

***Cladosporium herbarum* and *Pityrosporum ovale* Allergen Extracts Share Cross-Reacting Glycoproteins**

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Key Words

Allergen extracts · Cross-reactivity · Fungi · Glycoproteins · Mould · Yeast

Abstract

Background: Patients sensitized to airborne fungi such as *Alternaria alternata* and *Cladosporium herbarum* often also show positive skin prick test results and specific serum IgE antibodies to a yeast, *Pityrosporum ovale*. We examined whether part of the IgE binding to these fungi is explained by cross-reacting mould and yeast allergens. **Methods:** Serum samples from 36 patients with positive skin prick test to *A. alternata* or *C. herbarum* were analyzed for IgE antibodies to fungal extracts by ELISA and immunoblot analysis. Cross-reactivity between mould and yeast extracts was studied by ELISA and immunoblot inhibition assays. In further analysis, the mannan-containing glycoproteins were removed from the yeast extract by concanavalin A-Sepharose chromatography, and the IgE binding properties of the extracts were compared. **Results:** Serum IgE reactivity to *P. ovale* was found in 40% of the mould-sensitized patients. The IgE antibody binding to *A. alternata* and *C. herbarum* moulds was partially inhibited by the yeast

P. ovale in ELISA and immunoblot inhibition assays. When the glycoproteins were removed from the extract, cross-reactivity was markedly reduced. **Conclusion:** Part of the IgE binding to mould and yeast allergen extracts is due to cross-reacting glycoproteins. False-positive IgE and skin prick test results should be taken into account in the diagnosis of mould allergy.

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Introduction

Cladosporium herbarum and *Alternaria alternata* are two common allergenic moulds present indoors as well as outdoors. The airborne mould spores are ubiquitous in nature and vary in number depending on the season and humidity of the air [1]. Exposure to fungal spores is often associated with allergic respiratory diseases [2]. The most common manifestations are allergic rhinitis and allergic asthma [3]. Sensitization to moulds is usually tested with a skin prick test and specific IgE assays using commercially available allergen extracts. The prevalence of sensitization to fungi was 20–30% in an atopic population but only 3–6% in a non-atopic population [4, 5]. Atopic patients often demonstrate IgE antibodies to a broad

range of allergens, and people allergic to moulds usually exhibit IgE to several mould species [6]. Recently, it has been observed that they also exhibit specific serum IgE antibodies to a yeast, e.g. *Pityrosporum ovale* [7].

P. ovale, synonymous to *Malassezia* spp., is a saprophytic yeast that colonizes the skin and normally does not induce IgE production in healthy individuals. In atopic diseases such as atopic dermatitis (AD) with head and neck eczema, the skin barrier may be reduced and *Malassezia* growth favored [8]. Increased levels of *Malassezia* growth on the skin may trigger the IgE antibody production to the yeast and, as a consequence, AD patients often show elevated specific IgE antibodies to *Malassezia* spp. [9]. Part of these AD patients, however, also demonstrate positive skin and IgE antibody reactivity to moulds [10].

The purpose of this study was to investigate the possible allergenic cross-reactivity between clinically important mould and yeast extracts and to identify the cross-reacting components.

Patients and Methods

Patient Sera

The same serum samples from a study cohort comprising 40 patients (36 patients in this study due to limited serum amounts) were used as in our previous study [4]. The group represented mould-sensitized patients selected from an originally larger group of patients (6,376) who were tested for suspected allergy at the Skin and Allergy Hospital in Helsinki. Patients with a positive skin prick test for *C. herbarum* (175) were also tested for *A. alternata*, and a group of patients showing positive results (wheal diameter >4 mm) to *C. herbarum* or *A. alternata* were invited for further testing. Of these mould-sensitized patients, 40 living in southern and western Helsinki nearby the hospital participated. Serum samples from a group of 10–20 non-atopic persons, without positive skin prick results to any of the moulds studied, were used as controls. The control group is also the same as in our previous study.

Antigens

All fungal extracts used were commercial skin prick test extracts obtained from ALK, Abello (Madrid, Spain).

