Monoclonal antibody 3H8: a useful tool in the diagnosis of candidiasis

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In a previous series of experiments six mAbs were obtained against cell wall extracts of *Candida albicans* ATCC 26555. After several studies only one of them, designated 3H8, has been used to produce a commercial kit for the rapid diagnosis of candidiasis, Bichro-latex albicans (Fomouze Diagnostics). The present study involved the generation and characterization of this mAb as an immunoglobulin G1 which recognizes mannoproteins of high molecular mass present in the *C. albicans* cell wall. ELISA assays showed that the presence of the epitope recognized by mAb 3H8 was similar in both yeast and mycelial cell walls of *C. albicans*, in contrast to the epitope for mAb 1B12, which is mainly expressed in the yeast cell wall. The 3H8 epitope was located at the external surface in *C. albicans* ATCC 26555, whereas it is partially cryptic in the cell wall in other *C. albicans* strains. No reaction was observed with other *Candida* species. Immunohistochemical studies using this antibody demonstrated that it specifically recognized *C. albicans* in tissue, detecting mycelial forms and, to a lesser extent, blastospores, suggesting that it is also a valuable tool in the evaluation of fungal infections in paraffin-embedded tissue, particularly when identification is required.

Keywords: *Candida albicans*, monoclonal antibody, diagnosis, cell wall

INTRODUCTION

Other than dermatophytosis, candidiasis is the most common fungal infection in humans. Different fungal species cause disseminated, invasive and organ infections, the incidence of which has increased dramatically in the last few years with the introduction of broad-spectrum antibiotics and the rising number of immunocompromised patients (Fox, 1993). *Candida albicans* is the species most commonly isolated from these patients, and therefore its identification and prompt diagnosis would allow early antifungal therapy.

Identification of *C. albicans* in the clinical microbiology laboratory is usually based on the germ tube test, a rapid procedure consisting of the production of germ tubes at 37 °C in the presence of serum. This technique has the disadvantage of being affected by laboratory conditions and the relative expertise of the technician who performs the test (Fenn *et al.*, 1996; Salkin *et al.*, 1987). Recently, other systems based on enzymic activities have been

evaluated for rapid identification of *C. albicans* (Dealler, 1991; Perry *et al.*, 1990).

In the case of cultured yeast colonies, the use of immunological tests with specific antisera has been described using either polyclonal (Shinoda *et al.*, 1981; Taguchi *et al.*, 1979) or monoclonal (Brawner & Cutler, 1984; Cassone *et al.*, 1988; Chaffin *et al.*, 1988; Guinet *et al.*, 1991; Hopwood *et al.*, 1986; Miyakawa *et al.*, 1986; Polonelli & Morace, 1986) antibodies, but these tests either lacked specificity or were not commercially available.

Procedures suitable for both clinical isolates and cultured yeast are now emerging. They are based on genomic amplification methods, such as PCR, and seem to detect and identify a large number of related species of pathogenic yeast using distinct target DNA sequences from either rDNA or secreted proteinase genes (Flahaut *et al.*, 1998; Mannarelli & Kurtzman, 1998).

With the aim of characterizing molecules of the *C. albicans* cell wall, we obtained various mAbs reactive against cell wall components. We previously reported the mAb designated 1B12, which specifically detects

Abbreviation: IIF, indirect immunofluorescence.

mannoproteins from *C. albicans*, but does not cross-react with other species, proving to be useful as a diagnostic tool in immunohistological identification (Marcilla *et al.*, 1993; Monteagudo *et al.*, 1995). Another antibody produced in this series, designated 3H8, has been used for the production of a diagnostic kit, Bichro-latex albicans (Fomouze Diagnostics, Asnières, France). This test has proven to be highly specific and sensitive for *C. albicans* (Dromer *et al.*, 1996; Freydière *et al.*, 1997; Quindós *et al.*, 1997; Robert *et al.*, 1994). In the present study the characterization of this IgG1 mAb, which is reactive against the high-molecular-mass mannoproteinaceous material extracted from *C. albicans* cell walls, is presented. In ELISA assays mAb 3H8 detects material from both yeast and mycelial cell walls to a similar extent, in contrast to mAb 1B12, which reacts against mycelial extracts to a lesser extent than against yeast extracts. mAb 3H8 also shows specific and strong cell surface immunostaining of *C. albicans* in paraffin-embedded tissues, being particularly effective in the detection of mycelial forms. These results suggest that this mAb is a powerful tool in the diagnosis of distinct types of candidiasis.

