

## Characterization of *Candida albicans* Cell Wall Antigens with Monoclonal Antibodies

JOSE PONTON,<sup>1\*</sup> AGNES MAROT-LEBLOND,<sup>2</sup> PILAR A. EZKURRA,<sup>1</sup> BEGOÑA BARTUREN,<sup>1</sup>  
RAYMOND ROBERT,<sup>2</sup> AND JEAN MARCEL SENET<sup>2</sup>

*Departamento de Inmunología, Microbiología y Parasitología, Facultad de Medicina y Odontología, Universidad del País Vasco, Apartado 699, E-48080 Bilbao, Vizcaya, Spain,<sup>1</sup> and Laboratoire d'Immunologie-Parasitologie, Section Pharmacie, UFR des Sciences Médicales et Pharmaceutiques, 49100 Angers, France<sup>2</sup>*

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The antigenic composition of *Candida albicans* is very complex. In order to study the antigenic relationship between blastoconidia and germ tubes of *C. albicans*, we produced several monoclonal antibodies and analyzed their reactivity against cell wall antigens either in intact cells or in cells treated with dithiothreitol. Overall, four types of reactivity were found. Monoclonal antibodies 3D9 and 15C9 stained the germ tubes only when tested by indirect immunofluorescence. However, they showed a different reactivity by immunoblotting. Monoclonal antibody 3D9 reacted with antigens with molecular masses of >200 and 180 kDa specifically expressed in the germ tube. Monoclonal antibody 15C9 reacted with antigens of 87, 50, and 34 kDa present in the germ tube extract and with antigens of 92, 50, 34, and 32 kDa present in the blastoconidium extract. The reactivity of blastoconidia treated for different times with dithiothreitol with these monoclonal antibodies was also studied by enzyme-linked immunosorbent assay. The reactivity of monoclonal antibody 3D9 did not significantly change during the cell wall extraction. However, the reactivity of monoclonal antibody 15C9 was increased for blastoconidia extracted for 60 min and decreased markedly for blastoconidia extracted for 120 min. Monoclonal antibody G3B was nonreactive by indirect immunofluorescence but reacted with antigens of 47 and 38 kDa present in the germ tube extract and with an antigen of 47 kDa present in the blastoconidium extract. Monoclonal antibody B9E stained both morphological phases by indirect immunofluorescence. By immunoblotting, it reacted with antigens of >70 kDa present in the germ tube extract and with antigens of >63, 56, 47, and 38 kDa present in the blastoconidium extract. Based on the results presented in this study, four types of antigens are described. Type I antigens are expressed on the outermost layers of the germ tube cell wall only. Type II antigens are expressed both on the germ tube cell wall surface and within the blastoconidium cell wall. Type III antigens are found within the cell wall of both blastoconidia and germ tubes. Type IV antigens are expressed on both the blastoconidium and germ tube surface. Two types more can be hypothesized for antigens expressed on the blastoconidium cell surface and within the germ tube cell wall (type V) and for those expressed on the blastoconidium surface only (type VI).

*Candida albicans*, a member of the normal human flora, is emerging as a leading pathogen in immunocompromised patients (15). Diagnosis of invasive *C. albicans* infections presents unique problems since there are no pathognomonic signs of the disease, blood cultures are often negative, and the collection of biopsy tissue may not be feasible in some patients (1, 19). Antigens from both the blastoconidium and the germ tube, the two morphological phases of the fungus observed in infected tissues, have been used in a variety of serological tests in an attempt to improve diagnosis (10). However, most tests used in *Candida* serology lack specificity (17). Antigens expressed specifically on the germ tube cell wall surface could be the basis for more-specific tests (23). Indeed, specificity of serodiagnosis of invasive candidiasis has been improved when tests detecting antibodies against those antigens are used (24, 25).

