

## CLINICAL REPORT

# IgE Antibodies to *Malassezia furfur*, *M. sympodialis* and *Pityrosporum orbiculare* in Patients with Atopic Dermatitis, Seborrheic Eczema or Pityriasis Versicolor, and Identification of Respective Allergens

PETER MAYSER and ANTJE GROSS

Department of Dermatology and Andrology, Justus Liebig University, Giessen, Germany

*Malassezia* yeasts may be a trigger factor for atopic dermatitis. Following the recent reclassification of the genus, the presence of specific IgE antibodies was examined in the sera of patients with atopic dermatitis ( $n=223$ ), pityriasis versicolor ( $n=83$ ), seborrheic eczema ( $n=50$ ) and hymenoptera allergy ( $n=39$ ) and in controls without skin diseases ( $n=50$ ). In addition to using the commercially available radioallergosorbent test (RAST) for *Pityrosporum orbiculare* couplings were also made against the reference strains for *M. furfur* and *M. sympodialis*. To characterize the specificity and molecular weight of corresponding epitopes identical material was used for production of an immunoblot. Despite high total levels of IgE, controls and patients with pityriasis versicolor showed no specific IgE antibodies. Six patients (12%) with seborrheic eczema were positive while 78 patients (35%) with atopic dermatitis had specific IgE antibodies in higher RAST classes that differed between the *Malassezia* species. The molecular weights of the main antigens of *M. sympodialis* and *M. furfur* were determined to be 15, 22, 30, 37, 40, 58, 79, 92, 99 and 124 kDa and 15, 25, 27, 43, 58, 92, 99 and 107 kDa, respectively. Evaluated according to the location of their disease, patients with head and neck lesions most frequently showed *Malassezia*-specific IgE antibodies. However, there were differences between the *Malassezia* species tested, the previously used strain *P. orbiculare* being assignable to the species *M. sympodialis*. **Key words:** *Malassezia* yeasts; IgE antibodies; atopic dermatitis; immunoblot.

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Peter Maysers, MD, Department of Dermatology, Justus Liebig University Giessen, Gaffkystr. 14, D-35385 Giessen, Germany.

E-mail: Peter.Maysers@derma.med.uni-giessen.de

Yeasts of the genus *Malassezia* (previously *Pityrosporum*) belong to the resident flora of human skin; in addition, they play an important role in the pathogenesis of diseases such as pityriasis versicolor (PV), seborrheic eczema (SE) and *Malassezia* folliculitis (1–4).

Detection of the high-affinity FcER-1 receptor and the low-affinity FcER-II/CD23 receptor on Langerhans cells pointed to the importance of microbial antigens in the pathogenesis of atopic dermatitis via IgE-mediated mechanisms (5). In this connection, two micro-organisms have been particularly emphasized: *Staphylococcus aureus* and the lipophilic yeasts *Pityrosporum ovaleorbiculare* (6–13). *Pityrosporum* yeasts are

thought to be mainly involved in the pathogenesis of so-called head and neck dermatitis in younger patients (6, 11, 12). Especially in this patient group anti-*Pityrosporum* IgE antibodies were found, and 15–65% of these persons had positive skin tests with *Pityrosporum* extracts (7, 14). The possible relevance of these data was supported by the results of a controlled study in which 14 patients suffering from head and neck dermatitis improved during systemic antimycotic therapy with ketoconazole, while 5 with generalized skin lesions did not (15). Some of these studies were based on a radioallergosorbent test (RAST) against *P. orbiculare* developed by Pharmacia (Uppsala, Sweden) and still available as ImmunoCAP under the labelling “m 70” (10, 13). According to the manufacturer, this test uses the strain 42132 from the American Type Culture Collection (ATCC) which is stored there under the name *P. orbiculare/M. furfur*.

During the last decade, molecular biological methods have revealed a similarity between *Pityrosporum* and *Malassezia* but for historical reasons the term *Malassezia* takes priority. Furthermore, the genus *Malassezia* has been extended: in addition to the known species *M. furfur* and *M. pachydermatis*, the species *M. sympodialis* was described in 1990, followed by *M. globosa*, *M. obtusa*, *M. restricta* and *M. slooffiae* in 1996 (4). Biochemical methods are now available for their differentiation (16–18).

