Specific Immunoglobulin E Antibodies to Saprophytic Yeasts in Sera of Atopic Patients Allergic to House Dust Mites

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Abstract

Background: Atopic dermatitis (AD) is a complex disease caused by genetic and environmental interactions. AD impairs skin barrier function, enabling microorganisms to penetrate and interact with the immune system.

Objectives: This study aimed to investigate the presence of specific immunoglobulin (Ig) E antibodies to the yeast species Candida pelliculosa, Candida guilliermondii, Candida famata, and Rhodotorula rubra in the sera of AD patients and to evaluate possible cross-reactivity between yeasts and allergens of Dermatophagoides pteronyssinus (D1) and Dermatophagoides farinae (D2).

Methods: We analyzed serum samples from 14 healthy individuals and 34 AD patients: 19 were positive to D1 and D2 (Immulite) and 15 were negative. Determinations were made using enzyme-linked immunosorbent assay (ELISA), competitive ELISA, and the Immulite inhibition assay.

Results: The results of ELISA showed that all house dust mite–positive sera had specific IgE antibodies to the yeast species tested: 42% of these sera reacted with all 4 yeast species. The inhibition study demonstrated partial cross-reactivity between IgE class antibodies with the yeast species. This finding indicates that different Candida species and R. rubra have species-specific and cross-reactive antigens with partially overlapping epitopes, thus suggesting cross-reactivity with mite allergens. C. pelliculosa protein extract inhibited IgE binding to D1 (63.4%) and D2 (71%) allergens. The inhibition value for D1 showed a significant correlation with the inhibition value for D2 (r=0.669, P=0.03; Spearman rank correlation).

Conclusion: The results of the present study suggest that C. pelliculosa and house dust mites share common antigens.

Introduction

Atopic dermatitis (AD) is a common skin disease characterized by a chronic, relapsing form of skin inflammation, xeroderma, and immunoglobulin (Ig) E–mediated sensitization to food and environmental allergens, such as pollen, house dust mites, and certain microorganisms [1].

The importance of fungi as allergens has been increasing considerably over the last decade, and a commensal yeast of the mucous membranes Candida albicans has been found to play a role in allergic diseases [2-6]. Studies of the allergenic properties of C albicans have identified 6 allergens [7-12], 3 of which show IgE-mediated cross-reactivity between different fungal phyla. Another Candida species, Candida boidinii, is a methylotrophic yeast distributed in sand, seawater, and fermented olives [13,14]. Although C boidinii is not a source of known allergens, its proteins share IgE binding epitopes with the major allergen of Aspergillus fumigatus (Asp f 3) [15]. Many of the numerous fungal allergens described share a high degree of homology [16]. Polyvalent sensitizations have been reported between homologous proteins in different fungal species [17,18].

The specific IgE response to C albicans is associated with carriage of saprophytic C albicans, as C albicans inhibits the skin response and elevates the IgE response. The degree of simultaneous sensitization may be sufficient to produce allergic symptoms in atopic individuals during occasional overgrowth of C albicans [19].

In a previous study, we demonstrated that some yeast species and their carriage rate are associated with the age of AD patients and with the part of the body on which they are found [20]. Isolated yeast species belonged mainly to the genus Candida, with a predominance of Candida famata and Candida pelliculosa. Both these species are common in fruits, tree exudates, soil, vegetables, cheese, and other organic compounds [21]. They are rarely recovered from clinical specimens [22]. Another Candida species, Candida guilliermondii, is widely distributed in the environment and is routinely isolated from soil, plants, air, insects, seawater, and processed food [23]. Although a common constituent of normal human microflora, this yeast rarely causes fungal infections [22]. No data have been reported about the possible allergenic properties of these species. We hypothesized that cutaneous colonization by these species could be involved in exacerbation of AD due to cross-reactivity.

