

Cell surface expression of two major yeast allergens in the *Pityrosporum* genus

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Summary

Background We have previously identified two major allergens of *Pityrosporum orbiculare* and characterized these as 37 kDa and 67 kDa proteins.

Objective In the present study we have investigated the presence and subcellular location of the 37 kDa and 67 kDa allergen components in various members of the genus *Pityrosporum* as well as in *Candida albicans*, *Candida parapsilosis* and *Saccharomyces cerevisiae*.

Methods To detect both cell surface and intracellular expression of the allergens, flow cytometry and confocal laser scanning microscopy (CLSM) were used. The cells were stained with indirect immunofluorescent (IIF) or alkaline phosphatase anti-alkaline phosphatase (APAAP) methods using mouse monoclonal antibodies (MoAbs).

Results Ninety-five per cent of the *P. orbiculare* (*P. ovale*) cells cultured for 4 days showed cell surface-binding of the anti-37 kDa MoAb and 88% of the cells bound the anti-67 kDa MoAb when analysed with IIF and flow cytometry. It was found that the members of the genus *Pityrosporum* (*Malassezia*), *P. pachydermatis* and *M. sympodialis*, expressed the 37 kDa and 67 kDa allergens to a similar extent as did *P. orbiculare*. Less than 5% of the cells of the genus *Candida* and *S. cerevisiae* showed positive staining with the MoAbs. The CLSM revealed that the 37 kDa and the 67 kDa components were located to the cell wall and could not be detected inside the acetone fixed and APAAP stained yeast cells of the genus *Pityrosporum*. When the yeast cells were cultured for more than 4 days the expression of both allergens decreased significantly.

Conclusion All three members of the genus *Pityrosporum* express the 37 kDa and 67 kDa major allergens on the cell surface, whereas these proteins could virtually not be detected in the *Candida* genus and *S. cerevisiae*.

Keywords: *Candida albicans*, confocal microscopy, flow cytometry, *Malassezia sympodialis*, *Pityrosporum orbiculare*, *Pityrosporum pachydermatis*, *Saccharomyces cerevisiae*

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Introduction

Pityrosporum orbiculare (*P. orbiculare*) also denoted *P. ovale* is a lipophilic yeast which belongs to the genus *Pityrosporum* (synonym: *Malassezia*) [1]. Although *P. orbiculare* is a member of the human normal skin microflora, it can under predisposing conditions act as a pathogen and

play an important role in the pathogenesis of pityriasis versicolor, folliculitis and seborrheic dermatitis [2–4]. Several investigations have shown the presence of anti-*P. orbiculare* reactivity with skin tests and/or specific IgE serology in patients with atopic dermatitis (AD) [5–8]. Atopic sensitization to *P. orbiculare* is especially common in patients with eczema in the head-neck-face region [5]. This region is also where the yeast is most frequently found [9]. Ketoconazole treatment has improved the eczema and decreased the serum IgE-levels in AD patients [10].

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Recently, we found that extracts from *P. orbiculare* induced a significantly higher T-cell reactivity in AD patients than in healthy controls [11]. Furthermore, *P. orbiculare* reactive T-cell clones derived from lesional AD skin showed a TH₂- or TH₂/TH₀-like cytokine profile [11]. Several indications are thus present suggesting that *P. orbiculare* may play a role in maintaining IgE mediated skin inflammation.

Characterization of the allergens expressed by *P. orbiculare* with SDS-PAGE and Western blot has revealed a multitude of IgE-binding compounds ranging from 10 to 100 kDa [12–14]. We have previously raised mouse monoclonal antibodies against the 37 kDa and 67 kDa major allergens of *P. orbiculare* and characterized these allergens as proteins [15]. The concentrations of the 37 kDa and the 67 kDa proteins in the yeast extracts were found to decrease with prolonged culture [16].

In the present study we have investigated the subcellular location of the 37 kDa and 67 kDa allergen components of *P. orbiculare*. Knowledge about the location of these allergens may add new information on their biological properties and functions. This should be of value in the understanding of the pathogenesis of at least some forms of AD and perhaps also other diseases where the *Pityrosporum* genus is involved. We used flow cytometry and confocal laser scanning microscopy (CLSM) to allow detection both of allergens expressed on the cell surface and intracellularly. We also investigated whether the two allergens could be found in two other species of the same genus, *Pityrosporum pachydermatis* (*P. pachydermatis*) and *Malassezia sympodialis* (*M. sympodialis*), and in various yeasts such as *Candida albicans* (*C. albicans*), *Candida parapsilosis* (*C. parapsilosis*) and *Saccharomyces cerevisiae* (*S. cerevisiae*).