Because many workers who investigated the importance of *Malassezia/Pityrosporum* in atopic eczema did not use reference strains and those tested are often no longer accessible, the question arises how to categorize antigens known as *P. ovaleorbiculare* according to today's classification. Therefore, the present study compared special couplings against the reference strains for *M. furfur* (CBS 1878) and *M. sympodialis* (CBS 7222) with the commonly used commercial test. *M. sympodialis* was chosen because it probably belongs to the *Malassezia* species most frequently encountered on human skin (3).

Molecular weights and specificity of the antigens were characterized by means of a newly developed immunoblot. Furthermore, the possible relationship between head/neck/face locations of atopic dermatitis (AD) and *Malassezia*-specific antibodies was investigated.

## MATERIAL AND METHODS

### Patients

Serum samples were analyzed from patients with AD ( $n=223$ ; 82 males, 141 females; mean age 34.4 years), SE ( $n=50$ ; 36 males, 14

females; mean age 36.4 years), PV ( $n=83$ ; 47 males, 36 females; mean age 37 years) and hymenoptera allergy (HA) ( $n=39$ ; 18 males, 21 females; mean age 41.3 years) and from controls with healthy skin (HC) without clinically relevant signs of allergic rhinitis or bronchial asthma ( $n=50$ ; 34 males, 16 females, mean age 38.9 years). Diagnosis of AD was based on the criteria of Hanifin and Rajka (19); all patients with AD, SE and PV showed acute exacerbation of their disease. None of the selected persons had recently undergone topical treatment with corticoids. According to the location of their disease, patients with AD were divided into the following groups: (i) head/neck/face; (ii) extremities (flexural eczema, atopic hand/foot eczema, generalized affliction of the extremities); and (iii) multifocal involvement of the body. According to their serum IgE value, persons with healthy skin were grouped into 2 categories:  $<120$  and  $>120$  kU/l (elevated level).

#### Determination of specific IgE antibodies against *M. furfur*, *M. sympodialis* and *P. orbiculare*

In addition to using the commercially available RAST for *P. orbiculare*, couplings were made against the reference strains for *M. furfur* (CBS 1878) and *M. sympodialis* (CBS 7222). Yeast cells harvested after 4 days growth at 30°C on m-Dixon agar (3) were immediately frozen at 80°C, lyophilized (speed-vac; Bachhofer, Germany) and sent to Pharmacia for coupling. A 4-day growth period was strictly adhered to because longer growth may result in loss of particular antigen regions (20). A patient serum which showed high RAST classes for all 3 antigens served as a control and was used repeatedly in the assays. The evaluation was made according to the RAST classes defined by Pharmacia (21). Furthermore, the total IgE level was measured in each patient by means of the Pharmacia ImmunoCAP system RAST and the inhalant allergen diagnostic test SX1 (Pharmacia) was performed in order to reveal possible atopic disposition.

#### Typing of strain ATCC 42132 "*P. orbiculare*"

The strain ATCC 42132, defined as *P. orbiculare*, was purchased from the ATCC (Rockville, MD, USA) and differentiated using the following methods: morphology of the culture; micromorphology after 10 days of growth on modified m-Dixon agar; catalase reaction and Tween assimilation according to Guillot et al. (16); cremophor EL assimilation; esculin splitting; production of ethyl esters; and production of pigment according to Mayser and co-workers (17, 18, 22).

#### Determination of specific antigen regions of *M. furfur* and *M. sympodialis* by means of the immunoblot technique

Sera from 40 patients with AD and demonstrable IgE antibodies to *M. furfur* and *M. sympodialis*, 1 healthy person without demonstrable antibodies, 2 patients with SE and 1 with PV were examined for specific antibody-binding epitopes by means of the Ala-BLOT system (DPC-Biermann GmbH, Bad Nauheim, Germany). Immunoblots for *M. furfur* (CBS 1878) and *M. sympodialis* (CBS 7222) were made by DPC-Biermann and lyophilized antigen material was prepared in the same way as for the RAST. The test was carried out according to the Ala-Blot system (23) and the Quanti Scan system (DPC-Biermann) was used for assignment of the specific epitopes and for determination of molecular weights.