The yeast Rhodotorula rubra is widespread in terrestrial, aquatic, and marine habitats [21]. Human disease caused by Rhodotorula species is rare, but opportunistic cases have been reported [24]. Two R rubra allergens have been described: one is an enolase that shows high sequence identity with other fungal IgE-reactive enolases [25]; the other is a vacuolar serine protease that cross-reacts with other fungal vacuolar serine proteases [26].

This study aimed to investigate the presence of specific immunoglobulin (Ig) E antibodies to the yeast species C pelliculosa, C guilliermondii, C famata, and R rubra in the sera of AD patients. We observed that sera from individuals with house dust mite allergy reacted to yeast extracts, we evaluated cross-reactivity between yeasts and allergens of Dermatophagoides pteronyssinus (D1) and Dermatophagoides farinae (D2).

Materials and Methods

Participants

We analyzed 34 sera from patients with clinically diagnosed exacerbation of AD (Hanifin and Rajka criteria [27]). Samples were obtained at the Center for the Diagnosis and Treatment of Allergic Diseases in Vilnius, Lithuania (UAB, Alerginių susirgimų diagnostikos ir gydymo centras). Clinical severity of AD was evaluated as mild-to-moderate using the SCORing Atopic Dermatitis Index (SCORAD), which scored severity from 20 to 40 [28]. The results of the Immulite 2000 3GAllergy assay (Siemens Healthcare Diagnostics Products Ltd.) revealed that 19 patients (8 males and 11 females; mean [SD] age, 38.4 [9.8] years; mean SCORAD index, 32 [5.7]) were positive (>0.7 kU/L) for D1 and D2 and that 15 patients (7 males and 8 females; mean [SD] age, 34.8 [13.8] years; mean SCORAD index, 35 [4.8]) were negative (<0.35 kU/L) for D1 and D2. We extracted 14 serum samples from clinically healthy controls (10 males and 4 females; mean [SD] age, 33.6 [7.8] years; range, 16 to 65 years).

The Lithuanian Bioethics Committee approved the study, and all the patients gave their written informed consent to participate.

Preparation of Yeast Extracts

The yeast extracts were prepared using cultures from the collection of the Microbiology and Biotechnology Laboratory of Vilnius University, Vilnius, Lithuania (R rubra 345, C pelliculosa 18, C famata 94, and C guilliermondii 65). All strains were cultured in glucose, peptone, and yeast extract broth (glucose 40 g/L, peptone 5 g/L, yeast extract 5 g/L) at 35°C with shaking for 4 days. Yeast cells were disrupted using a BB\*24 Bullet Blender (Next Advance, Inc.) according to the manufacturer’s protocol for C albicans homogenization in the Bullet Blender. Fifty milliliters of yeast culture (OD600\*1.0) was collected, centrifuged, resuspended in ice-cold 20% trichloroacetic acid, and incubated on ice for 30 minutes. The cells were pelleted by centrifugation at 10000g for 10 minutes. A solution of alkaline-buffered acetone (3 parts of 3M Tris, pH 8.8, to 7 parts acetone) was used to wash the pellet twice. The pellet was air-dried and resuspended in 8M of urea. A volume of zirconium oxide beads (0.5 mm) equal to the volume of the pellet was added to the cell suspension, and the cells were lysed in the Bullet Blender. After yeast cell extraction, the homogenate was separated from the debris and beads by centrifugation at 800g for 5 minutes. The protein concentration of the extracts was determined by spectrophotometry at 280 nm and adjusted to 250 μg/mL in phosphate-buffered saline (PBS). The extracts were stored at −20°C.

