

Sensitization to the Yeast *Malassezia Symptodialis* Is Specific for Extrinsic and Intrinsic Atopic Eczema

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The opportunistic yeast *Malassezia symptodialis* belongs to the normal cutaneous flora but can also cause IgE-mediated sensitization in patients suffering from atopic eczema (AE). We investigated 706 individuals by ImmunoCAPm70 and skin-prick tests with a crude *M. symptodialis* extract. In AE patients, we further performed skin prick tests, atopy patch tests, ELISA, and peripheral blood mononuclear cells proliferation assays with recombinant *M. symptodialis* allergens (rMala s 1 and 5–9). In 52/97 patients with AE-specific IgE against *M. symptodialis* was detectable. Almost no reactivity to *M. symptodialis* was seen in patients suffering from other allergic diseases (4/571) and no reactivity at all was seen in healthy controls (0/38). Skin tests showed variable recognition patterns against the different molecular structures with a predominant sensitization to rMala s 1, 5, 6, and 9, confirmed also by specific serum IgE to these allergens. Interestingly, IgE- and T-cell-mediated reactivity against *M. symptodialis* was also found in patients with the intrinsic form of AE. Thus, sensitization to *M. symptodialis* is specific for AE patients and occurs in both the extrinsic and intrinsic variant of eczema. Recombinant yeast allergens represent a useful tool to study molecular structures and differential sensitization patterns in the pathogenesis of AE.

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INTRODUCTION

Atopic eczema (AE) is a chronically relapsing inflammatory skin disease, where the pathogenesis is not yet fully understood (Leung, 2000). However, a combination of genetic background, deregulation of the immune system, a defective skin barrier, and environmental factors contribute to the development of AE (Leung and Bieber, 2003). Approximately 80% of the adult AE patients show immediate-type skin reactions to environmental allergens together with elevated serum IgE levels. However, approximately 20% of patients with clinical signs of AE have low serum IgE levels and lack detectable specific serum IgE and positive skin prick test (SPT) reactions to such allergens. This subgroup is also defined as intrinsic, non-allergic AE or more recently non-atopic type of eczema (Oppel *et al.*, 2000; Schmid-Grendelmeier *et al.*, 2001; Johansson *et al.*, 2004b). As the serum level of IgE to aero- and food allergens in the extrinsic type can correlate with the severity of AE (Scalabrin *et al.*, 1999) and increased

number of IgE receptors are found on cutaneous antigen-presenting cells (Bruynzeel-Koomen *et al.*, 1986; Bieber *et al.*, 1992), it can be postulated that allergens can be involved in the onset and/or maintenance of the disease.

Microorganisms have also been particularly emphasized as pathogenetic factors; in particular, *Staphylococcus aureus* and the skin colonizing opportunistic yeast *Malassezia*, earlier denoted as *Pityrosporum* (Jensen-Jarolim *et al.*, 1992; Scalabrin *et al.*, 1999; Scheynius *et al.*, 2002). Recently, such microorganisms have been postulated to be pathogenetically relevant also in a proportion of patients with the intrinsic form of AE (Johansson *et al.*, 2003; Novak *et al.*, 2003). *M. symptodialis* has been reported as the most frequent species in both AE patients and healthy individuals (Sandstrom Falk *et al.*, 2005).

In previous studies, it was demonstrated that around 30–80% of patients with AE have positive SPT, positive atopy patch test (APT) reactions, and/or IgE antibodies against extract of *Malassezia* (Rokugo *et al.*, 1990; Nissen *et al.*, 1998; Mayser and Gross, 2000; Tengvall Linder *et al.*, 2000; Zargari *et al.*, 2001; Scheynius *et al.*, 2002; Johansson *et al.*, 2003; Johansson *et al.*, 2004a). Proliferative responses of peripheral blood mononuclear cells (PBMCs) after stimulation with *M. symptodialis* extract are significantly higher in AE patients with *Malassezia*-specific serum IgE than in AE patients lacking *Malassezia*-specific serum IgE and in non-atopic healthy controls (Tengvall Linder *et al.*, 1998; Johansson *et al.*, 2002; Scheynius *et al.*, 2002). All these data indicate that the sensitization to *M. symptodialis* is a phenomenon specific for AE. However, studies in larger populations addressing this question are still lacking, namely