Germ tube-specific antigens of 230 to 250, 200, 155, 35, 27, and 19 kDa have been identified by using polyclonal antisera adsorbed with heat-killed blastoconidia (14, 21, 29). By using monoclonal antibodies (MAbs), germ tube-specific components of 260, 180, 60, and 55 kDa have also been described (5, 18). However, there is some evidence suggesting the

presence of similar components in the blastoconidium either in a cryptic state (8, 27) or as the result of antigenic variability (4). In an attempt to test these two hypotheses, we have examined the reactivity of several MAbs with different *C. albicans* cell wall antigens.

### MATERIALS AND METHODS

**Organisms and culture conditions.** *C. albicans* serotype A (NCPF 3153) was obtained from the National Collection of Pathogenic Fungi (London, United Kingdom). It was maintained at 4°C on slants containing 20 g of glucose, 10 g of yeast extract, and 20 g of agar per liter. For most experiments, *C. albicans* blastoconidia and germ tubes were grown in medium 199 (Sigma Chemical Co., St. Louis, Mo.) as previously described (20). In some of the immunofluorescence studies, *C. albicans* was grown in different media (glucose-yeast extract-peptone, medium 199, modified Lee's medium, or 10% fetal calf serum) as described by Brawner et al. (4).

**Antigenic extraction.** Cell walls of *C. albicans* blastoconidia and germ tubes were extracted for 4 h in the presence of dithiothreitol (DTT) as described by Smail and Jones (27). Cell wall extracts from 24-h-old blastoconidia and 3-h-old germ tubes were labeled DTT-B and DTT-GT, re-

\* Corresponding author.

spectively. In some experiments, cell walls of blastoconidia or germ tubes were extracted with DTT for 0.5, 1, 1.5, 2, and 3 h. After extraction, organisms were washed twice in phosphate-buffered saline (PBS) and fixed either to the wells of Teflon-coated microscope slides or to 96-well vinyl plates (Costar Europe, Oud-Beijerland, The Netherlands).

**MAbs.** Eight immunoglobulin M (IgM) MAbs (15C9, 3D9, G3B, B9E, PA10F, 11C11, 21E6, and D9F), one IgG MAb (14.8), and one IgA MAb (N3B) were employed. MAbs were produced by standard methods. Briefly, BALB/c mice were immunized by subcutaneous injections of different *C. albicans* antigens or antigenic extracts: a partially purified antigen of 260 kDa from a DTT-GT extract (G3B, B9E, PA10F, and N3B), a partially purified antigen of 47 kDa from a blastoconidium cytoplasmic extract (D9F), germ tube cell wall components adhered to plastic petri dishes (21E6), the germ tube fibrinogen binding factor (3D9), a germ tube culture supernatant (11C11 and 15C9), and a blastoconidium water-soluble antigen (14.8). Fusion of splenocytes from the high-responding mouse from the SP<sub>2</sub>O or x653Ag8 cell line was performed with polyethylene glycol 4000 (Merck, Darmstadt, Germany). The culture supernatants were assayed for antibodies against a DTT or  $\beta$ -mercaptoethanol germ tube extract by an enzyme-linked immunosorbent assay (ELISA) (32) and against whole *C. albicans* germ tubes and blastoconidia by indirect immunofluorescence. Positive hybrids were subcloned twice by limiting dilution. Antibodies used in this study were contained in cell supernatant fluid harvested from 10- to 15-day-old cultures of the respective hybridoma cells grown in Iscove's medium (Flow Laboratories, Irvine, Scotland, United Kingdom) supplemented with 10% fetal bovine serum (Flow).

**Immunofluorescence.** Indirect immunofluorescence assays (IFA) were carried out as previously described (24). Briefly, 10- $\mu$ l aliquots of each MAb were applied to the wells of Teflon-coated microscope slides to which *C. albicans* blastoconidia and germ tubes had been fixed and incubated for 30 min at 37°C. After being washed, the slides were incubated with fluorescein isothiocyanate-conjugated, goat anti-mouse IgM, IgG, or IgA (Sigma) diluted 1:100 in PBS supplemented with Evans blue (0.001%) and Tween 20 (0.001%) for another 30 min under the same conditions. The slides were washed again, mounted with carbonate-glycerol mounting fluid, and examined with a microscope equipped to detect reflected fluorescence.

