Dynamic expression of cell-surface antigens probed with *Candida albicans*-specific monoclonal antibodies

N. Deslauriers, J. Michaud, B. Carré and C. Léveillé†

Author for correspondence: N. Deslauriers. Tel: +1 418 656 2067. Fax: +1 418 656 2861.
e-mail: Noella.Deslauriers@greb.ulaval.ca

Immunology Laboratory,
Groupe de Recherche en
Ecologie Buccale (GREB),
Faculty of Dental
Medicine, Université Laval,
Québec, Canada G1K 7P4

IgG hybridomas were produced which preferentially reacted with cell-surface antigens of either yeast cells or hyphae of *Candida albicans*. Four mAbs were used in an immunostaining procedure to follow the expression dynamics of these antigens in media supplemented with glucose or galactose. Yeast cell growth was analysed during the lag phase, the early- and late-exponential phases and the stationary phase, and mycelium formation was analysed between 0.5 and 24 h induction at 37 °C. It appears that yeast cell-surface antigens 5C11 and 2E11 are expressed throughout all phases of yeast cell growth as well as on young hyphae after up to 1 h induction. Longer hyphae only faintly react with these two mAbs as they switch to hyphal cell-surface antigens 2G8 and 4E1 after 3 h induction. The reactivity to mAbs 2G8 and 4E1 was induced after a 3 h temperature shift and was confined to the terminal third of growing mycelia. Growth and hyphae induction in galactose prolonged the reactivity of young hyphae with the two anti-yeast-cell mAbs, whereas the expression of surface antigens 2G8 and 2E11 appeared delayed and desynchronized on hyphae. Whereas a similar reactivity was found with ten ATCC strains of *C. albicans*, four clinical isolates had a unique pattern of reactivity. Immunoblot analyses of DTT extracts of cell-surface constituents indicated that the antigens were proteinaceous in nature and showed that yeast-cell antigens 5C11 and 2E11 are detected in four bands between 68 and 104 kDa, whereas mycelial antigens 4E1 and 2G8 are detected in 117 kDa and 104 kDa bands found in mycelial but not in yeast-cell extracts. Present data support the concept of a dynamic balance in the expression of phase-specific antigens in *C. albicans*.

Keywords: *Candida albicans*, monoclonal antibody, surface antigen

INTRODUCTION

Within the genus *Candida*, *Candida albicans* is of major medical importance, being the most commonly isolated species from various mucosal surfaces in healthy humans and from infectious sites in patients with candidiasis (Odds, 1988). *C. albicans* is a dimorphic opportunistic pathogen and may grow as budding yeasts (yeast cells) or filamentous chains (hyphae) depending on environmental conditions. There has been an enormous interest in *Candida* dimorphism because hyphae formation is believed to be a process of pathological significance (Cutler, 1991), and also because hyphal antigens may serve as specific

markers for *C. albicans* identification and candidiasis diagnosis (Poulain *et al.*, 1985). Cell-surface components expressed differentially in both morphological forms are likely to modulate host–parasite relationships, e.g. adherence to and invasion of host cells, cell signalling and interaction with phagocytes.

The composition of the *Candida* cell wall has been thoroughly studied over the past decade (reviewed in Garzon *et al.*, 1989), and, more recently, antigenic variations in cell-wall mannoproteins as a function of dimorphism were investigated with polyclonal antibodies and monoclonal antibodies (mAbs) (Chaffin *et al.*, 1988; Hopwood *et al.*, 1986; Poulain *et al.*, 1989; Sundstrom *et al.*, 1988; Li & Cutler, 1991; Torosantucci *et al.*, 1990; Brawner & Cutler, 1986; Casanova *et al.*, 1989; Ollert &

† Present address: CHUL, Ste-Foy, Quebec, Canada.

Calderone, 1990; Smail & Jones, 1984; Leusch, 1989; Ponton *et al.*, 1993; Sundstrom & Kenny, 1984; Chardes *et al.*, 1986). Some of these cell-surface antigens appear to be expressed on both yeast-cell and hyphal surfaces (Hopwood *et al.*, 1986; Poulain *et al.*, 1989; Sundstrom *et al.*, 1988; Li & Cutler, 1991; Torosantucci *et al.*, 1990), others being restricted either to yeast cells (Chaffin *et al.*, 1988; Sundstrom *et al.*, 1988) or to germ tubes (Casanova *et al.*, 1989; Ollert & Calderone, 1990; Smail & Jones, 1984; Leusch, 1989; Ponton *et al.*, 1993). In addition to suggesting that cell-surface antigens specific for yeast cells and hyphae may be identified, these studies have shown that these antigens are likely to be expressed dynamically during *Candida* growth and mycelium formation. Until now, most of the antigens identified appear to be carbohydrate in nature and, consequently, mAbs are typically IgM. Two reports have described IgG mAbs which react with polypeptides preferentially associated with mycelium-specific cell-wall mannoproteins (Li & Cutler, 1991; Ollert & Calderone, 1990). As polypeptide antigens are the best choice for the characterization of phase-specific epitopes after cloning and large-scale production, we sought to obtain immunological probes specific for surface antigens. IgG hybridomas were produced against *C. albicans* surface polypeptides, the expression of cell-surface determinants on individual cells was investigated during yeast-cell growth and hyphae formation and the molecular constituents were characterized by immunoblots.