Materials and methods

Yeast strains

P. orbiculare strain no. 42132 was obtained from American Type Culture Collection. *P. pachydermatis* strain no. 454 and *M. sympodialis* strain no. 314, were kindly provided by Dr Midgley, St John's Institute of Dermatology, London, UK. *C. albicans* strain no. 562, *C. parapsilosis* strain no. 604, *S. cerevisiae* strain no. 1171 were obtained from Centraal Bureau voor Schimmelcultures (CBS), Yeast Division, Delft, The Netherlands. *P. orbiculare*, *P. pachydermatis* and *M. sympodialis* were cultured on Dixon medium [17] at 32 °C. *C. albicans* and *C. parapsilosis* were cultured on Sabouraud dextrose agar at 37 °C. *S. cerevisiae* was also cultured on Sabouraud dextrose agar but at 32 °C. All the yeasts were incubated for 4 days. In addition *P. orbiculare* was cultured on glycerol monostearate olive oil (Go) medium [18] without yeast extracts, for 2, 4, 8, 15 and 22 days at 37 °C.

Mouse monoclonal antibodies (MoAbs)

Two MoAbs, designated 9G9 and 1D5, both of IgG₁ class, were produced against *P. orbiculare* by immunizing Balb/c mice with *P. orbiculare* extract or yeast cells [15]. Hybridoma cells were cultured in serum-free media and antibodies were isolated from the cell supernatant. MoAb 9G9 was protein A-purified to a concentration of 500 µg/mL and MoAb 1D5 was ammonium sulphate-purified to a concentration of 100 µg/mL. MoAbs 9G9 and 1D5 are directed against protein components of *P. orbiculare* and recognize a 37 kDa and a 67 kDa major allergen, respectively, [15]. A mouse IgG₁ MoAb (DAKO, Glostrup, Denmark) directed against *Aspergillus niger* glucose oxidase was used as negative control and a mouse IgG1 MoAb against yeast mitochondrial porin from Molecular Probe (Leiden, The Netherlands) was used as positive control.

Indirect immunofluorescent (IIF) staining

The yeast cells were harvested and suspended in distilled water. The viability of the cells was determined by Trypan blue exclusion (a minimum of 100 cells were counted), and in parallel three serial dilutions of the yeast suspensions in sterile distilled water were prepared and plated for viable count. The yeast cells to be analysed were washed in 0.15 M phosphate buffered saline (PBS), pH 7.4, containing 0.02% NaN₃, 0.1 mM EDTA and 0.5% BSA (PBS-EDTA-BSA). Thereafter cells were mixed (10⁶ cells/tube) with MoAbs diluted to a final concentration of 75 µg/mL in PBS-EDTA-BSA buffer and incubated for 1 h at room temperature (20–22 °C) and shaken occasionally. The irrelevant mouse IgG₁, used at comparable concentrations, served as a control for non-specific binding. Fluorescein isothiocyanate (FITC)-conjugated rabbit F(ab')₂ anti-mouse IgG₁ (RAM) (DAKO) was used as a secondary antibody (10 µg/mL) and incubated for 45 min on ice. After each incubation step the cells were washed twice with PBS-EDTA-BSA buffer and spun down at 400 × g for 6 min at 4 °C. The cells were finally suspended in 500 µL PBS-EDTA-BSA and kept on ice until analysed. No aggregation was observed in ordinary light microscopy prior to flow cytometry analyses.

Flow cytometry and UV microscopy

An Epics profile II or an Epics XL-MCL flow cytometer (Coulter, Inc., Hialeah, FL, USA) was used. The cells were distinguished by two values based on their light scattering properties; forward scatter (FS) reflects the cell size and side scatter (SS) reflects the complexity/granularity. In the present study, the yeast cells were detected in a two parameter scatter plot histogram with logarithmic amplification and a gate was set around the yeast cell populations. At least 5000 cells were accumulated within this gate when the cells were analysed. The results are expressed as the percentage of the counted cells that were positive. In the control experiments,

unlabelled yeast cells and cells incubated with only the secondary antibodies (FITC-RAM) were used, to exclude the possibility that yeast autofluorescence interfered with the assay.

The yeast cells were in parallel evaluated in a UV-microscope (Nikon Optiphot II) with a $\times 100$ plano-apochromate Nikon objective with oil-immersion. The cells were spun down and smeared on glass slides and mounted with 0.5% *p*-phenylenediamine (PPD) in glycerol (Sigma, St Louis, MO, USA) to reduce fading of immunofluorescence during microscopy [19]. Some of the yeast cells were further analysed with CLSM.