**ELISA.** The ELISA was carried out by a modification of the method of Sundstrom and Kenny (28). Briefly, each well of plates (Costar) was coated with 100  $\mu$ l of either DTT-treated blastoconidia ( $3 \times 10^6$ /ml) or germ tubes ( $1 \times 10^6$ /ml) suspended in PBS and incubated overnight at 4°C. Plates were blocked by adding 200  $\mu$ l of PBS containing 1% bovine serum albumin (BSA) (fraction V; Sigma) and 0.05% Tween 20 and 10% formaldehyde to each well for 1 h at 37°C. They were then incubated with the MAbs diluted from 1:30 to 1:1,000 in PBS-0.05% Tween 20 (100  $\mu$ l per well) for 1 h at 37°C. Plates were washed and incubated with biotin-conjugated, goat anti-mouse IgM, IgG, or IgA (Sigma) diluted 1:2,000 in PBS-BSA for 1 h at 37°C. Plates were washed again and incubated with peroxidase-conjugated ExtrAvidin (Sigma) diluted 1:80 for 1 h under the same conditions. After washing, 100  $\mu$ l of a solution containing 0.05% orthophenyldiamine dihydrochloride (Pierce Europe, Badhoevedorp, The Netherlands) and 40  $\mu$ l of 30% hydrogen peroxide in phosphate-citrate buffer 0.15 M (pH 5.0) were added to each well, and the plates were incubated in the dark at room temperature for 30 min. The reaction was stopped with 50  $\mu$ l

TABLE 1. Reactivity of MAbs against blastoconidium and germ tube antigens by IFA, ELISA, and Western blotting

MAb	IFA reactivity <sup>a</sup> of:		ELISA reactivity <sup>b</sup> of:				Western blot reactivity <sup>c</sup> of:	
	BL <sup>d</sup>	GT <sup>d</sup>	Untreated cells		DTT-treated cells		DTT B	DTT-GT
			BL	GT	BL	GT		
3D9	-	+	L	H	L	H	-	+
PA10F	-	+	L	H	H	H	+	+
15C9	-	+	L	H	H	H	+	+
11C11	-	+	L	H	H	H	+	+
B9E	+	+	H	H	H	H	+	+
N3B	+	+	H	L	H	H	+	+
14.8	-	-	L	L	H	H	+	+
D9F	-	-	L	L	H	H	+	+
G3B	-	-	L	L	H	H	+	+
21E6	-	-	L	L	L	H	+	+

<sup>a</sup> -, no fluorescence; +, fluorescence.

<sup>b</sup> L, low ( $A_{450}$  < 0.3); H, high ( $A_{450}$  > 0.3).

<sup>c</sup> -, no bands; +, one or more bands.

<sup>d</sup> BL, blastoconidia; GT, germ tubes.

of 1 N H<sub>2</sub>SO<sub>4</sub>, and optical densities were read with a Titertek Multiscan at 450 nm.

**SDS-PAGE and Western blotting (immunoblotting).** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (13) in a minigel system (Bio-Rad Laboratories, Richmond, Calif.). The total amount of protein loaded per lane was 5  $\mu$ g for each extract. Electrophoresis was carried out in 6 and 13% polyacrylamide slab gels at 200 V for 45 min. Standards and molecular weights were as follows: myosin, 200,000; *Escherichia coli*  $\beta$ -galactosidase, 116,250; rabbit muscle phosphorylase b, 97,400; BSA, 66,200; hen egg white ovalbumin, 45,000; bovine carbonic anhydrase, 31,000; soybean trypsin inhibitor, 21,500; and hen egg white lysozyme, 14,400. Subsequently, the gels either were stained with Coomassie blue or were electrophoretically transferred to a nitrocellulose membrane (Bio-Rad) for 30 min at 60 V, 10 W, and 5 mA/cm<sup>2</sup> according to the method of Towbin et al. (31). After the transfer, the nitrocellulose membranes were blocked in 10% nonfat dry milk in Tris-buffered saline, washed in Tris-buffered saline, incubated with 1:20 to 1:100 dilutions of the MAb, washed, and incubated with peroxidase-labeled, affinity-purified goat anti-mouse IgM (Sigma). Immunoreactive bands were visualized after staining for 30 min with a substrate solution (0.05% 4-chloro-1-naphthol [Sigma] and 0.015% H<sub>2</sub>O<sub>2</sub> in Tris-buffered saline).

