Allergen-Containing Drug from *Malassezia SPP.* Yeast

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Malassezia spp. yeast habituating the skin of healthy humans can be a source of allergens for patients with atopic dermatitis. We proposed a method for obtaining allergen-containing preparation by trimming outer cell wall proteins with 1% sodium dodecyl sulfate. The resultant preparation contained 36 and 67 kD proteins known as *Malassezia* allergens. IgE antibodies to these proteins were detected in the sera of young people with atopic dermatitis.

Key Words: *Malassezia; Pityrosporum; skin; allergen; atopic dermatitis; IgE antibodies*

Lipophilic *Malassezia* fungi (*Pityrosporum*) were previously considered as etiological agent of pityriasis versicolor [2]. Further studies showed that basidiomycete yeasts are present on the skin of healthy subjects and warm-blooded animals, predominantly the sites enriched with sebaceous glands. About 66% healthy humans, mainly aged 18-48 years, are *Malassezia* carriers (unpublished data). *Malassezia* carriers were detected among patients with atopic dermatitis (AD) in whom the skin is contaminated in younger age (about 10 years) and the percentage of carriers among patients (37%) is lower than among healthy subjects.

Protein allergens released by *M. furfur* and *M. sympodialis* induce the production of IgE antibodies in patients with AD [13]. These allergens are surface proteins of the yeast cells with molecular weights of 36 and 67 kD. DNA locus controlling the synthesis of these proteins was identified and the possibility of obtaining recombinant allergen proteins from *E. coli* was demonstrated.

According to modern views, sensitization to *Malassezia* is a diagnostic signs of AD in young adults [8], and therefore allergen-containing preparation from *Malassezia* will be helpful in clinical allergology. Molecular biological methods for obtaining this preparation are expensive, but classical physiological and biochemical methods of allergen isolation are available.

The purpose of this study was to prepare allergen-containing preparation from *Malassezia.*

MATERIALS AND METHODS

Of 32 *Malassezia spp.* cultures (collection of the I. I. Metchnikov Institute of Vaccines and Sera), a strain characterized by the most rapid growth in a wide range of temperatures was selected. This strain was previously identified by the J. Guillot and E. Gueho method [7] as *M. sympodialis,* as it possessed catalase activity, had characteristic sympodial buds, and grew on Tween-20, -40, -60, and -80 with the formation of typical clearly seen rings of colonies around the substrate well in a solid medium with Tween-20.

M. sympodialis were cultured in solid and liquid modified Dixon's medium [6], synthetic medium with asparagine [1], and semisynthetic medium containing bovine bile instead of sodium taurocholate.

The culture was centrifuged for 5 min at 5000 rpm, washed twice with distilled water, and cell precipitate was then incubated with 2-fold volume of isolating agent (w/v) for 5 or 30 min (Table 1). After extraction the suspension was again centrifuged for 5 min at 5000 rpm, the supernatant was filtered through a bacterial filter, and protein was measured by the Lowry method.

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The preparations were analyzed by gel electrophoresis in 10% PAAG with sodium dodecyl sulfate by the method of Laemmli modified by Gal'chenko [3]. The extracts were mixed 1:1 with buffer, heated at 100°C for 5 min, and applied to the gel (5-25 µl per row). A Sigma kit of molecular weight markers was used: myosin 205 kD, galactosidase 116 kD, phosphorylase b 97 kD, fructose-6-phosphate kinase 84 kD, albumin 66 kD, glutamate dehydrogenase 55 kD, ovalbumin 45 kD, and glyceraldehyde-3-phosphate dehydrogenase 36 kD. After electrophoresis the proteins were fixed in 50% trichloroacetic acid for 18 h and then stained with 0.01% Coumassi brilliant blue R-250. The gels were washed in 7% acetic acid until bleaching. Scanned images were analyzed using Adobe PhotoShop 5 software.

Allergen in the extract was detected by dot-blot analysis. To this end, concentrated extracts and negative control (Bovine serum albumin, 1 mg/ml) were applied to nitrocellulose (1 µl each) and dried overnight. Nitrocellulose was blocked for 1 h in buffered saline containing 10% fetal calf serum and incubated with serum samples from 10 patients with AD and normal human serum containing no IgE antibodies to fungal allergens (negative control) for 16 h at 18-20°C. Nitrocellulose was then treated as described elsewhere [12] with peroxidase-conjugated monoclonal antibodies to human IgE (IgE/11-1) [5,6] or Le 27 [9]; 4-chloro-1-naphthol was used as the chromogen in enzymatic reaction.

RESULTS

The structure of *Malassezia* yeast surface layer (cell envelope) is presented by external lamella, cell wall, and plasma membrane [11]. The lamella contains lipid components and is apparently the prototype of polysaccharide capsule characteristic of the majority of other fungi; it serves for ensuring adhesion to host cells and osmotolerance. Cell wall consists of several layers, the outer layer (according to electron microscopy data) consists of microfibrillar protein and is 40-80 nm thick [11]. Confocal laser scanning microscopy showed that *Malassezia* allergenic proteins with molecular weights 36 and 67 kD are located on the outer protein layer of the cell wall [13]. The role of these proteins for yeast cells is unclear. Protein with a molecular weight of 36 kD with a hydrophobic domain is a structural protein.