ELISA and Inhibition ELISA

IgE reactivity to fungal extracts was assessed by ELISA as described previously [11], with minor modifications. Briefly, microtiter plates (Immunoplate, Nunc, Roskilde Denmark) were coated (100 µl/well) with 15 µg/ml of fungal extract in 50 mmol/l carbonate buffer. The plate was pre-blocked for 1 h with 1% human serum albumin before the serum samples were added (diluted 1:10 in PBS-0.05% Tween and 0.2% human serum albumin). Biotinylated anti-human IgE (diluted 1:1,000; Vector Laboratories, Burlingame, Calif., USA) was added followed by alkaline phosphatase-streptavidin diluted 1:1,000 (Zymed, San Francisco, Calif., USA) and the phosphatase substrate (Sigma Diagnostics, St. Louis, Mo., USA). The

absorbance was measured at 405 nm with an automated ELISA reader (Titertek Multiscan; Turku, Finland). In inhibition ELISA, the serum samples (diluted 1:10) were pre-incubated with the inhibitor (concentrations ranged from 0.01 to 100 µg/ml) for 1 h at room temperature before being added to the plates. All the other steps were performed as described earlier for ELISA. The cutoff limit for a positive result was calculated from mean optical density values for the control sera plus three standard deviations.

Immunoblot

The allergen extracts were run on SDS-PAGE gels and the IgE binding properties analyzed by immunoblotting as described previously [11], with minor modifications. Briefly, the allergens were separated on a gel, transferred onto a PVDF membrane (Millipore, Bedford, Mass., USA) and the sera (diluted 1:5) were applied onto the membrane. The membrane was incubated in a mini-blotter apparatus (Intermedica, Stockholm, Sweden). Biotinylated anti-human IgE (diluted 1:1,000) and alkaline phosphatase-streptavidin were added, followed by the alkaline phosphatase conjugate substrate (Color Development kit, Bio-Rad Laboratories, Hercules, Calif., USA). In the inhibition immunoblot assay, the serum samples (diluted 1:5) were pre-incubated with the inhibitor (concentrations ranged from 0.01 to 100 µg/ml) for 1 h before being transferred to the blot. All the other steps were performed as described earlier for immunoblotting.

Enzymatic Deglycosylation of the Extracts

The extracts were enzymatically deglycosylated using the Enzymatic Protein Deglycosylation Kit (Sigma-Aldrich, St. Louis, Mo., USA), according to the manufacturer's instructions. The native protocol was used for the extracts used in ELISA, while extracts used in gel electrophoresis and immunoblotting were deglycosylated under denaturing conditions. Briefly, the extract was incubated with the enzymes (peptide-N-glycosidase F, O-glycosidase, α_2 -neuraminidase, β (1-4)-galactosidase and β -N-acetylglucosaminidase) from 3 h to 4 days, depending on the protocol, at 37°C. The enzymes cleave and remove all N-linked and simple as well as complex core 2 O-linked carbohydrates from glycoproteins. The extent of deglycosylation was assessed on SDS-PAGE gels, and protein concentration was measured before the deglycosylated extract was used as an inhibitor in ELISA and immunoblotting.

Separation of Glycoproteins from the Extract with Concanavalin A-Sepharose Affinity Chromatography

The carbohydrate part was removed from the fungal skin prick test extract by running the extract through a concanavalin A (ConA)-Sepharose affinity chromatography system (Amersham Pharmacia Biotech, Uppsala, Sweden). Briefly, the extract was diluted in the running buffer (0.01 mol/l Tris HCl, 0.5 mol/l NaCl, 0.01% NaN₃, pH 7.4, containing 1 mmol/l MgCl₂ and 1 mmol/l CaCl₂) according to the manufacturer's instruction and applied to a column packed with ConA-Sepharose. The unbound fractions were collected and stored as the extract free of carbohydrate parts. The column was washed and the bound carbohydrate part of the extract was eluted with a borate buffer (0.1 mol/l, pH 6.5). The fractions were desalted with Sephadex G25 (Amersham Pharmacia Biotech), concentrated by Speed-Vac evaporation, and protein concentration was measured.