Immunocytochemical staining

Alkaline phosphatase anti-alkaline phosphatase (APAAP) staining was chosen because besides being rather permanent, it allows assessment with both ordinary light microscopy and CLSM [20]. Cells taken from the same cultures as those used for IIF staining were also used for APAAP staining. The cells were washed with sterile distilled water and resuspended to a concentration of 10^7 cells per mL in 0.5 M Tris-HCl buffer containing 0.5 M NaCl pH 7.6 (TBS buffer) and smeared on 8-well glass slides (Novakemi AB, Stockholm, Sweden), 40 μ L per well. The slides were air-dried and thereafter kept at -70°C until used. At the beginning of each experiment the yeast cells were immediately fixed in 50% acetone for 30 s followed by 5 min in 100% acetone at room temperature (22°C). Thereafter the cells were incubated with normal rabbit serum (DAKO) diluted 1:10 in 4% BSA for 10 min to decrease non-specific binding. All the incubations were carried out in a humid chamber at room temperature and after each incubation the glass slides were washed three times for 5 min in TBS buffer. The two MoAbs against *P. orbiculare*, diluted to 50 $\mu\text{g/mL}$ in TBS buffer, were added and incubated for 30 min. The subclass-matched irrelevant MoAb was included as a control at the same concentration. Thereafter rabbit anti-mouse immunoglobulin (diluted 1:40) (DAKO) was added as a secondary antibody for 30 min, followed by the APAAP complex (DAKO). The intensity of the staining was greatly enhanced by repeating the last two steps for 10 min. The chromogenic substrate Fast-Red (DAKO) was added and incubated for 15–20 min until desired colour intensity had developed. The cells were mounted with glycerol and evaluated under an ordinary light microscope with a $\times 100$ plano-apochromate Leitz objective with oil-immersion. At least 400 cells were counted for each antibody, yeast strain and experiment ($n = 3$). Stained cells were scored as positive, whereas unstained cells with normal morphology were scored as negative. The number of positive cells was calculated as the percentage of the total number of counted cells.

Confocal Laser Scanning Microscopy (CLSM)

CLSM was used to analyse both freshly isolated yeast cells stained with the IIF method to detect surface labelled structures and acetone-fixed cells stained with the APAAP method to allow also evaluation of labelled structures inside the cells. We used a SARASTRO[®]2000 CLSM system (Molecular Dynamics, Sunnyvale, CA, USA) equipped with an argon ion laser and an upright Nikon microscope. The software for scanning and image processing was installed on a Silicon Graphics 4D/25 Personal Iris[®] workstation (Silicon Graphics Inc., Mountain View, CA, USA), which runs a UNIX-based operating system. The 488 nm and 514 nm lines were used for excitation of FITC and Fast-Red, respectively. FITC was detected with a beam-splitter reflecting at 510 nm, and in front of the detector we placed either a longpass filter at 510 nm or a bandpass filter reflecting light < 515 nm and > 555 nm. The use of a bandpass filter was necessary when scanning *C. albicans* and *M. sympodialis* due to their strong autofluorescence at longer wavelengths. Fast-Red was detected with a beamsplitter reflecting at 535 nm, with a longpass filter at 535 nm in front of the detector. A confocal aperture of 50 μm and a $\times 100$ plano-apochromate Nikon objective with oil-immersion was used.

Each yeast cell was scanned sequentially into 10–12 'optical sections' separated by 0.4 μm , starting at the top. The top was determined from a vertical scan. Extended focus projections were produced by adding all optical sections in the stack with each section in focus. To further visualize the binding pattern of the MoAbs, 'intensity map' projections were produced where the heights of the peaks were proportional to the pixel intensities (i.e. registered emitted light).

Statistics

The expression of the 37 kDa and 67 kDa proteins on *P. orbiculare* yeast cells at different culture periods was evaluated by analysis of variance (ANOVA) and Duncan's multiple comparison test. A *P*-value less than 0.05 was considered to be significant. A SOLO[®] (version 4.0 software package was used (BMDP Statistical Software, Inc. Los Angeles, CA, USA).