METHODS

Micro-organisms. *C. albicans* ATCC strains 32354, 28366, 26555, 11006 and 36803 were purchased from the American Type Culture Collection. *C. albicans* ATCC strains 36801, 44374, 10231, 44373, 36802, and *Candida guilliermondii* (ATCC 9390), *Candida krusei* (Laboratoire de Santé publique du Québec 0052, Canada), *Candida parapsilosis*, *Candida pseudotropicalis*, *Candida tropicalis* (LSPQ 0057) and *Candida glabrata* were provided by Dr Pierre Auger (Université de Montréal, Canada). *C. albicans* strain LAM-1 was isolated from a case of systemic candidiasis and identified at the Hôpital du St-Sacrement, Quebec City, Canada (Lacasse *et al.*, 1990). Four strains of *C. albicans* were isolated from patients with *Candida*-associated denture stomatitis by Dr R. Pérusse (Faculty of Dental Medicine, Université Laval, Canada) and identified in our laboratory. Stock cultures were grown in liquid Sabouraud medium and aliquots were stored at -70°C in 50% (v/v) glycerol.

Culture conditions and growth curves. Frozen aliquots of *Candida* were thawed and cultured in Iscove's modified Dulbecco medium (IMDM) (pH 7.3, Sigma) supplemented with glucose (8.5 g l^{-1}) for 48 h in a moderately shaking water bath at 25°C to obtain stationary phase yeast cells. These were diluted to $5 \times 10^6\text{ ml}^{-1}$ in fresh IMDM supplemented with glucose (69.4 mM final concentration) or galactose (500 mM). For generating growth curves, yeast cells were grown for 4–48 h at 25°C in a shaking water bath. For determining the germination kinetics of *C. albicans*, suspensions were made in prewarmed medium and incubated at 37°C for 0.5–24 h in a shaking water bath.

Antigen preparations. *C. albicans* 32354 was used as a source of immunogens for cell fusions. Stationary phase yeast cells were prepared as above and mycelial phase organisms were obtained after 4 h incubation at 37°C . After washing twice in 0.1 M Tris

buffer, pH 8.1, outer-cell-wall antigens were extracted in DTT as described by Smail & Jones (1984) to yield extracts referred to as DTT-Y and DTT-M, respectively. DTT-Y extracts contain exclusively yeast-cell surface antigens, whereas DTT-M extracts contain a mixture of yeast-cell and mycelial antigens because DTT-M extracts are derived from hyphae-bearing yeast cells. The dialysed extracts were filtered before being lyophilized. After the protein content was estimated, extracts ($1\text{ mg protein ml}^{-1}$) were dissolved in Tris buffer and stored at -70°C . These extracts were used for immunization and ELISA protocols. For one immunization protocol, washed yeast cells were killed by overnight incubation in 0.5% formaldehyde in PBS (0.145 M NaCl, 0.15 M sodium phosphate) at 4°C . Aliquots ($1 \times 10^8\text{ ml}^{-1}$) were stored at -20°C after thorough washing in PBS.

Production of anti-*C. albicans* mAbs. BALB/c mice, 6–8 weeks old, were obtained from Charles River Breeding Farms (St Constant, Québec, Canada). To obtain the hybridomas described here, two immunization protocols were used. In the first, mice were primed by subcutaneous injection of $100\text{ }\mu\text{g}$ DTT-M in complete Freund's adjuvant. Two booster doses were given by intraperitoneal injections of $100\text{ }\mu\text{g}$ DTT-M at 15 d intervals. In the second, mice were primed by intraperitoneal injection with 5×10^7 formalin-killed yeast cells. Two booster doses of $100\text{ }\mu\text{g}$ DTT-M were given intraperitoneally at 15 d intervals. Following both protocols, the mice were sacrificed 3 d after the third injection. Spleen cells were fused with SP2 mouse myeloma cells and cultured in selective medium using a previously described procedure (Bazin & Lemieux, 1989). After 11 d growth, supernatants were screened by ELISA for Ig secretion using heavy-chain-specific affinity-purified antibodies (Kirkegaard and Perry Laboratory; KPL) and whole yeast cells or hyphae as coating antigens (see below). Hybridomas were then cloned by limiting dilution and clones were expanded for the production of supernatants and further characterization.