## RESULTS

**IFA staining of blastoconidia and germ tubes.** Three types of reactivity were observed when the MAbs were tested by IFA against both blastoconidia and germ tubes grown in medium 199. MAbs 15C9, 3D9, PA10F, and 11C11 stained the germ tube surface only. MAbs B9E and N3B reacted with both blastoconidia and germ tubes. No fluorescence was observed when the cells were incubated with MAbs G3B, 14.8, 21E6, and D9F (Table 1).

In some experiments, the influences of growth in four different media (glucose-yeast extract-peptone, medium 199, modified Lee's medium, or 10% fetal calf serum), temperature (24 and 37°C), and length of incubation (0.25, 0.5, 1, 2, 3, 4, and 24 h) on the surface expression of the antigens

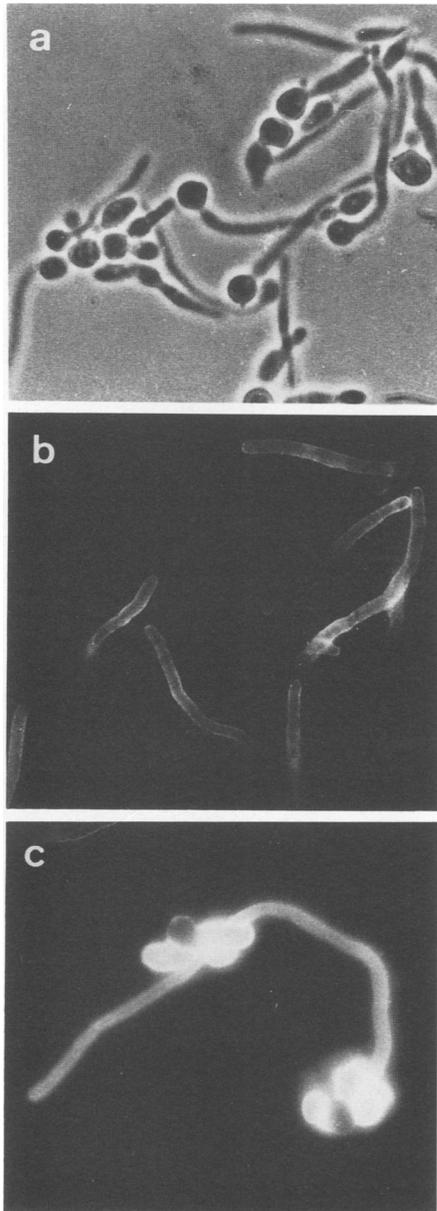


FIG. 1. Phase-contrast (a) and immunofluorescence (b) photographs, of the same microscopic field, stained with MAb 15C9. (c) Immunofluorescence photograph of blastoconidia and germ tubes stained with MAb B9E. Magnification,  $\times 1,000$ .

reacting with four of the MAbs were also investigated by IFA. Regardless of the conditions used, MAbs 3D9 and 15C9 showed a strong fluorescence, which was always restricted to the germ tubes (Fig. 1b). MAb B9E strongly reacted with both blastoconidia and germ tubes (Fig. 1c). On the contrary, no fluorescence was observed on both blastoconidia and germ tubes incubated with MAb G3B.