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Abbreviations: ACD, allergic contact dermatitis; AE, atopic eczema; APT, atopy patch test; OD, optical density; PBMC, peripheral blood mononuclear cells; SPT, skin prick test

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also in intrinsic AE. Thus, we investigated the differential reactivity to *M. sympodialis* in various conditions, including various immediate-type allergic diseases, urticaria, and allergic contact dermatitis (ACD) ($n=571$) using SPT with natural extract and ImmunoCAPm70. Furthermore, we performed in the patient group with AE SPT, APT, ELISA, and a PBMC proliferation assay with the six recombinant *M. sympodialis* allergens rMala s 1 and rMala s 5–9 to further characterize particular allergens involved in both extrinsic and intrinsic AE.

RESULTS

Response to *M. sympodialis* extract

From all the 706 tested individuals (97 AE patients, 571 patients with other diseases, and 38 healthy controls), only the patient group with AE showed a high percentage of sensitization to natural *M. sympodialis* extract. In 52 of 97 patients (54%) with AE specific IgE reactivity against *M. sympodialis* extract was detected either serologically with the ImmunoCAPm70 system (median 18 kU/l, range 0.39–79.6 kU/l) or in SPT. From the 571 patients with other allergic diseases, only four patients (two with rhinoconjunctivitis, two with asthma bronchiale) showed a weak IgE reaction to *M. sympodialis* extract either in ImmunoCAPm70 or in SPT (Table 1). The clinical investigation and the retrospective evaluation of the clinical history showed that two of these patients presented marginal signs for an AE and that the other two were previously afflicted with an AE. All healthy controls showed no IgE-mediated reaction to the *M. sympodialis* extract (Table 1). In the APT with *M. sympodialis* extract, 34 of the 69 tested AE patients (49%) showed a positive reaction (Table 3). Even 12 of the 16 tested patients (75%) reacted positive in the PBMC proliferation assay to *M. sympodialis* extract (Table 3).

In vivo response to rMala s allergens

Figure 1 shows the differential SPT and APT reactivity of the AE patients against the different recombinant *M. sympodialis*

allergens with a predominance of sensitization to rMala s 1, rMala s 5, rMala s 6, and rMala s 9 in both types of skin tests. Overall, 41 of 97 tested patients (42%) reacted to at least one rMala s protein (any rMala s) in the SPT, whereas 33 of 69 tested patients (48%) reacted to at least one rMala s protein (any rMala s) in the APT (Table 3). These results were just a little bit below the positive reactions detected by the natural extract (46 and 49%, respectively).

In vitro response to rMala s allergens

So far, the biological relevance of ELISA measurements of IgE reactivity with recombinant *M. sympodialis* allergens has not been determined. To assess the approximate cutoff values of the optical density (OD) for a given allergen in terms of biological activity, the quantitative OD measurements have to be correlated to the *in vivo* results. The patients were divided into four groups (SPT + APT+, SPT + APT–,

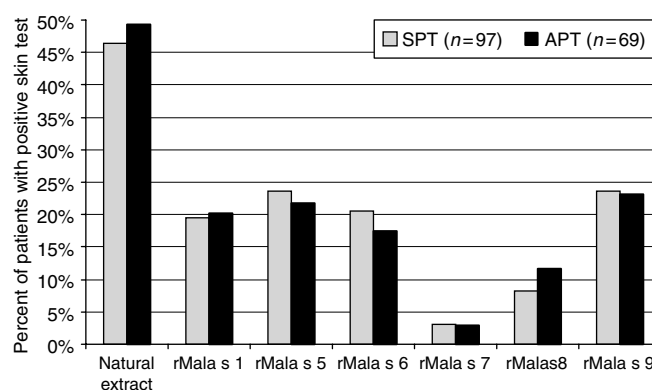


Figure 1. SPT and APT reactions with rMala s allergens in patients with AE.

