

A standardized densitometric immunoblotting analysis of *Candida albicans* protein allergens

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Summary

Sera from 196 patients with a strong skin reactivity to *Candida albicans* and a history of atopic disease were used for *C. albicans* IgE-immunoblotting. The IgE-immunoblots were analysed by densitometry and integration of the densitogram peaks. A standardized reference disc system allowed parallel analysis of several different immunoblotting experiments. Altogether 105 patients had *C. albicans*-specific IgE antibodies in immunoblotting. The IgE-response against *C. albicans* was extremely polyspecific and was directed against 42 different bands. The most important IgE binding bands were 46 kDa and 27 kDa bands against which 44 (42%) and 29 (28%) patients, respectively, had IgE responses. A combination of IgE-immunoblotting and densitometry was found practical in analysis of large number of patient sera allowing a profound and accurate analysis and characterization of *C. albicans* allergen extract. This data evolved from a sufficiently large patient population can be utilized in further standardization of *C. albicans* extracts.

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Introduction

Presently, a certain scepticism is, and should be, retained on the concept of *Candida albicans* allergy. *C. albicans* hypersensitivity has been the subject of several studies connecting *C. albicans* hypersensitivity with asthma, allergic rhinitis, urticaria and atopic dermatitis [1–4]. Furthermore, allergy to *C. albicans* has been suggested to underlie chronic or recurrent infections caused by *C. albicans* [5,6]. However, only limited postulations on the subject can be made, mainly because the composition of the antigenic preparations used in the studies is undefined. The individual allergens have remained unidentified until recently and the standardization requirements consequently have remained unfulfilled [7].

The crude cytoplasmic fraction of *C. albicans* has been shown by the RAST-technique to bind human IgE [8] and several IgE-binding bands have been detected with immunoblotting [4,9–11]. Mannan, a mannose polymer,

is the major antigenic determinant of the *C. albicans* cell surface and also binds IgE [12–15].

Immunoblotting analyses utilizing small patient populations have revealed different panels of allergens with different dominant allergens [4,9–11]. The purpose of the present study was to identify the antigens of *C. albicans* recognized by human IgE antibodies in a combined large population of atopic individuals and to determine the most important IgE-binding components by immunoblotting. A semiquantitative classification for the strength of the IgE-binding to individual allergenic bands was established with standardized densitometer assessment.

Materials and methods

Antigens

Yeast cultures Two strains of *Candida albicans* were used in the study. One strain was obtained from the Centraalbureau voor Schimmelcultures, Delft, Netherlands (CBS 5982, serotype A) and cultured on agar plates on synthetic medium as previously described [15]. The

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other strain was supplied and typed by the Mycological Laboratory of the Department of Dermatology, University of Turku (strain no 72024) and grown in a fermentor in peptone-supplemented medium as previously described [16].

Extraction The crude cytoplasmic extract of the CBS 5982 yeast cells was prepared after disruption in X-press as previously described [15,17] and the crude cytoplasmic extract of the Turku University strain 72024 by disruption of the yeast cells with Manton-Gaulin homogenizer after extensive washing as previously described [16]. There were no significant differences in the allergen composition of these two cytoplasmic extracts [10,11,18]. Both extracts were also prepared in carbohydrate free form by ConA affinity chromatography purification as previously described [10,15].

Patient sera Sera from 196 patients were used for *C. albicans* IgE-immunoblotting. The patients had a history of atopic disease and a diagnosis of bronchial asthma (48 patients), atopic eczema (169 patients) or allergic rhinitis (76 patients), mainly in combinations. These diagnoses were determined at the University Central Hospital of Turku. Ninety-one of the patients were male and 105 female. The mean age of the patients was 25.8 years (SD 11.2). All patients had a histamine equivalent or stronger immediate skin reaction to *C. albicans* tested according to the nordic recommendations with laboratory generated

extracts as previously described [10,16]. Parts of this large material have been previously published [4,10,11]. Subjects with no history of atopic disease served as immunoblotting controls.

