

IgE-binding Components in *Pityrosporum orbiculare* Identified by an Immunoblotting Technique

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IgE-binding components in crude extracts of *Pityrosporum orbiculare* were separated by sodium dodecylsulphate polyacrylamide gel electrophoresis and transferred to Immobilon membrane. The components were detected by *P. orbiculare* radioallergosorbent (RAST) positive sera (class 2–4) from 37 patients with atopic dermatitis and visualized by enzyme-labelled anti-human IgE antibodies in chromogenic substrate. Numerous IgE-binding compounds were demonstrated in the molecular weight (MW) range of 14–94 kD. Eight of them were characterized to molecular size, frequency of reacting sera and intensity of bands. Six of the defined components were found to be major allergens reacting with more than 50% of the sera. They had MWs 86, 76, 67, 28, 17 and 13 kD. Thirty-six of the 37 patients had elevated total serum IgE. Two other patient categories were studied; 11 patients with lymphatic filariasis (high total serum IgE, >1,300 kU/l) and 15 pityriasis versicolor patients (normal total serum IgE, <122 kU/l). The *P. orbiculare* RAST values for these sera showed good accordance with the reactivity in immunoblotting experiments. **Key words:** Atopic dermatitis; Immediate hypersensitivity; Allergens; Yeasts.

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During recent years the role of *Pityrosporum orbiculare* as a pathogenic factor in atopic eczema has been discussed. *P. orbiculare* is a lipophilic yeast which colonizes the skin of most healthy adults (90–100%) (1). Patients with “head-neck” dermatitis and type I hypersensitivity to this fungus were improved when treated with ketoconazole (2). In a retrospective study with 731 consecutive patients, one patient category with the same type of dermatitis showed a high proportion of positive skin prick tests (SPT) to an aqueous extract of *P. orbiculare* when compared with patients with other allergic diseases (3).

Recently we have studied children with different

atopic diseases and found that the occurrence of serum IgE antibodies to *P. orbiculare* measured by a radioallergosorbent test (RAST) had the best explanatory value for eczema in comparison with SPT data and RAST data for six other fungi. These were five common moulds (*Penicillium notatum*, *Cladosporium herbarum*, *Aspergillus fumigatus*, *Mucor racemosus*, *Alternaria alternata*) and the most common yeast in human infections, *Candida albicans* (4). In the present study, we made a screening of IgE binding components in *P. orbiculare* using RAST positive sera from patients with atopic dermatitis and a RAST negative control serum from a patient with pea-nut allergy. Two other patient groups were studied: 11 patients with lymphatic filariasis and 15 pityriasis versicolor patients.

MATERIAL AND METHODS

Serum samples

Serum samples from 37 patients (mean age 34 years) with atopic dermatitis and RAST positive to *P. orbiculare* were selected for immunoblotting analysis. Three *P. orbiculare* RAST negative sera were used as negative controls, one from a patient with pea-nut allergy and two from healthy adult volunteers.

Two other patient groups were studied: 11 sera from African patients with a parasite infection, lymphatic filariasis, and 15 sera from pityriasis versicolor patients (mean age 37 years). Dilutions of myeloma IgE added to a serum pool were also investigated.

Extracts for RAST discs

An isolate of *P. orbiculare* (no. 42132) from the American Type Culture Collection (ATCC) was grown for 4 days on a selective agar medium containing, among others, glycerol monostearate, olive oil and Tween 80, as described earlier (5), with the exception that no yeast extract was added to the substrate. The *P. orbiculare* cells were carefully harvested from the solid agar medium and a thick yeast cell suspension in sterile distilled water was obtained. After freeze-drying the cells were sonicated in 0.05 M phosphate buffered saline, pH 7.4, and extracted overnight. To 100 cyanogen bromide-activated paper discs, 0.25 ml of a 1/10 w/v extract was coupled as previously described (6, 7). For coupling of the culture medium (GO substrate) to discs the same concentration was used.