In this figure, we show the percentage of patients who reacted positive in the SPT (performed in all patients, $n=97$) or the APT (performed in $n=69$ patients) to rMala s allergens. SPT was considered as positive by a wheal diameter of ≥ 3 mm surrounded by erythema. APT was considered as positive when there was at least an erythema and infiltration (+) after 48 and/or 72 hours.

Table 1. Characteristics of study population and sensitization to *M. sympodialis* extract

	Totally tested	Allergic manifestations other than stated	Gender (M/F) ¹	Median age years (range)	Pos. "m70" ² (≥ 0.35 kU/l)	Pos. SPT to nat. extract
Asthma	83	69 rhinitis/conjunctivitis	0.8	23 (5–72)	1	1
Rhinitis/conjunctivitis	378	55 asthma, 20 food allergy	0.9	27 (6–63)	0	2
AE	97	—	0.8	32 (17–56)	51	45
Urticaria	31	2 food allergy	0.2	42 (15–48)	0	0
Food allergy	24	—	0.4	26 (11–52)	0	0
Insect sting allergy	42	—	0.5	35 (9–64)	0	0
ACD	13	—	0.6	33 (17–63)	0	0
Healthy controls	38	—	0.4	29 (15–61)	0	0
Total	706	—	0.6	31 (5–72)	52	48

AE, atopic eczema; ACD, allergic contact dermatitis; F, female; "m70", ImmunoCAPm70; M, male; SPT, skin prick test.

¹Ratios refer to male divided by female.

²"m70" is the commercially available ImmunoCap test for *M. sympodialis* ATCC yeast strain 42132.

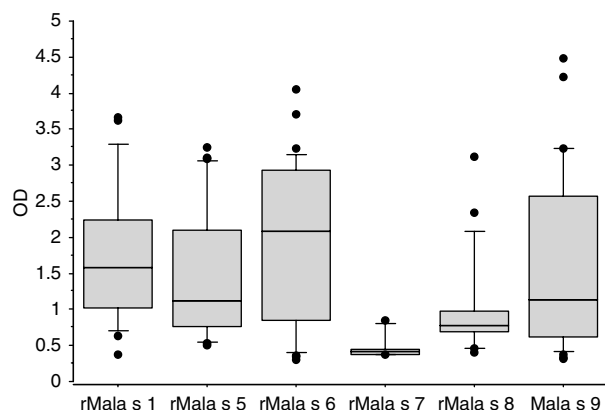


Figure 2. IgE binding against the various r allergens determined by ELISA in all patients with AE ($n=97$). In this figure, we show only reactions with an OD >0.3 . The line shows the median, the body of the boxes show the 25–75th percentile, and the whiskers show the limit of the 90th, respective the 10th percentile. All observations >90 th or <10 th percentile are illustrated as dots.

SPT–APT+, SPT–APT–) based on the *in vivo* tests to the natural extract. Because the mean OD of the SPT+/APT+ tested patients was 0.9 and the mean OD of the SPT–/APT– tested patients was 0.09, we defined 0.3 as the limit for positive ELISA reactions for rMala s (Figure S1). Overall, 53% (51/97) reacted to one of the recombinant *M. sympodialis* allergens with a predominance of sensitization to rMala s 1, rMala s 5, rMala s 6, and rMala s 9 (Table 3). Almost no reaction to rMala s 7 and rMala s 8 was observed (Figure 2). In the patient group, where we performed PBMC proliferation assays ($n=16$), we could induce a positive proliferation with all rMala s allergens, except for rMala s 5. A predominance of T-cell sensitization was seen to rMala s 1, rMala s 6, and rMala s 9. In all individuals of the negative control group ($n=3$), the PBMC proliferation assay was negative (Table 3).