Sodium Dodecyl Sulfate — Polyacrylamide Gel Electrophoresis (SDS-PAGE) and immunoblotting

SDS-PAGE All extracts and molecular weight markers (Electrophoresis Low Molecular Weight Calibration Kit, Pharmacia Fine Chemicals, Uppsala, Sweden) were separated by SDS-PAGE, a high resolution technique, according to the method of Laemmli [19] in a large 5–17.5% gradient gel.

Blotting SDS-PAGE-separated antigens were transferred to nitrocellulose sheets (Millipore 0.22 µm, Millipore Co, Bedford, Mass, USA) in Transphor apparatus (LKB, Bromma, Sweden) according to Towbin *et al.* [20]. After blotting antigens onto the nitrocellulose, sheets from the large gel were cut in strips, for incubation with individual sera. The transferred bands were visualized by Amido-Black staining of one strip.

IgE detection IgE-detection was done according to the method of Bengtson *et al.* [21]. The nitrocellulose strips were incubated in 1:4 diluted individual patient sera. After washing three times with NaCl-Tween, the blotted

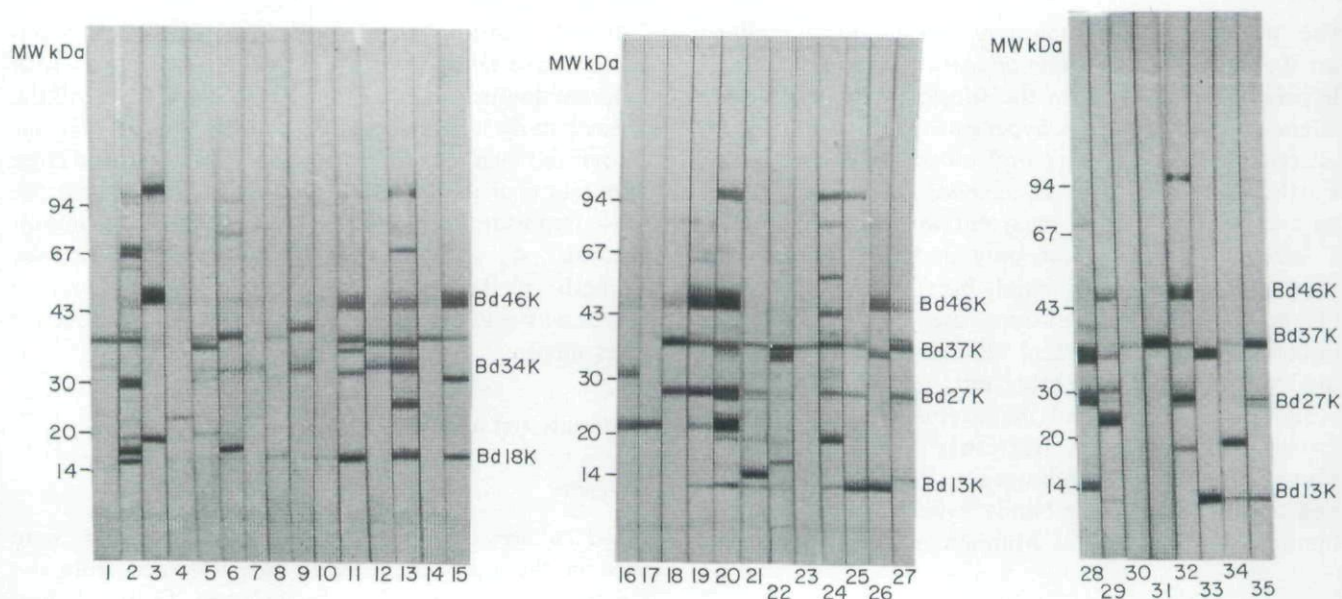
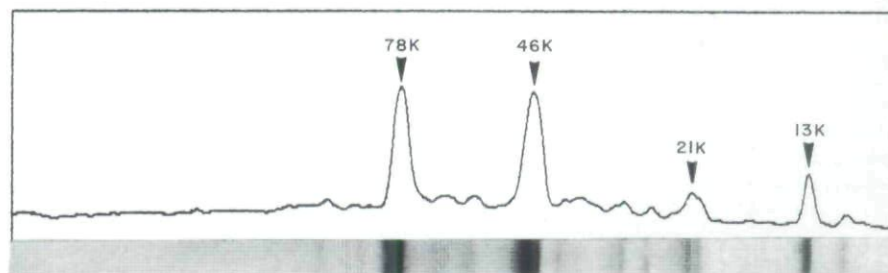


Fig. 1. IgE immunoblots of *C. albicans* skin-prick tests positive atopic patient sera. The most important IgE-binding bands are indicated on the right.

