

Antigenic components of *Malassezia* species for immunoglobulin E antibodies in sera of patients with atopic dermatitis

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Abstract

Antigenic components of *Malassezia furfur*, *M. globosa*, *M. restricta*, *M. slooffiae*, and *M. sympodialis* were studied for immunoglobulin E antibodies in sera of patients with atopic dermatitis (AD). Antigenic components were extracted from *Malassezia* cells by treatment with β -mercaptoethanol, referred to as 2-ME extract. CBB staining and lectin blots using Con A, LCA, PHA-E4, PNA or RCA120 showed that the 2-ME extracts contained several species-dependent components that differed quantitatively and qualitatively among the *Malassezia* species at the protein level. In the Western blot with the 2-ME extracts, of 54 sera of the patients with AD (54 patients), the patients' IgE antibodies most frequently recognized components showing molecular weights of 43–46 kDa for *M. slooffiae*, 12–22 kDa for *M. sympodialis*, 35–40 kDa for *M. restricta*, 45–50 kDa for *M. globosa*, and of 67–72 kDa for *M. furfur*, respectively. In the correlative study, in which the total band intensities generated for each extract in Western blot were compared among the *Malassezia* species, the intensity for *M. globosa* was well correlated with that for *M. sympodialis* ($r = 0.756$). In the Western blot inhibition test, the 2-ME extract of *M. globosa* partially inhibited the reaction of the antigenic components of other *Malassezia* species with the patient's IgE antibodies. These results indicated that *Malassezia* species contained both species-specific and common antigenic components at the IgE antibody level. © 2001 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Atopic dermatitis; IgE antibodies; *Malassezia* species; Western blot

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1. Introduction

The lipophilic fungus *Malassezia furfur* (also called *Pityrosporum orbiculare* and *P. ovale*) is often isolated as a common commensal from the surface of healthy human skin [1]. Recently, the genus *Malassezia* was classified into seven species, *M. sympodialis*, *M. globosa*, *M. restricta*, *M. slooffiae*, *M. furfur*, *M. obtusa*, and *M. pachydermatis* based on their morphological, biochemical, and genetic characteristics [2–4].

Immunoglobulin E (IgE) antibodies for *M. furfur* are frequently found in patients with atopic dermatitis (AD), which is a chronic allergic disease of the skin [5,6]. In addition, the results of prick tests [7], histamine release assay [7,8], patch tests [9] and responses to antimycotic treatment [10,11] in patients with AD have indicated that *M. furfur* is involved in the pathogenesis of AD. Therefore, many studies on the fungal allergens have been focused on *M. furfur*, and several antigenic components of this fungus have been reported as possible major allergens in AD [8,12–15]. However, these studies have been performed under the old classification of *Malassezia* species. Recently, we isolated five *Malassezia* species from patients and identified them based on the new criteria for classification [2,3], and found that IgE antibodies for *M. globosa* and *M. sympodialis* were the species most frequently detected in sera of patients with AD [16]. Furthermore, we isolated the 46 kDa mannoprotein from *M. globosa* and reported that this molecule is a major antigenic component of this fungus for IgE antibodies in patients with AD [17]. However, the major antigenic components of the other species of *Malassezia* have not been clarified.

In this study, to study the major antigenic components of the other *Malassezia* species, *M. restricta*, *M. sympodialis*, *M. furfur* and *M. slooffiae*, we extracted the antigenic components from these species and compared their antigenicity for IgE antibodies in sera of patients with AD.

2. Materials and methods

2.1. Microorganisms, culture conditions and antigen preparation

Three strains of *M. furfur* (NUM 6014, NUM 6030, NUM 6074), two strains of *M. sympodialis* (NUM 6004, NUM 6013), two strains of *M. restricta* (NUM 6005, NUM 6071), three strains of *M. globosa* (NUM 6006, NUM 6061, NUM 6062) and *M. slooffiae* NUM 6003, which were clinically isolated, were used in this study. *M. furfur*, *M. slooffiae* and *M. sympodialis* were cultured in GYEP broth (2% glucose, 0.3% yeast extract, 1% peptone) containing 1% olive oil for 7 days at 30°C under aeration by shaking at 100 strokes per min or on potato dextrose agar (PDA) containing 0.3% yeast extract and 1% peptone (YEP) plate for 7 days at 30°C in the presence of olive oil. *M. restricta* and *M. globosa* were inoculated onto PDA-YEP plates and allowed to grow for 7 days at 30°C in the presence of olive oil.