Intrinsic AE patient group

From the 97 patients with AE, 41 had a head-and-neck type whereas 56 had a more generalized form. Eighty-three were of the extrinsic type (36 with head-and-neck type) and 14 of the intrinsic type (five with head-and-neck type) (Table 2). All intrinsic AE patients had a total IgE level below 350 kU/l. Interestingly, also the patient group with the so-called intrinsic type of AE (because of the absence of specific IgE to the common aero- and food allergens) showed *in vivo* skin test reactivity to *M. sympodialis* extract (SPT 36%, APT 38%) and also to the different rMala s proteins except for rMala s 7 (Table 4). Furthermore, 7/14 (50%) showed positive ImmunoCAPm70 reactions, respective positive ELISA reactions to any rMala s proteins. In the PBMC proliferation assay, 5/8 (63%) reacted to the natural *M. sympodialis* extract. Also the rMala s allergens could induce a positive proliferation assay, except rMala s 5 and rMala s 7 (Table 4).

DISCUSSION

Our study fully confirms in a large patient number that sensitization to *M. sympodialis* is found exclusively in

Table 2. Characteristics of the AE patients

	Extrinsic AE ($n=83$)	Intrinsic AE ($n=14$) ¹
Median age in years (range)	32 (17–56)	29 (19–53)
Gender M/F	35/48	7/7
SCORAD median (range)	31 (7–76.5)	27 (8.3–79.5)
Total IgE median (kU/l) (range)	2,086 (55–16,400)	194 (18–349)
Inhalation allergies ²	37 (45%)	0 (0%)
Head-neck type	36 (43%)	5 (36%)
Sx1 (0.35 kU/l)	81 (99%)	0 (0%)
Fx5 (0.35 kU/l)	18 (23%)	0 (0%)

¹Intr. AE=absence of respiratory allergy, negative Sx1 and Fx5, total IgE <350 kU/l; F, female; Fx5, screening for food-allergies; M, male; Sx1, screening for respiratory allergies.

²Inhalation allergies=either rhinoconjunctivitis or allergic asthma and pos. Sx1.

patients with AE, but not in other hypersensitive diseases such as patients with inhalant, food or insect venom allergies, urticaria, or ACD. Thus, sensitization against *M. sympodialis* can be regarded as specific or almost pathognomonic for AE, both in generalized and in the head-and-neck type. According to the literature, where *Malassezia*-specific serum IgE was detected with a frequency of 32–68% and positive SPT to *Malassezia* was found in approximately 30–80% of AE patients (Nordvall *et al.*, 1992; Scalabrin *et al.*, 1999; Tengvall Linder *et al.*, 2000; Zargari *et al.*, 2001; Johansson *et al.*, 2002; Scheynius *et al.*, 2002), we can confirm this type I sensitization in 53% (51/97) of the AE patients tested by the ImmunoCAPm70-system and in 46% (45/97) tested by SPT (Table 1). Also in the APT, 49% (34/69) of the patients reacted positively to natural extract (Table 3), this was in accordance with previous studies, where positive APT to *Malassezia* was found in 41–64% of the AE patients (Rokugo *et al.*, 1990; Tengvall Linder *et al.*, 2000; Johansson *et al.*, 2003).

It is not yet fully understood why sensitization against *M. sympodialis* occurs only in the patient group with AE, but not in patients with exclusively inhalant symptoms such as rhinoconjunctivitis and/or asthma. A possible cause is the high, long-standing cutaneous colonization with *Malassezia*. Antimicrobial peptides known as cathelicidins (LL-37) and beta-defensins (HBD-2) are deficient in skin lesions from patients with AE (Ong *et al.*, 2002), which might also contribute to a higher colonization with *Malassezia* in this patient group. Furthermore, the selective sensitization in AE patients might be favored by the disrupted skin barrier in AE. Whole yeast cells and allergenic components of the yeast are likely to pass the skin barrier and come in contact with professional antigen-presenting cells, such as Langerhans cells in the epidermis (Buentke *et al.*, 2000, 2001). Antigen-presenting cells in the skin of AE patients also have an increased ability to react to those allergens (Gabrielsson *et al.*, 2004).