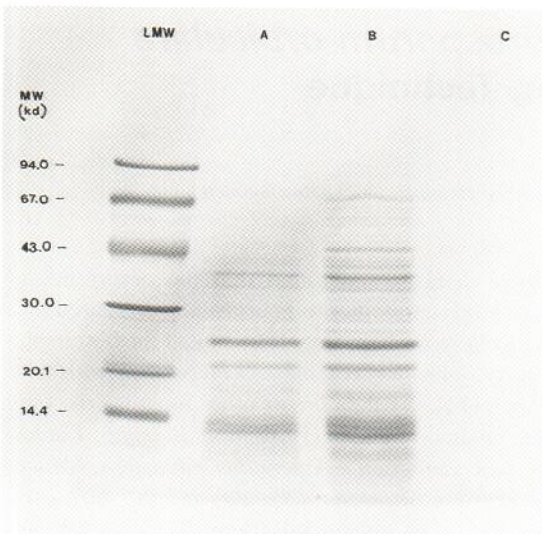


Fig. 1. CBB-staining of SDS-PAGE separation of extracts of two different culture batches of *P. orbiculare*, lanes A and B. Lane C is extract of the culture medium (GO substrate).

Radioallergosorbent test (RAST)

IgE antibodies were determined by RAST (6, 7) using commercially available Phadebas RAST kits (Pharmacia Diagnostics AB, Uppsala, Sweden) and following the recommendations of the manufacturer. The sera were tested in duplicates with *P. orbiculare* discs and GO substrate discs, respectively, and the results expressed in Phadebas RAST units (PRU/ml).

Total IgE determinations

Levels of total serum IgE were measured by IgE RIA (8, 9) (Pharmacia Diagnostics AB) following the recommendations of the manufacturer. The sera were tested in duplicates and the results given in kU/l.

Extracts for immunoblotting

P. orbiculare was cultured as described above. After freeze-drying the cells were sonicated in 0.15 M NaCl as 1/10 w/v and extracted at 4°C overnight. The extract was centrifuged at 6,600 g for 10 min, filtrated through a 0.8 µm Millex-HA filter (Millipore, Bedford, Mass., USA), desalted on a Sephadex G-25 column (Pharmacia, BTG) and lyophilized. Two extracts were prepared from different culture batches of *P. orbiculare* and one extract from the GO substrate and stored at -20°C until tested.

Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Extracts were subjected to a polyacrylamide (PAA) gradient gel (7.5%-20%) at an approximate concentration of 100 µg dry weight/mm gel. The electrophoresis was carried out essentially as described by Neville (10) and Johansson et al. (11). Low-molecular-weight proteins (LMW, Electro-

phoresis calibration kit, Pharmacia) were used in one well. The electrophoresis was run at 6 mA/gel for 16 h. The gel in Fig. 1 was stained with Coomassie brilliant blue R-250 (CBB).

Electrophoretic transfer

The separated proteins were transferred from the SDS separation gel to a polyvinylidene difluoride membrane (Immobilon™, Millipore) in a Biorad Trans-blot cell. The Immobilon membrane is hydrophobic and is made hydrophilic with methanol treatment according to the manufacturer. Blotting buffer containing 25 mM Tris, 192 mM glycine and 20% methanol, pH 8.3, was used to equilibrate the gel for at least 30 min at 4°C and the Immobilon membrane for a few minutes prior to the transfer which was run at 450 mA for 4 h at 10°C with voltage decreasing from 160–140 V. The quality of the transfer was checked by protein staining (CBB) of the LMW-standard blotted onto the Immobilon membrane. The destain was performed in 90% methanol.

Quenching and immunological detection

Protein binding sites still available on the Immobilon membrane after the transfer were blocked by 0.5% Tween 20 in 0.05 M phosphate buffer, pH 7.4, containing 0.2% bovine serum albumin and 0.02% NaN₃ (PSA-Tween) for 60 min at 37°C. For immunological detection the Immobilon membrane was cut into narrow strips and incubated for 16–18 h with 250 µl of serum diluted to 1 ml in PSA-Tween buffer. After washing they were incubated for 4 h with 1 ml of a mixture of rabbit antihuman IgE (diluted 1/2000) (Miab, Knivsta, Uppsala, Sweden) and β-galactosidase-labelled sheep antirabbit IgG (diluted 1/200) (Miab). After rinsing, visualization of IgE-binding components was performed in a substrate containing 2-N-β-D-galactopyranosidemonohydrate and Fast Blue B salt. The molecular weights of eight IgE-binding compounds were determined by comparison of their relative mobility with that of CBB-stained low-molecular-weight proteins on Immobilon membrane.