Fig. 2. An example of an IgE immunoblot and the corresponding densitometric curve. Four allergens are indicated with molecular weights.



membranes were incubated with beta-galactosidase-labelled anti-IgE antibodies (Phadezym-RAST, Pharmacia Diagnostics, Uppsala, Sweden) diluted 1:4 in PBS-TWEEN. Membranes were then washed three times with NaCl-Tween and twice with NaCl. Bands were visualized using a chromogenic substrate containing 2-*N*-beta-*D*-galactopyranoside monohydrate (Sigma N-2259) and Fast Blue B-Diazonium salt (Dajac 5331, Trevone, PA, USA). The membranes were immersed in substrate solution for 15 s and finally laid on a filter paper pre-wetted with the substrate solution. The nitrocellulose was allowed to lay on the filter paper until no additional bands appeared.

Reference discs To standardize the development of the beta-galactosidase stain a system of reference discs was developed with the CRIE reference discs as a model [22]. This was necessary for the elimination of the differences in repeated experiments due to aging of the conjugate and possible differences in substrate incubation time and conditions. Serial dilutions were made from Phadezym conjugate in PBS with 1:2 dilutions ranging from undiluted to 1:64 and finally with PBS alone. Nitrocellulose discs were made from the same nitrocellulose as in the blotting experiments and were incubated in the serial dilutions of the conjugate and as a control in PBS at +37°C for exactly 1 h. Thereafter, the discs were washed three times with water and laid in the substrate exactly the same way and same time as the immunoblotted strips. The discs were allowed to stay in substrate exactly as long as the immunoblotted strips. The discs were thereafter read with the densitometer simultaneously with the strips.

Densitometry

Basic principles of densitometry A Bio-Rad densitometer in a reflectance mode was used to analyse the individual immunoblot strips. The settings were adjusted to achieve maximum peak height with minimum background and to avoid too detailed densitometric pattern. This was done by fully opened lens, minimized enhancement

and an unboosted filter frequency of 2.00 lines/mm. The densitometer was thus used for the sole purpose of quantitation. Additional software was used for integration and quantitation. The same densitometer settings were used in analysing all scanned strips. Basic cut-off was the background baseline level taken from the low molecular part of the strip. The corresponding baseline level of the reference discs was the disc incubated with PBS alone, without any conjugate.

Results

Immunoblots with affinity purified *C. albicans* antigen are presented in Fig 1. The IgE-staining of protein bands was assessed by densitometry as shown in an example in Fig. 2. Microheterogeneity of some proteins caused widening of some bands which made direct comparison of the peak heights impossible. This was eliminated by integration of the densitogram. After integration of the densitogram peaks data was expressed as ODmm (x-axis: mm, y-axis: OD) values. The background varying from strip to strip was standardized by use of a basic, strip-specific, cut-off and background variation of one single strip by use of an individual band-specific cut-off. The overlapping bands were also taken into account. The basic principles used for integration and calculation of the density peaks and corresponding backgrounds are presented in Figs 3, 4 and 5.

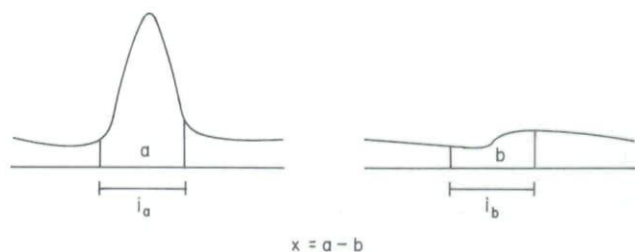


Fig. 3. The background (b) measured from a negative strip at the same interval ($i_a = i_b$) was subtracted from the integrated study peak (a) to give an integrated value (x) in ODmm for the assessed specific binding.