Results

Freshly isolated Pityrosporum cells express the 37 kDa and 67 kDa allergens on the surface

P. orbiculare cells cultured for 4 days were analysed with IIF and flow cytometry. In three different experiments it was found that 95% of the cells showed positive staining with the anti-37 kDa MoAb and 88% with the anti-67 kDa MoAb

Table 1. Expression of the 37-kDa and 67-kDa proteins of various yeast strains cultured for 4 days

Strain	Expression of the 37 kDa protein pos cells (%)		Expression of the 67 kDa protein pos cells (%)	
	Flow cytometry*	Microscopy**	Flow cytometry*	Microscopy**
<i>P. orbiculare</i>	95 ± 0.5	91 ± 2	88 ± 3	79 ± 3
<i>P. pachydermatis</i>	82 ± 3	81 ± 2	71 ± 7	75 ± 1
<i>M. sympodialis</i>	91 ± 4	89 ± 0.4	89 ± 4	87 ± 3
<i>C. albicans</i>	4 ± 1	2 ± 1	2 ± 1	0.3 ± 0.1
<i>C. parapsilosis</i>	3 ± 0.5	1 ± 0.3	2 ± 0.3	0.7 ± 0.4
<i>S. cerevisiae</i>	4 ± 0.3	1 ± 0.5	2 ± 0.5	2 ± 1

* The expression was analysed with IIF in an Epics XL-MCL flow cytometer. A minimum of 5000 cells was analysed, and the background value was subtracted.

** The expression was evaluated with APAAP staining and analysed in an ordinary light microscope. At least 400 cells were counted. Mean value ± standard error of the mean are given for three different experiments.

(Table 1), indicating that both proteins are exposed on the cell surface. The distribution of the fluorescence intensity of the *P. orbiculare* yeast cells incubated with the two MoAbs is shown in Fig. 1.

The other members of the genus *Pityrosporum*, *P. pachydermatis* and *M. sympodialis*, also expressed the 37 kDa and 67 kDa allergens on the cell surface, and to a similar extent as did *P. orbiculare* (Table 1). Less than 5% of the cells of

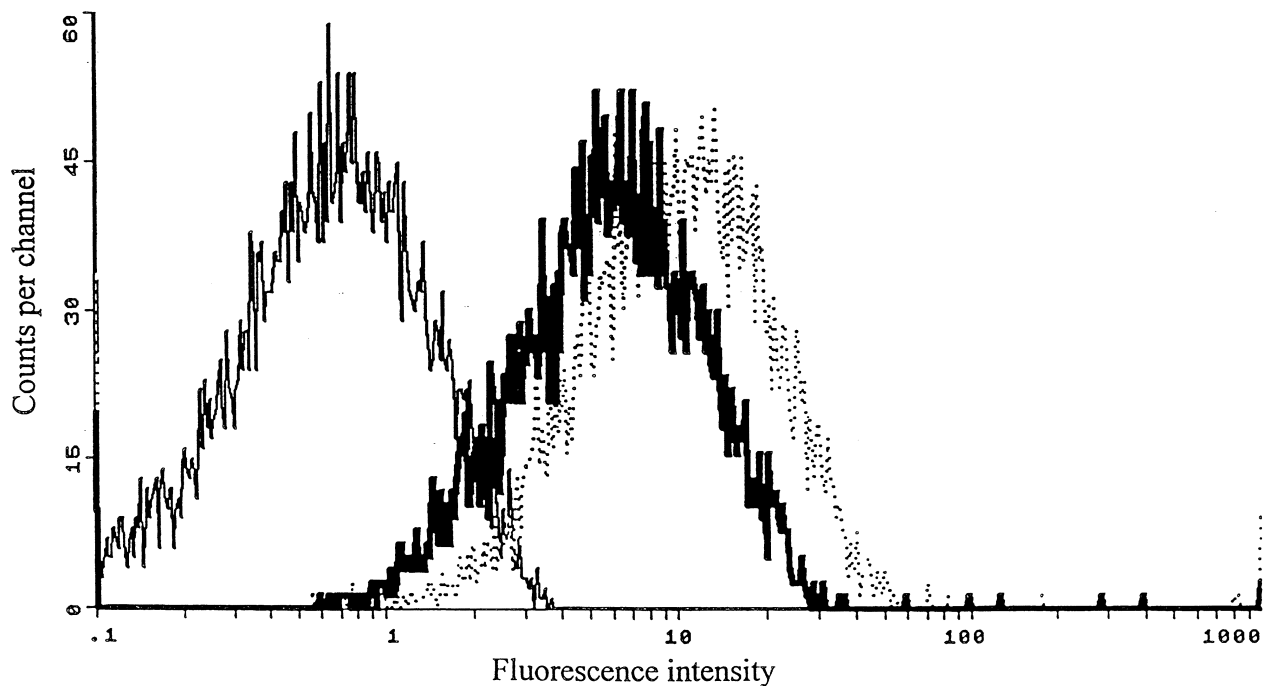


Fig. 1. Expression of the 37 kDa and 67 kDa proteins on unfixed cells incubated with MoAb 9G9 (anti-37 kDa) and MoAb 1D5 (anti-67 kDa —) and stained with IIF. The results are given as fluorescence intensity. The figure shows a representative histogram with logarithmic amplification of the fluorescence. Irrelevant mouse IgG1 (---) was used as background control. A minimum of 5000 cells were analysed for each MoAb.