A Zargari [13] described only one method for isolation of soluble fraction from *Malassezia* cells: yeast culturing on agarized medium, washout with distilled water, ultrasonic extraction of protein in 0.05 M phosphate buffer saline (pH 7.4) for 3 min, and subsequent overnight incubation at 4°C. The cells were removed by centrifugation and the resultant supernatant was filtered through a membrane filter. It is most likely that some cells were destroyed during this procedure, and the extract contained cytoplasmic proteins. The presence of numerous electrophoretic bands in extracts from young (2-day) cultures and few protein bands obtained from 6-15-day cultures with more rigid cell walls confirmed this hypothesis.

We therefore assumed that mild isolation procedure will provide the yield of more pure allergen-containing preparations from *Malassezia spp.* These methods were described by S. Lortan *et al.* [10], who demonstrated the possibility of isolating protein S-layers from the surface of *Lactobacillus helveticus* without destruction of bacterial cells. After treatment with sodium dodecyl sulfate and lithium chloride the cells remained intact.

Treatment of *Malassezia* cells with 1% sodium dodecyl sulfate at 50-55°C for 25 min was the best, as this procedure did not destroy yeast cells and the extraction of protein components from the outer layer of cell wall was effective (Table 1). We used this extraction method in further studies.

Interestingly, half of the precipitate wet mass was present in the supernatant after extraction, *i. e.* essen-

TABLE 1. Characteristics of Extracts Obtained by Treatment of *M. sympodialis* Cells by Different Agents

Parameters	Distilled water	LiCl 5 M, 20°C	Triton X-100, 1%	Sodium dodecyl sulfate, 1%		
				10°C, 30 min	50-55°C, 30 min	100°C, 5 min
Loss of precipitate mass after extraction, %	5	7	7	24	48	59
Data of the precipitate microscopy	Whole cells	Whole cells	Whole cells	Whole cells	Whole cells	Cells+debris
Characteristics of extracts						
appearance	White transparent	White transparent	Light-yellow opalescent	White transparent	Light-yellow transparent	Deep-yellow transparent
protein content, µg/ml	170	230	210	295	690	450

TABLE 2. Characteristics of Preparations Obtained from *M. sympodialis* Cells after Culturing in Various Media during Growth Deceleration and Stationary Phases

Parameter	Culture medium			
	solid mD	liquid mD	semisynthetic	synthetic
Wet biomass yield, g/liter	14.6/18.9	35.4/55.1	24.2/31.2	25.5/28.7
Protein content in extract, μ/ml	800/690	1950/1275	1700/800	750/900
Protein yield, mg/g wet biomass	1.64/1.22	3.86/2.00	3.56/1.17	1.55/1.36

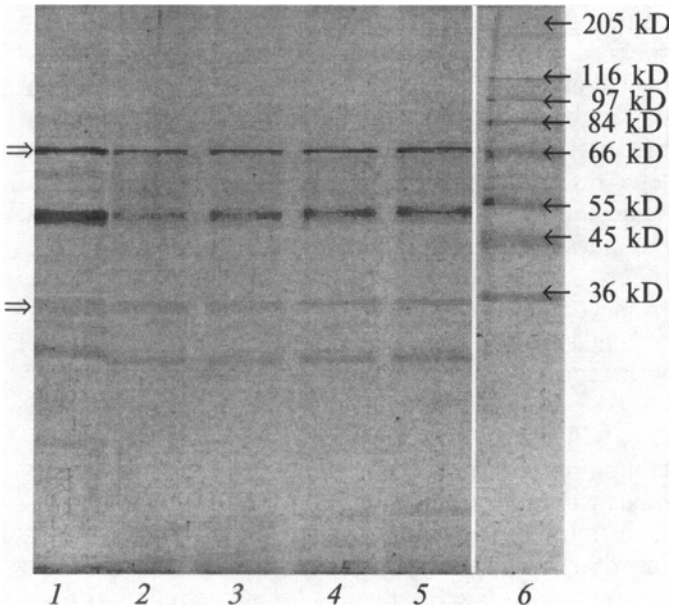

Fig. 1. Separation of *Malassezia* surface proteins in PAAG. *1*) preparation from cells grown in agarized medium; 2-5) preparation from cells grown in liquid synthetic medium. The volume of added protein 34, 12, 15, 19, and 23 μg, respectively. Arrows show proteins with mol. weight 67 (upper) and 36 kD (lower).

tial amounts of components soluble in surfactants were present on the surface of *Malassezia* cells. It seems that these components ensure the optimal interactions with host skin cells.

Maximum yield of biomass was obtained during the stationary phase of culture growth in different culture media, especially in the multicomponent liquid nutrient medium mD (Table 2). However the yield of surface protein considerably decreased during transition from the lag to stationary phase. Since our method of extraction did not lead to destruction of even young (2-day) *Malassezia* cells, this phenomenon can be explained by decreased content of protein components in surface layers in the course of cell aging.

PAAG electrophoresis showed that extracts of *Malassezia* grown in mD medium and in synthetic medium contained strips corresponding to proteins with molecular weights about 36 and 67 kD (Fig. 1) before growth deceleration phase. Interestingly that the major strips were fewer than described by A. Zargari [13] for 2-day cultures extracted by ultrasonic destruction.

According to the results of dot-blot analysis, 3 sera of patients with AD aged being 10, 19, and 28 years contained IgE antibodies to the studied preparation. Other sera (from patients aged 3.5-12 years) contained no IgE antibodies to the extract. Such a correlation between the age of patients and presence of IgE antibodies to *Malassezia* proteins is easily explained. Our previous study showed that *Malassezia* are detected in patients with AD starting from the age of 10 years, while the contamination of the skin reaches the maximum by 18-23 years (unpublished data).

Hence, allergen-containing preparation obtained from *Malassezia* can be helpful in identification of the source of allergen in young patients with AD.

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