RESULTS

The proteins in extracts from two culture batches of *P. orbiculare* and one culture medium extract were separated by SDS-PAGE and the CBB stained protein profiles are shown in Fig. 1. In comparison, extracts A and B showed almost total accordance with the exception of strength. At least 30 protein bands of varying intensity could be observed in lane B in the molecular weight (MW) range of about 10–70 kD. No bands could be detected by this staining technique in the lane with GO substrate preparation (lane C).

In order to characterize the IgE-binding components in the extracts, the proteins, after having been transferred to Immobilon membrane, were reacted with *P. orbiculare* RAST positive sera obtained from

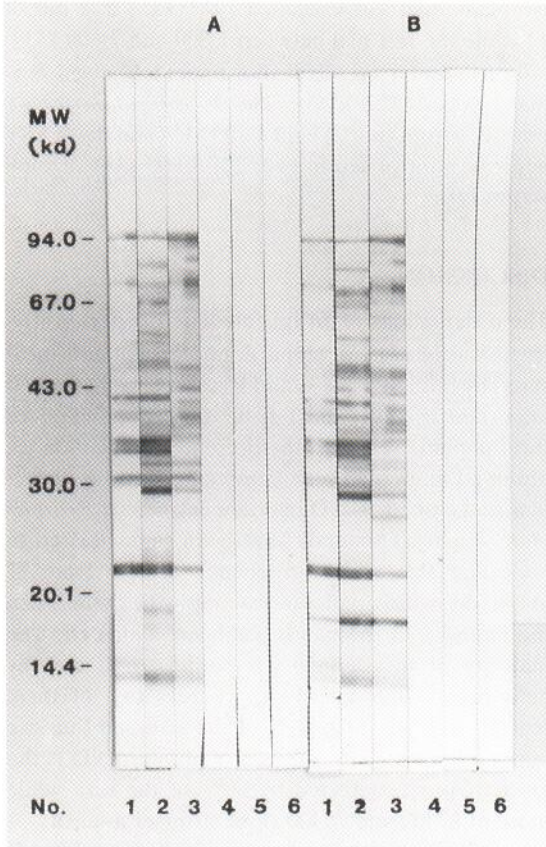


Fig. 2. IgE-binding components in two different extracts of *P. orbiculare*, on Immobilon membrane strips, detected by sera from 3 patients with atopic dermatitis (nos. 1-3), one patient with non-specified dermatitis (no. 4) and two healthy controls (nos. 5-6).

patients with atopic dermatitis and *P. orbiculare* RAST negative control sera. Detection was performed by a mixture of enzyme-labelled conjugate containing anti-human IgE (Figs. 2, 3 and 4). Fig. 2 illustrates IgE-binding proteins in the two extracts of *P. orbiculare* (A and B). Numerous IgE-binding compounds are detected when three sera from RAST positive atopic dermatitis patients (sera nos. 1-3) are used. One apparent discrepancy is noticed; extract B exhibits a stronger and more distinct 17 kD band. Extract B was used in a screening experiment with *P. orbiculare* RAST positive sera (class 2-4) from 37 atopic dermatitis patients (sera nos. 1-3, 7-22, 24-41) and one *P. orbiculare* RAST negative serum (no. 23) (Figs. 3 and 4). All strips but one, no. 38, show varying numbers of IgE-binding components. In the MW range of about 30-67 kD the protein bands on many strips are difficult to distinguish from each other.

We have therefore chosen to calculate the MWs for those which are both frequent and relatively easy to distinguish. They are of MWs 94, 86, 76, 67, 28, 21, 17 and 13 kD. Fig. 5 shows the frequency of sera reacting with these IgE-binding compounds and the intensity of the bands. Most of the sera exhibit binding towards the IgE-binding components of MWs 67 kD (32/37), 86 kD (29/37), 13 kD (27/37), 76 kD (26/37) and also those of 17 kD (22/37) and 28 kD (21/37) which are major allergens while those of 21 kD (16/37) and 94 kD (11/37) are minor allergens.