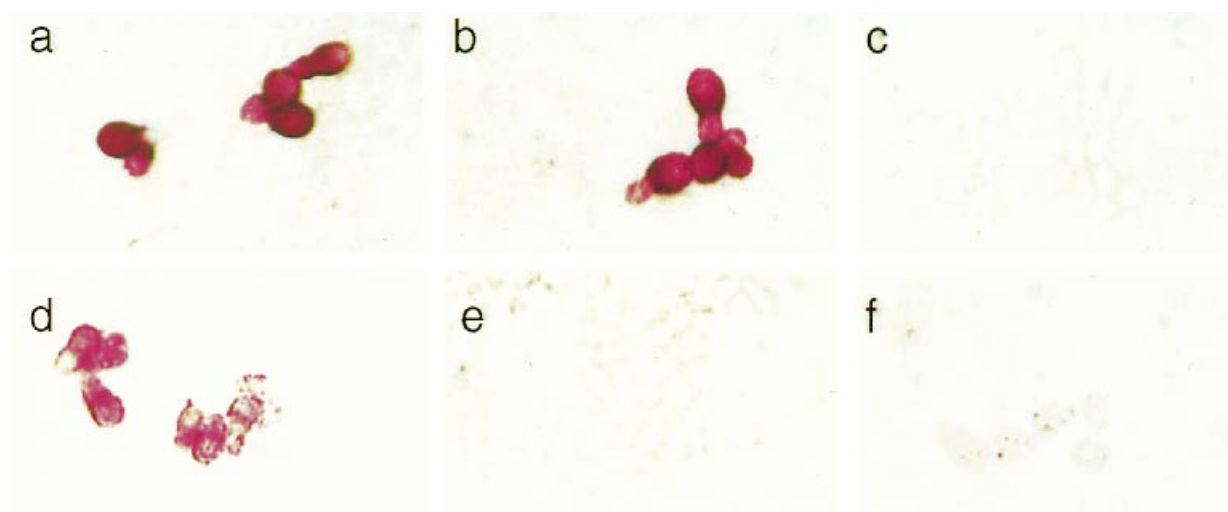


Fig. 2. Expression of the 37 kDa and 67 kDa proteins in acetone fixed yeast cells of 4 days culture. The yeast cells of *P. orbicularis* (a, b and c), *P. pachydermatis* (d and e) and *C. albicans* (f) were probed with anti-37 kDa (a and f), anti-67 kDa (b and d) and subclass matched irrelevant (c and e) monoclonal antibodies. Staining was performed with APAAP method (see methods section). Magnification $\times 1400$ used for ordinary light microscopy.

Table 2. Flow cytometric analysis of *P. orbicularis* yeast cells cultured for different time periods

Culture period (days)	Viability* (%)	Expression of the 37 kDa protein pos cells (%)**	Expression of the 67 kDa protein pos cells (%)**
2	100 $\pm$ 0.0	77 $\pm$ 6	79 $\pm$ 5
4	100 $\pm$ 0.0	85 $\pm$ 5	81 $\pm$ 2
8#	98 $\pm$ 0.3	76 $\pm$ 4	64 $\pm$ 4
15#	92 $\pm$ 1	62 $\pm$ 7	53 $\pm$ 2
22#	69 $\pm$ 1	63 $\pm$ 6	40 $\pm$ 2