Homologous and heterologous inhibition tests were performed for further specification of the epitopes and detection of possible cross-reactions between the two *Malassezia* species. One sample of each of the sera showing high IgE antibody concentrations against *M. furfur* (RAST class 3; 8.4 kU/l) and *M. sympodialis* (RAST class 6; 101 kU/l) were incubated with different concentrations of the homologous or heterologous lyophilizate in phosphate-buffered saline pH 7.4, followed by re-evaluation in the immunoblot. Extract concentrations of 10, 50, 100 and 200 mg/ml were used for heterologous inhibition; the homologous inhibition test was performed with 2.5, 5, 10 and 50 mg/ml.

#### Statistical analyses

Statistical evaluation was performed by means of the  $\chi^2$  test, Spearman's correlation coefficient and the Mann-Whitney test. The program SPSS 8.0 was used for all statistical calculations.

## RESULTS

### Rast

The results obtained with the RAST examinations are shown in Table I. The individual groups did not show significant differences with regard to total IgE (Mann-Whitney test), but varied considerably in terms of specific IgE antibodies to *Malassezia/Pityrosporum*.

The largest number of positive results was found in patients with AD (78; 35%), as against 12% in patients with SE and 8% in those with HA. Although patients with PV have their

Table I. Number of positive RAST results within the different patient groups

Characteristic	SE	AD	HA	PV	Controls	Controls <sup>a</sup>
Number of patients	50	223	39	83	17	33
Mean age (range)	36.4 (11–70)	34.4 (2–88)	38.9 (8–73)	37.0 (11–78)	43.0 (24–77)	37.2 (15–83)
Median IgE (kU/l) (range)	409 (2–7,684)	440 (1–22,222)	328 (2–9,556)	52(2–11,311)	16 (2–69)	1258 (125–7,892)
Number of positive sera (RAST class $\geq 1$ )	6 (12%)	78 (35%)	3 (8%)	0 (0%)	0 (0%)	0 (0%)
All 3 yeasts positive	2	52	2	0	0	0
P.o. and M.s. positive	1	13	0	0	0	0
M.f. and M.s. positive	2	3	0	0	0	0
P.o. and M.f. positive	0	0	0	0	0	0
Only P.o. positive	0	4	1	0	0	0
Only M. f. positive	1	2	0	0	0	0
Only M.s. positive	0	4	0	0	0	0
Inhalant allergen test positive	16 (32%)	173 (78%)	28 (72%)	19 (23%)	2 (12%)	13 (39%)

P.o.: *Pityrosporum orbiculare*; M.s.: *Malassezia sympodialis*; M.f.: *Malassezia furfur*; SE: seborrheic eczema; AD: atopic dermatitis; HA: hymenoptera allergy; PV: Pityriasis versicolor.

<sup>a</sup> Controls with healthy skin and without clinically relevant signs of allergic rhinitis or asthma, but with increased total IgE ( $>120$  kU/l) and partially positive inhalant allergen test, possibly indicating an atopic constitution.

skin colonized with *M. furfur*, none was demonstrated to be sensitized in the RAST. The absolute level of total IgE in 1 patient was correlated with the demonstration of specific antibodies, but not all patients with a high total IgE value were found to be sensitized.

The correlation coefficient between total IgE level and occurrence of *Malassezia/Pityrosporum*-specific IgE antibodies was  $r = 0.496$  ( $p < 0.05$ ) for *P. orbiculare*,  $r = 0.534$  ( $p < 0.05$ ) for *M. furfur* and  $r = 0.485$  ( $p < 0.05$ ) for *M. sympodialis*.

Differences in the type and extent of sensitization were also found among the *Malassezia/Pityrosporum* yeasts. *M. sympodialis* ( $n = 79$ ) and *P. orbiculare* ( $n = 75$ ) were almost equally positive, while the frequency of sensitization to *M. furfur* ( $n = 62$ ) was lower. Approximately 64% of all positive sera ( $n = 87$ ) were positive for all 3 yeasts ( $n = 56$ ), 16% for *P. orbiculare* and *M. sympodialis* ( $n = 14$ ) and 6% for *M. sympodialis* and *M. furfur* ( $n = 5$ ); however, none were positive for *P. orbiculare* and *M. furfur* (Table I).