ELISA for anti-*Candida* reactivity. Hybridoma supernatants were screened by ELISA shortly after fusion and after cloning by limiting dilution. Briefly, 96-well round bottom plates (Immulon II; Costar) were treated with $50\text{ }\mu\text{g}$ poly-L-lysine ($1\text{ }\mu\text{g ml}^{-1}$ in 0.1 M PBS, pH 7.3) for 2 h at room temperature and washed thoroughly with PBS. Stationary phase yeast cells and hyphae of *C. albicans* ATCC 32354 prepared as above were suspended in 0.1 M PBS, pH 5.0, and distributed at 3×10^5 per well and 1×10^5 per well, respectively. Coating proceeded while drying overnight at 4°C . Plates were blocked with 1% (w/v) BSA in PBS and supernatants were added in $50\text{ }\mu\text{l}$ aliquots and incubated for 2 h at 37°C . Affinity-purified peroxidase-labelled goat anti-mouse IgG (KPL) was used at a 1/1000 dilution in PBS. 2,2'-Azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (KPL) was used as the substrate at 1 mg ml^{-1} in citrate buffer. The A_{405} values were read after 30 min. PBS (pH 7) plus Tween 20 (0.05%) was used for the washes between each step. In a series of experiments involving different species of *Candida* and different strains of *C. albicans*, whole micro-organisms were prepared in the same manner for coating the plates. Further characterization of the mAbs was carried out using $50\text{ }\mu\text{l}$ DTT-Y and $50\text{ }\mu\text{l}$ DTT-M (1 mg ml^{-1}) as coating antigens.

Immunostaining of the *Candida* cell surface with mAbs on multiwell microscope slides. The wells of multiwell microscope slides (Flow) were filled with a $20\text{--}30\text{ }\mu\text{l}$ aliquot of *C. albicans* suspensions (yeast cells or hyphae diluted to $5 \times 10^6\text{ ml}^{-1}$) in PBS. Slides were first treated with 0.1% poly-L-lysine in double-distilled water for 15 min to ensure strong adhesion of the micro-organisms. Samples were slowly

evaporated by warming the slides at 45 °C. The slides were stored at -20 °C or the samples were rehydrated in blocking buffer as follows. Yeast cells were incubated in TBS, pH 7.5, supplemented with 18% (w/v) glucose, 10% (v/v) glycerol, 0.75% Tween 20 and 2.5% (w/v) BSA. Mycelial-phase micro-organisms were incubated in TBS supplemented with 0.1% gelatin (J. T. Baker). Hybridoma supernatants were added in 30 µl aliquots and incubation and revelation of bound antibodies proceeded as in the ELISA assays using diaminobenzidine as the substrate. After washing, the slides were counterstained in 1% (w/v) toluidine blue in water for 10 min. Micro-organisms were also assayed by immunofluorescence using affinity-purified fluorescein and rhodamine-labelled goat anti-IgG antibodies (Sigma) diluted 1/200 in PBS supplemented with 0.5% Tween 20, 0.25% gelatin and 1% BSA. Counterstaining was done in 0.005% Evans blue. Negative controls were done by omitting each reagent in turn.

SDS-PAGE and Western blotting (immunoblotting). SDS-PAGE was performed using a minigel system (Bio-Rad): 150 µg volumes of DTT-M extracts were loaded and the electrophoresis was carried out using 7.5% (w/v) polyacrylamide gels at 100 V for 90 min. Gels were silver-stained (Ansorge, 1985) or electrophoretically transferred to a 0.45 µm nitrocellulose membrane (Bio-Rad) for 60 min at 100 V (Towbin *et al.*, 1979). After the transfer, the nitrocellulose membranes were blocked in TBS as described above before being sliced into strips for staining using a three-layer immunoenzymic technique. Strips were incubated in turn with mAbs (crude supernatants), biotin-labelled goat anti-mouse IgG diluted 1/500 (Sigma), biotin-labelled donkey anti-goat IgG diluted 1/500 (BioCan) and streptavidin alkaline phosphatase diluted 1/500 (Cedarlane). Strips were developed for 8 min in a nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate solution (Gibco). Negative controls included omitting each reagent in turn and positive controls included developing with mAb A2C7 (Stockbine *et al.*, 1984), which reacts with the *C. albicans* enolase (ATCC HB 8397). In preliminary experiments, thymol-sulfuric acid (Gerard, 1990) was used for staining membrane polysaccharides. In two experiments, DTT extracts were submitted to enzymic cleavage before electrophoresis or after protein transfer to a nitrocellulose membrane using a 2 h proteinase K treatment or N-glycosidase F (Boehringer) digestion (Marot-Leblond *et al.*, 1993). Immunoblots were digitally processed after scanning (Ofoto, Apple) to enhance contrast using Photoshop version 2.0 software (Adobe systems).