Table 3. Reactivity to the *M. sympodialis* extract and different recombinant *M. sympodialis* allergens in SPT, APT, and ELISA, and comparison between the reactions to recombinant allergens in various analysis techniques divided for ImmunoCAPm70 positive and negative results

		Natural extract		rMala s 1		rMala s 5		rMala s 6		rMala s 7		rMala s 8		rMala s 9		Any rMala ¹	
ImmunoCAPm70		n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%
SPT (n=97)	CAP pos. (n=51)	44	86	19	37	22	43	19	37	3	6	8	16	23	45	39	76
	CAP neg. (n=46)	1	2	0	0	1	2	1	2	0	0	0	0	0	0	2	4
	Total (n=97)	45	46	19	20	23	24	20	21	3	3	8	8	23	24	41	42
APT (n=69)	CAP pos. (n=42)	31	74	14	33	15	36	12	29	2	5	8	19	16	38	33	79
	CAP neg. (n=27)	3	11	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Total (n=69)	34	49	14	20	15	22	12	17	2	3	8	12	16	23	33	48
ELISA ² (n=97)	CAP pos. (n=51)	NA	NA	20	39	24	47	28	55	5	10	16	31	31	61	45	88
	CAP neg. (n=46)	NA	NA	0	0	2	4	3	7	1	2	1	2	0	0	6	13
	Total (n=97)	NA	NA	20	21	26	27	31	32	6	6	17	18	31	32	51	53
PBMC ³ proliferation (n=16)	CAP pos. (n=13)	12	92	6	46	0	0	5	38	1	8	4	31	7	54	11	85
	CAP neg. (n=3)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Total (n=16)	12	75	6	38	0	0	5	31	1	6	4	25	7	44	11	69

APT, atopy patch test; PBMC, peripheral blood mononuclear cells; SPT, skin prick test.

¹Any rMala=accumulated results with any recombinant *M. sympodialis* allergen (rMala s1, rMala s 5–9).

²Positive reaction in the ELISA were defined as an OD >0.3.

³Positive reaction in the PBMC assay were defined as stimulation index >2.5 (results obtained with an allergen concentration of 1 µg/ml after 6 days of cell culture are shown).

Table 4. Reactivity against the *M. sympodialis* extract and various recombinant allergens in patients with intrinsic AE

	SPT pos. (%), n=14	APT pos. (%), n=13	ELISA pos ¹ . (median OD, range ³), n=14	PBMC proliferation pos ² . (median SI, range ³), n=13
<i>M. sympodialis</i> extract	5 (36%)	5 (38%)	7 ⁴	5 (5.8, 3.1–8.9)
rMala s 1	3 (21%)	2 (15%)	2 (1.13, 1.04–1.21)	4 (3.3, 2.8–3.7)
rMala s 5	2 (14%)	1 (8%)	3 (0.98, 0.87–1.10)	0
rMala s 6	3 (21%)	0	4 (2.74, 2.03–3.71)	2 (5.3, 4.2–6.4)
rMala s 7	0	0	1 (0.37)	0
rMala s 8	3 (21%)	3 (23%)	3 (0.68, 0.41–0.92)	2 (4.5, 3.2–5.8)
rMala s 9	4 (29%)	2 (15%)	5 (1.62, 0.32–4.49)	4 (4.5, 2.9–6.3)

AE, atopic eczema; APT, atopy patch test; PBMC, peripheral blood mononuclear cells; OD, optical density; SPT, skin prick test.

¹Positive reaction in the ELISA were defined as an OD >0.3.

²Positive reaction in the PBMC assay were defined as stimulation index >2.5 (results obtained with an allergen concentration of 1 µg/ml after 6 days of cell culture are shown).

³Median and range is given for positive only.

⁴IgE analysis for the *M. sympodialis* extract was carried out with ImmunoCAP (kU/1).