**Immunoblot analysis using the MAbs.** When the MAbs were tested by immunoblotting, different reactivities were observed. However, they could be divided into two groups: those reacting with both DTT-GT and DTT-B extracts and those reacting with DTT-GT extracts only. Most of the MAbs reacted with antigens present in both antigenic extracts, MAbs 15C9, G3B, and B9E being representative of

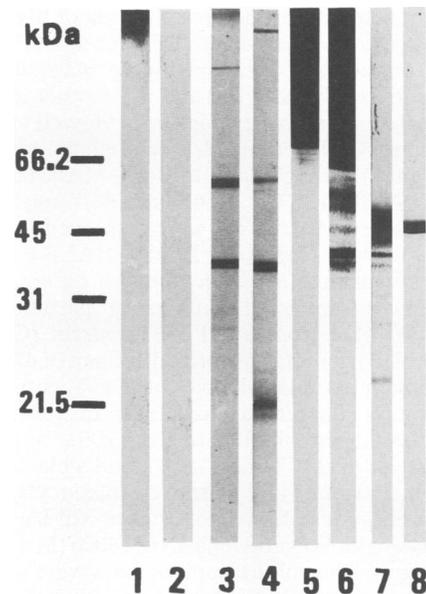


FIG. 2. Western blots of 13% polyacrylamide slab gels loaded with DTT extracts from germ tubes (lanes 1, 3, 5, and 7) or blastoconidia (lanes 2, 4, 6, and 8), stained with MAbs 3D9 (lanes 1 and 2), 15C9 (lanes 3 and 4), B9E (lanes 5 and 6), and G3B (lanes 7 and 8). Molecular masses of standard proteins are listed to the left of the gel.

this group (Table 1). MAb 15C9 reacted with antigens of 87, 50, and 34 kDa present in the DTT-GT extract and with antigens of 92, 50, 34, and 22 kDa present in the DTT-B extract. MAb G3B reacted with antigens of 47 and 38 kDa present in the DTT-GT extract and with an antigen of 47 kDa present in the DTT-B extract. MAb B9E reacted with antigens of  $>70$  kDa present in the DTT-GT extract and with antigens of  $>63$ , 56, 47, and 38 kDa present in the DTT-B extract. MAb 3D9 reacted with antigens specifically expressed in the germ tube and not resolved in an SDS-13% PAGE gel (Fig. 2). Their molecular masses in an SDS-6% PAGE gel were  $>200$  and 180 kDa.

**Expression of antigens through the cell wall.** The reactivity of MAb 15C9 was surprising, since it reacted with antigens present in both DTT-GT and DTT-B extracts by immunoblotting but only the germ tubes were stained when studied by IFA. A possible explanation for this observation is that antigens reacting with MAb 15C9 are expressed on the surface of the germ tube cell wall and within the blastoconidium cell wall. In an attempt to demonstrate this possibility, the cell wall of blastoconidia was treated with DTT for different times and the reactivity of the blastoconidia with the MAb was studied by both IFA and ELISA. Although no reactivity was observed by IFA, an increase in reactivity with MAb 15C9 was seen in blastoconidia extracted with DTT for 1 h. This reactivity disappeared in blastoconidia extracted for longer time (Fig. 3). No changes in reactivity were observed when the treated blastoconidia were incubated with MAb 3D9.

The same approach was also used to study the location of other antigens in the cell wall. A summary of the reactivity of the MAbs is shown in Table 1. In most cases, the reactivity of germ tubes increased with DTT treatment, suggesting an increase in the exposition of the antigens. An example of such reactivity is presented by MAb N3B (Fig. 4). However,