RESULTS

Immunoreactivity of four mAbs against *Candida* spp. yeast cells and *C. albicans* hyphae

Four IgG clones obtained using two immunization protocols were studied. As the preliminary screening was done with solid-phase whole micro-organisms, mAbs that reacted with surface determinants were selected. Furthermore, the ELISA screening protocol was designed to detect clones that produced mAbs which reacted with germ-tube-bearing micro-organisms but not with yeast cells alone (putative germ-tube-specific clones), or whose reactions were much stronger in wells coated with yeast cells than in wells containing hyphae-bearing yeast cells (putative yeast-cell-specific clones). Of the 237 IgG-secreting clones tested, 19 were positive in ELISA using whole *C. albicans* cells (strain ATCC 32354 cells, i.e. the immunogen). Two clones produced after the first

immunization protocol (2G8 and 4E1) reacted with the hyphae-bearing yeast cells of seven strains of *C. albicans* but not with *C. albicans* yeast cells alone, nor with yeast cells from the other *Candida* spp. Two clones produced after the second immunization protocol (5C11 and 2E11) reacted with yeast cells and with hyphae-bearing yeast cells of seven strains of *C. albicans* and with *C. guilliermondii* and *C. tropicalis* yeast cells.

When DTT extracts of *C. albicans* (ATCC 32354) yeast cells (DTT-Y) and of hyphae-bearing yeast cells (DTT-M) were used as coating antigens, the reactivities of 2G8 and 4E1 were restricted to wells coated with DTT-M extract, indicating that corresponding immunogens were recovered in extracts containing mycelial antigens but not in extracts containing only yeast-cell antigens. mAbs 5C11 and 2E11 reacted in wells coated with both DTT-M and DTT-Y extracts, indicating that corresponding immunogens were present in both extracts and were thus of yeast-cell origin. As expected, the reactions of mAbs 5C11 and 2E11 were much stronger with the DTT-Y extracts than with DTT-M extracts. This suggested that clones 5C11 and 2E11 might be yeast-cell-specific and that clones 2G8 and 4E1 might be hyphae-specific. An immunofluorescent analysis of these mAbs was carried out using these same strains and species. This qualitative study confirmed the ELISA data and supported our hypothesis that clones 5C11 and 2E11 react preferentially with yeast cells, whereas clones 2G8 and 4E1 react preferentially with hyphae (Fig. 1). Table 1 summarizes the data obtained after screening the mAbs with eight strains of *C. albicans* and six other *Candida* species.

Dynamic expression of yeast cell-surface antigens recognized by mAbs

C. albicans ATCC 32354 yeast cells were cultured in a synthetic medium, and samples corresponding to the lag phase (4 h), to the early-, middle- and late-exponential phases (10, 16 and 20 h, respectively) and to the early- and late-stationary phases (24 and 48 h, respectively) were removed. mAbs 5C11 and 2E11 (anti-yeast-cell) reacted with virtually all the yeast cells throughout the entire growth cycle. At the beginning of the exponential phase, young budding yeast cells tended to remain clustered. A small proportion of the aggregated cells in these clusters (5–20%) did not react with the mAbs between 10 and 16 h. Subsequently, the cells tended to dissociate and yeast-cell reactivity increased to 100% (Table 2).

Dynamic expression of mycelial-surface antigens recognized by mAbs

Hyphae formation by *C. albicans* ATCC 32354 yeast cells was induced by incubation at 37 °C for increasing periods of time. Throughout the germination process, mother yeast cells retained the cell-surface antigen recognized by mAbs 5C11 and 2E11 (anti-yeast-cell) (Fig. 2a). Hyphae formation started after 1 h and young hyphae still expressed yeast antigens. After 5 h induction, longer hyphae no longer reacted with mAb 5C11, whereas reactivity with mAb 2E11 started to decrease and

Fig. 1. Immunofluorescent staining of *C. albicans* with mAbs. (a) mAb 5C11 preferentially reacts with *C. albicans* yeast cells. (b) Bright-field illumination of (a). (c) mAb 2G8 preferentially reacts with *C. albicans* hyphae. Hyphae-bearing yeast cells were harvested after 4 h induction at 37 °C. (d) Bright-field illumination of (c). Bars, 10 µm.

disappeared after 24 h incubation. Anti-mycelium antibodies 2G8 and 4E1 reacted strongly with the entire surface of young hyphae and, thereafter, with the terminal third of the tip of growing mycelia (Fig. 2b, c). Quantitative assessment of the proportion of micro-organisms stained with these two mAbs at any time-point suggested that the antigens are expressed synchronously in virtually the entire population throughout the growth kinetics (Table 2).