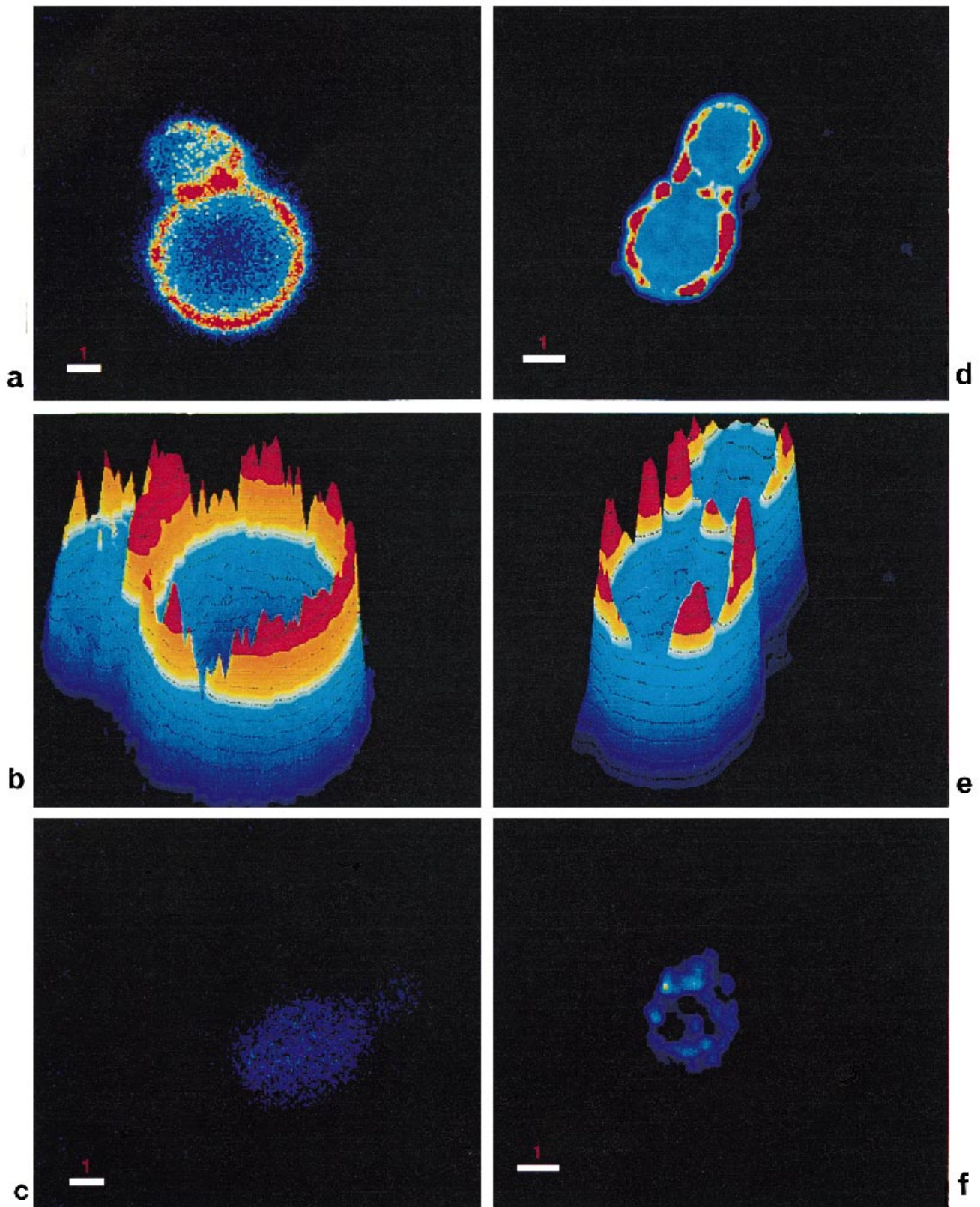
* Viability was estimated with Trypan blue exclusion. A minimum of 100 cells was counted.

** The expression was analysed with IIF in an Epics profile II flow cytometer. A minimum of 5000 cells was analysed, and the background value was subtracted.

A statistically significant decrease in the expression of the 37 kDa and 67 kDa component was found between day 2 or 4 and day 8, 15 and 22 ($P < 0.05$).

Mean value $\pm$ standard error of the mean are given for three different experiments.

Fig. 3 Expression of the 37 kDa allergen of *P. orbicularis* is restricted to the cell wall. *P. orbicularis* cells cultured for 4 days were analysed with CLSM after being probed with MoAb 9G9 (anti-37 kDa) and stained with either IIF method on fresh unfixed cells (a and b) or with the APAAP method using Fast Red on acetone fixed cells (d and e). The specificity test comprised replacement with a subclass-matched MoAb which showed negative staining with both methods (c and f). The cells were scanned with a $\times 100$ oil-immersion objective, with a pixel size $0.0025 \mu\text{m}^2$ and a stepsize of $0.4 \mu\text{m}$. Extended focus projections are shown from the summation of four serial optical sections in (a, c, d, and f). The intensity of staining is colour-banded (range red-yellow-green-blue-black, where red is the strongest). In (b and e) the staining is visualized with an 'intensity map projection' of (a and d). The heights of the peaks are proportional to pixel intensities (i.e. detected emitted light). The positive staining of the cell wall is clearly visible. Bar = $1 \mu\text{m}$.



the genus *Candida* and *S. cerevisiae* showed positive staining with the MoAbs.