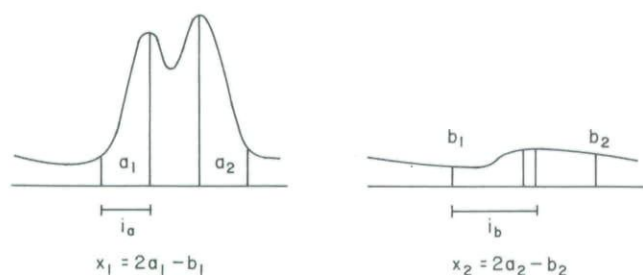


Fig. 4. When peaks of equal size overlapped, half of the peak from the opposite side of the overlapping peak was integrated (a_1). The value was duplicated ($2a_1$) and subtracted with the background (b_1), which was integrated from the whole interval of the peak ($2i_a = i_b$).

The uneven background caused distortion of the densitograms; and subtracting the background caused a variation in the assessed values from one subtraction to another. This was resolved by determining limits for the positive response. The limit for defining positive responses was based, on one hand, on the range of the distortion and on the variation of the assessed optical density (0–0.02 OD) and, on the other hand, on the mean width of all the bands (5 mm, range 3–7 mm). The lower limit for a positive response was thus, by definition, 0.1 ODmm (0.02 OD $\times$ 5 mm). All bands were classified using the same ODmm-limitation scheme. Such an approach assured the simultaneous evaluation of not only the amount of protein but also the strength of the IgE-response in assessing the importance of a band as an allergen.

The development of the stain in conjugate incubation was measured and standardized in each incubation with the reference discs. The development of the stain in the reference discs is described in Fig. 6. In order to classify IgE-binding bands, the integrated ODmm values were

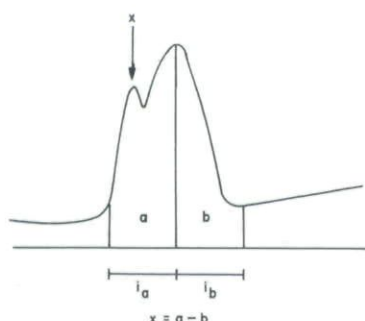


Fig. 5. When a minor peak mixed with a major peak, the major peak was divided to minor peak-positive (a) and -negative (b) halves ($i_a = i_b$). The area of the minor peak was then calculated.

converted to three intensity classes for semiquantitative analysis. The ODmm-limits for the classes depended on comparison with the reference discs guaranteeing classification of the stain independently from the incubation time. The arbitrarily chosen limits, based on several earlier experiments and attempts of classification were as follows. The lower limits for the responses were in $\ln(\text{conc})$: -4 for class I, -2.8 for class II and -0.6 for class III. The reproducibility of the standardized assessment and classification was controlled with reanalysis of several serum samples. The inter-experimental changes were found to be mainly within the class-limits.

The data collected from all immunoblotting experiments with *C. albicans* with 196 patients are presented in Fig. 7. A total of 105 patients were IgE-positive in immunoblotting. The extremely polyspecific response is directed against 42 different protein bands. The most important allergens are the 46 kDa and 27 kDa bands against which over 25% of the patients had an IgE-response. No major allergen (>50% response) was detected and against the most dominant allergen, the 46 kDa band, 42% of the patients had an IgE-response.