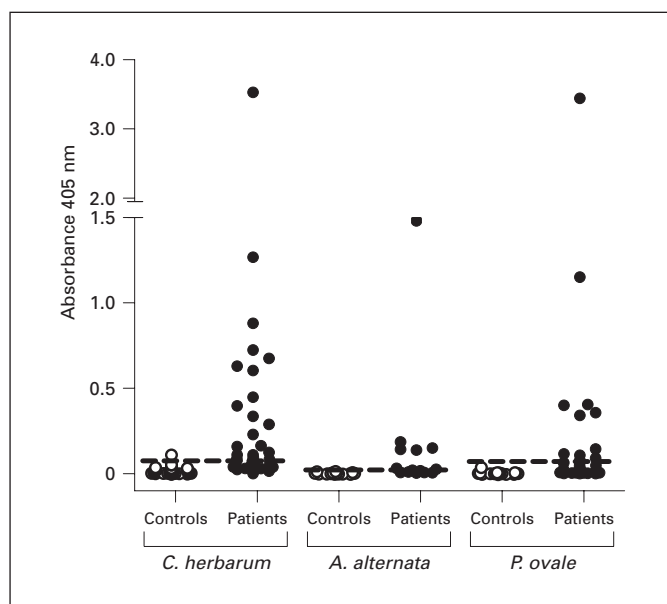


Fig. 1. The IgE binding to fungal allergen extracts [*C. herbarum* and *A. alternata* (moulds) and *P. ovale* (yeast)] in sera from mould-sensitized patients and non-atopic controls. ELISA values (absorbance units) above the mean value of the controls +3 SD (indicated by a dashed line in the graph) were considered positive.

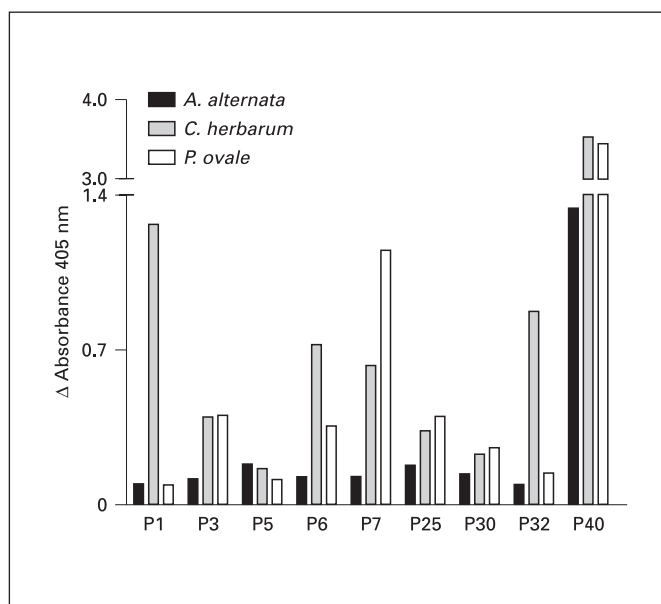


Fig. 2. IgE antibody levels in ELISA to *C. herbarum*, *A. alternata* and *P. ovale* in individual serum samples from mould-sensitized patients. Fungal extracts (15 µg/ml) were coated on microtiter plates and incubated with the sera. Nine patients showed IgE binding to all the fungal extracts but with different intensities. P = Patient.

Results

IgE Binding to Mould and Yeast Extracts

The patients studied had a positive skin prick test to *A. alternata* and/or *C. herbarum*. Of these 36 patients, 20 patients (55%) had specific IgE antibodies to *C. herbarum* and 12 patients (33%) to *A. alternata* in ELISA (fig. 1). Fifteen patients (42%) showed also specific IgE antibodies to *P. ovale*. Sera from non-atopic controls were negative to the fungal extracts in ELISA.

Seven of the 36 mould-sensitized patients showed IgE binding to only one fungus, 5 to two fungi, whereas the sera of 9 patients exhibited IgE binding simultaneously to all fungi. The IgE binding values of these 9 patients are shown in figure 2. A representative group of 6 patients showing simultaneous IgE binding to all fungi but with different intensities was studied in more detail. All 6 patients had atopic eczema and rhinoconjunctivitis and 5 of them also had asthma. Patients 1 and 32 showed strong IgE binding to *C. herbarum* but weak IgE binding to *A. alternata* and *P. ovale* (fig. 2). Patients 25 and 3 (P3) showed moderate or weak IgE binding to all fungi studied. On the contrary, patient 7 showed intense IgE bind-

ing to two fungal extracts and patient 40 showed strong IgE binding to all extracts studied.

