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Monoclonal Antibodies against *Candida tropicalis* Mannan: Antigen Detection by Enzyme Immunoassay and Immunofluorescence

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Three strains of mice were immunized with *Candida tropicalis* cell walls, and antibodies against mannan were detected by indirect enzyme immunoassay (EIA) in 3 of 9 BALB/c mice, 4 of 11 C57BL/6 mice, and 4 of 8 CFW mice. Responding mice produced immunoglobulin M (IgM), but IgG was not detected in their sera. Fusion of the high-responder BALB/c mouse with a plasmacytoma cell line resulted in 41 clones secreting antimannan monoclonal antibodies (MAbs). Four clones selected for propagation included one IgM and one IgG MAb that reacted with mannans of *Candida albicans* serotypes A and B and of *C. tropicalis* and two IgM MAbs specific for an epitope only in the mannans of *C. albicans* serotype A and *C. tropicalis*. One of the IgM MAbs, CB6, was an effective substitute for rabbit antibodies in the double-antibody sandwich EIA to detect antigenemia produced in rabbits infected with *C. albicans* A or *C. tropicalis*. It could function either as the peroxidase-conjugated indicator antibody or as the capture antibody. Two MAbs, CB6 (*C. tropicalis* and *C. albicans* A specific) and AC3 (*C. tropicalis* and *C. albicans* A and B specific), functioned in place of polyclonal antisera in the serotyping of *C. albicans* by immunofluorescence. There was 95.8% agreement in the results of serotyping using MAbs as reagents compared with rabbit antisera. Competitive inhibition in EIA between CB6 and monospecific antisera against *C. albicans* factors 1, 4, and 6 indicated that CB6 binds to an epitope which is probably factor 6. Serologic similarity between factor 4 and the binding site of MAb AC3 was also determined.

Mannan, an α-linked branched homopolymer of mannose linked to protein, resides on the surface of *Candida albicans* (6). A numerical taxonomy of surface factors of the pathogenic candidae was proposed by Tsuchiya et al. (27) on the basis of agglutinin adsorptions. The corresponding monofactorial sera were purified from rabbit antisera and made generally available (24). It was found that *C. albicans* serotype A contained major specific factor 6 and, rarely, cofactor 13b, *Candida tropicalis* also contained major factor 6, and *C. albicans* B was devoid of factor 6 but contained factor 13b. The epitopes to which these factor sera bind are not known, although one study bears on this point (26). Several investigations, reviewed by de Repentigny and Reiss (8), showed that antigenemia occurs in disseminated candidiasis, one antigen that circulates has the characteristics of mannan, and its detection forms the basis of a specific immunoassay.

Monoclonal antibodies (MAbs) have the capacity to substitute for polyclonal antisera as more specific probes for investigating cell surface topography, for elucidating the molecular basis of serotypes, and to act as indicator antibodies for the detection of antigenemia. This report presents preliminary findings about the production, characterization, and use of MAbs against *C. tropicalis* cell walls. *C. tropicalis* was selected because it is most frequently isolated from the deep tissues and blood cultures of patients with invasive candidiasis (8).

MATERIALS AND METHODS

Antigens. Mannan. *C. tropicalis* 83-48062, a 1983 human blood isolate, was grown in a 16-liter culture at 22.5°C for 48 h on yeast extract-neopeptone-glucose broth (Difco Laboratories, Detroit, Mich.) (23). The organisms were killed with 0.2% formaldehyde. The washed blastoconidia (315 g [wet weight]) were suspended in 1 liter of sodium citrate-citric acid buffer (0.02 M; pH 7.0) and autoclaved at 121°C and 5.1 kg/cm^2 for 1 h. The cooled suspension was centrifuged (4,400 × *g*, 30 min), and the supernatant was reserved. The blastoconidia were resuspended in citrate buffer and autoclaved for another hour. The first autoclaved supernatant was concentrated at reduced pressure and ≤37°C to 220 ml, mixed with an equal volume of absolute ethanol, and left overnight at 4°C. The precipitate was collected by centrifugation (900 × *g*, 15 min), and the supernatant was reprecipitated with two and three volumes of cold ethanol. The precipitates were washed with absolute ethanol and acetone and then dried in a vacuum desiccator. Mannan was precipitated as the borate-cetyltrimethylammonium bromide complex (Fig. 1) (17). Total carbohydrate was determined with phenol-sulfuric acid (2), phosphorus was determined with ammonium molybdate and hydrazine sulfate (10), nitrogen was determined by the micro-Nessler procedure (1), and protein was determined by Coomassie blue dye binding (Bio-Rad laboratories, Richmond, Calif.) (5). Mannans of *C. albicans* 20A (serotype A) and *C. albicans* 526B (serotype B) were prepared by the method of Peat et al. (19). Cell walls of *Candida* species were prepared as previously described (23).