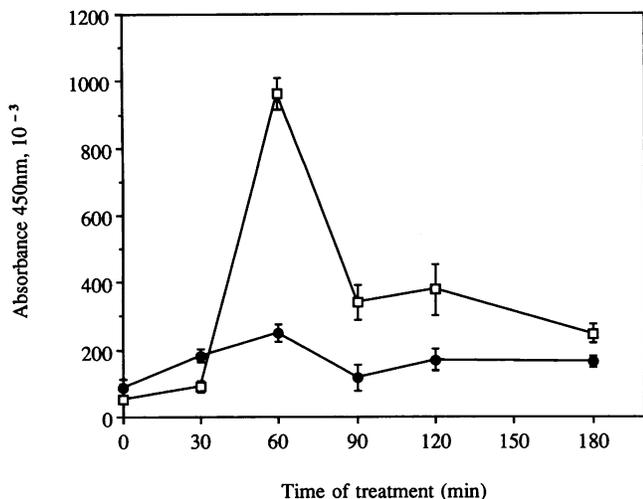


FIG. 3. Reactivity of DTT-treated blastoconidia with MAbs 3D9 (●) and 15C9 (□) measured by ELISA. Datum points represent the means of triplicate determinations  $\pm$  standard errors of the means.

the reactivity of one of the MAbs (B9E) with germ tubes was very high and not altered with DTT treatment (Fig. 4). Other MAbs, such as B9E and N3B, showed a decrease in reactivity during the initial treatment of blastoconidia with DTT, reaching a minimum at 60 min. The subsequent treatment of the cell wall produced an increase in reactivity, which reached values similar to those of untreated blastoconidia by the end of treatment.

## DISCUSSION

The cell wall of *C. albicans* is a multilayered structure (23). Immunoelectron microscopy, the best method to locate the antigens in the *C. albicans* cell wall, has been used in some studies of MAbs (2, 3, 16). However, it requires expertise as well as sophisticated equipment, and it is difficult to apply to some antigens. The sequential treatment of the cell wall with DTT or other agents can be used as a preliminary approach to study the distribution of the cell wall antigens. With this technique, we have been able to demonstrate that some antigens are expressed uniformly throughout the cell wall but some others appear to be irregularly distributed within this structure. Particularly interesting is the increase in reactivity observed in germ tubes incubated with most MAbs reacting with germ tube antigens studied, suggesting an increase in the antigen content deeper in the germ tube cell wall. By contrast, the reactivity of some MAbs with the blastoconidium seems to rise and fall as DTT extraction proceeds, suggesting that the content of those antigens is lower in some layers of the blastoconidium cell wall. However, studies to confirm by immunoelectron microscopy the distribution of antigens observed by ELISA in DTT-treated *C. albicans* cells as well as the purification and chemical characterization of the antigens are needed to draw definitive conclusions.

The antigenic composition of the cell wall of *C. albicans* has been extensively studied in an attempt to identify antigens of diagnostic relevance (17). Among them, those expressed in the germ tube are thought to be important since the filamentous phase is present in infected tissues (23). Several germ tube-specific antigens have been described by using both polyclonal antisera adsorbed with heat-killed

blastoconidia and MAbs (5, 14, 18, 21, 29), and detection of antibodies against antigens specifically or primarily expressed in the germ tube has been used in serodiagnosis of candidiasis (9, 12, 25). Despite their potential for serodiagnosis, the identities of the antigens responsible for the induction of anti-germ tube antibodies are not fully known, and therefore, no test based on purified germ tube-specific antigens has been developed.

By using a polyclonal antiserum eluted from the surface of whole germ tubes, which was postulated to contain the anti-germ tube antibodies which Quindós et al. (25) detected in the IFA used in the serodiagnosis of invasive candidiasis, Regúlez et al. (26) have identified four antigens of 230 to 250, 62, 43, and 41 kDa in DTT-GT extracts. Surprisingly, the same antigens were also observed in DTT-B extracts. Evidence suggesting the presence of germ tube-specific antigens in the blastoconidium either in a cryptic state or as the result of antigenic variability or cross-reactivity has been presented in different studies (4, 8, 27, 29).