Allergenic Cross-Reactivity between Fungal Extracts

Cross-reactivity between yeast and mould extract was examined by ELISA and immunoblot inhibition. The IgE binding to *C. herbarum* was inhibited only weakly (<9%) in patients 1, 32 and 25 when *P. ovale* was used as an inhibitor. Patients 1 and 32 represented patients with high IgE antibody levels to only one of the three fungi studied while patient 25 exhibited moderate levels of IgE to all fungi. On the contrary, IgE binding to *C. herbarum* was inhibited up to 100% by *P. ovale* in sera from patients 3, 7 and 40 showing moderate or intense IgE reactivity to all fungi. Comparable results were seen for *A. alternata* in the solid phase, although the binding values were low (data not shown). Similarly, the IgE binding to *P. ovale* was inhibited when moulds (*A. alternata* or *C. herbarum*) were used as inhibitors (table 1). However, the degree of inhibition of the IgE binding to *P. ovale* was generally lower with the mould extracts. Although sera from patients showing moderate or intense IgE reactivity to all fungi (patients 7 and 40) were used, the binding was al-

Fig. 3. Inhibition immunoblotting was performed with selected sera that showed IgE reactivity to all three fungi studied. Mould or yeast extract was separated on a gel and transferred onto a PVDF membrane. Serum and inhibitor, in concentrations of 0, 1, 10 and 100 µg/ml, were pre-incubated for 1 h at room temperature before being applied onto the membrane. **a** *C. herbarum* in the solid phase and *P. ovale* as an inhibitor using sera from patients 25 and 40. **b** *P. ovale* in the solid phase and *C. herbarum* as an inhibitor using sera from patients 7 and 40.

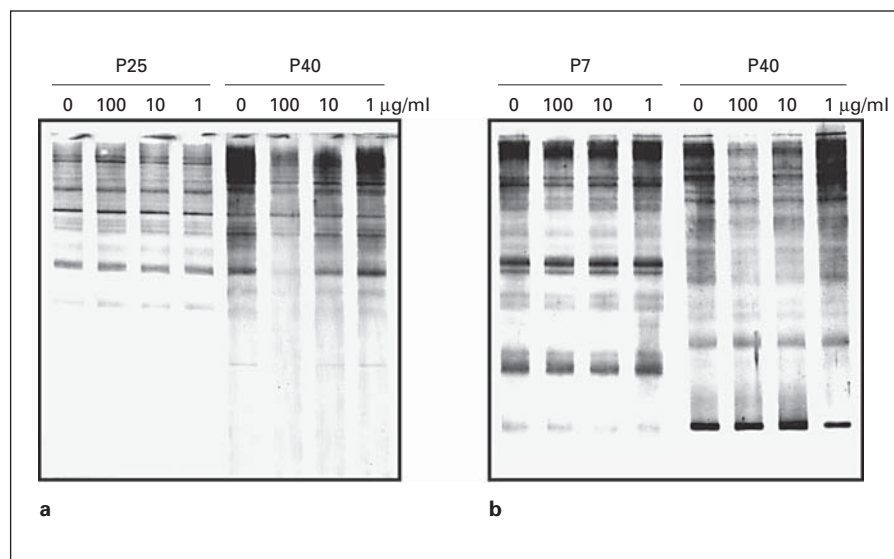


Table 1. The degree of inhibition of IgE binding to fungal extracts in sera from mould-sensitized patients pre-incubated with a potential inhibitor extract

	Solid phase: Inhibitor:	Inhibition, %		
		<i>C. herbarum</i> <i>P. ovale</i>	<i>P. ovale</i> <i>A. alternata</i>	<i>P. ovale</i> <i>C. herbarum</i>
Patient	1	0	96	87
	3	100	100	97
	5	74	66	85
	6	81	35	31
	7	82	49	52
	25	2	8	12
	30	100	46	46
	32	9	22	24
	40	85	78	74

Microtiter plates were coated with mould (*C. herbarum*) or yeast (*P. ovale*) extracts (15 µg/ml). Serum and inhibitor (*P. ovale*, *A. alternata* and *C. herbarum*), in a concentration of 100 µg/ml, were pre incubated for 1 h at room temperature before being applied onto the plate.

ways lower compared to when *P. ovale* was used as an inhibitor.

