70+/SPT+/APT+ (Fig. 1a). In general, the seven m70-/SPT+/APT+ patients with AD had stronger APT reactions to *M. sympodialis* than the 12 SPT-/APT+ patients with AD (m70- or m70+). No correlation was found between the APT (0–3+) and SPT (mm) reactions to *M. sympodialis* extract or between m70 (kU L⁻¹) and

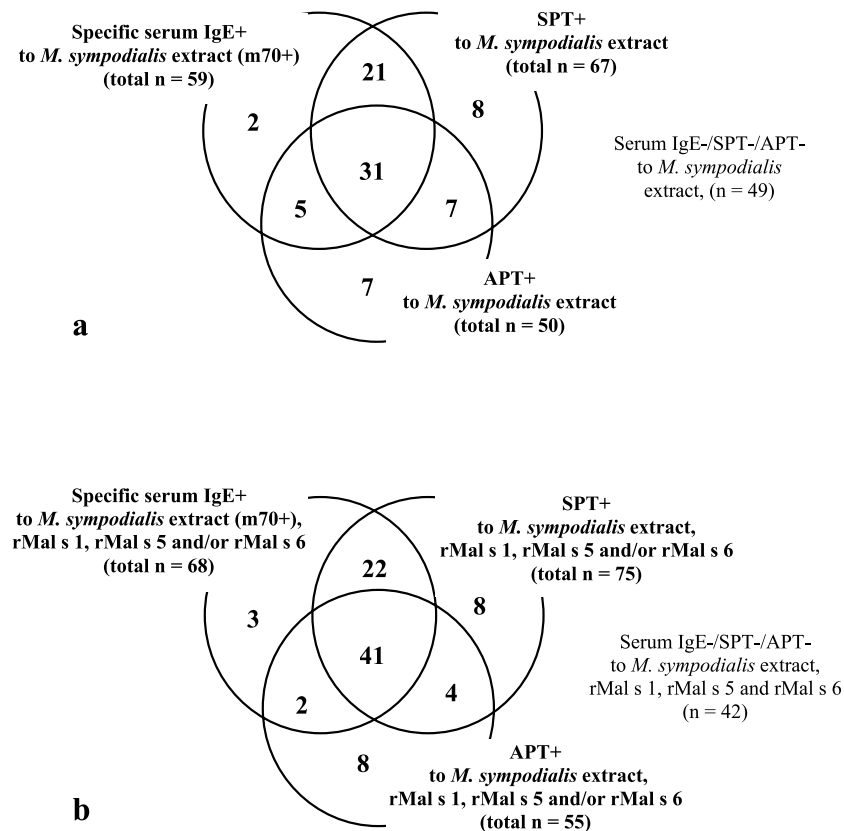


Figure 1. Concordance between specific serum IgE, skin prick test (SPT) and atopy patch test (APT) reactions to *Malassezia sympodialis* extract and recombinant allergens in 130 patients with atopic dermatitis (AD). Two patients who were not tested for specific serum IgE are not included in the figure. They were SPT-/APT- to *M. sympodialis* extract, rMal s 1, rMal s 5 and rMal s 6.

Table 2. Characteristics of patients with atopic dermatitis (AD) with different *in vivo* reactions to *Malassezia sympodialis* extract

	SPT+/APT+ (n = 38)	SPT+/APT- (n = 29)	SPT-/APT+ (n = 12)	SPT-/APT- (n = 53)
Eczema score ^a	44	36	41	27
median (range)	(22-86)	(8-59)	(24-82)	(8-66)
Head and neck dermatitis	84%	79%	67%	67% ^e
Rhinoconjunctivitis and/or asthma	84%	93%	75%	68%
Total serum IgE ^b > 122 kU L ⁻¹	87%	93%	67%	43% ^f
Total serum IgE ^b (kU L ⁻¹)	1800	800	580	73 ^f
median (range)	(16-12 600)	(79-6300)	(35-23 800)	(2-2807)
m70+ ^c > 0.35 kU L ⁻¹	82%	72%	42%	4% ^f
m70 ^c (kU L ⁻¹)	5.2	1.4	0	0 ^f
median (range)	(0-48.0)	(0-22.0)	(0-42.0)	(0-15.4)
APT reactivity ^d 3+/2+/1+	10/45/45%	-	0/8/92%	-

SPT, skin prick test; APT, atopy patch test. ^aAssessed by SCORAD.³¹ ^bImmunoCAPTM (Pharmacia Diagnostics AB), reference range 1.6-122 kU L⁻¹. ^cSerum IgE to *M. sympodialis* extract (m70), ImmunoCAPTM (Pharmacia Diagnostics AB), reference range <0.35 kU L⁻¹. ^dPercentage of individuals with 3+, 2+ or 1+ APT reactivity to *M. sympodialis* extract (the highest score, regardless of extract concentration, is given for each individual). ^eRecorded in all but one AD patient. ^fTested in all but two AD patients.

the APT reaction to *M. sympodialis* extract in the AD patients positive in one or both of the tests compared.