For antigen preparation, the *Malassezia* cells grown were washed three times with distilled water and treated with 300 mM β -mercaptoethanol (2-ME) in 100 mM ethylenediamine tetraacetate (EDTA) (pH 9.0) for 60 min at 20–25°C [18]. The resultant supernatants were dialyzed against distilled water for 48 h at 4–6°C. Dialysates were lyophilized and were referred to as 2-ME extract. The protein content of each 2-ME extract was measured by a Bio-Rad protein assay (Bio-Rad Lab. Richmond, CA) and was adjusted to 400 μ g/ml. Bovine serum albumin (BSA) was used as a standard.

2.2. Patients' sera

Sera from 54 patients with atopic dermatitis AD (29 male and 25 female aged 13–61 years), who were diagnosed based on the "Japanese Dermatological Association Criteria for the diagnosis of atopic dermatitis" [19], were used in this study. Most of the patients with AD were treated with topical corticosteroid ointments and/or oral antihistamine drugs. None of the patients with AD received systemic corticosteroid or anti-fungal agent therapy. For a control study, sera from four

patients with seborrheic dermatitis (SD) and from two patients with chronic urticaria (CU) were used. None of them received systemic corticosteroid or anti-fungal agent therapy. All sera were stored at -80°C before use.

2.3. SDS-PAGE and lectin blots

Ten microliters of the 2-ME extract (400 μg of protein/ml) were loaded on a 10% polyacrylamide gel. The gel was stained with Coomassie Brilliant Blue R (CBB) (Sigma Chemical Co., St. Louis, MO) for proteins [20].

For the lectin blots, horse-radish peroxidase (HRP)-conjugated concanavalin A (Con A) (from *Canavalia ensiformis*, specific for α -D-Man and α -D-Glc), lentil lectin (LCA) (from *Lens culinaris*, specific for α -D-Man and α -D-Glc), phytohemagglutinin (PHA-E4) (from *Phaseolus vulgaris*, specific for GlcNAc), peanut lectin (PNA) (from *Arachis hypogaea*, specific for Gal β 1-4GlcNAc), castor-bean lectin (RCA120) (from *Ricinus communis*, specific for Gal β 1-4GlcNAc), or wheat-germ agglutinin (WGA) (from *Triticum vulgaris*, specific for GlcNAc β 1-4Man β 1-4GlcNAc β 1-4GlcNAc) (Honen Co., Tokyo, Japan) was used to define carbohydrate chains in the molecules. The lectin blots were made using the method described previously [20].

2.4. Western blots and Western blot inhibition tests

A Western blot was performed to determine the antigenic components of the *Malassezia* species for IgE antibodies in sera of the patients with AD. The procedures of the Western blot were essentially the same as that described by Koyama et al. [17].

For Western blot inhibition tests, a patient's serum that reacted with all *Malassezia* species was diluted 1:20 in 15 mM Tris-HCl buffer containing 0.5 N NaCl (TBS) buffer that contained 1% skim milk, and preincubated with various concentrations of the 2-ME extract of *M. globosa* (0, 50, 100, 250, 500 μg of protein/ml) for 2 h at 30°C . The treated serum was added to the polyvinylidene difluoride (PVDF) (Millipore, Bedford, MA)

sheet that was electroblotted with antigenic components of each *Malassezia* species; or, the patient's serum was preincubated with each of the 2-ME extracts of the *Malassezia* species (100 μg of protein/ml each) for 2 h at 30°C , and the preincubated serum was then added to the PVDF sheet, which was electroblotted with the 2-ME extract of *M. globosa*. The sheets were incubated overnight at $4-6^{\circ}\text{C}$ followed by incubation in a mixture of Nitroblue Tetrazolium (NBT) and 5-bromo-4 chloro-3 incoyl phosphate (BCIP), as described previously [17].