Immunoblotting revealed a dose-dependent inhibition of IgE binding to *C. herbarum* when *P. ovale* was used as an inhibitor, and sera showing moderate or intense (patient 40) IgE reactivity to all fungi were used (fig. 3a).

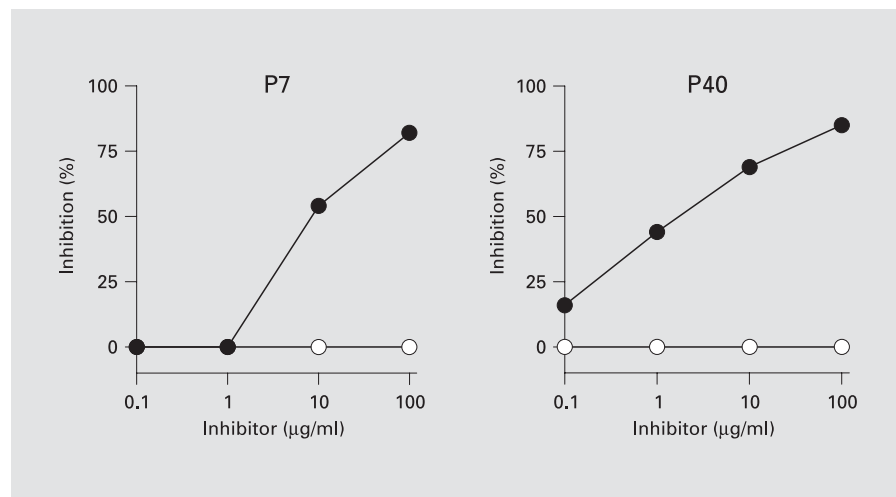
Sera from patients with high IgE levels to one fungus only (patients 1 and 32, data not shown) or sera with moderate reactivity to all three fungi (patient 25), showed no inhibition of IgE binding to *C. herbarum* when *P. ovale* was used as an inhibitor (fig. 3a). Similar inhibition patterns for IgE binding were also seen to *P. ovale* when *C. herbarum* was used as an inhibitor. Inhibition of IgE binding is shown in figure 3b for sera showing moderate or intense IgE reactivity to all fungi used.

Glycoprotein Removal from the Extracts Reduced the Inhibition of IgE Binding

Since the inhibited part of the IgE binding was not a sharp but a smeary band, it indicated involvement of glycoproteins. After enzymatic deglycosylation of the extract, a shift in the mobility of the IgE binding proteins was seen on the immunoblot, which confirmed the presence of glycoproteins in the extracts (data not shown).

We therefore separated, using Con A-Sepharose, the mannan-containing glycoproteins from *P. ovale* extract and compared its inhibitory capacity before and after glycoprotein removal. As can be seen in figure 4, there was a dose-dependent inhibition of IgE binding in ELISA to *C. herbarum* with untreated *P. ovale* extract, while no inhibition was seen after the glycoprotein elimination. The same kind of reduced inhibition was observed on immunoblots when glycoprotein-free extract was used (data not shown).

Fig. 4. IgE-ELISA inhibition results in sera from the mould-sensitized patients 7 and 40 before and after the glycoprotein had been removed from the extract using ConA-Sepharose chromatography. *C. herbarum* mould extract was coated on microtiter plates. Serum and inhibitor [*P. ovale* extract still containing the glycoproteins (●) and *P. ovale* without glycoproteins (○)], in concentrations of 0.1, 1, 10 and 100 µg/ml, were pre-incubated for 1 h at room temperature before being applied onto the plate.



Discussion

We examined a group of mould-sensitized patients and observed that more than 40% of these patients also had IgE antibodies to the yeast *P. ovale*. In order to investigate possible allergenic cross-reactivity between moulds and yeasts, we characterized the IgE reactivity to these fungi in detail. Our results suggest that the IgE binding to mould and yeast can partially be explained by allergenic cross-reactivity between glycoproteins in the extracts.