Evaluation of the yeast cells in the UV microscope confirmed that almost all cells in the *Pityrosporum* genus stained positive. Positive cells were not found in the genus *Candida* or *S. cerevisiae*. The isotype-matched control MoAb showed negative staining for all yeasts.

Viability of the cells was in all experiments above 97% as estimated with Trypan blue exclusion and was confirmed with colony count of the cultured yeasts.

Evaluation of the allergen components in acetone-fixed yeasts stained with the APAAP method

To investigate whether the 37 kDa and 67 kDa proteins could be detected inside the cells as well as on the surface, the cells were permeabilized with acetone and stained with the APAAP method. *P. orbiculare*, *P. pachydermatis* and *M. sympodialis* yeast cells bound the anti-37 kDa and anti-67 kDa MoAbs to a similar degree as found with IIF and flow cytometry and showed negative staining for the control MoAb (Table 1 and Fig. 2). The staining intensity was stronger for the mother cells than the daughter cells (Fig. 2 a, b and d). Staining of *P. pachydermatis* was somewhat weaker for both MoAbs than that of *P. orbiculare* and *M. sympodialis* (Fig. 2 d). Specific staining was not observed for *C. albicans* (Fig. 2 f), *C. parapsilosis*, or *S. cerevisiae* with the two MoAbs used (Table 1).

Localization of the allergens with CLSM

After evaluation of the yeast cells with light microscopy, selected cells stained either with the IIF or the APAAP method were further analysed with CLSM. The CLSM revealed that the 37 kDa and the 67 kDa components were confined to the cell wall and could not be detected inside the acetone-fixed yeast cells of the genus *Pityrosporum* (Fig. 3a, b, d and e). The control irrelevant IgG1 MoAb showed negative staining (Fig. 3c and f). No staining was observed in the *Candida* genus and *S. cerevisiae*. The MoAb against mitochondrial porin showed positive intracellular staining, thus demonstrating that the acetone-fixation permeabilized the yeast cell wall and allowed penetration of the antibodies (data not shown).

Prolonged culture time is accompanied by decreased expression of the 37 kDa and 67 kDa allergens on P. orbiculare

In this series of experiments *P. orbiculare* was cultured for periods ranging from 2 to 22 days. The culture medium was glycerol monostearate olive oil [18] without yeast extracts instead of Dixon medium [17] and the incubation temperature raised from 32 °C to 37 °C. These changes induced no statistically significant difference in the expression of the

37 kDa and 67 kDa proteins on cells cultured for 4 days as analysed with flow cytometry ($P > 0.05$, Tables 1 and 2).

After more than 4 days of culture there was a statistically significant decrease in the expression of the 37 kDa and 67 kDa components of *P. orbiculare* ($P < 0.05$, Table 2). There was no statistically significant difference in the sensitivity of the two allergens to prolongation of the culture period. The viability of the yeast cells had decreased to around 70% at day 22 (Table 2).

Discussion

The lipophilic yeast *P. orbiculare* has attracted much interest during recent years, as it may play an important role in the pathogenesis of AD. Thus, in 40–65% of AD patients anti-*P. orbiculare* reactivity has been demonstrated with skin tests and/or specific IgE antibodies in serum [5–7, 21, 22]. We have raised MoAbs against two major allergens of this yeast and characterized them as 37 kDa and 67 kDa proteins [15]. In this study we identified the subcellular location of the two major 37 kDa and 67 kDa allergen components of *P. orbiculare* with flow cytometry and CLSM. Our results showed that these two proteins were expressed on the cell surface. We also investigated the presence of the allergens in the close relatives of *P. orbiculare*, the non-obligatory lipophilic yeast *P. pachydermatis*, isolated from animals and rarely found in humans [23], and the new member of the genus *Pityrosporum*, *M. sympodialis*, isolated from the ear canal of a healthy male and from the scalp of an AIDS patient with tinea capitis [24]. Our results indicate that all three members of the genus *Pityrosporum* expressed the 37 kDa and 67 kDa proteins on the cell surface as analysed with flow cytometry.