METHODS

Strains, cell culture and isolation of cell walls. *Candida albicans* ATCC 26555 (serotype A) and the clinical isolates 44195 (serotype A) and 89165 (serotype B) (kindly provided by Dr J. Guarro, Hospital del Mar, Barcelona) were used in this study, together with *Aspergillus fumigatus* 2071 and *Saccharomyces cerevisiae* X21801A, obtained from the CECT (Spanish Type Culture Collection). The identification of clinical strains had been confirmed by sugar assimilation tests and by serological assays using Candida-Check (Iatron Laboratories) (Monteagudo *et al.*, 1995). All strains were maintained by subculturing every 2–3 weeks on 1.5% Bacto agar slopes of Sabouraud dextrose medium. *Candida* species were propagated at 28 °C as yeasts in Lee's medium (Lee *et al.*, 1975), and the conversion of yeast to mycelium for *C. albicans* strains was carried out at 37 °C after cold starvation as described previously (Elorza *et al.*, 1988). *S. cerevisiae* was grown in YPD medium (1% Bacto yeast extract, 2% Bacto peptone and 2% glucose).

The isolation of cell walls of *C. albicans* ATCC 26555 after homogenization with glass beads, treatment with SDS and digestion with Zymolyase 20T, mainly a 1,3- β -glucanase (Seikagaku), has been previously described (Marcilla *et al.*, 1993).

Antibodies. 3H8, an IgG1 murine mAb raised against a Zymolyase-solubilized preparation from blastoconidia cell walls of *C. albicans* ATCC 26555, was made in female BALB/c mice (8–10 weeks old) immunized with intraperitoneal injections of 100 μ g antigen, measured as total sugar (Dubois *et al.*, 1956), emulsified in Freund's complete adjuvant. Two and four weeks later, the mice received a booster intraperitoneal injection with the same amount of antigen emulsified in Freund's incomplete adjuvant. One week after the last injection, mice were tail-bled and the reactivity of sera was tested for anti-antigen antibody titre by ELISA. Finally, mice were given a soluble intraperitoneal injection of 100 μ g antigen; 3–4 d later, three animals were killed, and their spleens were used as the source of B lymphocytes for fusion.

Myeloma cells were cultured in high-glucose Dulbecco's Modified Eagle's medium (Gibco) supplemented with 2 mM glutamine, non-essential amino acids, penicillin (100 U ml⁻¹), streptomycin (100 μ g ml⁻¹) and 15% (w/v) foetal bovine serum (s-DMEM). Cell fusion and selection of hybrids were carried out essentially as described by Nowinsky *et al.* (1979). Spleen lymphocytes from immunized mice were fused with P3-X63/Ag 8.653 murine myeloma cells (ATCC) at a 5:1 ratio using PEG 1500 as the fusing agent. The fused cells were distributed in 96-well culture plates (Cell-Cult) at an approximate density of 4×10^5 cells in 100 μ l s-DMEM per well; 24 h after plating, 100 μ l HAT selection medium (s-DMEM supplemented with hypoxanthine, aminopterin, thymidine) was added to each well. On day 10 post-fusion, the culture supernatants were screened for the presence of antibodies against the antigen. Positive hybridomas were cloned by limiting dilution on a feeder layer of BALB/c thymocytes (about 10^6 cells per well) and peritoneal macrophages (about 5000 cells per well). Cells were grown in HAT medium for 2 weeks, and the HAT was substituted by HT medium (HAT medium without aminopterin), and maintained in this medium. Positive clones were cultivated and ascites were obtained by injecting hybridoma cells (about 5×10^6 per mouse) into the peritoneal cavity of pristane-treated BALB/c mice. mAbs were purified from clarified ascites by (NH₄)₂SO₄ precipitation followed by ion-exchange chromatography on DEAE-Sepharose. mAb classes and subclasses were determined by ELISA using a Bio-Rad isotyping kit according to the manufacturer's instructions. The titre of antibodies during immunization and purification processes was determined by ELISA. mAb 1B12 (Marcilla *et al.*, 1993) was used as a control in some experiments.