Expression of cell-surface antigens in galactose-supplemented medium

As high concentrations of galactose in defined medium stimulates both the synthesis and immunoreactivity of the

fibrillar material, including adhesins, on the *Candida* cell surface (McCourtie & Douglas, 1984, 1985; Critchley & Douglas, 1987), a kinetic study was also carried out in galactose-supplemented medium. mAbs 5C11 and 2E11 (anti-yeast-cell) exhibited the same reactivity with *C. albicans* ATCC 32354 yeast cells under these conditions. However, 2E11 reactivity with young hyphae is conserved longer in galactose-supplemented medium, being observed even after 24 h induction over the entire surface of growing hyphae. On the other hand, cell-surface structures that react with mAbs 2G8 and 4E1 (anti-mycelium) are expressed on a lower proportion of hyphae when germination is induced in medium supplemented with galactose (< 50 % after 6 h). Even after 24 h, less than half of the hyphae stained positive with these mAbs.

Table 1. Reactivity of four mAbs with whole *Candida* yeast cells and mycelia assayed by immunofluorescence on hyphae-bearing yeast cells

Mycelial induction was at 37 °C in glucose-supplemented IMDM for 4–6 h. Y, Reactivity with mother yeast cells; M, reactivity with growing mycelia.

Candida strain/species	mAbs							
	5C11		2E11		2G8		4E1	
	Y	M	Y	M	Y	M	Y	M
<i>C. albicans</i>								
ATCC 32354 (serotype A)	+	–	+	–	–	+	–	+
ATCC 36801 (serotype A)	+	–	+	–	–	+	–	+
ATCC 36802 (serotype B)	+	–	+	–	–	+	–	+
ATCC 44373 (serotype B)	+	–	+	–	–	+	–	+
ATCC 28366 (serotype unknown)	+	–	+	–	–	+	–	+
ATCC 26555 (serotype unknown)	+	–	+	–	–	–	–	–
ATCC 36803 (serotype B)	–	–	–	–	–	+	–	+
LAM-1 (serotype unknown)	+	–	+	–	–	+	–	+
Other <i>Candida</i> spp.								
<i>C. guilliermondii</i>		+		+		–		–
<i>C. pseudotropicalis</i>		–		–		–		–
<i>C. tropicalis</i>		+		+		–		–
<i>C. glabrata</i>		–		–		–		–
<i>C. krusei</i>		–		–		–		–
<i>C. parapsilosis</i>		–		–		–		–

Table 2. Kinetic study of mAb reactivity with *C. albicans* ATCC 32354 cell-surface antigens

Results shown are a semi-quantitative assessment of the percentage of positive cells: $\pm$, ~ 25%; +, ~ 50%; ++, ~ 75%; +++, 100%. Note that hyphae are not present at the beginning of the kinetics. Y, Reactivity with mother yeast cells; M, reactivity with growing hyphae (mycelia).

Time (h)*	mAbs							
	5C11		2E11		2G8		4E1	
	Y	M	Y	M	Y	M	Y	M
0	+++		+++		–		–	
2	+++	+++	+++	+++	–	–	–	–
3	+++	++	+++	++	–	$\pm$	–	$\pm$
4	+++	$\pm$	+++	++	–	$\pm$	–	+
5	+++	–	+++	++	–	+	–	++
6	+++	–	+++	+	–	++	–	+++
24	+++	–	+++	–	–	+++	–	+++

* Time of mycelial induction at 37 °C in glucose-supplemented IMDM.