As has been shown previously, the prevalence for mould sensitization varies depending on the clinical atopic status of the patient. In this study, 58% of the mould-allergic patients had atopic eczema or dermatitis, 44% had asthma and 31% had rhinitis [4]. In an atopic population, especially in patients with atopic eczema or dermatitis, high total IgE serum levels with weak IgE reactivity to multiple allergens are often observed. Two explanations for this reactivity have been suggested: (1) sensitization has occurred either independently and in parallel to several individual allergens or (2) the reactivity is due to cross-reactivity between shared allergenic structures [12]. Differences in IgE binding patterns were observed between our 6 patients. One group of them exhibited high IgE levels to one mould with almost no inhibition of binding by any of the other extracts tested. Thus, these patients were most likely specifically sensitized to the mould. In patients with IgE antibodies to all three fungal extracts, two different reactivity patterns were distinguished. In one patient the IgE binding showed no inhibition by any of the other extracts, suggesting sensitization to all these fungi independently. In another reactiv-

ity pattern, the IgE binding was inhibited by the other fungal extracts, suggesting allergenic cross-reactivity.

Cross-reactivity between fungal allergens has been observed previously, especially between different mould species [13, 14] but also between yeast species [15]. Furthermore, it has been shown that part of the allergen cross-reactivity between yeast species is due to binding to common yeast cell wall carbohydrate parts, such as mannan [16]. In the present study, the inhibited part of the IgE binding looked like a smeary band suggesting that carbohydrates may play a part. In order to elucidate the role of carbohydrates or glycoproteins as cross-reacting agents in mould and yeast allergen extracts, we removed them from the yeast extract using lectin-binding ConA-Sepharose chromatography. When this yeast extract was used as an inhibitor, no inhibition of IgE binding to solid-phase mould was seen although the protein concentration of the extracts was adjusted the same. Since ConA binds α -D-mannosyl and α -D-glucosyl residues, our results suggest that a significant part of the cross-reactivity is due to binding to glycoproteins or free carbohydrate parts.

In order to find out if the cross-reacting part was the carbohydrate itself or a protein bound to a carbohydrate, we enzymatically deglycosylated the extracts. The enzymatic deglycosylation removes N- and O-linked glycans from glycoproteins. However, the deglycosylated extracts still inhibited the IgE binding to the other extracts. This indicates that the protein part of the cross-reacting glycoprotein plays the major role in the binding of IgE antibodies.

Parts of the fungal allergens are known to be glycoproteins. The major allergen of *A. alternata*, Alt a1, is a 31-

kDa glycoprotein [17]. Among the *C. herbarum* allergens, a 54-kDa glycoprotein, designated Cla h II, has been characterized in detail for its protein and carbohydrate parts [18]. Yeasts, on the other hand, have been shown to share cross-reacting carbohydrate parts such as mannan [16]. However, the carbohydrate-containing allergens or glycoproteins that showed cross-reactivity between the extracts seen in this study were larger than the known glycoproteins in *A. alternata* and *C. herbarum* extracts. The diffuse carbohydrate bands inhibited by another fungal extract were in the size range of 60–200 kDa. Our results therefore imply that previously unrecognized cross-reacting high-molecular-weight glycoproteins may be present in mould and yeast allergen extracts.

An association has been observed between AD and IgE reactivity to certain allergens such as house dust mite but also to moulds [7]. It is not clear whether this is due to the occurrence of high IgE levels with reactivity to a broad range of allergens or linked to the pathogenesis of AD. A substantial number of our mould-sensitized patients also

suffered from atopic eczema and exhibited IgE antibodies to a yeast, leaving open the question which of these fungi the patients were originally sensitized to. Interestingly, the yeast extract used in the present study had stronger capacity to inhibit IgE binding to mould than vice versa, suggesting that yeast might be the primary sensitizer through skin. Therefore, the potential cross-reactivity between mould and yeast species in mould allergy tests should be borne in mind in order to avoid false-positive results. Particular attention should be paid to AD patients with antibodies to *P. ovale* which may also show IgE reactivity to moulds possibly without sensitization.

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