ELISA. Determinations were made as described by Voller & Bidwell (1986) with the following modifications. Wells of Nunc-Immunoplate I (A/S Nunc) plates were coated with 10 μ g antigen (mannoproteinaceous material released by Zymolyase from yeast or mycelial cell walls) measured as total sugar (Dubois *et al.*, 1956). In some assays purified mannan (lacking proteinaceous material), obtained as previously described by Iranzo *et al.* (1994), was used in 50 μ l 0.03 M sodium carbonate buffer (pH 9.6) and incubated at 4 °C for 18 h. The plate was rinsed three times with PBS (0.01 M KH₂PO₄, pH 7.4; 0.15 M NaCl) containing 1% (w/v) BSA and 0.05% Tween 20 (PBST). mAbs 1B12 and 3H8 purified from ascites fluid were diluted 1:4000 with PBST, added to the appropriate wells and incubated at 37 °C for 1 h in a moist chamber. The wells were then rinsed three times with PBST and 50 μ l of a 1:100 dilution in PBST of goat anti-mouse polyvalent immunoglobulins conjugated with peroxidase (Bio-Rad) was added to each well. After incubation for 1 h at 37 °C, the wells were rinsed with o-phenylenediamine, the plate was incubated in the dark for 10 min, and the reaction was stopped by the addition of 25 μ l 3 M H₂SO₄ to each well. The colour intensity was determined at 492 nm with an automated plate reader (Titertek Multiskan Plus MKII).

SDS-PAGE and Western blot analysis. These techniques were essentially carried out as previously described (Marcilla *et al.*, 1993), using mAb 3H8 at a dilution of 1:2500.

Indirect immunofluorescence (IIF). The reactivity of 3H8 towards fungal cells in culture was assayed by IIF essentially as described by Casanova *et al.* (1989), using a dilution of 1:200 of the mAb, and examined with a Zeiss Photomicroscope III equipped for epifluorescence. Assays were carried out with cells from clinical isolates that were incubated at 28 and 37 °C, as we found that some wall antigens had different surface locations at the two different temperatures (see below).

Tissues and immunohistochemistry. Tissue samples from patients with well-characterized systemic and superficial candidiasis were analysed with mAb 3H8 according to the avidin-biotin immunoperoxidase technique described by Hsu *et al.* (1981). Specific anti-*C. albicans* mAb 1B12 (Marcilla *et al.*, 1993) was used as a positive control. In addition, samples from normal human tissues and from pulmonary aspergillosis were also included in the study as a negative control. Tissues (kidney, lung, thyroid, oesophagus and small bowel) were processed for immunohistochemistry staining as previously described (Monteagudo *et al.*, 1995), using a 1:200 dilution of the mAb (3H8). PBS was used for all washes between steps and for antibody dilutions. Negative control slides were made by replacing the primary antibody with PBS.

RESULTS

Characterization of the epitope reacting with mAb 3H8

We previously produced six different mAbs against the material covalently linked to the cell wall of *C. albicans* and released by Zymolyase. Three of them, designated 1B12, 3H8 and 3G10, were obtained using material from blastoconidia cell walls. mAb 1B12, an IgM, has been previously reported to be a valuable tool in the evaluation of candidal infections by immunohistochemical techniques (Marcilla *et al.*, 1993; Monteagudo *et al.*, 1995). To characterize mAb 3H8, we compared the immunoreactivity of this antibody with that obtained with mAb 1B12. Different materials solubilized from isolated yeast and mycelial cell walls from *C. albicans*, together with the material secreted by regenerating protoplasts, were assayed for their reactivity by ELISA. As shown in Table 1, mAb 3H8 exhibited strong reactivity against the material obtained from cell walls of both morphologies of *C. albicans*, whereas no reactivity against solubilized materials from *S. cerevisiae* was observed. In contrast, mAb 1B12 was less reactive against the material solubilized from mycelial cell walls (Table 1). Negative results were

obtained with both antibodies when preparations from other fungi were used (Marcilla *et al.*, 1993; Monteagudo *et al.*, 1995; Robert *et al.*, 1994), indicating that both antibodies were specific for *C. albicans*. After testing both mAbs, mAb 3H8 had been chosen for the design of a kit for rapid identification of colonies of *C. albicans* (Robert *et al.*, 1994), indicating its better performance in this identification system.

The nature of the epitope recognized by mAb 1B12 is mannoproteinaceous (protein moieties carrying short chains of O-linked sugars) (Marcilla *et al.*, 1993). To determine the nature of the epitope detected by mAb 3H8, oligosaccharide fractions of the mannoproteins present in the cell wall were obtained as previously described (Iranzo *et al.*, 1994), and tested by ELISA using both mAbs. mAbs 3H8 and 1B12 exhibited reduced levels of reactivity with mannan from *C. albicans* yeast, and did not react with mannan obtained from either the *C. albicans* mycelial cell wall or the *S. cerevisiae* cell wall (Table 1; Marcilla *et al.*, 1993). Moreover, when the materials secreted by regenerating protoplasts were analysed, an increase in the reactivity was observed in the presence of tunicamycin, an antibiotic which blocks the N-glycosylation of mannoproteins (Tackz & Lampen, 1975). These results suggest that mAb 3H8 recognized either a proteinaceous epitope or an O-linked mannoproteinaceous epitope, but did not detect the sugar moiety alone (mannan).