Since we have previously successfully localized major allergens in mites with CLSM [25], we decided also to use this technique. CLSM has several advantages over light microscopy [26], such as significant reduction of stray light, thus improving contrast, and increased resolution in both lateral and vertical dimensions. Furthermore, a specimen can be scanned sequentially at different depths, both horizontally and vertically. CLSM analyses of *P. orbiculare* revealed that the 37 kDa and the 67 kDa allergens could not be detected inside the protoplasm, but that they were located to the cell wall. The lateral resolution limit of CLSM as used in the present study is $\approx 0.16 \mu\text{m}$; thus more precise localization requires electron microscopy techniques. To ascertain that the MoAbs did have access to intracellular components in the acetone-fixed yeast cells, we included a MoAb against yeast mitochondrial porin. This MoAb showed a positive intracellular staining as analysed with 'optical sectioning' in the CLSM, thus demonstrating that MoAbs can penetrate into the acetone-fixed yeast cells. We can, however, not exclude that the negative intracellular

staining with the anti-37 kDa and anti-67 kDa MoAbs is due to destruction or alteration of their intracellular epitopes by the acetone-fixation.

The two proteins located in the cell wall may play a structural role in maintaining cell morphology and/or play a physiological role as enzymes. The lower expression of these proteins in the daughter-cells has to be explained; this will necessitate further experiments to clarify where in the cell cycle different compounds are built up. So far we have determined the complete cDNA of the 37 kDa allergen denoted Pit o 1. Computer comparisons with common data banks show that Pit o 1 is a novel protein [27]. It has a hydrophobic region of sufficient length at the *N*-terminal to be a cell wall protein (Schmidt *et al.* unpublished).

The enzyme enolase from *S. cerevisiae* is a major allergenic component and has allergenic crossreactivity with a *C. albicans* extract [28]. Savolainen *et al.* showed that the 46 kDa protein of *C. albicans* was the most important IgE-binding component found in a group of 156 subjects with AD [29]. The same investigators demonstrated parallel IgE antibody reactivity between this protein and *S. cerevisiae* enolase [30]. Ito *et al.* reported the crossreaction between *C. albicans* and *S. cerevisiae* and suggested that *C. albicans* enolase contains several IgE-binding determinants some of which crossreact with *S. cerevisiae* enolase [31]. Allergenic crossreaction has also been found between *P. orbiculare* and *C. albicans* [14, 32 and 33]. Therefore, we investigated the presence of the 37 kDa and 67 kDa proteins in other common yeasts such as *C. albicans*, *C. parapsilosis* and *S. cerevisiae*. Flow cytometry analysis and light microscopy revealed that less than 5% of the cells express the 37 kDa and 67 kDa proteins in these yeasts. Since, *Candida* and *Saccharomyces* grow faster than *P. orbiculare*, we have also investigated the presence of the 37 kDa and 67 kDa components in the early log phase of growth of these yeasts. Flow cytometry analysis revealed almost no differences in the expressions of the 37 kDa and 67 kDa proteins between 2-day-old and 4-day-old cultures of *Candida* and *Saccharomyces* (data not shown). Thus, the 37 kDa and 67 kDa components are not significantly produced in the early stage of life of these yeasts.

We have previously shown with SDS-PAGE and immunoblotting that the amount of allergenic protein in the *P. orbiculare* extract decreases with prolonged culture [16]. In the present study we used IIF and flow cytometry to investigate the presence of the 37 kDa and 67 kDa major allergens in *P. orbiculare* cultured for different periods. The result was consistent with our previous finding that the 37 kDa and 67 kDa expression decreases if the culture period exceeds 4 days. We therefore suggest that extracts obtained from the exponential phase of the yeast culture should be preferred in studies on IgE response to *P. orbiculare*.

As shown in Table 2 decrease in viability is accompanied

by a decrease in percentage *P. orbiculare* positive cells for the 37 kDa and 67 kDa allergens. We have also analysed cells which were cultured for 65 days with only 4% viability. Around 50% of the cells were reactive with the anti-37 kDa MoAb and about 25% of the cells were positive with the anti-67 kDa MoAb indicating that also dead cells can express the allergens (data not shown). We assume that the proteins are constantly produced by living cells, transported to the cell surface and then later released into the medium or degraded. One possible explanation for the expression of the allergens by dead cells could be that the cells die due to lack of nutrition in different stages of their life cycle before they are able to release the allergens into the environment. It still remains to be elucidated to what extent *P. orbiculare* cells on human skin express the allergens. We have preliminary data from seven patients with AD where *P. orbiculare* was isolated from lesional skin and found to react with our MoAbs against the 37 kDa and 67 kDa proteins, suggesting that our results reflect the *in vivo* situation (unpublished observation).

In conclusion, the 37 kDa and 67 kDa major allergens of *P. orbiculare* are expressed on the surface of the cells, a factor which is probably of significance in host–parasite interactions and suggests an active role of the yeast in the pathogenesis of AD.

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