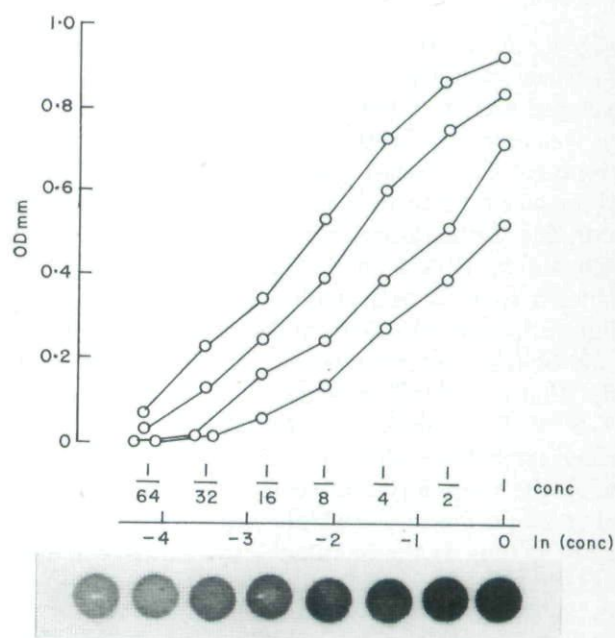


Fig. 6. Densitometric assessment of the optic density of the conjugate reference discs coated with different concentrations of conjugate (conc: 1–1 : 64 and $\ln(\text{conc})$: -4–0) and PBS alone (OD = 0) incubated in substrate for different time periods. The four plotted curves joining individual ODmm values (○) represent measurements after four different incubation times: 12 (photograph), 10, 6 and 4 h. The lower limits for classification were in $\ln(\text{conc})$: -4 for class I, -2.8 for class II and -0.6 for class III. conc. Concentration.

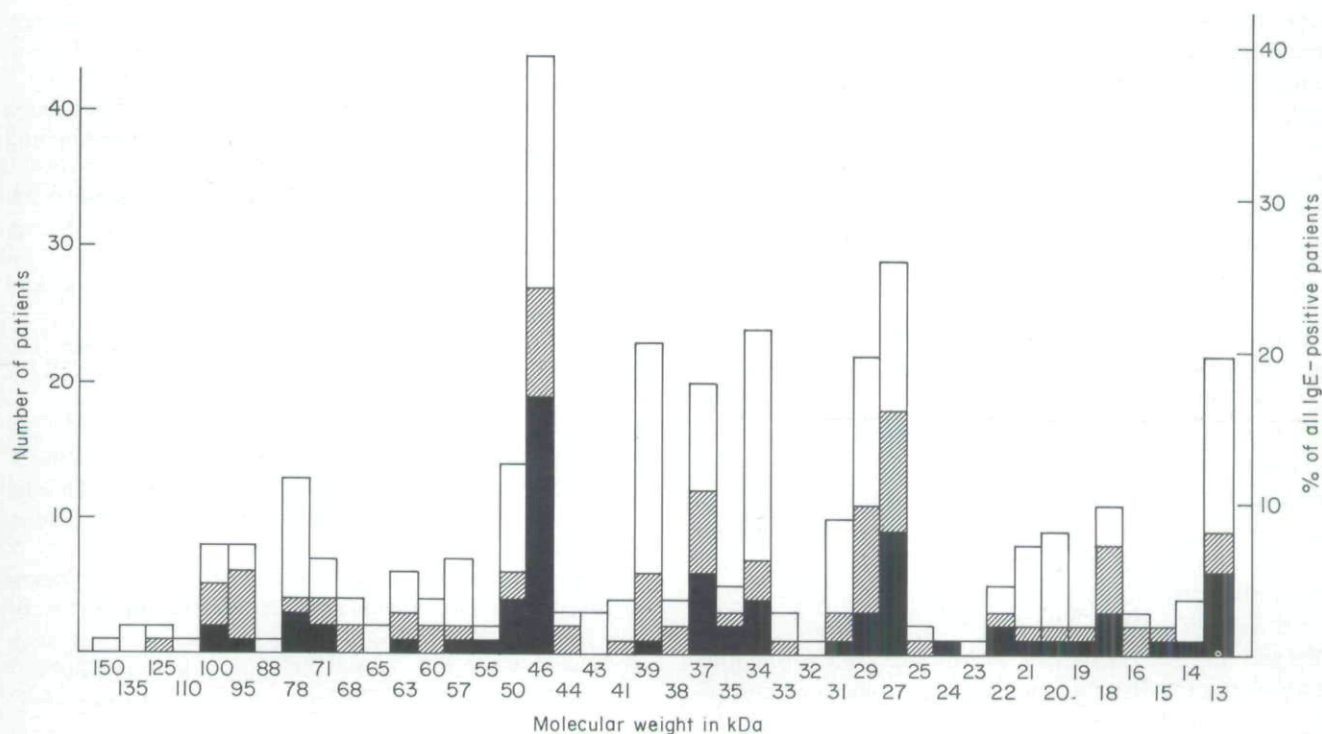


Fig. 7. A schematic summary of the IgE-binding bands detected with immunoblotting using 105 IgE-positive patient sera. The grading of the density of the immunostain was performed with densitometer and the classification was performed according to the reference disc system. IgE-response class: □. I. ▨. II. ■. III. kDa. Kilodalton.

