

Immediate hypersensitivity to *Malassezia furfur* in patients with atopic dermatitis

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Summary

Atopic dermatitis (AD) is a chronic pruritic dermatitis that has unknown aetiology. It seems that *Malassezia furfur* has a role in pathogenesis of AD. The purpose of this study was to evaluate skin responses to *M. furfur* antigens in AD patients. *Malassezia furfur* was grown and the yeasts were broken. Cells were centrifuged and supernatants were used as crude extracts (CE). Protein components of CE were separated by sodium dodecylsulphate–polyacrylamide gel electrophoresis (SDS–PAGE). In addition, to fractionate CE antigens, gel filtration chromatography was performed. One hundred and fifteen AD patients were selected for skin-prick test (SPT). In SDS–PAGE, CE showed a total of 19 different protein bands (10–100 kDa). Chromatographic gel filtration with *M. furfur* proteins showed four major fractions (F). The protein pattern of F₁ (tube no. 40) was between 22 and 100 kDa and it was selected for SPT. In SPT, 49.6% and 42.6% patients showed positive reactions with CE and F₁ antigens respectively. The most positive results were obtained in 20–29 aged group ($P < 0.001$). The allergens of *M. furfur* may have a role in AD signs; it is suggested to use F₁ antigens in allergy tests.

Key word: *Malassezia furfur*, allergens, atopic dermatitis, skin-prick test.

Introduction

Atopic dermatitis (AD) is a chronic multifactorial inflammatory skin disease characterised by a chronically relapsing course, and severe pruritis which has increased during the past few decades.^{1–3} The pathogenesis is still not fully understood. As approximately 80% of patients with AD have elevated serum immunoglobulin (Ig)E and skin-prick test (SPT) reactions to a wide variety of allergens, it is assumed that allergens are involved in maintenance of the disease.^{4,5} 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.³ The genus *Malassezia* comprises

yeasts that are natural habitants of the human skin and warm-blooded animals.⁶ *Malassezia* species are associated with several cutaneous and systemic diseases, either as the causative agent of these conditions or associated with lesions. Diseases caused by *Malassezia* species include pityriasis versicolor, seborrheic dermatitis, dandruff, folliculitis, atopic dermatitis, confluent reticulate papillomatosis and seborrheic blepharitis.^{7–9} *Malassezia* species have also been shown to cause more deep-seated infections, including mastitis, sinusitis, septic arthritis, malignant otitis externa, fungaemia, pulmonary vasculitis, peritonitis and meningitis.^{10–12} The yeast *Malassezia furfur*, earlier denoted *Pityrosporum ovale*, is considered to be one of the triggering allergens that can contribute to AD.⁷ A number of IgE-binding components have been found in *Malassezia* species.^{13–15} Patients usually have an increased sensitisation to *Malassezia* species, via both the cellular and humoral immune responses. The cellular responses tend to be of Th₂ type, with higher levels of interleukin (IL)₄ and IL₅ production by the T cells of patients than of healthy

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controls. These cytokines are associated with IgE-mediated reactions and so may drive the continued production of IgE.^{16,17} The aim of this study was to evaluate skin responses to *M. furfur* antigens [crude extract (CE) and F₁ antigens] in AD patients.

Patients and methods

Yeast strain, culture condition and CE preparation

Malassezia furfur (ATCC 44344) was cultured at 30 °C for 4 days on a special glucose–neopepton agar containing olive oil (2%), monostearate (2.5 g l⁻¹) and Tween 80 (0.2%) as described by Faergemann & Fredriksson [18]. The *M. furfur* cells were harvested from the surface of the medium. After freeze-drying, 5 g of the yeasts was suspended in 10 ml of 0.05 mol l⁻¹ phosphate-buffered saline (PBS), pH 7.4. Extraction was obtained using glass beads (diameter, 1 mm) on a vortex mixer for 5 min. The suspension containing disrupted organisms was then centrifuged in 25 000 *g* for 1 h and 30 min and the supernatant was isolated and dialysed overnight at 4 °C against distilled water (cutoff = 12 kDa, Sigma, Poole, UK). Finally, CE was lyophilised and stored at -20 °C. In this study, all general chemical materials were purchased from Merk Co. (Tehran, Iran).

Gel filtration chromatography

In order to fractionate CE antigens, gel filtration chromatography was performed using Sephadex G-100 (Sigma, St Louis, MO, USA). The sample (2 ml of CE containing 30 mg proteins) was injected onto the Sephadex G-100 column (100 × 1.6 cm; Bio-Rad Laboratories, Hercules, CA, USA). The material was eluted with 0.15 mol l⁻¹ PBS, pH 7.2, at a flow rate of 6 ml h⁻¹ and a total of 135 tubes containing 2 ml of elution was collected. Subsequently, the optical density of each fraction in tube was read at 280 nm.