2.5. Band intensities and assessment of major antigenic components

To determine the degree of reaction of antigenic components of *Malassezia* with IgE antibodies, the intensities (arbitrary units) of each band reacted with IgE antibodies were measured with a Digital Image System IS 1000 instrument (Alpha Innotech Co., San Leandro, CA). The total intensity of the bands was recorded as the total intensity for each *Malassezia* species [20].

For assessment of the major antigenic components in each of the *Malassezia* species, the approximate molecular weights of the five most intense bands in each *Malassezia* species were measured in all serum samples.

3. Results

3.1. Cell components of *Malassezia* species

To compare the protein components of the *Malassezia* species, the 2-ME extracts were loaded on a polyacrylamide gel, and the gel was stained with CBB. The 2-ME extracts contained several proteins that showed different staining profiles among the species of *Malassezia* (Fig. 1A). The c. 68 kDa band was commonly seen in all extracts except *M. restricta* (Fig. 1A, arrow). Similar results were found in 2-ME extracts that were prepared from *M. furfur*, *M. slooffiae* and *M. sympodialis* grown on PDA-YEP plates (data not shown).

The 2-ME extracts were characterized by lectin blot, in which HRP-conjugated Con A, LCA, PHA-E4, WGA, or PNA was reacted with PVDF sheets electroblotted with the 2-ME extract. Most of the CBB-stained bands reacted with Con A (Fig. 1B) and a similar result was obtained when LCA was used in a lectin blot (data not shown). In contrast, PHA-E4 reacted only with the 55 kDa component of the 2-ME extract from *M. sympodialis* (Fig. 1C), and WGA reacted only with the 45 kDa bands of *M. slooffiae* (Fig. 1D). Neither PNA nor RCA 120 reacted with any components of any of the *Malassezia* species (data not shown).

3.2. Antigenic components of *Malassezia* species for IgE antibodies

Western blot was used to study the antigenic components of the *Malassezia* species for IgE antibodies in sera of the patients with AD. The

Western blot showed that each of the 2-ME extracts contained several antigenic components for IgE antibodies, which were apparently species-dependent (Fig. 2). In all species, no differences were observed among the same species on the IgE-Western blot (data not shown). In control experiments to test the specificities of the positive bands generated for each *Malassezia* antigenic component, no binding of IgE antibodies to the antigen fractions was observed with the secondary antibody only or with sera from any of the patients with SD or CU (data not shown). Therefore, the positive bands generated for each *Malassezia* species were specific to the *Malassezia* antigens and sera from AD patients.

Of 54 patients' sera, 45 (83%) reacted simultaneously with one or more species of *Malassezia*. Furthermore, 27 (50%), 40 (74%), 30 (56%), 45 (83%) and 35 (65%) reacted with the extracts of *M. slooffiae*, *M. sympodialis*, *M. restricta*, *M. globosa*, and *M. furfur*, respectively.