Furthermore, skin-homing activated T-cells bearing the cutaneous lymphocytic antigen show increased reactivity in AE and may contribute to the formation of specific IgE (Akdis *et al.*, 1997). All these observations support the hypothesis that penetration of *M. sympodialis* in AE is possible and can

induce an allergic reaction with a consequent production of specific IgE via B-cell stimulation in patients with AE.

Interestingly, also in the intrinsic type of AE, we found a high IgE- and T-cell-mediated sensitization to *M. sympodialis* (Table 4). These patients have neither associated respiratory

or food allergies nor detectable IgE-mediated sensitization to common inhalant or food allergens. However, they also show a disrupted skin barrier and T cells from those patients equally respond to superantigen, staphylococcal enterotoxin B, and produce similar cytokines (Akdis *et al.*, 1997; Novak and Bieber, 2003). One could speculate that this patient group – so far defined by the absence of sensitization to standard aero-/food allergens – is in fact a group of patients that nevertheless is at risk to get similarly sensitized to other agents with primary cutaneous exposure such as skin colonizing microbes as *Staphylococci* or *Malassezia*. As already described elsewhere, microbes such as *M. sympodialis* might indeed play a role as trigger factors also in intrinsic AE (Johansson *et al.*, 2003; Novak *et al.*, 2003). The term intrinsic AE is therefore misleading to a certain extent (Johansson *et al.*, 2004b). Thus, our results also call into question the relevance of IgE in the pathogenesis of AE.

Furthermore, we could show a very clear concordance on the one hand *in vivo* between SPT and APT (Figure 1), and on the other hand between the *in vivo* and *in vitro* findings (Table 3). Hence, 86% of the 51 ImmunoCAPm70-positive tested patients reacted positively in the SPT to natural extract, and 74% in the APT. This is most probably owing to the fact that the same allergen extract was used in all *in vivo* and *in vitro* tests (ATCC yeast strain no. 42132).

Malassezia extracts contain a wide range of IgE-binding components (Zargari *et al.*, 1994; Lintu *et al.*, 1997), but variations in allergenic contents, difficulties in standardization of the extract (Zargari *et al.*, 1995) as well as large batch to batch variation (Vailes *et al.*, 2001; Zargari *et al.*, 2001) may be solved by the use of highly pure recombinant proteins. By investigating in this study six of the known recombinant *M. sympodialis* allergens (rMala s 1 and rMala s 5–9) (Schmidt *et al.*, 1997; Lindborg *et al.*, 1999; Rasool *et al.*, 2000; Andersson *et al.*, 2004), we found a strong correlation between positive SPT and APT to each recombinant allergen with a predominance of sensitization to rMala s 1, rMala s 5, rMala s 6, and rMala s 9 (Figure 1, Table 3). There was a significant reduced reaction to rMala s 7 in both *in vivo* tests (ATP and SPT), also detectable in the ELISA (Figure 2) and in the PBMC proliferation assay (Table 3).

Of the recombinant *M. sympodialis* allergens used, only rMala s 5 and rMala s 6 show sequence similarity to other proteins; the others, rMala s 1, rMala s 7 to rMala s 9, are unique structures (Table S1). Hence owing to structural relationship of fungal manganese superoxide dismutase (rMala s11) with the human form of this enzyme, IgE- and T-cell-mediated autoreactivity can result in AE patients (Schmid-Grendelmeier *et al.*, 2005). Thus, high cross-reactivity between rMala s 6 and human cyclophilin, a protein found in many organisms and proteins of human (Fluckiger *et al.*, 2002) and showing the highest sensitization rate in our study (Table 3, Figure 2), might also induce similar autoreactivity phenomena in AE.