Vaccines, immunization, and hydridoma production. Vaccines were prepared with cell walls of *C. albicans* 20A or *C. tropicalis* 83-48062. BALB/c, C57BL/6, and CFW mice, 8- to 10-week-old females, were immunized intraperitoneally with 50 μg of walls in 0.2 ml of incomplete Freund adjuvant. Seven days later, 50 μg of the same antigen, in saline, was injected intraperitoneally at weekly intervals for 6 weeks. Finally, 25 μg of walls in saline was injected intravenously 3

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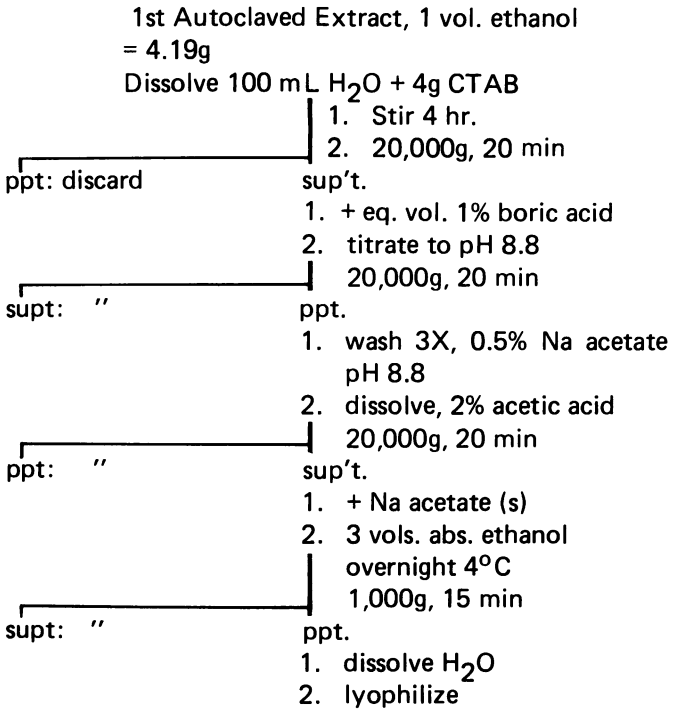

FIG. 1. Flow diagram showing precipitation of *C. tropicalis* mannan as the borate-cetyltrimethylammonium bromide complex (CTAB), followed by the recovery of purified mannan.

days later. Animals were bled before reinforcing doses, and antibodies against mannan of *C. albicans* 20A or 526B or *C. tropicalis* were quantitated by indirect enzyme immunoassay (EIA) (13). Fusion of the splenocytes of a high-responding BALB/c mouse with the SP2/0-Ag14 plasmacytoma cell line was performed by a standard protocol (30). Hybridoma supernatants were assayed for antibodies against mannan by indirect EIA (13). The isotype of immunoglobulins was determined by the immunodot variation of the indirect EIA (15). Clones propagated as ascites tumors were an immunoglobulin M (IgM; AC3) and an IgG (DC5) MAb that recognized mannan of *C. albicans* 20A and 526B and *C. tropicalis* and two IgM MAbs (BC4 and CB6) that were reactive with mannan of *C. albicans* 20A and *C. tropicalis* but not with mannan of *C. albicans* 526B. An IgM MAb (CDE344), produced by immunization of mice with a mixture of live and killed blastoconidia of *C. albicans* A and B serotypes, was kindly provided by Gunars Valkirs, Hybritech, Inc., Torrey Pines, Calif.

BALB/c mice were pretreated with 0.5-ml intraperitoneal injections of pristane 2 weeks before the injection of 10^6 MAb-producing cells. The ascitic fluids were collected by paracentesis and stored at −70°C.