Reactions to Malassezia sympodialis extract in relation to total serum IgE levels and clinical features

Clinical features and serum IgE levels for the AD patients grouped according to their SPT/APT reactions to *M. sympodialis* extract are shown in Table 2. No differences in age or gender were seen between the groups. The pattern of positive reaction to Phadiatop[®] followed the pattern for elevated total serum IgE (data not shown). The m70 levels correlated with total serum IgE levels in the AD patients ($r_s = 0.76$, $P < 0.001$, $n = 130$). However, the ratio of m70 to total serum IgE also seemed to have an impact on the outcome of the *in vivo* tests: seven m70+ patients with

a low m70/total serum IgE ratio responded with a negative SPT reaction, while 14 patients with total serum IgE levels < 500 kU L⁻¹ and m70- responded with a positive SPT reaction (data not shown). Figure 2 shows a clear difference between the two groups of patients with conflicting results in their m70/SPT reactivity. The small group of m70+/SPT- patients

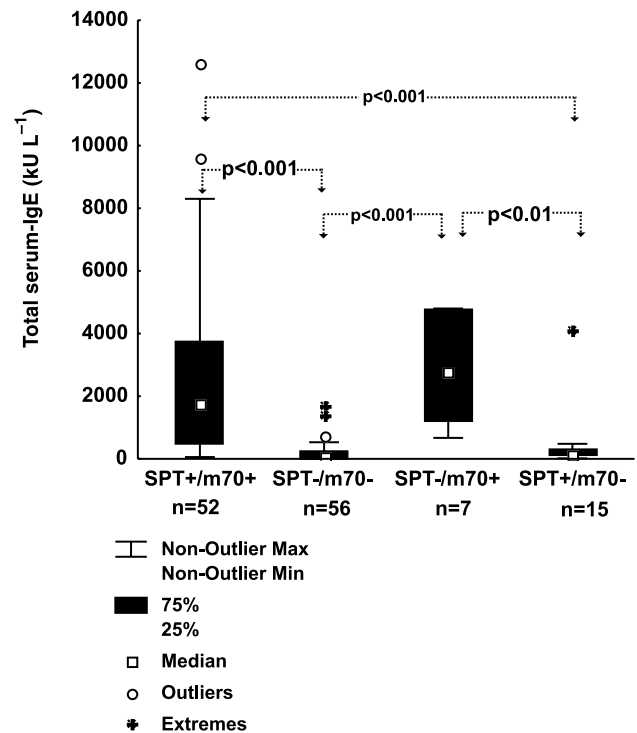


Figure 2. Total serum IgE levels in patients with atopic dermatitis (AD) with different patterns of m70 and skin prick test (SPT) reaction to *Malassezia sympodialis*. Total serum IgE levels (ImmunoCAPTM, Pharmacia Diagnostics AB; reference range 1.6-122 kU L⁻¹) compared between AD patients with m70+/SPT+, m70-/SPT-, m70+/SPT- and m70-/SPT+ reactivity to *M. sympodialis* extract. SPT reactions to *M. sympodialis* extract of ≥ 3 mm were regarded as positive (see Materials and methods). m70 = specific serum IgE to *M. sympodialis* extract (ImmunoCAPTM, Pharmacia Diagnostics AB; reference range <0.35 kU L⁻¹). Data were analysed by Kruskal-Wallis ANOVA by ranks ($P < 0.001$), *post hoc* comparisons made by the Mann-Whitney *U*-test corrected for multiple comparisons by the Bonferroni method. Arrows indicate statistically significant differences between pairs. One extreme total serum IgE value (23 800 kU L⁻¹) in the m70+/SPT- group is not shown in the figure.

(APT+, $n = 5$; APT-, $n = 2$; total, $n = 7$) did not differ significantly from the m70+/SPT+ group either in total serum IgE levels (Fig. 2) or in m70 levels ($P = 0.83$), but had a significantly lower m70/total serum IgE ratio ($P < 0.05$, data not shown). In contrast, the m70-/SPT+ patients (APT+, $n = 7$; APT-, $n = 8$; total, $n = 15$) had significantly lower total serum IgE than both the m70+/SPT+ and the m70+/SPT- patients (Fig. 2). Notably, seven of these patients had positive APT reactions (m70-/SPT+/APT+), five of them at 2+.