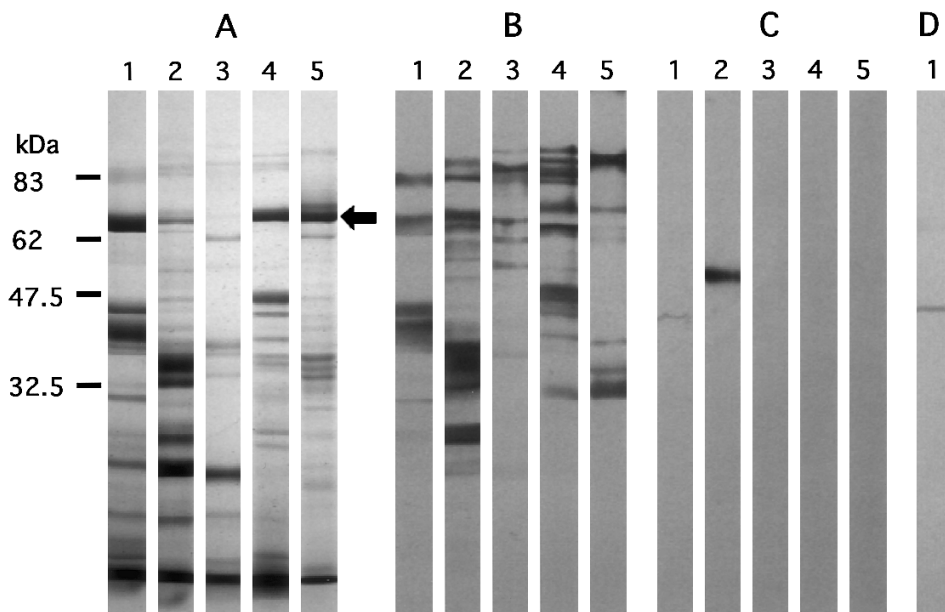


Fig. 1. CBB staining and lectin blot profiles of the 2-ME extract of *Malassezia* species. Cell components in the extract were characterized by CBB stain (A) and lectin blots using HRP-Con A (B), HRP-PHA (C), or HRP-WGA (D). The 2-ME extracts contain species-dependent components. A c. 68 kDa band was commonly seen among *Malassezia* species (arrow). The mobilities of the molecular mass markers (kDa) are indicated on the left. Lane 1, *M. slooffiae*; lane 2, *M. sympodialis*; lane 3, *M. restricta*; lane 4, *M. globosa*; lane 5, *M. furfur*.

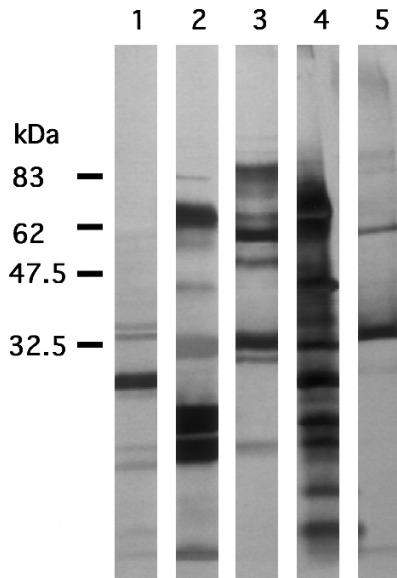


Fig. 2. An example of Western blot profiles of the 2-ME extracts for IgE antibodies in serum of an AD patient. The 2-ME extracts contain several antigenic components for IgE antibodies, which are apparently species-dependent. The mobilities of the molecular mass markers (in kilodaltons) are indicated on the left. Lane 1, *M. slooffiae*; lane 2, *M. sympodialis*; lane 3, *M. restricta*; lane 4, *M. globosa*; lane 5, *M. furfur*.

3.3. Distribution of antigenic components of *Malassezia* species for IgE antibodies

To define the possible major antigenic components of each of the *Malassezia* species for IgE antibodies in patients with AD, the molecular masses of the five most intense bands were recorded for all of the positive sera. The profiles of the major antigens based on the molecular masses of the bands were apparently species-dependent (Fig. 3). The molecular masses of the bands that were most frequently recognized by the patients' IgE antibodies were 43–46 kDa ($n = 11$) for *M. slooffiae*, 19–22 kDa ($n = 23$) for *M. sympodialis*, 35–40 kDa ($n = 21$) for *M. restricta*, 45–50 kDa ($n = 43$) for *M. globosa*, and 67–72 kDa ($n = 15$) for *M. furfur*, respectively (Fig. 3).

3.4. Cross-reactivity of *M. globosa* with other *Malassezia* species

In the correlation study, the total intensities

generated for each species of *Malassezia* in the Western blot were compared among the *Malassezia* species. A good correlation was obtained between *M. globosa* and other *Malassezia* species except *M. furfur*, and the highest correlation was found with *M. sympodialis* ($r = 0.756$) (Table 1).