Preparation and conjugation of antisera and MAbs. Antisera against cell walls of *C. albicans* 20A or 526B were produced in New Zealand White rabbits by injecting 500 μg of antigen intravenously twice a week for 44 days, for a total dose of 7 mg of dry walls. The globulins were precipitated with half-saturated $(NH_4)_2SO_4$ and chromatographed on DEAE-Sephadex A-50 (Pharmacia Fine Chemicals, Piscataway, N.J.). The IgG fractions were adjusted to 10 mg/ml by using the extinction coefficient, $E_{280}^{mM} = 211$. MAbs were purified by class: IgM was chromatographed on a column (2.5 by 75 cm) of Sephacryl S300 (Pharmacia). The IgM was recovered in the void volume and concentrated to 5 mg/ml by ultrafiltration over a YM 30 membrane (Amicon Corp., Lexington, Mass.). The IgG MAb was precipitated at half-saturation with $(NH_4)_2SO_4$ and dialyzed versus potassium phosphate buffer (0.1 M; pH 8.0). The IgG was purified on a staphylococcal protein A-Sepharose column (1 by 10 cm; Pharmacia) by elution with a pH gradient from pH 8.0 (potassium phosphate, 0.1 M) to pH 3.1 (citric acid-sodium citrate, 0.1 M). The IgG was dialyzed versus phosphate-buffered saline (PBS) and adjusted to 5 mg/ml.

Immunoglobulins were conjugated to horseradish peroxidase (EC 1.11.1.7; type VI; Sigma Chemical Co., St. Louis, Mo.) by the periodate method (28). The CB6 MAb (5.9 mg) was conjugated to 158 μg of fluorescein isothiocyanate (11). Monofactorial rabbit antisera specific for *Candida* species factors 1, 4, 5, 6, and 13b were obtained from Iatron Laboratories, Chiyoda, Tokyo, Japan (Candida Check) (24).

Immunosuppression and infection. New Zealand White rabbits, 3-kg females, were immunosuppressed with cortisone acetate (subcutaneously in the flank, 10 mg/kg on days −2, −1, and 0) and infected intravenously with 10^7 blastoconidia of either *C. albicans* 3181A, *C. albicans* 526B, or *C. tropicalis* 83-48062 (7). Blood was drawn after 3 days, and on day 4 the rabbits were anesthetized and exsanguinated. Both kidneys were removed, weighed, and homogenized in saline, and the CFU in dilutions from each kidney were enumerated on Sabouraud glucose medium. Other details of this rabbit model were reported previously (7, 21).

EIA and immunofluorescence. An indirect EIA was used to detect antibodies against mannan in rabbit antisera, in ascitic fluids, and in supernatants of hybridomas cultivated in vitro (13). Heavy-chain-specific (μ or γ) peroxidase-conjugated anti-rabbit and anti-mouse immunoglobulins (Cooper Biomedical, Inc., West Chester, Pa.) were used at a 1/1,000 dilution. Positive results were recorded as an A_{490} of >0.2 after subtraction of the negative control.

Sera from immunosuppressed, infected rabbits were dissociated by boiling 1-ml samples in an equal volume of disodium EDTA (0.1 M; pH 7.2) for 3 min, and then they were centrifuged at 20,000 × *g* for 30 min. The double-antibody sandwich EIA to detect mannan was conducted as previously described (12), except that microtiter plates were coated with rabbit anti-*C. albicans* IgG obtained by immunization with cell walls of serotype A (isolate 20A) or serotype B (isolate 526B) at coating dilutions of 1/4,000 and 1/2,000, respectively, made from 10-mg/ml immunoglobulin solutions. Optimal concentrations of MAbs, for both the capture and peroxidase-labeled indicator antibodies, were experimental variables.

Competitive binding EIAs were conducted to test the ability of Iatron monofactorial antisera to inhibit the binding of MAb-peroxidase conjugates AC3 and CB6 to blastoconidia or mannans adsorbed to the microtitration plates. *C. albicans* serotype A, strain 65; *C. albicans* serotype B, strains Lecocq and Q 16; or *C. parapsilosis* B390 was the source of blastoconidia, and optimal coating concentrations were determined with dilutions of 1:1 (vol/vol) stock solutions in saline. The coating suspension, 100 μl per well, was allowed to dry at 23°C. First, Iatron reagents and control antibodies, diluted in PBS (pH 7.2) containing 0.05% Tween 20 were added and incubated for 1 h at 23°C, and the plates were washed with the diluent. The diluted enzyme-MAb conjugates were added, and after another 15-min incubation and washing cycle the absorbance in the presence of a chromogenic substrate was recorded (13). Further EIA inhibitions were conducted with mannan of *C. tropicalis*, *C.*

albicans serotype B, or *C. parapsilosis* as the solid phase-adsorbed antigen. Results were expressed as percent inhibition = 100 (1 − A_{490} of inhibitor/A_{490} of normal rabbit serum). The binding of Iatron reagents to the blastoconidia was determined by using as the indicator antibodies 1/1,000 dilutions of goat anti-rabbit IgG, γ specific, or anti-IgM, μ specific, peroxidase conjugates (Cooper).