Although a higher proportion of positive APTs to *M. sympodialis* (41%) was found among patients with head and neck dermatitis, quite a high proportion of *M. sympodialis*-positive APTs (30%) was also found among patients without head and neck dermatitis, despite a lower proportion of individuals with m70+ among the latter (Table 3). The high proportion of the *M. sympodialis*-reactive AD patients with head and neck dermatitis (Table 2) can partly be explained by the fact that 74% of the investigated AD patients had head and neck dermatitis.

A high prevalence of both m70+ and APT+ reactions was found among the AD patients with very high

total serum IgE levels ($> 4000 \text{ kU L}^{-1}$, Table 3). In the AD patients with total serum IgE levels $\leq 500 \text{ kU L}^{-1}$ a lower prevalence of m70+ was found. Interestingly, the number of SPT+ and APT+ reactions to *M. sympodialis* in these patients was quite high. Twenty-three per cent of the AD patients with total serum IgE levels $\leq 122 \text{ kU L}^{-1}$ had APT+ reactions to *M. sympodialis* extract (Table 3).

A connection between SCORAD and *M. sympodialis* reactivity was seen (Table 3), in that a high reactivity frequency was found in the AD patients with the highest score and a low reactivity frequency among the patients with a low score (Table 3). However, in the majority of AD patients, with a SCORAD between 24 and 59 ($n = 92$), no correlation between SCORAD and reactivity to *M. sympodialis* was seen.

Comparisons between the reactions to *Malassezia sympodialis* extract and recombinant allergens

The use of rMal s 1, rMal s 5 and rMal s 6, tested as single allergens and evaluated together, gave a positive reaction in 73% of the patients with m70+, 70% with a

Subgroup	m70+ ^a (%)	SPT+ ^b (%)	APT+ ^b (%)	APT score ^c 3+/2+/1+ (% of APT+)
Head and neck AD+ ($n = 98$)	55 ^f	56	41	10/38/52
Head and neck AD- ($n = 33$)	19 ^f	36	30	0/30/70
Total serum IgE ^d 4001–23 800 kU L^{-1} ($n = 16$)	94	81	87	7/36/57
Total serum IgE 501–4000 kU L^{-1} ($n = 36$)	89	75	44	12/38/50
Total serum IgE 123–500 kU L^{-1} ($n = 38$)	26	53	29	9/18/73
Total serum IgE $\leq 122 \text{ kU L}^{-1}$ ($n = 40$)	8	18	23	0/56/44
SCORAD ^e 60–86 ($n = 14$)	64 ^f	57	79	9/27/64
SCORAD 24–59 ($n = 92$)	52 ^f	54	41	8/39/53
SCORAD 8–23 ($n = 26$)	24	35	4	0/0/100

Table 3. Reactivity to *Malassezia sympodialis* extract in selected groups of patients with atopic dermatitis (AD)

SPT, skin prick test; APT, atopy patch test. ^aPercentage of individuals with specific serum IgE to *M. sympodialis* extract (m70), ImmunoCAPTM (Pharmacia Diagnostics AB), reference range $< 0.35 \text{ kU L}^{-1}$. ^bPercentage of individuals with positive SPT or APT reaction to *M. sympodialis* extract, respectively. ^cPercentage of individuals with 3+, 2+ or 1+ APT reactivity within the *M. sympodialis* extract APT+ patients (the highest score, regardless of extract concentration, is given for each individual). ^dImmunoCAPTM (Pharmacia Diagnostics AB), reference range $1.6\text{--}122 \text{ kU L}^{-1}$. ^eAssessed by SCORAD.³¹ ^fNot tested in one AD patient due to lack of serum.

Table 4. Comparison between reactivity to *Malassezia sympodialis* extract and recombinant *Malassezia* allergens

	rMal s 1		rMal s 5		rMal s 6		rMal s 1 + rMal s 5 + rMal s 6 ^b		'rMal s mix' ^c	
	Extract+	Extract-	Extract+	Extract-	Extract+	Extract-	Extract+	Extract-	Extract+	Extract-
Specific serum IgE ^a	25/59 ^d (42%)	0/71 ^e (0%)	32/59 (54%)	5/71 (7%)	28/59 (47%)	4/71 (6%)	43/59 (73%)	9/71 (13%)	ND	ND
SPT+	17/67 (25%)	2/65 (3%)	36/67 (54%)	2/65 (3%)	27/67 (40%)	7/65 (11%)	47/67 (70%)	8/65 (12%)	39/67 (58%)	7/65 (11%)
APT+	8/50 (16%)	0/82 (0%)	17/50 (34%)	3/82 (4%)	11/50 (22%)	2/82 (2%)	24/50 (48%)	5/82 (6%)	20/50 (40%)	2/82 (2%)