Determination of protein

Protein concentration of lyophilised sample was determined after reconstituting in 50 and 100 µl of distilled water by Bradford method.¹⁹

Patients and control

A total of 115 patients with AD were enrolled in this study. The diagnosis of AD was based on the criteria of Hanfin & Rajka [20] which was established by

dermatologists from the Department of Dermatology, Imam Khomeini Hospital. Patients were 81 females and 34 males. Age varied from 7 to 69 years old (mean age 27.6 years). In addition, 55 other healthy controls with no personal history of skin or allergic diseases were run in experiments.

Sodium dodecylsulphate–polyacrylamide gel electrophoresis

Components of CE antigens and different protein fractions were separated according to molecular weight in a 16% separation gel with a 5% stacking gel in a discontinuous buffer system according to the method of Laemmli [21]. The extracts were boiled for 5 min with a reducing sample buffer (containing 2-mercaptoethanol) and were run at a concentration of 25 µg ml⁻¹ of freeze-dried extracts per mm gel. Molecular weight standard markers (Rainbow markers, 14.3–97.4 kDa; Amersham International PLC, Aylesbury, UK) were loaded on each gel to facilitate comparison of the bands obtained. The gels were stained with the brilliant blue staining method.

Skin-prick test

Crude extract and F₁ antigens of *M. furfur* were diluted with 0.05 mol l⁻¹ PBS buffer, pH 7.4, and containing 50% glycerin and then sterilised by means of filter (0.2 µm; Sartorius, Westbury, NY, USA). Skin-prick tests were performed with 3 × 10⁻⁶ ml of CE and F₁ antigens of *M. furfur* (protein concentration, 20 µg ml⁻¹) on the volar aspect of the forearm and pricked with a 27 gauge needle. A negative control solution containing 0.05 mol l⁻¹ PBS buffer, pH 7.4, with 50% glycerin and a positive control solution containing 1% histamine were applied in the same way. The tests were read after 15 min and any reaction with a mean wheal diameter at least 3 mm greater than negative control was taken as positive.

Statistical analysis

Data were analysed by chi-square and multiple logistic regression tests. A *P* < 0.05 was considered significant.

Results

Gel filtration chromatography

A total of four major fractions were obtained from *M. furfur* extract [F₁ (tube no. 40), F₂ (tube no. 53), F₃ (tube no. 74), F₄ (tube no. 102)]. The protein content of

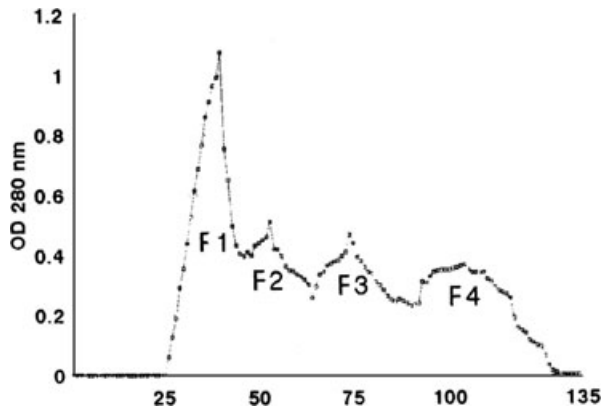


Figure 1 Gel filtration chromatogram of crude extract of *Malassezia furfur*.

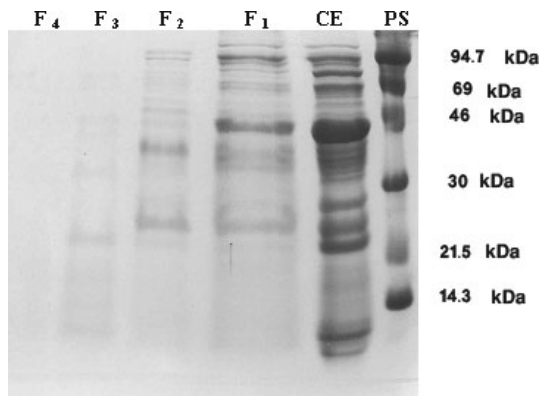


Figure 2 Protein components of crude extract of *Malassezia furfur* by sodium dodecylsulphate–polyacrylamide gel electrophoresis; *S, standard.

F₁, F₂, F₃, F₄ fractions, measured by means of Bradford method, were 1.35, 0.9 and 0.55 mg ml⁻¹, respectively, whereas the protein content of F₄ fraction was undetectable (Fig. 1).