Specific IgE ELISA

Yeast protein extracts were adjusted to a protein concentration of 25 μg/mL with PBS (pH 7.2). The ELISA plates (Greiner Bio-One) were coated with 100 μL of each extract, sealed, and incubated at 4°C overnight. The content of the wells was aspirated, and the free surface was blocked with 150 μL of 1% bovine serum albumin (BSA) in PBS.
for 1 hour at room temperature. The test sera were diluted (1:10) in PBS buffer containing 0.1% Tween 20 (PBS-T), distributed at 50 µL/well, and incubated overnight at 4°C. The bound antibodies were detected using biotin-labeled mouse anti-human IgE antibodies (BD Biosciences) and streptavidin–horseradish peroxidase (HRP) (Thermo Fisher Scientific Inc.). Bound conjugate was detected calorimetrically using o-phenylenediamine/H2O2 substrate (100 µL per well). The reaction was stopped with 2M H2SO4, and absorbance was measured with a Synergy 2 microtiter plate reader (BioTek) at 490 nm. The plates were washed 3 times with PBS-T between each step. Several negative controls were included to estimate nonspecific binding. PBS buffer was used for the first control instead of the test serum. The second negative control was analyzed for each tested serum separately: the uncoated wells were blocked and incubated with each serum, biotin-labeled mouse anti-human IgE antibodies, and streptavidin-HRP conjugate. Nonspecific binding was always lower in the second negative control than in the first one. Thus, we used the first control to calculate the results. Blank wells were coated with PBS buffer (instead of the yeast extract) and incubated with PBS buffer (instead of the test serum). Assays with test sera and negative controls were performed in duplicate. The results were calculated as follows: [mean OD490 of the 2 wells with test serum – OD of the blank wells] – [mean OD490 of the 2 wells with PBS instead of test serum – OD of the blank wells].

The Mann-Whitney test was used to investigate possible differences between the groups. A P value of <.05 was considered statistically significant.

Competitive ELISA to Determine Cross-reactivity Between the Yeast Species

ELISA competition tests were carried out to evaluate possible crossed antigenicity between the 4 yeast species. The inhibiting fluid phase antigens comprised 75 µg/mL of protein extracts in PBS from the 4 yeast species. The serum samples from 3 patients (reacting with all 4 yeast species) were pooled and mixed (1:10) with each yeast extract. The pooled sera mixed with PBS buffer instead of the yeast extract were used to estimate maximal binding. The mixtures were preincubated overnight at 4°C and transferred into the wells of the ELISA plate coated with yeast extracts (solid phase antigens) as described above. The plates were incubated overnight at 4°C. Plates were further incubated with biotin-labeled mouse anti-human IgE antibodies and streptavidin-HRP as described above. PBS was included as a blank control instead of the sera–yeast extract mixture.

The percentage of inhibition was calculated as follows:

\[
\text{Inhibition \%} = \frac{A}{B} \times 100 \quad \text{(where } A = \text{OD}_{490} \text{ with inhibiting fluid phase antigens – blank OD}_{490}, B = \text{OD}_{490} \text{ without inhibiting fluid phase antigens – blank OD}_{490}).
\]

Immulite Inhibition Assay to Evaluate Cross-reactivity Between Yeasts and Mites

In order to assess possible cross-reactivity between the 4 yeast species and house dust mites, 2 mite-positive sera were pooled, mixed with each yeast extract, and transferred into the wells of an ELISA plate coated with the same yeast extract (100 µL of 25 µg/mL yeast extract was added into the microtiter wells and incubated overnight at 4°C) with the aim of absorbing the yeast-specific antibodies (binding of antibodies to the solid phase antigen) or blocking the yeast-specific epitopes of remaining antibodies (binding of antibodies to the antigen in the liquid phase). A pooled sera sample preincubated with PBS (instead of the yeast extract) served as a control. The sera with adsorbed yeast-specific antibodies (or blocked yeast-binding epitopes) were analyzed using Immulite 2000 3GAllergy for IgE antibody to D1 and D2 according to the manufacturer’s instructions.

Additionally, 10 D1-positive and D2-positive sera were selected. Microtiter wells were coated with 100 µL of C. pelliculosa extract (25 µg/mL). The selected serum samples were added, and the wells were incubated overnight at 4°C. Serum samples preincubated with PBS (instead of the yeast extract) served as controls. The IgE antibody value against D1 and D2 was determined using Immulite 2000 3GAllergy.