SPT, skin prick test; ATP, atopy patch test; ND, not done. ^aImmunoCAPTM (Pharmacia Diagnostics AB), reference range <0.35 kU L⁻¹. ^bPositive reaction to one or more of the single recombinant allergens rMal s 1, rMal s 5 and rMal s 6. ^cPositive reaction to the mixture of rMal s 1, rMal s 5 and rMal s 6. ^dNumber of individuals with a positive reaction to the stated rMal s allergen within the atopic dermatitis (AD) patients with a positive reaction to *M. sympodialis* extract. ^eNumber of individuals with a positive reaction to the stated rMal s allergen within the AD patients with a negative reaction to *M. sympodialis* extract.

positive SPT reaction and 48% with a positive APT reaction to *M. sympodialis* extract. In addition, the rMal s allergens identified a few AD patients who had a negative reaction to the *M. sympodialis* extract (Table 4). The concordance for specific serum IgE, SPT and APT reactions tested with the three rMal s allergens together with the *M. sympodialis* extract is shown in Figure 1(b).

The SPT reactions to the 'rMal s mix' were in good concordance with the reactions to the single rMal s allergens (Table 4). Positive SPT reactions to 'rMal s mix' were found in 84% of the 55 patients with positive SPT reactions to one or more of the single rMal s allergens, but in none of the patients with negative SPT reactions to all of the single rMal s allergens. However, the APT reactions to 'rMal s mix' did not agree very well with the APT reactions to the single rMal s allergens (Table 4). Nevertheless, the strong APT reactions to 'rMal s mix' showed quite good concordance with the APT reactions to the single rMal s allergens.

Of the three tested rMal s allergens, rMal s 5 was most efficient in identifying individuals also positive to *M. sympodialis* extract (Table 4). The pattern of reactions to rMal s 5 differed significantly from the two other rMal s allergens. This was most evident for the APT where 20 of 29 patients reacting with a positive APT to any of the rMal s allergens were APT positive to rMal s 5 (Table 4). Fifteen of the 20 AD patients with a positive APT to rMal s 5 were also SPT positive and had specific serum IgE to rMal s 5, in contrast to rMal s 1 and rMal s 6, where this pattern

was found in only one of eight and four of 13 patients, respectively. More AD patients reacted with a 2+ and 3+ APT score in response to rMal s 5 than to the other rMal s allergens.

Correlations were found between SPT reactivity (mm) and specific serum IgE (kU L⁻¹) for rMal s 1 ($r_s = 0.67$, $P < 0.001$, $n = 27$), rMal s 5 ($r_s = 0.75$, $P < 0.001$, $n = 46$) and rMal s 6 ($r_s = 0.69$, $P < 0.001$, $n = 38$). No positive correlations were found between the APT (0-3+) and SPT reactions or between the APT reactions and specific serum IgE to rMal s 1, rMal s 5 or rMal s 6. The correlations were in all cases calculated for patients positive in one or both of the tests compared.

No specific serum IgE, positive SPT or APT reactions to the rMal s allergens were found in the SD patients or healthy controls. Positive SPT and/or APT reactions to the irrelevant recombinant control allergen, rAca s 13, were found in 11 of the AD patients, but in none of the SD patients or healthy controls. In those patients with positive reactions to rAca s 13, the SPT and APT reactivity ranged from none to all of the rMal s allergens, without any specific pattern. This indicates hypersensitivity to this allergen in a few of the AD patients rather than a non-specific cross-reactivity between the recombinant allergens.

Discussion

In this study we found sensitization to *Malassezia* (*M. sympodialis* extract, rMal s 1, rMal s 5 and/or rMal s 6) measured as specific serum IgE levels, SPT and APT

reactions, in two-thirds of the investigated adult AD patients. The levels of m70 correlated with the total serum IgE levels and the highest proportion of AD patients with elevated m70 levels was found among patients with high total serum IgE levels, among patients with head and neck dermatitis and among patients with a high eczema score. Those findings are in agreement with earlier investigations.^{3,9,36} More importantly, the addition of the APT to the test battery used in this study reveals a previously overlooked impact of *Malassezia* hypersensitivity in certain subgroups of AD patients (Table 3): *M. sympodialis* APT positivity was found in 30% of the AD patients without head and neck dermatitis, quite a high figure compared with 41% among the AD patients with head and neck dermatitis. It was earlier argued that *Malassezia* allergy was seen preferentially in AD patients with head and neck dermatitis^{3,9,21} on the grounds that *Malassezia* mainly colonizes sebum-rich parts of the body.