Discussion

In the investigation presented herein, the densitometer-aided IgE-immunoblotting technique proved suitable for the characterization of *C. albicans* allergen extracts. The immunoblotting technique allowed a large number of patient samples to be studied. The use of the densitometer allowed an objective semiquantitative evaluation of IgE-binding to allergens. As individual sera were repeatedly analysed, samples from separate experiments could be run in parallel, thus making possible the comparison of many samples from different subpopulations.

The varying background staining and total serum IgE made the use of a single horizontal baseline as a background impossible. Therefore in addition to the basic strip-specific cut-off, which eliminated the general background interference, an individual band-specific cut-off was also established to further eliminate the possible background stain variation. Background caused by different length of the incubation was resolved by the use of reference disc in same way as in CRIE as described by Aas [22]. In addition to length of conjugate incubation no effect on reproducibility of the results was caused by used sera, age of the conjugate, the

nitrocellulose used or conditions of SDS-PAGE. The remaining factors influencing the reproducibility of the results were the quantity and quality of antigen and the time used for the electric transfer onto nitrocellulose. The blotting conditions and quantity of the antigen are, however, relatively easy to standardize from experiment to experiment. The quality of the antigen and patient sera thus remain the varying parameters studied.

Freely soluble cytoplasmic proteins are extracted from disrupted *C. albicans* cells and a total of 40–78 antigens have been detected in the cytoplasmic extract by crossed immunoelectrophoresis (CIE) [8,23] and two-dimensional polyacrylamide gel electrophoresis has revealed 168 methionine-labelled protein components [24]. PCA-tests and the RAST-technique already proved the purified cytoplasmic protein fraction to be allergenic and the individual protein allergens of *C. albicans*, have been analysed with immunoblotting in several studies [4,8–11,25]. The dominant allergens and allergen profiles in different studies vary markedly. Different genetic basis for antibody production, differences of the yeast extracts or simply small patient populations used may explain the results. In this study the largest available material is presented. The human IgE-response against *C. albicans*

appeared polyspecific and was directed against 42 different peptide bands, most often against the 46 kDa cytoplasmic protein. The dominant 46 kDa allergen has earlier been recognized in two studies with small predominantly asthmatic patient populations [10,26]. According to its amino acid composition the 46 kDa band is likely to be an enolase enzyme [26]. The most important protein allergen of *S.cerevisiae* also appears to be the enolase enzyme with a molecular weight of 48–52 kDa [27,28].

It is of interest that a cytoplasmic protein, with a molecular weight of 47 kDa in SDS-PAGE, seems to be an especially stimulatory determinant in the immune response of systemic candidiasis patients. In fact, antibodies to this protein have been associated with recovery from the infection [29]. This antigen has been established as a breakdown product of a fungal heat shock protein 90 [30]. Another immunodominant 47 kDa antigen for IgG antibodies in invasive candidiasis has been recognized as enolase [31–33]. The most important protein allergen in our study, in accordance with previous studies, was the 46 kDa band [10,26]. Amino acid homology and simultaneous IgE binding with *S. cerevisiae* enolase suggest this major allergen to be enolase [26,28].

C. albicans mannan polysaccharide has been shown to bind IgE in sera of atopic patients [14,15,34,35]. In IgE immunoblotting mannan exhibits a typical diffuse high molecular weight stain [10,15]. The sensitivity of immunoblotting to detect IgE antibodies against mannan seems low and therefore the analysis of the anti-mannan IgE in patient populations does not seem reliable with immunoblotting. Quantitation of the anti-mannan IgE-response with immunoblotting and densitometry is difficult and inaccurate. In order to classify IgE-binding to mannan a nitrocellulose-based RAST has been developed [35].

The combination of immunoblotting and densitometry proved to be an invaluable analytical tool for the screening of large numbers of patients. This constitutes significant progress towards one of the most challenging requirements in allergy standardization.

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