For indirect immunofluorescence, the MAbs as ascitic fluids were diluted fourfold in PBS–0.5% bovine serum albumin; these included CB6 (*C. albicans* A and *C. tropicalis* specific), AC3 (*C. albicans* A and B and *C. tropicalis* specific), and, as the negative control, P3X63, an IgG active against ovine erythrocytes. *C. albicans* 65 serotype A and *C. albicans* Lecocq serotype B were the positive control cultures, and *C. parapsilosis* B390 was the negative control. Smears of the blastoconidial suspensions on microscope slides received fourfold-diluted ascitic fluids and were incubated at 23°C for 35 min. The slides were washed in PBS, goat anti-mouse IgM-fluorescein conjugate (Cooper) was applied, and incubation was continued for 35 min. Washed smears were examined in a fluorescence microscope. If the *C. albicans* culture reacted positively with AC3 and CB6 MAbs, it was recorded as serotype A. Blastoconidia of serotype B isolates reacted positively with AC3 but not with the CB6 MAb.

Cultures. Sixty isolates of *C. albicans* (equal numbers of serotypes A and B) were coded and submitted by one of us (P.A.). These isolates were previously serotyped with antisera by indirect immunofluorescence (3). Additional cultures of *C. albicans* and isolates of *C. tropicalis* 83-48062 and *C. parapsilosis* B390 were obtained from the Division of Mycotic Diseases, Centers for Disease Control. All cultures were tested blindly by indirect immunofluorescence. Discrepant cultures were checked by resubmitting them to the laboratory at the University of Montreal. A smaller panel of *C. albicans* isolates was tested by direct immunofluorescence. Identification numbers of all *Candida* species cultures used are available upon request.

RESULTS

Mannan. The *C. tropicalis* culture yielded 315 g of packed blastoconidia. The recovery of ethanol-precipitated material from the extract of autoclaved whole cells is shown in Table 1. Most of the polysaccharide was extracted after autoclave treatment 1 and was precipitated with 1 volume of ethanol. This fraction, containing 2.56 g of polysaccharide and 92 mg of protein, was purified by borate-cetyltrimethylammonium bromide complex precipitation to yield 860 mg of mannan (20.5% recovery). It had a total nitrogen of 1.52% and a total phosphorus of 1.05%. This mannan was used to coat microtiter plates and to generate a standard curve (1 to 50 ng/ml) for the sandwich EIA.

TABLE 1. Effect of autoclaving and ethanol precipitations on preparation of mannan from *C. tropicalis* 83-48062 blastoconidia

Autoclaved extract	Ethanol (vol)	Yield (g [dry wt])	Protein		Polysaccharide	
			%	Yield (mg)	%	Yield (g)
1	1	4.19	2.2	92	61	2.56
	2	1.21	6.0	72	24.2	0.29
	3	1.42	2.5	36	14.6	0.21
2	1	1.59	4.0	63	16	0.254
	2	2.10		0	3	0.06
	3	1.84		0	2	0.04

TABLE 2. Immunization of mice with *Candida* species cell walls for fusions

Mice	No./ group	Cell walls	No. of responders[a]/ group	Anti-mannan titer^{-1}	Class	
					IgG	IgM
BALB/c	9	*C. tropicalis*	3	640, 1,280, —[b]	0	3
	7	*C. albicans*	1	640	0	1
C57BL/6	11	*C. tropicalis*	4	640, 640, 1,280, 2,560	0	4
	7	*C. albicans*	0			
CFW	8	*C. tropicalis*	4	640, 640, 640, 2,560	2	4
	8	*C. albicans*	5	640, 640, 1,280, 1,280, 2,560	0	5

[a] Determined by indirect EIA against homologous mannan.
[b] —, Quantity not sufficient.

Immunization of mice with cell walls and characterization of cell fusion products. The mice responded to *Candida* species cell walls with serum antibodies against mannan in decreasing order: CFW > C57BL/6 > BALB/c (Table 2). Moreover, the *C. tropicalis* walls were more immunogenic in the two inbred strains of mice than were those of *C. albicans* 20 serotype A. Sera from all responders contained antimannan IgM; only the CFW mice showed, in addition, antimannan IgG.