The 223 patients with AD divided into subgroups according to the location of their disease showed multifocal involvement of the body in 21% of cases, lesions predominantly on the arms in 38% of cases and lesions in the head/neck region in 41% of cases (Fig. 1). A similar division of the 78 patients with AD in whom the RAST had revealed sensitization to *Malassezia/Pityrosporum* yeasts shows that the relative proportion of patients with involvement of the extremities only was less than that with generalized affliction or with head/neck location (10%, 34% and 55%, respectively) (Fig. 2).

*Differentiation of ATCC 42132*

All the tests performed suggested that the strain ATCC 42132 "P. orbiculare" belongs to the species *M. sympodialis*.

*Immunoblot results*

The immunoblot developed showed a variety of antigens for *M. sympodialis* (27 bands) and *M. furfur* (27 bands) in the 40 patient sera examined. The molecular weights of the most important bands (present in > 5 different sera) were 15, 22, 30, 37, 40, 58, 79, 92, 99 and 124 kDa for *M. sympodialis* and 15, 25, 27, 43, 58, 92, 99 and 107 kDa for *M. furfur*. The

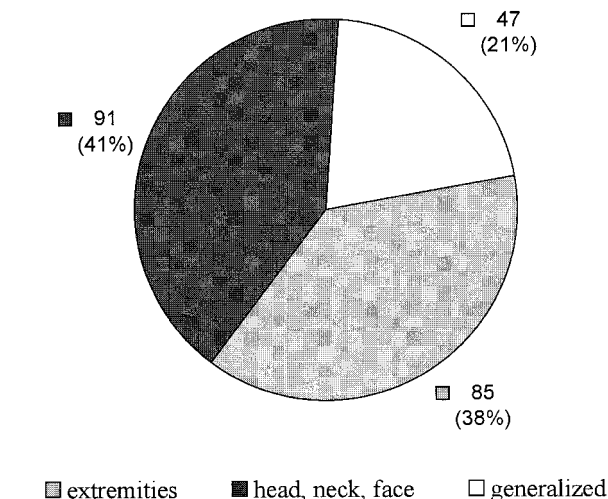


Fig. 1. Localization of lesions in patients with AD ( $n = 223$ ).

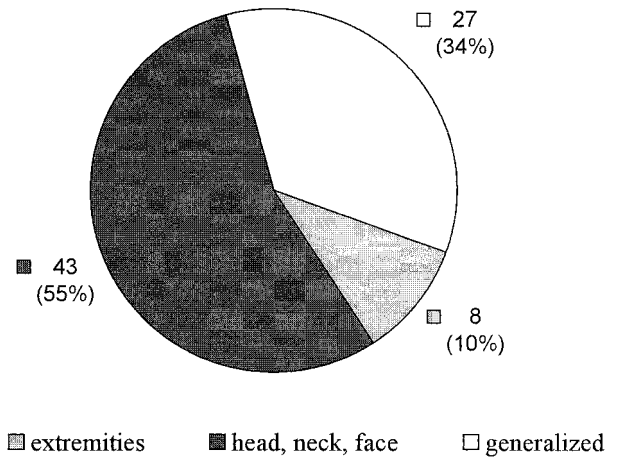


Fig. 2. Localization of lesions in patients with AD and RAST positivity for *Malassezia/Pityrosporum* ( $n = 78$ ).

results of the immunoblot are shown in Fig. 3. No bands were demonstrated on examination of the negative control (RAST class 0, total IgE level 12 kU/l) and the sera from patients with SE (2 patients) and PV (1).