To identify the actual epitope recognized by mAb 3H8, we next analysed by SDS-PAGE and Western blotting the same preparations as those analysed by ELISA. mAb 3H8 detected a high-molecular-mass component (> 150 kDa) in the *C. albicans* cell wall extracts (Fig. 1, lanes 2 and 3). As expected, it failed to detect any material from *S. cerevisiae*, which was used as a control (lane 1). The antibody recognized two main polydisperse components secreted by regenerating protoplasts at 28 °C (lane 4), which were less polydisperse when grown in the presence of tunicamycin (lane 5). These results

Table 1. Comparison of the immunoreactivity of mAbs 3H8 and 1B12 against different antigenic preparations from *C. albicans* and *S. cerevisiae* by ELISA

Values indicate the A_{492} and are the mean of three experiments; the SD was never higher than $\pm 10\%$. ND, Not determined.

Antigen	mAb 3H8		mAb 1B12	
	28 °C	37 °C	28 °C	37 °C
Zymolyase-released material from <i>C. albicans</i> cell walls	1.71	1.60	1.20*	0.37
Zymolyase-released material from <i>S. cerevisiae</i> cell walls	0.07	ND	0.05	ND
Supernatant of regenerating control protoplasts	0.27	ND	0.48	ND
Supernatant of regenerating protoplasts in the presence of tunicamycin	0.86	ND	0.67	ND
Mannan from <i>C. albicans</i> cell walls	0.25	0.03	0.15*	0.05
Mannan from <i>S. cerevisiae</i> cell walls	0.03	ND	0.04	ND

* Similar results to previous reports (Marcilla *et al.*, 1993).

Fig. 1. Western blot stained with mAb 3H8 of cell wall mannoproteins and materials secreted by regenerating protoplasts. Samples containing 200 µg carbohydrate were analysed by SDS-PAGE (8%). Zymolyase-released materials from cell walls of *S. cerevisiae* (lane 1) and *C. albicans* ATCC 26555, grown either at 28 °C (lane 2) or 37 °C (lane 3), were obtained and analysed by Western blotting. Materials secreted by *C. albicans* protoplasts regenerating for 5 h at 28 °C in either the absence (lane 4) or the presence (lane 5) of 20 µg tunicamycin ml⁻¹ were obtained and processed identically.

confirmed the ELISA data and support the notion of a mannoproteinaceous epitope recognized by mAb 3H8 which contains a small O-linked oligosaccharide moiety.

Fig. 3. Western blot detection with mAb 3H8 of materials released by Zymolyase 20T from purified cell walls. Lanes: 1 and 4, *C. albicans* ATCC 26555; 2 and 5, *C. albicans* 44195; 3 and 6, *C. albicans* 89165. The organisms were grown at either 28 °C (lanes 1–3) or 37 °C (lanes 4–6).

Localization of the epitope detected by mAb 3H8 in the cell wall of *C. albicans*

Because the epitope recognized by mAb 3H8 was present in the *C. albicans* cell wall and not in *S. cerevisiae*, we then analysed its specificity against other *Candida* species by IIF. Cultures from four different clinical strains of *C. albicans*, together with cultures of *Candida tropicalis* and *Candida guilliermondii*, were grown at either 28 or 37 °C (to induce mycelium formation).

Fig. 2. Phase-contrast (a, c) and IIF detection (b, d) with mAb 3H8 of *C. albicans* strain 44195, grown either at 28 °C (a, b) or 37 °C (c, d). The experiment was reproduced three times. Bars, 10 µm.

Fig. 4. Immunohistochemical detection of *C. albicans* using mAb 3H8. Samples from kidney (a, b), thyroid gland from patients suffering from candidiasis (c) and thyroid gland from a healthy individual as a control (d) were obtained and processed as described in Methods. Arrows in (b) indicate blastospores negative for detection. Bars, 10 μ m.

