IgE and IgG Antibodies to *Malassezia spp.* Yeast Extract in Patients with Atopic Dermatitis

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The presence of immunoglobulins to *Malassezia spp.* surface proteins in the sera from patients with atopic dermatitis and healthy subjects was studied. It was found that 28% of 25 examined patients with atopic dermatitis had IgE antibodies to *Malassezia spp.* surface protein preparation. All patients and 5 healthy subjects had IgG antibodies to this preparation. The presence and concentration of specific IgE antibodies in patients with atopic dermatitis correlated with reverse titers of IgG antibodies to this preparation (r=0.782). The medians of values reciprocal to IgG antibody titers in patients with atopic dermatitis with and without specific IgE antibodies to the preparation and in healthy subjects were 64, 1024, and 16, respectively. The preparation derived from *Candida albicans* (candidine) and previously derived preparation from *Malassezia* did not cross-react. According to immunoblotting data, the preparation contains allergens presented by proteins with molecular weights 15, 36, 52-56, and 78.4 kDa.

Key Words: *Malassezia; skin; allergen; atopic dermatitis; IgE-AB; IgG-AB*

Malassezia yeast, a component of normal human skin microflora, can become a triggering factor in atopic dermatitis (AD) [4]. Clinical diagnosis of sensitization to *Malassezia spp.* requires a high-quality preparation of these lower fungi, containing no cross epitopes with other fungi and consisting of maximum possible number of potentially allergenic proteins. Our recent studies were aimed at the development of this preparation: purification of *Malassezia spp.* cultures, creation of synthetic and semisynthetic media, screening for strains rapidly growing in complete and synthetic nutrient media, development of a method for preparation of allergenic proteins and evaluation of their sensitizing activity [1,2]. Now we have a preparation of *Malassezia spp.* cell surface proteins containing proteins with molecular weights of 36 and 67 kDa, known as allergens [8], and reacting (according to the dot-blot analysis) with class E antibodies (AB) from the serum of AD patients [2]. However, we did not identified proteins inducing production of IgE-AB. Bearing in mind the perspective of obtaining not only diagnostic, but also therapeutic preparations from *Malassezia spp.*, we deemed it useful to evaluate activity of this preparation with specific IgG-AB.

The aim of this study was to detect IgG-AB to antigens of *Malassezia spp.* preparations in patients with AD and healthy subjects and to study binding of proteins with different molecular weight with specific IgE-AB.

MATERIALS AND METHODS

Sera from 40 humans were analyzed: 25 AD patients aged 14-63 years and 10 individuals with IgE-AB to commercial *Candida albicans* preparation candidine (class 2-3 in enzyme immunoassay), and 5 adult subjects aged over 30 years without allergic diseases.

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Malassezia spp. cell surface protein preparation was made and dot-blot analysis of the sera for IgE-AB was carried out as described previously [2].

Specific IgE-AB were detected by enzyme-linked immunosorbent assay developed at Laboratory of Allergodiagnosis, I. I. Mechnikov Institute of Vaccines and Sera [3]. Protein preparation in a concentration of 200 μg/ml was adsorbed on Nunc plates. Immobilization was carried out in carbonate-bicarbonate buffer (pH 9.6±0.1) at 37°C for 1 h and then 16 h at 4°C. Phosphate buffered saline (PBS, pH 7.4±0.1) served as the negative control. The test sera diluted 1:1 (pH 7.4±0.1) were incubated (in duplicates) for 1 h at 37°C, washed 3 times with PBS, and incubated with peroxidase-conjugated monoclonal antibodies to human IgE-AB for 1 h at 37°C. After washing in PBS (3 times) the reaction with the substrate mixture (citrate buffer, pH 4.2±0.2, orthophenylene diamine chromogen, and 33% hydrogen peroxide) was carried out for 15 min. The reaction was stopped with 50% H_2SO_4 and the plates were read on an AKI-Ts-01 spectrophotometer at λ=490-492 nm. The content of allergospecific IgE-AB in the sera was expressed in optical density units.