To examine the presence of *M. globosa*-specific antigenic components or components cross-reacting

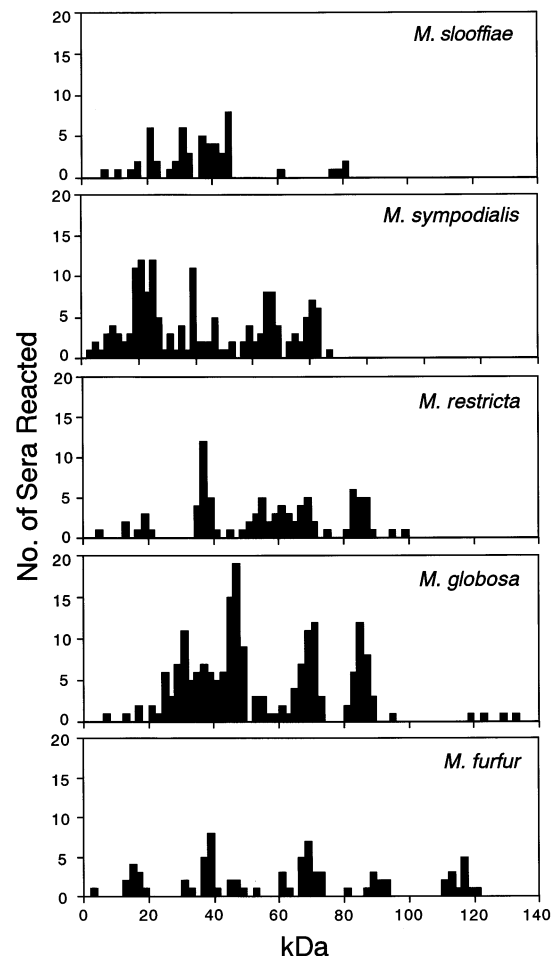


Fig. 3. Molecular size distribution of possible major antigenic components of *Malassezia* species to IgE antibodies of the patients with AD. *Malassezia* species contain several possible major antigenic components for IgE antibodies, whose molecular sizes are variable among the species. The bands with the five highest intensities were recorded for all positive sera in the Western blots, and their molecular masses were plotted.

Table 1

Correlation among the *Malassezia* species based on total intensities of the positive bands for IgE antibodies^a

	<i>M. slooffiae</i>	<i>M. sympodialis</i>	<i>M. restricta</i>	<i>M. globosa</i>	<i>M. furfur</i>
<i>M. slooffiae</i>	1.000				
<i>M. sympodialis</i>	0.643	1.000			
<i>M. restricta</i>	0.612	0.628	1.000		
<i>M. globosa</i>	0.598	0.756	0.587	1.000	
<i>M. furfur</i>	0.273	0.291	0.279	0.369	1.000

^a Total intensities of the bands generated for each of the *Malassezia* species in the Western blot by a Digital Image System IS 1000 instrument (see Section 2). The total intensities for the *Malassezia* species were recorded for all of the positive sera and analyzed for correlations among the *Malassezia* species.

tive with other species, Western blot inhibition tests were done. When a patient's serum was preincubated with various concentrations of the 2-ME extract of *M. globosa*, the subsequent reaction of IgE antibodies to *M. globosa* was inhibited dose-dependently (data not shown) and completely inhibited at 500 µg of protein/ml of the extract (Fig. 4B, lane 4). The reaction of IgE antibodies to *M. slooffiae*, *M. sympodialis*, and *M. restricta* was partially inhibited at 500 µg of protein/ml (Fig. 4B, lanes 1–3) but the reaction to *M. furfur* was not inhibited (Fig. 4B, lane 5). When a patient's serum was preincubated with each of the 2-ME extracts of the *Malassezia* species (100 µg of protein/ml), the subsequent reaction of IgE antibodies to the extract of *M. globosa* was strongly inhibited only when the extract of *M. globosa* was used in the preincubation (data not shown).

4. Discussion

In this study, we demonstrated the species variation of the major antigenic components of *M. slooffiae*, *M. sympodialis*, *M. restricta*, *M. globosa*, and *M. furfur* for IgE antibodies in the sera of patients with AD.

In the CBB stain, the 2-ME extracts contained several components, most of which showed different mobilities depending on the *Malassezia* species. Similar results were obtained when the 2-ME extracts prepared from cells grown under different culture condition (see Section 2) were used (data not shown). Furthermore, in the lectin blot tests,

PHA reacted only with the extract of *M. sympodialis*, and WGA reacted only with the extract of *M. slooffiae*. Similar results were found in the preliminary experiment in which the CBB staining patterns of the 2-ME extracts of *Malassezia* species were compared with cell extracts prepared by