Existence of different types of germ tube surface antigens could explain the conflicting results observed by different groups. The data presented in this paper confirm this possibility. At least three different types of antigens can be described on the *C. albicans* germ tube surface. Type I antigens are truly germ tube specific and are expressed on the germ tube cell wall surface only. Antigens reacting with our MAb 3D9 and the components of 260 and 180 kDa described by Casanova et al. (5) belong to this type. Type II antigens seem to be expressed on both the germ tube cell surface and within the blastoconidium cell wall. These antigens would appear as germ tube specific if tested by indirect immunofluorescence. However, they can be extracted from the blastoconidium cell wall with treatments which remove the outermost layers. Antigens reacting with MAbs 15C9, PA10F, and 11C11 and possibly the antigen described by Ollert and Calderone (18) and the C3d receptor of *C. albicans* described by Kanbe et al. (11) belong to this type. The presence of type II antigens within the blastoconidium cell wall would explain the induction of anti-germ tube antibodies in rabbits immunized with DTT-B, heat-

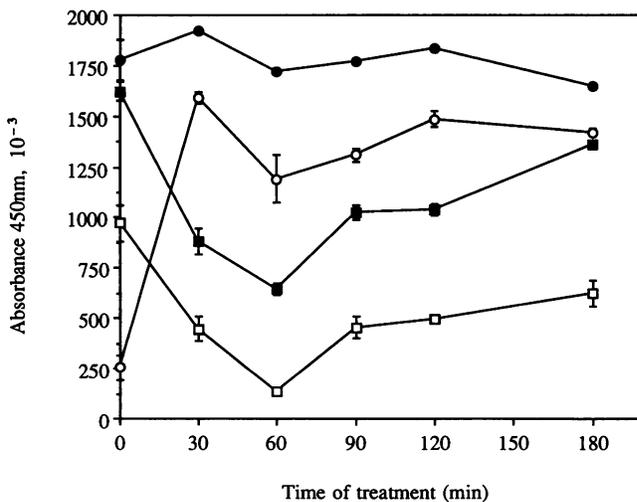


FIG. 4. Reactivity of DTT-treated blastoconidia (squares) or germ tubes (circles) with MAbs B9E (closed symbols) and N3B (open symbols) measured by ELISA. Datum points represent the means of triplicate determinations  $\pm$  standard errors of the means.

killed blastoconidia, and formalin-killed blastoconidia (26) or in both patients (25) and rabbits (22) infected with *Candida* spp. different from *C. albicans* which are unable to produce germ tubes in serum.

Type III antigens seem to be expressed on both the blastoconidium and germ tube surface. Antigens reacting with MAbs B9E and N3B and the mannans belong to this type. MAbs G3B, 21E6, D9F, and 14.8 defined a fourth type of antigen expressed within the cell wall of both blastoconidia and germ tubes. MAb C6 described by Brawner and Cutler (3), MAbs 3B7 and 3G6 described by Fortier et al. (7), and MAb AF-1 described by Cassone et al. (6) may belong to this type. In addition to the types already described, two types more can be hypothesized for antigens expressed on the blastoconidium cell surface and within the germ tube cell wall (type V) and for those expressed on the blastoconidium surface only (type VI). Sundstrom et al. (30) have described three MAbs which reacted with the blastoconidium by IFA but failed to react with germ tubes.

On the basis of the results presented in this paper it is likely that the polyclonal antisera adsorbed with heat-killed blastoconidia initially used to identify the *C. albicans* germ tube-specific antigens were detecting both type I and II antigens. Differentiation of these two types of antigens may have practical implications since detection of anti-*C. albicans* germ tube antibodies is useful for the serodiagnosis of invasive candidiasis (25). However, to detect the anti-germ tube antibodies, the sera have to be adsorbed, a process that takes time and needs large amounts of heat-killed blastoconidia. A test based on the detection of antibodies against type I antigens may not need the adsorption of the sera and therefore would facilitate serodiagnosis of invasive candidiasis. Its potential for serodiagnosis of invasive candidiasis is under study.

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