The inhibition percentage was calculated as \([A/B] \times 100\) (where \(A = \text{value of serum preincubated with yeast extract and } B = \text{value of serum preincubated with PBS}\)).

Correlations were analyzed using the Spearman rank correlation test.

Results

Candida- and R. rubra–Specific IgE Antibodies in Sera of Patients With AD

Specific IgE antibodies to different yeast species were
IgE Reactivity to Yeasts and House Dust Mites

Figure 2. Results of enzyme-linked immunosorbent assay showing specific IgE antibodies to 4 yeast species in the sera of patients with atopic dermatitis. The D1-/D2-negative group contains sera from patients with no specific IgE antibodies to house dust mites (<0.35 kUA/L), whereas the D1-/D2-positive group contains sera from patients with specific IgE to house dust mites (>0.7 kUA/L). The control group comprises sera from clinically healthy individuals. D1 indicates Dermatophagoides pteronyssinus; D2, Dermatophagoides farinae; OD, optical density; neg, negative; pos, positive.

A reduction of 40% and 51% in the specific IgE reactivity in homologous inhibition was obtained, respectively, for C guilliermondii and R rubra.

Table 1. Cross-inhibition (%) of Immunoglobulin E Binding to Solid Phase Antigens After Preincubation With Fluid Phase Antigens

<table>
<thead>
<tr>
<th>Solid Phase Antigen</th>
<th>Candida guilliermondii</th>
<th>Candida famata</th>
<th>Candida pelliculosa</th>
<th>Rhodotorula rubra</th>
</tr>
</thead>
<tbody>
<tr>
<td>Candida guilliermondii</td>
<td>40 (11.5)</td>
<td>168 (13)</td>
<td>130 (8)</td>
<td>100 (1)</td>
</tr>
<tr>
<td>Candida famata</td>
<td>55 (4)</td>
<td>152 (12)</td>
<td>129 (5)</td>
<td>121 (2)</td>
</tr>
<tr>
<td>Candida pelliculosa</td>
<td>40 (7)</td>
<td>107 (9)</td>
<td>97 (12)</td>
<td>63 (9)</td>
</tr>
<tr>
<td>Rhodotorula rubra</td>
<td>105 (4)</td>
<td>124 (5)</td>
<td>22 (18)</td>
<td>51 (21)</td>
</tr>
</tbody>
</table>

*Data are shown as mean (SD) obtained from the 2 experiments with pooled sera of 3 patients with atopic dermatitis.
In all cases, the *C. pelliculosa* extract inhibited IgE binding to both or neither of the D1 and D2 allergens. The inhibition value against the D1 antigen showed a significant correlation with the inhibition value against D2 (p=0.669, P=0.03; Spearman correlation coefficient).

### Discussion

Our previous study showed that colonization of the skin by *Candida* species (especially *C. famata* and *C. pelliculosa*) is more frequent in patients with mild-to-moderate AD [20]. In the present study, we investigated the presence of specific IgE antibodies to 3 *Candida* species and *R. rubra* in the sera of patients with AD. We found that specific IgE antibodies to the yeast species investigated are often present in the sera of patients who also have specific IgE antibodies to house dust mite allergens (Figure 2). Additionally, testing of specific IgE antibodies to *C. pelliculosa* extract showed cross-inhibition with house dust mite allergens (Table 2). House dust mite is recognized as the most important risk factor in indoor allergies, with *D. pteronyssinus* and *D. farinae* as the most abundant species in house dust throughout the world [29]. It has been shown that the most important allergenic proteins in these mites are highly homologous and react with 80% of sera from mite-sensitive patients. Mite allergens exhibit variable degrees of cross-reactivity between different mite and other invertebrate species [30,31]. More than half of all cross-reactive fungal allergens have homologous IgE-reactive proteins in nonfungal species [16]. Therefore, we hypothesized that the yeast species investigated in our research may be involved in exacerbation of AD as a result of cross-reactivity with house dust mites.