Seven of the 11 sera from patients with lymphatic filariasis demonstrated positive RAST values to *P. orbiculare* (class 1-2) and also reacted with some of the IgE-binding components in the *P. orbiculare* extract while the RAST negative sera did not. The dilutions of myeloma IgE were found to be *P. orbiculare* RAST negative and showed no reactivity to IgE-binding compounds. All the sera from pityriasis

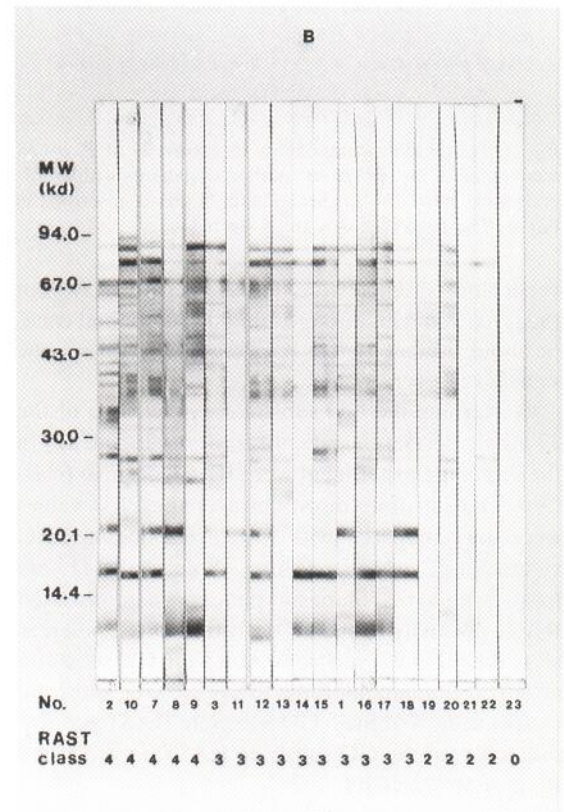


Fig. 3. IgE-binding components in extract B of *P. orbiculare* detected by sera from 19 patients with atopic dermatitis (nos. 1-3 and 7-22) and one control (no. 23). The RAST classes are given below.

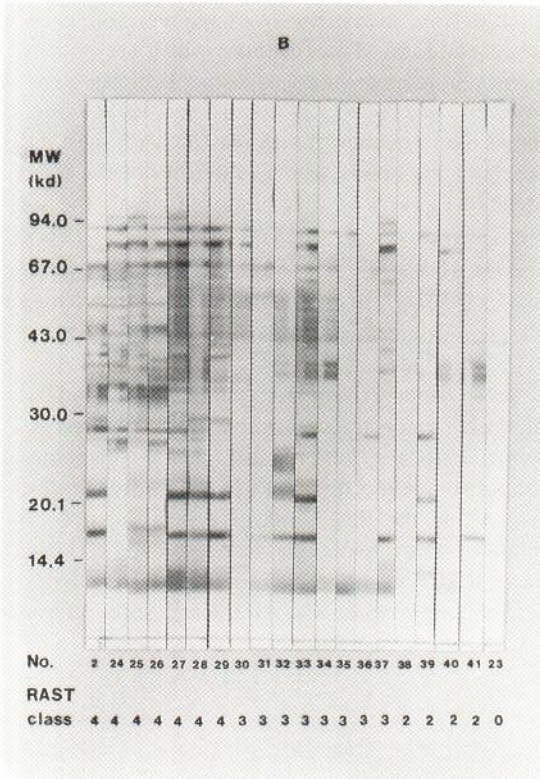


Fig. 4. IgE-binding components in extract B of *P. orbiculare* detected by sera from another 18 patients with atopic dermatitis (nos. 24–41). Sera 2 and 23 are as described in Fig. 3. The RAST classes are given below.

versicolor patients were RAST negative; two of them reacted with one IgE-binding compound each, of about 90 and 13 kD respectively; however, the signals were very weak.

Concerning the GO substrate extract, two of the 37 atopic dermatitis patient sera (nos. 13 and 18) in the screening experiment were RAST positive (class 2) to GO substrate discs; however the PRU values were low compared with the corresponding PRU values for *P. orbiculare*. Also, three of the 11 sera from lymphatic filariasis patients demonstrated RAST positivity to the GO substrate discs, whereas all of the pityriasis versicolor sera were GO substrate negative. Immunoblotting test with GO substrate extract using 4 of the 5 GO substrate RAST positive sera and 8 of the GO substrate RAST negative sera showed no reactivity.

Thirty-two of the 37 patients included in the screening experiment had total serum IgE levels >500 kU/l and 20 of them had >5,000 kU/l. The amounts of total serum IgE in the patients with

lymphatic filariasis exceeded 7,000 kU/l in 7 out of 11 while the rest had between 1,300 and 3,600 kU/l. All of the pityriasis versicolor patients showed normal levels (<122 kU/l). The *P. orbiculare* RAST positive serum (no. 2) held 4,100 kU/l and the RAST negative control serum (no. 23) 3,900 kU/l of total serum IgE.