Sodium dodecylsulphate–polyacrylamide gel electrophoresis test

In sodium dodecylsulphate–polyacrylamide gel electrophoresis (SDS–PAGE), CE showed a total of 19 different protein bands (10–100 kDa). The protein pattern of F₁, based on molecular weight, was between 22 and 100 kDa. As F₁ lacked non-specific proteins, it was therefore selected for SPT (Fig. 2).

Skin-prick test

Of 115 patients with AD, 57 (49.6%) and 49 (42.6%) patients had positive reactions against CE and F₁

Table 1 Comparison of positive prick test results with CE and F₁ of *Malassezia furfur* with several factors observed in patients with atopic dermatitis.

Factors	No. (%) positive results	
	CE	F ₁
Sex		
Female	38/81 (46.9)	31/81 (38.3)
Male	19/34 (55.9)	18/34 (52.9)
Age		
≤9	1/6 (16.7)	1/6 (16.7)
10–19	6/20 (30.0)	5/20 (25.0)
20–29	33/42 (78.6)	28/42 (66.7)
30–39	12/28 (42.9)	11/28 (39.3)
40–49	3/13 (23.1)	3/13 (23.1)
≥50	2/6 (33.3)	1/6 (16.7)
History of familial AD		
Present	36/72 (50.0)	31/72 (43.1)
Absent	21/43 (48.8)	18/43 (41.9)
Time of starting of AD signs		
Infantile	12/19 (63.2)	12/19 (63.2)
More than 1 year	31/64 (48.4)	24/64 (37.5)
Less than 1 year	14/32 (43.8)	13/32 (40.6)
Association with other atopic diseases		
Patients with other atopic diseases	12/29 (41.4)	8/29 (27.6)
Patients without other atopic diseases	45/86 (52.3)	41/86 (47.6)

CE, crude extract; F₁, fraction 1 (tube no. 40); AD, atopic dermatitis.

antigens of *M. furfur*, respectively, whereas in healthy control only one subject (6.7%) had positive reaction to both antigens (CE and F₁). Positive SPT to *M. furfur* antigens were mostly observed in patients between 20 and 29 years old. Significant differences were observed in 20–29 aged group with other groups ($P < 0.001$) in both CE and F₁ antigens. There were no significant differences in SPT reactions to CE and F₁ antigens between patients with respect to sex, familial history, time of starting of AD signs, and various atopic diseases (Table 1).

Discussion

Atopic dermatitis is a chronic, itching, inflammatory skin disease which is associated with asthma and/or hay fever and a familial occurrence of these conditions.²⁰ *Malassezia* species are members of the normal human cutaneous flora, and a defect in the skin barrier may facilitate contact of these yeasts with the immune system.⁷ The goal of our study was to evaluate *M. furfur* antigens on SPT reactions of patients with AD. SDS–PAGE test showed that CE contained proteins in molecular weight range of 10–100 kDa. In general, the results of this study and other studies^{22–24} show

little differences in molecular weights of proteins extracted from *M. furfur* that it is probably related to the strain of *Malassezia* used, disruption methods, length of the culture period, and using standard proteins and stability of the extracts is also influenced by the storage period and conditions. In gel filtration chromatography, four major fractions were obtained. The protein pattern of F₁ was between 22 and 100 kDa which presented in CE, whereas it had no protein components less than 22 kDa. This is the first study in which purified antigens (F₁) along with CE of *M. furfur* were used in SPT. In this study, 49.6% and 42.6% patients with AD showed positive reactions against CE and F₁ antigens respectively. Young *et al.* demonstrated that 59% of patients with AD reacted positively to *M. furfur* CE.²⁵ Moreover, Rokugo [26] showed that SPT reactions to CE were positive in 54% of patients with AD. The relationship between several background factors of patients with AD and the results of the SPT with the CE and F₁ antigens of *M. furfur* were investigated. As shown in Table 1, sex, history of infantile seborrheic eczema, or association with other atopic diseases had no significant influence on the SPT results, whereas significant difference was observed in 20–29 aged subjects with other groups ($P < 0.001$) using CE and F₁ antigens, which is similar to the findings of Young [25] and Kieffer *et al.* [12] findings. As the frequency and density of colonisation of individuals are related to age and sebaceous glands activity, with respect to increased activity of the glands and concentration of skin lipids during adolescence and postpuberty,¹¹ the colonisation of *M. furfur* most commonly occurs in this period. These results show that *M. furfur* can play a role in eliciting and maintaining allergic reactions in patients with AD. As F₁ antigens, in comparison with CE, have the most allergenic components, it is suggested to use F₁ antigens in allergic tests.