We found more AD patients with a positive SPT reaction to *M. sympodialis* extract (51%) than with m70 positivity (45%). The SPT may be more sensitive—and also more relevant for AD patients—than measurement of specific serum IgE. By the use of *M. sympodialis* extract we found the ratio between specific serum IgE and total serum IgE to have an impact on the outcome of the SPT: some AD patients with specific serum IgE together with high levels of total serum IgE reacted with a negative SPT, whereas positive SPT reactions in the absence of measurable specific serum IgE were found among the AD patients with low total serum IgE (Fig. 2). This might be due to an influence of the number of allergen-specific IgE molecules bound to each mast cell. A relatively high proportion of *M. sympodialis* APT positivity was also found among patients without high total serum IgE levels, despite a low proportion of m70+ and *M. sympodialis* SPT+ individuals. Positive *M. sympodialis* APT reactions were found in 23% of the AD patients with total serum IgE $\leq 122 \text{ kU L}^{-1}$ (Table 3). Four AD patients who would have been classified as having 'non-IgE-associated allergic atopic eczema/dermatitis syndrome' according to their low total serum IgE levels, m70 negativity and negative Phadiatop^{®37} all had a positive SPT and a 2+ APT to *M. sympodialis*. Their reaction pattern could be explained by the presence in skin of allergen-specific IgE that was not detected in the serum.

There is no 'gold standard' for the relevance of an allergen in AD. However, Darsow *et al.* showed, by the use of 'patients reported history' as the true positive

control, a higher specificity but a lower sensitivity in the APT compared with specific serum IgE or positive SPT reactions.³⁸ There is evidence for both an inhalation route and penetration through the skin as possibilities for an allergen to elicit an eczematous reaction, with the APT mimicking the skin penetration route.²⁶ By its growth on human skin *Malassezia* could be suspected mainly to elicit eczema through the skin. Thus, the APT may be considered as a clinically relevant and important test for the diagnosis of *Malassezia* hypersensitivity.

Malassezia extracts contain a wide range of IgE binding components,^{10,11} but variations in allergenic contents and difficulties in standardization of extract³⁹ may be solved by the use of recombinant allergens. In this study, we included rMal s 1, rMal s 5 and rMal s 6. They were sufficient to detect approximately 70% of the AD patients with specific serum IgE or SPT positivity to *M. sympodialis* extract and, interestingly, an additional 13% within the AD patients who were m70– (Table 4). However, their efficiency in detecting patients with a positive APT to *M. sympodialis* extract was somewhat lower (48%), which may be explained by different mechanisms behind the reactions. Both the ImmunoCAP[™] method for detecting specific serum IgE and the SPT are designed to demonstrate specific IgE. The initiation of an APT reaction, on the other hand, is thought to be dependent on antigen presentation mediated by specific IgE bound to epidermal Langerhans cells.^{40,41} Less is known about the later events in the reaction, but allergen-specific T cells^{42,43} with a Th2-like cytokine profile⁴³ also seem to be of importance. In addition, eosinophils belong to the early skin-infiltrating cells in the APT reaction.⁸ This is in accordance with the cellular response in chronic allergic asthma or rhinitis and could be classified as a Th2-dominated type IV allergic reaction.⁴⁴ There is still no explanation for the occurrence of positive APT reactions in patients without detectable allergen-specific IgE. One could speculate about minute amounts of allergen-specific IgE, not detected by SPT but sufficient for antigen presentation, a Th2-dominated type IV reaction initiated in the absence of specific IgE, or even a classical Th1-dominated type IV reaction. The complexity in the reaction may imply a need for several allergens or support from adjuvant factors in a full allergen extract to elicit a positive APT reaction. Of the three tested recombinant allergens we found most positive reactions to rMal s 5, which also had the best concordance with the *M. sympodialis* extract in its reactivity pattern. The 'rMal s mix', tested in parallel

with the single recombinant allergen in the SPT and APT, was less efficient than the rMal s allergens used separately. This may partly be explained by the fact that the protein concentration for each single rMal s in the 'rMal s mix' was only one-third of the concentration used for the tests with single rMal s allergens. Taken together, our results suggest that a proper mixture of rMal allergens might be a valuable tool for detection of specific serum IgE and SPT reactions in the future.

In summary, our results support that *Malassezia* can play a role in eliciting and maintaining eczema in patients with AD. The APT reveals an impact of *Malassezia* hypersensitivity in additional subgroups of AD patients.

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