Dynamic expression of cell-surface antigens on various strains of *C. albicans*

The kinetic study with *C. albicans* ATCC 32354 was compared to studies of three ATCC strains and four clinical isolates (Table 3). Two out of these three ATCC

strains and all the clinical isolates reacted much like *C. albicans* ATCC 32354 with 5C11 (anti-yeast-cell), 2G8 and 4E1 (anti-mycelium). This suggested that the time-regulated expression of the cell-surface antigens defined by these three mAbs may be a feature common to most *C. albicans* isolates. mAb 2E11 had a unique pattern of

Fig. 2. Immunoenzymic staining of cell-surface antigens on *C. albicans* using mAbs. (a) Reactivity of mother yeast cells with mAb 5C11 (anti-yeast-cells) in the late stage of the germination process (between 5 and 24 h induction). Parts of some hyphae still faintly react with this mAb. (b) Reactivity of young mycelium (3 h induction) with anti-hyphae mAb 2G8. (c) Reactivity of longer mycelium (5–24 h induction) with mAb 2G8. Bars, 10 µm.

Table 3. mAb reactivity with yeast cells and mycelia of various strains of *C. albicans* as shown by immunoenzymic staining

Results shown are a semi-quantitative assessment of the percentage of positive cells: —, 0%; +, <30%; ++, 30–60%; +++, >60%. Y, Stationary phase yeast cells grown in glucose-supplemented IMDM; M, hyphae after 24 h induction at 37 °C in glucose-supplemented IMDM.

Strains of <i>C. albicans</i>	mAbs							
	5C11		2E11		2G8		4E1	
	Y	M	Y	M	Y	M	Y	M
ATCC 32354	+++	—	+++	—	—	+++	—	+++
ATCC 10231	+++	—	+++	—	—	+++	—	++
ATCC 36802	+++	—	++	—	—	+++	—	+++
ATCC 44374	—	—	—	—	—	++	—	++
Patient 1 isolate	+++	—	+++	+++	—	+++	—	+++
Patient 2 isolate	+++	—	+++	+++	—	+++	—	+++
Patient 3 isolate	+++	—	+++	+++	—	+++	—	++
Patient 4 isolate	+++	—	+++	+++	—	+	—	++

reactivity with the four clinical isolates as over 90% of hyphae stained along their full length from 7 to 24 h after induction. This contrasted with the reactivity of this mAb with the other *C. albicans* strains, which was restricted to yeast cells and to young hyphae (see above), but is reminiscent of the staining pattern obtained using this mAb with *C. albicans* ATCC 32354 yeast cells grown in galactose-supplemented medium (see above).

Extraction of cell-surface antigens with DTT

Cell-surface antigens that reacted with the four mAbs were released following mild extraction with DTT (see above). DTT-Y and DTT-M extracts no longer reacted

with any of the mAbs (data not shown). Under the conditions used, DTT-Y and DTT-M extracts were resolved by SDS-PAGE into approximately 25 major bands as revealed by protein staining (Fig. 3a). Polysaccharides and proteins larger than 200 kDa did not enter the gel. Quantitative differences were observed for most bands resolved from DTT-Y and DTT-M extracts, but the presence of protein bands unique to one or the other extract could not be unambiguously demonstrated.

Attempts to identify the molecular constituents recognized by the mAbs were made using immunoblots of DTT-M and DTT-Y extracts of *C. albicans* ATCC 32354. In immunoblots, the yeast-cell antigens 5C11 and 2E11 appeared to be carried by four bands of between 68

Fig. 3. Immunoblot analysis of *C. albicans* antigens with mAbs. (a) Electrophoretic pattern of DTT-Y (lane 1) and DTT-M (lane 2) extracts after silver staining. Lane 3, electrophoretic pattern of DTT-M extract after thymol-sulfuric acid staining. (b) DTT-M extracts were separated and transferred to nitrocellulose sheets and reacted with mAb 4E1 (lane 1) or mAb 5C11 (lane 3). The same mAbs were used to stain proteinase-K-treated nitrocellulose transfers (lane 2, mAb 4E1; lane 4, mAb 5C11). Treating the membrane with glycosidase before the reaction with the mAb did not change the pattern of lanes 1 and 3. DTT-Y extracts were separated and transferred to nitrocellulose sheets and reacted with mAb 5C11 before (lane 6) or after (lane 5) proteinase K treatment of the membrane. Treating the membrane with glycosidase before the reaction with the mAbs did not change the pattern of lane 6. Lane 7, control mAb A2C7 (anti-enolase). Immunoblots were digitally processed after scanning to enhance contrast.

and 104 kDa (Fig. 3b). Similar patterns of reactivity were observed with the two mAbs whether DTT-Y or DTT-M extracts were used, indicating that yeast-cell antigens 5C11 and 2E11 are still present on hyphae-bearing yeast

cells and confirming immunostaining data that yeast cells retain their reactivity with 5C11 and 2E11 mAbs irrespective of their growth cycle and of the germination process. There appeared to be no correlation with the presence in SDS-PAGE of a 117 kDa band found in much higher concentrations in DTT-Y extracts than in DTT-M extracts (Fig. 3a) and the reactivity of the two anti-yeast-cell mAbs with one or the other of the DTT-Y and DTT-M extracts (Fig. 3b), since both showed the same pattern of reactivity. On the other hand, the 96 and 82 kDa bands were major components in DTT extracts separated by electrophoresis and silver-stained (Fig. 3a), while the 104 and 68 kDa immunogens detected in immunoblots (Fig. 3b) with both 5C11 and 2E11 mAbs were only faintly revealed by silver staining (Fig. 3a).