In the homologous inhibition test high concentrations of the corresponding homologous extract were required for extinction of the specific bands. The patterns were only reduced by concentrations > 50 mg/ml. The heterologous inhibition test was therefore performed with higher concentrations of the extract to detect possible cross-reactions of the antibodies. Pre-incubation of *M. sympodialis*-positive serum with *M. furfur* extract (10–200 mg/ml) caused no change in, or extinction of, the bands between 15 and 124 kDa. In contrast, extinction did result from pre-incubation of *M.*

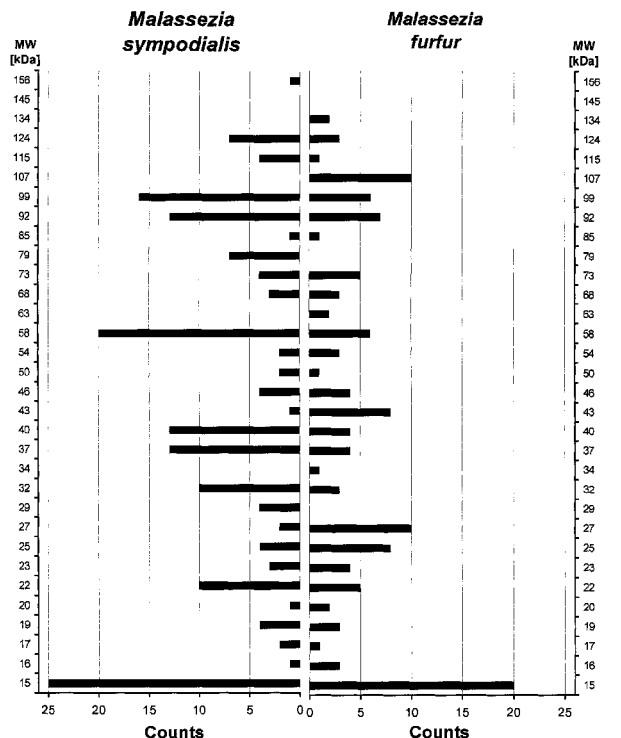


Fig. 3. Frequency of bands in immunoblots for *M. furfur* and *M. sympodialis*.

*furfur*-positive sera with *M. sympodialis* independent of the amount of heterologous substrate. The clear band at 15 kDa disappeared after addition of 10 mg/ml extract, indicating a possible cross-reaction of the antibodies.

## DISCUSSION

This study is one of the first to determine IgE-mediated sensitization to *Malassezia/Pityrosporum* yeasts using antigen material according to the new classification of these yeasts. Reference strains for *M. furfur* (CBS 1878) and *M. sympodialis* (CBS 7222) were used and, for comparison, the commercially available test of Pharmacia, which uses *P. orbiculare/M. furfur* (ATCC 42132) as the antigen source, was also utilized. A culture period of 4 days was chosen because examinations by Zargari et al. (20) had shown that the spectrum of allergens is essentially dependent on the duration of culture.

Sensitization of the immediate reaction type was demonstrated in 87 of 445 sera (19.6%), with extents varying among the 3 antigens and patient/control groups. In >64% of positive sera (56/87) all 3 antigen sources (*M. furfur*, *M. sympodialis* and *P. orbiculare/M. furfur*) were positive, suggesting group antigens for IgE antibodies within the genus *Malassezia*. However, the frequency of sensitization to *M. furfur* (CBS 1878) was lower than that achieved with the 2 other test antigens (64, vs. 79 for *M. sympodialis* and 75 for the commercial test), and the RAST classes were often lower. The test results for *M. sympodialis* were highly correlated with those obtained from the commercial test, which is based on *P. orbiculare/M. furfur* (ATCC 42132). Apart from the 56 sera in which sensitization to all 3 antigen sources was demonstrated, specific antibodies to *P. orbiculare/M. furfur* were detected in another 14 cases. This result can be explained by biochemical characterization of the strain ATCC 42132 which is the basis of the commercial test. Currently available methods (16–18) show that the strain ATCC 42132 named *P. orbiculare/M. furfur* can be classified as *M. sympodialis*. Therefore, a test system based on *M. sympodialis* is more suitable for screening for immediate-type sensitization to *Malassezia* yeasts than a system based on *M. furfur*, all the more so as the former is more frequently observed on human skin than the latter (3). However, no final recommendation can be made because of the absence of test systems and data for the other recently described species (*M. globosa*, *M. obtusa*, *M. restricta* and *M. slooffiae*). To assess their actual importance, epidemiological details for these *Malassezia* species on healthy and diseased skin are required.