Specific IgG-AB were also detected by enzyme-linked immunosorbent assay [4]. The same protein preparation from *Malassezia spp.* in a concentration of 20 μg/ml was adsorbed (in carbonate bicarbonate buffer, pH 9.6±0.1) on plates as the antigen. The plates were incubated at 37°C for 1 and 16 h at 4°C. PBS (pH 7.4±0.1, negative control) and the test sera in serial 1:1 dilutions (from 1:8 to 1:16) were added to wells and incubated at 37°C for 1 h, washed 3 times with PBS, and incubated with anti-IgG-peroxidase conjugate (manufactured by N. F. Gamaleya Institute of Epidemiology) for 1 h at 37°C. After washout (PBS, 3 times) the reaction with orthophenylenediamine and 33% hydrogen peroxide in citrate buffer (pH 4.2±0.2) was carried out. The reaction was stopped by adding 50% H_2SO_4. The results were recorded on an AKI-C-01 spectrophotometer at λ=490-492 nm. The content of specific IgG-AB was expressed in inverse titers.

Immunoblotting was carried out as follows. Electrophoresis after Laemmli was carried out in 5-20% polyacrylamide gradient with sodium dodecyl sulfate. The samples were prepared under nonreducing conditions (without mercaptoethanol and heating): *Malassezia spp.* allergen extract (2 mg/ml protein) was diluted 1:1 with the buffer and applied onto the gel (about 10 μl per row). After electrophoresis the proteins were transferred to nitrocellulose in a Novablot semidry transfer device (Amersham—Pharmacia) in accordance with the manufacturer's instruction. After transfer the nitrocellulose was blocked with 10% fetal calf serum in buffered saline (BS) for 1 h at 20°C (all subsequent incubations were carried out at this temperature). The gel was cut into strips and incubated overnight with nondiluted patients' sera. In the morning the strips were washed 3 times with BS with 1% Twin-20, once with BS, then peroxidase-conjugated anti-IgE-AB in BS with 1% BSA for 1.5 h. After incubation with the conjugate the strips were washed and stained with 4-chloro-1-naphthol [2].

Specific IgE-AB bound to allergens were detected using a mixture of monoclonal anti-IgE-AB Le 27 and BSU 17 conjugates (a gift from Prof. A. De Weck, Switzerland) from Immunodot kit for detection of specific IgE-AB (CMG-HESKA).

RESULTS

The main epitopes responsible for cross-reactivity between *Candida albicans*, *Saccharomyces cerevisiae*, and *Malassezia spp.* yeasts are cell wall mannoproteins and enolases (Krebs cycle enzymes with a mole-

TABLE 1. IgE-AB and IgG-AB to Preparation from *Malassezia spp.* Surface Proteins in Sera from AD Patients

Serum No.	IgE-AB, opt. density units	IgG-AB, inverse titers	IgE-AB, dot-blot
1	0.064	64	n
2	0.069	32	n
3	0.073	32	n
4	0.076	256	n
5	0.081	32	n
6	0.084	64	—
7	0.085	32	n
8	0.092	32	n
9	0.092	256	n
10	0.093	256	n
11	0.098	128	n
12	0.102	32	—
13	0.102	256	n
14	0.117	64	n
15	0.117	128	n
16	0.123	256	—
17	0.127	256	—
18	0.128	32	—
19	0.131	512	+
20	0.138	64	+
21	0.140	1024	+
22	0.181	512	+
23	0.188	1024	+
24	0.239	1024	+
25	0.986	1024	+

Note. "n": dot-blot analysis with these sera was not carried out.

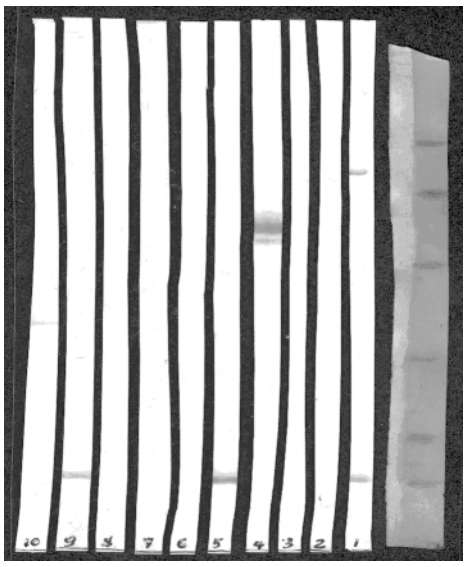

Fig. 1. IgE-binding components of extract of *Malassezia spp.* surface proteins detected using sera from patients with atopic dermatitis.