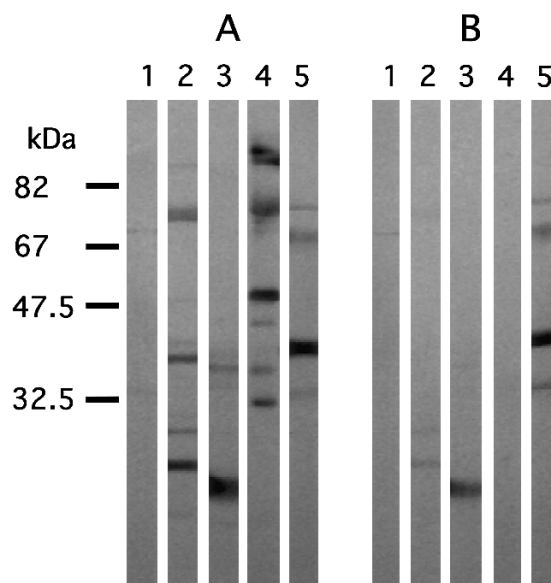


Fig. 4. Cross-reactivity of *M. globosa* with other *Malassezia* species by Western blot inhibition tests. (A) indicates the Western blot profile of the 2-ME extracts of *Malassezia* species with a patient's serum. (B) indicates the result of the Western blot profiles of the 2-ME extracts of *Malassezia* species with the patient's serum that was pretreated with the 2-ME extract of *M. globosa* (500 µg/ml). The mobilities of the molecular mass markers (kDa) are indicated on the left. Lane 1, *M. slooffiae*; lane 2, *M. sympodialis*; lane 3, *M. restricta*; lane 4, *M. globosa*; lane 5, *M. furfur*.

heating for 10 min at 100°C (data not shown). Thus, these results indicated the presence of species-dependent components among the *Malassezia* species. Previously, we reported that the 46 kDa mannoprotein, which may be a major component of *M. globosa*, was specifically expressed in this species [17]. Zargari et al. [21] reported that prolonged culture periods decreased the antigenic components of *M. furfur*, suggesting that the loss of antigenicities depended on quantitative changes of the antigenic components, not on qualitative changes. In this study, the proteins in each extract were adjusted to the same concentration, which would exclude the influence of the quantitative loss of the antigenic components.

Studies concerning the major allergens of *Malassezia* have focused on *M. furfur*, and several possible allergens have been reported [12–15]. In the present study, the 37–42 and 67–72 kDa components reacted the most frequently with the patients' sera, and these components showed similar sizes to those reported by Huang et al. [14]. However, the molecular masses of the possible major allergens obtained in the present study were not completely in agreement with those of previous reports. This is because previous studies on the allergenic components of *M. furfur* were not based on the new classification for *Malassezia* species, so they might reflect those of the other *Malassezia* species or differences in the preparation protocol. The distribution of the molecular sizes of the possible major antigenic components that reacted with IgE antibodies in the sera of the patients showed a species dependency (Fig. 3). This suggests the presence of species-dependent major antigenic components for IgE antibodies at the protein level. We reported that the 46 kDa mannoprotein of *M. globosa*, a species-specific component, reacted the most frequently with IgE antibodies of patients with AD [17]. Lindborg et al. [22] cloned five possible allergens of *M. furfur* by a phage-display technique. We believe that molecular-cloning techniques can yield the sizes of allergens and illuminate details of the major allergens of *Malassezia* species.

In the IgE-Western blot using the 2-ME extracts, the patients' sera reacted the most frequently with the 2-ME extract of *M. globosa*,

followed by *M. sympodialis*, and most of the patients' sera that were positive for both *Malassezia* species reacted simultaneously with the extracts of other *Malassezia* species. These findings suggested two possibilities: the patients with AD have been sensitized independently with each of the *Malassezia* species or with antigenic components common among the *Malassezia* species. Nakabayashi et al. reported that *M. globosa* was isolated most frequently from both normal subjects and patients with AD [23], which can suggest that AD patients have many opportunities to be sensitized by *M. globosa*. In this study, however, the Western blot inhibition tests showed the presence of antigenic components common among the *Malassezia* species except *M. furfur*. Thus, neither possibility can be excluded. The identification of species-specific or common major antigenic components of *Malassezia* species is now under investigation.

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