DISCUSSION

The observations of an IgE mediated hypersensitivity to *P. orbiculare* in atopic dermatitis patients measured by both SPT (2, 3) and RAST (4) have made it important to study the IgE-binding components of this lipophilic yeast. In the present investigation we observed numerous such components in the molecular range of 14–94 kD in crude extracts of *P. orbiculare*. Eight compounds have been characterized to molecular size and to frequency of sera from 37 atopic dermatitis patients reacting with them. Also the intensity of the bands has been evaluated. The majority of the defined compounds (6/8) are, by definition, major allergens, whereas two of them reacted with less than 50% of the sera and thus are minor allergens. The allergen of about 67 kD is the most frequent, detected by 86% of the sera while those of 17 kD and 76 kD show stronger intensity. It is possible that both frequency and intensity of bands would have increased if the reacting sera had been less diluted. The difficulties to distinguish the bands in the region between 30 and 67 kD may possibly be overcome by separating the proteins in homogeneous polyacryl amide gels of different densities covering this special region. The band of about 13 kD is broad and diffuse and could perhaps be split into two

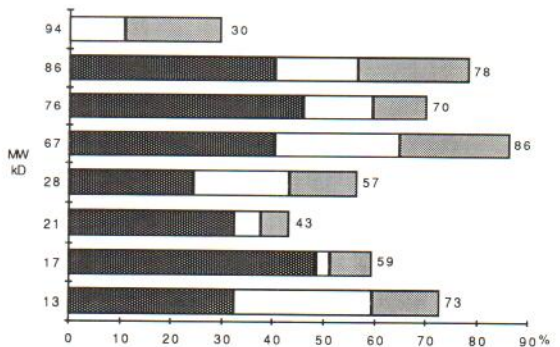


Fig. 5. Frequency (%) of sera from 37 patients with atopic dermatitis containing reactive IgE antibodies to 8 defined allergenic components in *P. orbiculare* extract. Intensity of the bands: ■ weak □ moderate ■ strong

or more compounds by means of a homogeneous gel.

It is known that total serum IgE levels are elevated in a majority of patients with atopic dermatitis (12). Another patient category with high serum IgE are those with parasite infections. In this study, three of the 11 sera from patients with lymphatic filariasis had extremely high total serum IgE levels (19,000, 32,000 and 39,000 kU/l). They were *P. orbiculare* RAST positive (class 2, about 1 PRU/ml each) and showed fewer and less intense bands than the positive control serum (no. 2) tested in the same run. No agreement was thus observed between the frequency and intensity for these bands and the content of total serum IgE. Moreover, no reaction was found when dilutions of myeloma IgE (total serum IgE from about 300 up to 30,000 kU/l) were tested.

Until now few investigators have studied the antigenic composition of *P. orbiculare*. A complex antigenic structure of *P. orbiculare* has been demonstrated by Bruneau & Guinet (13) using different *Pityrosporum* strains and rabbit antisera in a crossed immunoelectrophoresis (CIE). IgG-binding antigens in *P. orbiculare* have been examined by SDS-PAGE and immunoblotting by means of reacting sera from seborrhoeic dermatitis patients, controls and subjects known to have high antibody titres to fungi (referred to as allergic subjects) (14). Four sera from allergic subjects were tested and showed a strong positive reaction to a band in the region of 66 kD. This band may be identical with that of about 67 kD found to be the most frequent IgE-binding component in our study. Allergenic components can induce IgG antibodies, which has been shown in a study with *C. albicans* (15).

A major question that arises after this characterization of allergens in *P. orbiculare* is the mode of sensitization. Regarding their molecular sizes, three of the defined major allergens, 13 kD, 17 kD and 28 kD, and one of the minor allergen, 21 kD, may be inhalant allergens. As *P. orbiculare* is a skin colonizing microorganism and also occurs in sebaceous glands and deep in hair follicles, it might be assumed that this fungus causes sensitization via the skin. However, cross-reactions with other fungi are likely to occur. Allergenic cross-reactivity between different species of fungi has been described (16, 17).

Further characterization of IgE-binding components in *P. orbiculare* is needed before a proper allergen extract can be prepared. The allergens identified in this study can be used to optimize reagents

for diagnosis and to produce monoclonal antibodies for studies on cross-reactivity and for environmental investigations.

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