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References

- 1 Rajka G. *Essential Aspects of Atopic Dermatitis*. Berlin: Springer-Verlag, 1989: 172–7.
- 2 Isaac A. The international study of asthma and allergies in childhood steering committee: world wide variation in prevalence of symptoms of asthma, allergic rhinoconjunctivitis, and atopic eczema. *Lancet* 1998; **351**: 1225–32.
- 3 Wollenberg A, Biber T. Atopic dermatitis: from the genes to skin lesions. *Allergy* 2000; **55**: 205–13.
- 4 OÈhman S, Johansson SGO. Immunoglobulins in atopic dermatitis. *Acta Derm Venereol (Stockh)* 1974; **54**: 193–202.
- 5 Werfel T, Kapp A. Environmental and other major provocation factors in atopic dermatitis. *Allergy* 1998; **53**: 731–9.
- 6 Midgley G. The diversity of *Pityrosporum* (*Malassezia*) yeasts *in vivo* and *in vitro*. *Mycopathologia* 1989; **106**: 143–53.
- 7 Faergemann J. *Pityrosporum* species as a cause of allergy and infection. *Allergy* 1999; **54**: 413–9.
- 8 Faergemann J, Bergbrant IM, Dohse M, Scott A, Westgate G. Seborrheic dermatitis and *Pityrosporum* (*Malassezia*) folliculitis: characterization of inflammatory cells and mediators in the skin by immuno-histochemistry. *Br J Dermatol* 2001; **144**: 549–56.
- 9 Crespo Erchiga V, Ojeda Martos A, Vera Casano A, Crespo Erchiga A, Sanchez Fajardo F. *Malassezia globosa* as the causative agent of pityriasis versicolor. *Br J Dermatol* 2000; **143**: 799–803.
- 10 Gupta AK, Kohli Y, Faergemann J, Summerbell RC. Epidemiology of *Malassezia* yeasts associated with pityriasis versicolor in Ontario, Canada. *Med Mycol* 2001; **39**: 199–206.
- 11 Waersted A, Hjorth N. *Pityrosporum orbiculare* a pathogenic factor in atopic dermatitis of the face, scalp and neck. *Acta Derm Venereol (Stockh)* 1985; **114**: 146–148.
- 12 Kieffer M, Bergbrant IM, Faergemann J. Immune reactions to *Pityrosporum ovale* in adult patients with atopic and seborrheic dermatitis. *J Am Acad Dermatol* 1990; **22**: 739–42.
- 13 Lintu P, Savolainen J, Kalimo K. IgE antibodies to protein and mannan antigens of *Pityrosporum ovale* in atopic dermatitis patients. *Clin Exp Allergy* 1997; **27**: 87–95.
- 14 Zargari A, HaÈrfast B, Johansson S, Johansson SG, Scheynius A. Identification of allergen components of the opportunistic yeast *Pityrosporum orbiculare* by monoclonal antibodies. *Allergy* 1994; **49**: 50–56.
- 15 Tengvall Linder M, Johansson C, Bengtsson AÈ, Holm L, HaÈrfast B, Scheynius A. *Pityrosporum orbiculare* reactive T cell lines in atopic dermatitis patients and healthy individuals. *Scand J Immunol* 1998; **47**: 152–8.
- 16 Tengvall Linder M, Johansson C, Zargari A. Detection of *Pityrosporum orbiculare* reactive T cells from skin and blood in atopic dermatitis and characterisation of their cytokine profiles. *Clin Exp Allergy* 1996; **26**: 1286–97.
- 17 Savolainen J, Lintu P, Kosonen J. *Pityrosporum* and *Candida* specific and non-specific humoral, cellular and cytokine responses in atopic dermatitis patients. *Clin Exp Allergy* 2001; **31**: 125–34.
- 18 Faergmen J, Fredricksson T. Age incidence of *Malassezia furfur* on human skin. *Acta Derm Venereol* 1998; **60**: 531–3.
- 19 Bradford MM. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the

- principle of protein-dye binding. *Anal Biochem* 1976; **72**: 248–54.
- 20 Hanfin JM, Rajka G. Diagnostic features of atopic dermatitis. *Acta Derm Venereol* 1980; **92**: 44–47.
- 21 Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T₄. *Nature* 1970; **227**: 680–5.
- 22 Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* 1979; **76**: 4350–4.
- 23 Jensen-Jarolim E. Atopic dermatitis of the face, scalp, and neck. *J Allergy Clin Immunol* 1992; **89**: 44–51.
- 24 Johansson S, Karlstrom K. IgE binding components in *M. furfur* identified by an immunoblotting technique. *Acta Derm Venereol* 1991; **71**: 11–6.
- 25 Young E. Intracutaneous tests with *Pityrosporum* extract in atopic dermatitis. *Acta Derm Venereol* 1989; **144**: 122–4.
- 26 Rokugo M. Contact sensitivity to *P. ovale* in patients with atopic dermatitis. *Arch Dermatol* 1990; **126**: 627–32.