Other fungi, such as *A. fumigatus* and *S. cerevisiae*, were also analysed. No reactivity was obtained when species other than *C. albicans* were assayed (data not shown). Identical results had been reported when using the Bichro-latex albicans kit, with only 1.13% of false negative results (Quindós *et al.*, 1997; Robert *et al.*, 1994). As shown in Fig. 2, a clinical isolate of *C. albicans* (*C. albicans* 44195) surprisingly exhibited reactivity by IIF only when grown at 37 °C (Fig. 2d), and not at 28 °C (Fig. 2b). Identical results were obtained when *C. albicans* 89165 was used. These observations raised the question as to whether the epitope was not expressed in some strains under these culture conditions, or whether it was present but localized internally in the cell wall. When isolated cell walls from *C. albicans* ATCC 26555 and clinical strains 44195 and 89165 grown at either 28 or 37 °C were obtained, and Zymolyase extracts were

analysed by SDS-PAGE and Western blotting, the epitope was detected in all extracts (Fig. 3), demonstrating that it was probably internally located in the cell wall in a clinical strain. These results explain the previous empirical observation that adding a dissociating agent (including enzymes like Zymolyase) is needed in the Bichro-latex albicans kit to improve the reactivity against yeast colonies (Robert *et al.*, 1994).

Immunohistochemical detection of *C. albicans* in human tissues

In previous studies we had shown the usefulness of mAb 1B12 in other diagnostic techniques such as immunohistochemistry (Monteagudo *et al.*, 1995). Because this mAb 1B12 showed less reactivity against solubilized material from *C. albicans* mycelial cell walls (Table 1),

we hypothesized that mAb 3H8 could be more sensitive in immunohistochemistry techniques, since both morphologies of *C. albicans* are found in infections (Fox, 1993). mAb 3H8 was effective in detecting *C. albicans* in paraffin-embedded human tissue sections (Fig. 4). Cell surface 3H8 immunoreactivity was present in most (>90%) pseudomycelial or filamentous forms of *C. albicans* (Fig. 4a), but surprisingly only in a minority (about 20%) of yeast cells (Fig. 4b, c), where most unreactive yeasts were immunostained with mAb 1B12 (Monteagudo *et al.*, 1995; and data not shown), in spite of the fact that both antibodies were obtained against the yeast cell wall (Marcilla *et al.*, 1993; see above). On the other hand, immunoreactivity with normal human tissues (Fig. 4d) or *Aspergillus* species was not detected (data not shown). These observations suggest that mAb 3H8 is better than mAb 1B12 at identifying filamentous forms of *C. albicans* in infected tissues, without lacking reactivity against blastospores. Future assays will address whether the use of a combination of both mAbs could have a synergistic effect in the sensitivity of the immunohistochemistry technique.

DISCUSSION

The results of this study confirm that mAb 3H8 is a powerful tool in the identification of *C. albicans* by different techniques. Its use in the Bichro-latex albicans kit, which is a rapid identification system for colonies, has been documented, showing it to be highly specific for *C. albicans*, ranging from 99.87 to 100% depending on the study: it is also very sensitive (Dromer *et al.*, 1996; Freydière *et al.*, 1997; Quindós *et al.*, 1997; Robert *et al.*, 1994). This kit is reliable and can also be used directly on blood culture bottles, allowing rapid identification of *C. albicans* fungaemia (Laurent *et al.*, 1996). Our results demonstrate that even in cases when systemic candidiasis is suspected but blood cultures are negative (Jones, 1990), mAb 3H8 can be a useful tool in the diagnosis of candidiasis. Thus, histological identification is a method of choice in some cases, and the availability of specific mAbs, such as 3H8, can be useful in the detection of *C. albicans* in tissue by immunofluorescence or immunohistochemistry. The use of specific reagents avoids the complication of identifying other pathogenic fungi difficult to distinguish from *C. albicans*, such as *Aspergillus*, *Cryptococcus* and *Histoplasma* (Chandler *et al.*, 1989; Kovacs *et al.*, 1988). The absence of immunoreactivity of normal human tissues, together with the fact that no immunostaining of *Aspergillus* has been found, supports the value of this antibody in the diagnosis of filamentous forms of fungi in tissue samples, especially in systemic mycoses, since *Candida* and *Aspergillus* are the most commonly isolated fungi in these processes (Jones, 1990). Future investigations will address the usefulness of mAb 3H8 in other clinical non-invasive techniques such as ELISA capture assays for measuring antigens from blood samples.

mAb 3H8 recognizes a mannoproteinaceous epitope of the *C. albicans* cell wall, which may account for its high

specificity. Other antibodies which react exclusively against the carbohydrate moieties of mannoproteins also react with other *Candida* species (Calderone & Braun, 1991; Iranzo *et al.*, 1994). Here we demonstrate that the weak (or non) reactivity in some strains in IIF was due to inaccessibility of the epitope, as shown by Western blotting (Fig. 3). This inconvenience can easily be overcome in the identification of colonies by the Bichro-latex albicans kit by adding a small amount of hydrolytic enzymes to the reaction mixture, as had been done empirically (Robert *et al.*, 1994).

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