cular weight ~46 kDa) [5,7]. The study of *Malassezia spp.* crude cell homogenates with monoclonal antibodies showed that the main specific allergenic components are proteins with molecular weights of 36 and 67 kDa [8]. These two proteins are located on the cell surface; in addition, the authors obtained recombinant 36-kDa protein. However, we believe that use of diagnostic allergenic agents based on individual proteins can lead to misdiagnosis, because the possibility of detection of specific IgE-AB decreases with removal of each potential specific allergen. Proceeding from this assumption, we concentrated our efforts on the study of a complex preparation obtained by selective extraction of *Malassezia spp.* cell surface proteins [2].

The sera from 25 patients with AD were tested by ELISA for specific IgE-AB and IgG-AB to this preparation and some sera were tested for IgE-AB by dot-blot analysis (Table 1).

It was found that the sera of AD patients showing negative results in dot-blot analysis and optical density of 0.064-0.128 in ELISA for specific IgE-AB contained specific IgG-AB to *Malassezia* preparations in 1:32-1:256 dilutions. The presence of specific IgE-AB (optical density >0.131 and positive dot-blot test) in the sera correlated with high titers of IgG-AB to the same preparation (1:64-1:1024). Pierson coefficient reflecting linear correlation between optical density (IgE-AB level) and titer of IgG-AB for sera 1-24 was r=0.782.

In the sera from humans without allergic diseases (n=5) the titers of IgG-AB to *Malassezia spp.* preparation were 1:16-1:64 and optical density in ELISA for specific IgE-AB was 0.008-0.056.

Testing of 10 sera containing IgE-AB to commercial *Candida albicans* preparation candidine (class 2-3) showed the absence of IgE-AB to the studied *Malassezia* preparation (according to the dot-blot analysis), but the presence of IgG-AB in titers 1:16-1:64. The medians of inverse titers for IgG-AB in AD patients with and without specific IgE-AB and in healthy subjects were 64, 1024, and 16, respectively.

In order to detect epitopes binding IgE-AB, sera Nos. 19, 20, 22, 24, and 25 (experimental) and Nos. 1, 2, 6, 11, and 12 (control) were tested by immunoblotting (Fig. 1). Control sera contained no IgE-AB to the preparation. IgE-AB from experimental sera reacted with the following proteins: 15 kDa with sera Nos. 20, 22, and 24; 36 kDa with sera Nos. 19 and 24; 52 and 56 kDa with serum No. 25; and 78.4 kDa with serum No. 22.

Major bands corresponding to proteins with molecular weights of 15, 28, 36, 55, and 67 kDa were previously detected in extract of cell surface proteins of *Malassezia spp.* grown in complete medium [2]. Hence, the results of immunoblotting indicate that at least 3 of these proteins are allergens. However, despite published data, none of the tested sera contained IgE-AB to 67 kDa protein. On the other hand, we found no reports about allergenic potential of *Malassezia spp.* surface proteins with molecular weights 52-56 or 78 kDa. Some reports indicate that *Malassezia* proteins with a molecular weight of 14-15 kDa extracted from crude cell homogenates are cross-allergens and are located in the cell wall [7,8]. The probability that in our experiments we detected these proteins is low, because we used a mild extraction method preserving membrane intactness and cell viability [2].

Hence, 28% patients with AD in our study had IgE-AB to preparation of *Malassezia spp.* cell surface proteins. All examined patients and a group of donors had IgG-AB to this preparation, which can be attributed to high prevalence of *Malassezia spp.* carriership in adult humans. The correlation between the levels of specific IgE-AB and IgG-AB can be used in creation of a therapeutic preparation of *Malassezia* allergen.

The absence of cross-reactions between commercial *Candida albicans* preparation and our *Malassezia* preparation indicates that our preparation contains no mannoprotein and other nonspecific protein admixtures. The resultant protein preparation of *Malassezia* yeast contains, in addition to the known allergens, allergens heretofore not described.

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