The mycelial antigens 4E1 and 2G8 appeared to be carried predominantly by the 117 and 104 kDa components found in DTT-M extracts (Fig. 3b), but not in DTT-Y extracts. Both 4E1 and 2G8 mAbs showed the same pattern of reactivity with DTT-M immunoblots and both mAbs failed to react with DTT-Y immunoblots. Even though a 117 kDa protein was present at high concentrations in DTT-Y extracts and at very low concentrations in DTT-M extracts on SDS-PAGE silver-stained profiles, the reactivity of anti-mycelial mAbs with DTT-M immunoblots but not with DTT-Y immunoblots suggests that it is independent of this protein component. On the other hand, the 104 kDa immunogen detected by anti-mycelial mAbs on DTT-M immunoblots appeared to be absent from DTT-Y immunoblots, even though an immunogen of similar molecular mass is the basis of anti-yeast-cell mAb 5C11 and 2E11 reactivity.

None of the immunoreactive bands were revealed by polysaccharide staining of the membrane, thymol-sulfuric-acid-positive material being confined to the top of the gel (>200 kDa) (Fig. 3b). Glycosidase treatment of the nitrocellulose sheets had no effect, while proteinase K abolished the reactions with all the mAbs (Fig. 3b).

DISCUSSION

Four mAbs were analysed by immunostaining, ELISA and immunoblotting for their reactivity with *C. albicans* and other *Candida* species. Hybridomas were produced and screened to identify *C. albicans*-specific mAbs and phase-specific mAbs, avoiding cross-reactivity with common antigenic structures present in other *Candida* spp. Mannan is an immunodominant cell-surface antigen (Summers *et al.*, 1964) and *C. albicans* isolates can be divided into two highly cross-reactive serotypes, designated A and B, on the basis of this polysaccharide (Hasenclever & Mitchell, 1961). *C. tropicalis* contains cross-reacting antigens as well but appears more closely related to *C. albicans* serotype A than to serotype B (Hasenclever *et al.*, 1961). The specificity of the mAbs that we describe appeared to be serotype-independent and not related to mannan, as suggested by the negative reaction of two strains of *C. albicans* and most other species of *Candida*. However, anti-yeast-cell mAbs reacted with a common determinant on nine out of eleven ATCC strains

of *C. albicans*, four clinical oral *C. albicans* isolates, one *C. tropicalis* strain and one *C. guilliermondii* strain. A similar pattern of reactivity of polyclonal antibodies and mAbs with *C. albicans*, *C. guilliermondii* and *C. tropicalis*, but not with *C. krusei*, *C. parapsilosis* and *C. glabrata*, was reported by other groups (Chaffin *et al.*, 1988; Miyakawa *et al.*, 1986; Tsuchiya *et al.*, 1974; Cassone *et al.*, 1988). Even though this panel of species does not correspond to any of the major *Candida* groups in the serological classification scheme of Tsuchiya *et al.* (1974), these pathogenic species may possess common epitopes. However, this does not imply that the same antigenic determinant is involved, as discussed by Chaffin *et al.* (1988). In fact, carbohydrate epitopes were proposed as recognition structures for the aforementioned antibodies, whereas peptidic epitopes are probably involved in reactions with our mAbs. In any case, this particular pattern of reactivity restricts their specificity to a narrow range of surface antigens that deserves further attention.

When intact organisms are treated with DTT, a complex array of proteins, glycoproteins and polysaccharides is extracted and the surface of the micro-organisms appears roughened (reviewed in Jones, 1989). Such treatment abolished the reactivity of whole yeast cells with mAbs 5C11 and 2E11, the antigens being recovered in the DTT soluble extracts. That the two anti-yeast-cell mAbs shared the same pattern in immunoblots while 2E11 reacted more strongly with growing hyphae (older than 7 h induction), especially in galactose-supplemented medium, and with mycelia from clinical isolates suggests that these two mAbs recognized different epitopes on a common protein or that their affinity for a common epitope was different. It is possible that the expression of antigens 2E11 and 5C11 would not be restricted to yeast cells but rather would be quantitatively different on the mycelial surface.