Although *Malassezia* species belong to the resident skin flora and are normally found there after puberty (4), it appears clinically remarkable that none of the 50 persons with clinically healthy skin had demonstrable IgE antibodies to these yeasts, even though 33 had elevated levels for serum IgE and 15 had a positive SX1 test as a marker for atopic constitution. However, in diseases where *Malassezia* yeasts play a pathogenetic role and are found in higher numbers on the skin, such as PV or SE, sensitization was found either rarely (SE, 12%) or not at all (PV, 83 patients) despite occasionally very high levels of total IgE. It appears that other mechanisms are involved in the pathogenesis of these diseases (4, 22, 24–26). Among the groups selected, patients with AD were most frequently sensitized (78/223; 34%).

However, allergic/atopic disposition is not necessarily a prerequisite for sensitization. Of the 39 patients with HA only 2 were weakly sensitized (8%), although the SX1 test indicating atopic disposition was positive nearly as often as in the group of atopics (72% vs. 78%). Moreover, the frequency of sensitization in AD was markedly correlated with the location of the disease, especially in those with head/neck/face location ( $p < 0.05$ ). As *Malassezia* yeasts occur primarily in these body areas, they might be a trigger factor. It is possible that skin colonization with *Pityrosporum/Malassezia* yeasts leads to ongoing immunostimulation in patients with AD (27–29). Increased presentation of the antigen is thought to result from inflammatory processes in these areas, augmented by chronic itching and scratching and followed by elevated IgE production of specific antibodies (5, 12). This sensitization might influence the further course of the disease, particularly as *in vitro* studies have shown that extracts of *P. ovale* can markedly increase IgE synthesis and release of interleukin-4 and interleukin-10 in patients with AD (12, 26). Furthermore, atopics showed specific IgE antibodies to *Pityrosporum/Malassezia* significantly more often than other patients, while total IgE levels were elevated ( $p < 0.05$ ). This connection was also noted in previous studies (8–11, 14). According to Waerstedt & Hjorth (6), unspecific sensitization as an expression of general hyperreagibility is less likely in view of the finding that patients with respiratory allergy are seldom sensitized to *Malassezia* antigens. Also, patients with a head/neck disease location had more frequently positive results (10, 12, 14).

In children, sensitization to *Malassezia* yeasts was correlated with increased activity of AD and more nocturnal itching (13). In particular, the effective antimycotic treatment of lesions in the head/neck region is an argument in favor of the pathogenetic importance of these yeasts (15, 30). However, these findings could not be confirmed in all studies (31).

Interesting results were also obtained by determining the antibody-binding epitopes of *Malassezia* yeasts. Both qualitative and quantitative agreements between the RAST and immunoblotting were good. In our study, the following epitopes with a high binding frequency were detected: proteins with molecular weights of 15, 25, 27, 43, 58, 92, 99 and 107 kDa for *M. furfur* CBS 1878 and 15, 22, 30, 37, 40, 58, 79, 92, 99 and 124 kDa for *M. sympodialis* CBS 7222. The demonstrated epitopes are not consistent with the binding sites reported in the literature (29, 32–34), but again a question arises concerning the identity of the species. In *P. orbiculare* Zargari et al. (32) detected IgE-binding proteins with molecular weights of 37 and 67 kDa. Johanssen & Karlström (34) demonstrated proteins with molecular weights of 86, 76, 67, 28, 17 and 13 kDa by means of an immunoblot technique and sodium dodecyl sulfate-polyacrylamide gel electrophoresis, 67 kDa being the major antigen determinant. Lintu et al. (33) emphasized particularly the importance of the 9, 20 and 96 kDa binding regions.

In summary, it was shown that patients with manifest AD are more often sensitized to *Malassezia* yeasts than other patient groups and healthy persons. Furthermore, it was confirmed that patients with a head/neck disease location are most frequently sensitized to *Malassezia* yeasts. The immunoblot results are indicative of a variety of group antigens between *M. furfur* and *M. sympodialis*, but antigen material

obtained from *M. sympodialis* is most suitable for screening tests. Based on these findings, extended examinations of prick, intracutaneous and atopy patch tests could help to distinguish a group of atopics who might benefit from antimycotic therapy.

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