In conclusion we could demonstrate in a large study population, that sensitization to *M. sympodialis* is highly specific for patients with AE and does not occur in patients with only inhalant allergies, urticaria, or ACD. Regarding the

high frequency of sensitization to *M. sympodialis*, one could argue that these potential microbial allergens should be added in the diagnostic workup of AE patients, namely also in the intrinsic type of AE. This might be also relevant for therapeutic purposes, as various studies have reported beneficial effects of anti-fungals in some AE patients (Back *et al.*, 1995; Back and Bartosik, 2001; Lintu *et al.*, 2001; Ikezawa *et al.*, 2004; Svejgaard *et al.*, 2004). Diagnosis with recombinant yeast allergens proved as a highly useful tool to further characterize the allergens involved, and to determine their clinical relevance in AE.

MATERIALS AND METHODS

Patient selection

Totally, 668 individuals with different allergic diseases (AE, allergic asthma, rhinitis and/or conjunctivitis, urticaria, food allergy, insect sting allergy, and ACD), and 38 healthy controls were investigated (Table 1). Diagnosis of AE was made according to the criteria of Hanifin and Rajka (Hanifin, 1980). Disease activity for AE was estimated using SCORAD (severity scoring of atopic dermatitis) (Consensus Report, 1993). Furthermore, we divided the patients with AE in the clinical subtype of generalized or head-and-neck type of AE and also extrinsic and intrinsic type (Table 2) (Wüthrich, 1989; Johansson *et al.*, 2001). The group of intrinsic AE was defined by the absence of other atopic diseases such as allergic rhinoconjunctivitis or allergic bronchial asthma and a low or moderate total IgE level (<350 kU/l) in combination with negative *in vitro* IgE screening for aeroallergens and food allergens (e.g., sx1 and fx5, Pharmacia ImmunoCAP System, Pharmacia Diagnostics AB, Sweden). Patients with allergic rhinitis and/or conjunctivitis had all a typical history of seasonal or perennial symptoms and positive SPT with pollen or perennial allergens (house dust mites, animal dander from ALK, Denmark). All asthmatic subjects had in addition a history of wheezing and dyspnea during allergen exposure (American Thoracic Society, 1995). Patients with food allergy showed a typical history of food-induced allergy and had either a positive SPT or detectable levels of food-specific serum IgE. Diagnosis in insect sting allergic patients was based on clinical history, intracutaneous skin tests, and specific IgE against bee or wasp venom in the serum (Pharmacia ImmunoCAP System). The urticaria diagnosis was carried out on the basis of clinical investigation and the typical history. Patients with ACD had a clear clinical history of contact dermatitis after exposure to a certain substance (such as nickel, cobaltchloride, and fragrance mix) and a corresponding positive patch test with the relevant substance, but no history or clinical signs of atopic dermatitis. Eleven out of the 13 patients had active ACD at the time of testing. Affected areas were in all cases areas other than the back tested and localized mostly to face or hands. The 38 healthy controls without clinical history of atopy were not sensitized to common allergens as controlled by SPT and serologic screenings (fx5 and sx1) and had a median total IgE of 50.6 kU/l (range 0.35–78). The study was approved by the Ethical Committee of the University of Zurich and was conducted according to the Declaration of Helsinki Principles. All patients and controls gave written informed consent.

Allergens and allergen extracts

A crude *M. sympodialis* (ATCC; Strain No. 42132) extract was prepared as described previously (Zargari *et al.*, 1994). Recombinant

M. sympodialis allergens rMala s 1 and rMala s 5–9 were produced in *Escherichia coli* as described previously (Schmidt *et al.*, 1997; Lindborg *et al.*, 1999; Rasool *et al.*, 2000; Andersson *et al.*, 2004). Limulus amebocyte lysate test for purity was performed and endotoxin doses (lipopolysaccharide) in all recombinant allergens and the natural extract were well under the cutoff value (Pedersen *et al.*, 1994).

Determination of total and allergen-specific IgE

Specific serum IgE to *M. sympodialis* extract (ImmunoCAPm70) and total serum IgE were determined for all patients and controls by the ImmunoCAP system as prescribed by the manufacturer (Pharmacia Diagnostics AB, Sweden). In the patient group with AE specific IgE to common inhalant allergens (sx1, Pharmacia) and food allergens (fx5, Pharmacia) were determined. IgE values ≥ 0.35 kU/l was defined as atopic sensitization. In addition, an ELISA was established to determine specific IgE to rMala s 1 and rMala s 5–9 as described previously (Moser *et al.*, 1994).