On the other hand, the two mAbs (2G8, 4E1) that have a similar reactivity with hyphae appear to detect a mycelium-specific antigen as no reaction was ever observed with yeast cells from *C. albicans* or any other *Candida* sp. by immunofluorescent/immunoenzymic staining or in ELISA/immunoblots of DTT-Y extracts. This antigen appeared early during the germination process and persisted on the distal portion of older hyphae. With the exception of strain 44374, where only 50% of hyphae reacted with mAbs 2G8 and 4E1, the antigens were expressed on virtually every germ tube in other strains when grown in glucose-supplemented medium. A high concentration of galactose in defined medium has been shown to stimulate both the synthesis and immunoreactivity of the fibrillar material on the *Candida* cell surface, including adhesion molecules (McCourtie & Douglas, 1984, 1985; Critchley & Douglas, 1987). However, our study shows that the percentage of reactive hyphae was both lowered and delayed, if not abolished, when grown on galactose, suggesting that either a denser mucous layer (Garzon *et al.*, 1989) would hamper the access of relevant epitopes to the mAbs, or the expression of the antigens is downregulated under these culture conditions. It cannot be excluded that the

shedding (McCourtie & Douglas, 1985) of 2G8 and 4E1 during germ-tube development in galactose-supplemented medium would decrease their density at the cell surface and preclude their detection by immunostaining.

As summarized recently by Ponton *et al.* (1993), different types of germ-tube surface antigens have been studied by different groups using polyclonal antisera adsorbed with yeast cells or using mAbs. True germ-tube-specific antigens (type I antigens) appear to be scarce, however, because most hyphae-specific mAbs also react with either DTT-treated yeast cells (type II antigens) or both yeast cells and germ tubes (type IV antigens). We believe that 2G8 and 4E1 are type I antigens because no reactivity has ever been demonstrated with these mAbs against yeast cells of any *Candida* strain tested before or after DTT treatment, and because DTT-Y extracts tested negative in ELISA and in immunoblots probed with the 2G8 and 4E1 mAbs. The reactivity of these two mAbs with determinants found at growing hyphal tips from the early initiation of germination to up to 24–48 h induction is reminiscent of mAbs H9 and C6 described by Brawner & Cutler (1986). However, these mAbs are of the IgM isotype and are thought to be directed against carbohydrate rather than protein antigens expressed continuously or periodically on both yeast cells and hyphae.

The notion of a dynamic balance in the expression of phase-specific antigens, which would be environmentally regulated, is supported by the fact that mycelial antigens are preferentially expressed at the growing tip of hyphae, that anti-yeast-cell mAbs react with emerging hyphae but not with older hyphae and that expression of both mycelial and yeast antigens is modified in galactose-supplemented medium. Moreover, that the expression of yeast-cell antigen 2E11 is retained on the surface of growing hyphae in clinical isolates points to an *in vivo* correspondence with an *in vitro* regulated shift. *In vivo* reactivity of these mAbs against *C. albicans* was previously tested in our experimental mouse model (Lacasse *et al.*, 1990). Mice were topically infected with *C. albicans* LAM-1 and imprints were taken at the peak of the acute infection for immunofluorescent staining (Lamy *et al.*, 1992). Antigens 5C11, 2E11, 2G8 and 4E1 were expressed within the tissue microenvironment, indicating that the specificity of the mAbs for probing *C. albicans* under physiological conditions was conserved.

The identity of the antigens inducing phase-specific mAbs is not known, although efforts have been made to characterize them. Until now, most of the mycelial-phase-specific antigens identified by polyclonal antisera have been found to have molecular masses ≥ 155 kDa (Leusch, 1989; Ponton & Jones, 1985; Sundstrom & Kenny, 1985), and those identified by mAbs have been found to have an even higher molecular mass (180–260 kDa) (Casanova *et al.*, 1989; Marot-Leblond *et al.*, 1993; Torosantucci *et al.*, 1990; Ponton *et al.*, 1993). Most of these antigens are carbohydrates. Surface proteins with molecular masses between 50 and 68 kDa were detected with germ-tube-specific mAbs (Ollert & Calderone, 1990;

Linehan *et al.*, 1988). As our mAbs 2G8 and 4E1 recognize 104 and 117 kDa proteins on the surface of mycelial cells, they thus differ from all other mycelial-phase-specific mAbs produced until now. To further characterize and purify the mycelial cell-surface antigens 2G8 and 4E1, and also to monitor the differential expression of these antigens in hyphae and yeast cells, cloning of the relevant peptidic epitopes is critical, and immunological probes are invaluable for this purpose. This work is in progress in our laboratory.

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