![Figure 3. Inhibition of IgE binding to house dust mite allergens using 4 yeast species protein extracts (Immulite inhibition assay).](image)

<table>
<thead>
<tr>
<th>Serum No.</th>
<th>Inhibition to <em>Dermatophagoides pteronyssinus</em>, %</th>
<th>Inhibition to <em>Dermatophagoides farinae</em>, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>22</td>
<td>70</td>
<td>63</td>
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<tr>
<td>47</td>
<td>73</td>
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<td>49</td>
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</table>

Our findings are consistent with those of other investigations, which demonstrated that moderate-to-severe AD is strongly associated with sensitization to house dust mites and fungi [32].

We found no correlation between specific IgE values with the yeast species analyzed and total IgE values in sera from AD patients. These data are consistent with the results of Mimura et al [33]. Strong correlations between total IgE and specific IgE antibody levels to yeast, particularly *C. albicans* and the genus *Malassezia*, have been reported [32,34]. *Malassezia* is a part of the normal human cutaneous commensal flora; however, under certain conditions, it is an important trigger for AD. Although numerous *Candida* allergens have been described, their potential role in AD remains open to debate. The frequency of specific IgE to *Candida* antigens varies from study to study. A possible explanation could be that *Candida* has weak allergenic properties and probably acts more as an aggravating factor as a result of cross-reactivity with other allergens. Increased immediate-type reactions with specific IgE synthesis and diminished delayed skin reactions are observed in some atopic patients. The depressed delayed immune response leads to growth of saprophytic *C. albicans*. Continuous exposure to saprophytic yeast could induce synthesis of specific IgE [19].

Most of the sera tested tended to react with all 4 yeast species or with none. This finding is consistent with the results of previous studies indicating that sensitization to multiple fungal species is common among sensitized individuals [35,36]. We hypothesized that this multiple binding is due to common epitopes. Our inhibition study demonstrated partial cross-reactivity between IgE class antibodies and the yeasts analyzed. Our data show that different *Candida* species and *R. rubra* have species-specific and shared antigens. It has been suggested that, if an individual develops IgE reactivity to a specific allergenic component (panallergen), the patient’s serum could react with cross-reactive components in various unrelated species [37]. Although this hypothesis was originally put forward for plant allergens, we believe it can be applied to fungi.

We used flow cytometry to determine whether serum specific IgE antibodies to the yeast extract could recognize...
yeast surface antigens (data not shown). The antibodies tested did not bind to the yeast surface, indicating that they recognized some intracellular antigens. This observation is consistent with the results of other studies, which indicate that most identified fungal allergens are intracellular proteins [16].

Skin barrier defects play a primary role in the pathogenesis of AD. Genetic mutations in the filaggrin gene, which encodes a skin barrier protein, are strongly associated with the development of AD and asthma associated with AD [38,39]. A defective function of the permeability barrier enables environmental allergens from grass pollen, house dust mite, and microorganisms to penetrate the skin and initiate an immune response. The results of another investigation using an animal model suggest that repeated epidermal stimulation with ovalbumin induces ovalbumin-specific IgE and eczematous lesions at the application site [40]. The yeasts we investigated are commensal microorganisms that could be present in soil, food, or elsewhere in the environment. If these yeasts come into contact with a previously sensitized individual, cross-reactive epitopes could lead to aggravation of the allergic skin symptoms. We recommend that specific attention be paid to cutaneous colonization by saprophytic yeasts, since an immune response to their allergens could further exacerbate allergic inflammation.

In conclusion, the results of our study suggest that specific IgE antibodies to saprophytic yeasts are often present in the sera of patients who also have specific IgE antibodies to house dust mite. Competitive ELISA tests revealed that these yeasts have species-specific and shared antigens with partially overlapping epitopes. Specific IgE antibodies to a C. pelliculosa extract showed cross-inhibition with house dust mites, indicating that C. pelliculosa and house dust mites might share some allergenic epitopes.

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