Skin tests

SPT was performed with *M. sympodialis* extract and with the recombinant allergens rMala s 1 and rMala s 5–9. The proteins were diluted in 0.9% sterile NaCl solution in a concentration of 100 μ g/ml. 0.9% NaCl and histamine hydrochloride 0.01% (ALK, Denmark) served as negative and positive control, respectively. All skin tests were performed in double on the volar forearm and applied in the two opposite directions, using Stallerpointe® devices (Trimedal, Switzerland). The tests were evaluated after 15 minutes. Wheals and flares were pen-marked and then transferred with scotch tape to a paper. The mean wheal diameter was calculated by the addition of the maximal longitudinal (*d*1) and transversal (*d*2) diameter divided by two [(*d*1 + *d*2)/2]. A wheal diameter of ≥ 3 mm surrounded by erythema was considered positive.

APT was performed under open conditions in 69 AE patients and six healthy controls. No signs of eczema were allowed at the patch test sites (on the back). The skin was tape-stripped 10 times with scotch tape (2 cm, 3 M, Germany) as described previously (Darsow *et al.*, 1995; Langeveld-Wildschut *et al.*, 1995). Thirty microliter of each recombinant allergen (rMala s 1 and rMala s 5–9) at a concentration of 100 μ g/ml were applied on paper discs in Finn chambers (12 mm, Epitest Ltd, Hermal, Germany), in parallel with 30 μ l *M. sympodialis* extract at a concentration of 5 mg/ml. NaCl 0.9% was used as a negative control. Oral corticosteroids were discontinued for at least 6 weeks before testing. Topical corticosteroids, calcineurin inhibitors, or UV therapy had not been used in the last 3 weeks at test application sites. Reactions were read and documented after 48 and 72 hours by a dermatologist. The APT was considered as positive when there was at least an erythema and infiltration (+) after 48 and/or 72 hours, according to the criteria of conventional contact allergy patch testing (Wilkinson *et al.*, 1970) and corresponding at least with erythema and infiltration (a one+ reaction) considering the European Task force of atopic dermatitis (EFTAD) guidelines for atopy patch testing (Darsow *et al.*, 2004).

Proliferative response of PBMC

In 16 AE patients (eight extrinsic and eight intrinsic) and three healthy controls PBMCs were isolated from heparinized peripheral

venous blood by Ficoll density gradient centrifugation, washed three times, and resuspended in Rosewell Park Memorial Institute media 1640 supplemented with 1 mM sodium pyruvate, 2 mM L-glutamine, 50 μ M 2-mercaptoethanol, 1% MEM non-essential amino acids and vitamins, 100 μ g/ml streptomycin, 100 U/ml penicillin (all from Life Technologies, Switzerland), and 10% heat-inactivated fetal calf serum (Sera-Lab, UK). Samples of 5×10^5 cells/100 μ l were stimulated in round-bottomed 96-well plates with different concentrations, ranging from 0.01 to 10 μ g/ml of rMala s 1 and rMala s 5–9 or *M. sympodialis* extract, respectively, in quadruplicates for 4 and 6 days. Phytohemagglutinin (10 μ g/ml, Life Technologies, Switzerland) was used as a positive control and BSA served as a negative control. Proliferation was measured as incorporation of thymidine (DuPont-NEN, Switzerland) during the final 8 hours of culture. The results were expressed as mean c.p.m. for quadruplicates and as stimulation index calculated as mean c.p.m. for stimulated cells divided by mean c.p.m. for unstimulated cells. A stimulation index > 2.5 was considered as positive.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIALS:

Table S1. Allergens identified in *Malassezia sympodialis* species.

Figure S1. ELISA investigations with recombinant allergens in response to the *in vivo* reaction to *M. sympodialis* extract in patients with AE.

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