The high-responding BALB/c mouse immunized with *C. tropicalis* walls was selected for fusion. Screening of hybrid clone supernatants by indirect EIA for MAbs against mannan of *C. albicans* serotypes A and B and *C. tropicalis* resulted in 41 mannan-reactive clones. Of these, only one produced IgG; the remainder produced IgM. Four clones were selected for propagation as ascites tumors. Two clones (BC4 and CB6) were particularly interesting, because they reacted with mannan from *C. tropicalis* and *C. albicans* serotype A but not with that from *C. albicans* serotype B. The MAbs of the remaining productive hybridomas reacted with mannan from all three yeasts.

MAbs as indicator antibodies to detect candida antigenemia. The ability of these antimannan MAbs to act as capture and indicator antibodies in the double-antibody sandwich EIA was assessed with mannan of *C. tropicalis* added to normal human serum. Either clone AC3 or CDE acted efficiently as a capture antibody, but no additional benefit was achieved by using a different MAb as the peroxidase-labeled indicator antibody (Table 3).

The ability of MAbs to detect antigen in the sera from immunosuppressed rabbits infected with *C. albicans* A or B was evaluated (Table 4). Antigenemia was detected effi-

TABLE 3. MAbs as capture and indicator antibodies in the double-antibody sandwich EIA to detect mannan of *C. tropicalis* (100 ng/ml) added to normal human serum

Unlabeled capture MAb[a]	A_{490}		
	Peroxidase-conjugated indicator MAb[a]		
	AC3 IgM (2.1 mg/ml; 1/4,000)	DC5 IgG (1 mg/ml; 1/1,000)	CDE 344 IgM (1.2 mg/ml; 1/4,000)
AC3 (18 mg/ml; 1/4,000)	0.97	0.04	0.44
DC5 (11.5 mg/ml; 1/500)	0.44	0.44	0.56
CDE 344 (7.7 mg/ml; 1/16,000)	0.41	0.04	0.89

[a] The stock concentration in milligrams of protein per milliliter and the working dilution in PBS-Tween are shown in parentheses.

TABLE 4. Effect of MAbs on detection of antigenemia by double-antibody sandwich EIA in rabbits infected with *C. albicans* 3181 serotype A or *C. albicans* 526 serotype B.

Capture antibody[a]	Indicator antibody[b]	Mannanemia (ng/ml [mean ± SD])[c] in rabbits infected with:	
		Type A	Type B[d]
Expt 1			
Antiserum	Antiserum	62 ± 7.0	7.3 ± 1.0
Antiserum	CDE 344	44.7 ± 5.8	0
Antiserum	DC5	0	1.5 ± 0.3
Antiserum	AC3	3.1 ± 2.6	22.3 ± 3.4
CDE 344	CDE 344	0	16.6 ± 6.2
DC5	DC5	0	9.0 ± 1.9
AC3	AC3	4.5 ± 0.7	26.9 ± 2.7
Expt 2			
Antiserum	Antiserum	594 ± 35.1	0
CB6	Antiserum	435 ± 47.5	0
AC3, CDE 344, or DC5	Antiserum	0	0

[a] Microtitration plates coated with concentrations shown in Table 3; Antiserum, IgG of rabbits immunized with cell walls of *C. albicans* A or B.

[b] Horseradish-peroxidase-conjugated antibodies; for concentrations, see Table 3 and reference 26.

[c] Concentrations are means of six to nine replicates (two experiments) from one rabbit infected with each isolate; CFU per kidney: Experiment 1, type A, 4.2×10^8; type B, 8.7×10^8; experiment 2, type A, 9.9×10^8.

[d] Dissociated serum concentrated 10-fold by ultrafiltration over a YM 10 membrane (experiment 1 only; experiment 2, unconcentrated).

ciently only when polyvalent rabbit IgG or MAb CB6 was used as the capture antibody. Furthermore, antigenemia in experiment 1 caused by infection with *C. albicans* A was detected only with the CDE-344-peroxidase conjugate, which was comparable to a polyclonal rabbit IgG-peroxidase indicator. Antigenemia resulting from *C. albicans* type B infection could not be detected with these MAbs unless the sera from these infected rabbits were concentrated 10-fold by ultrafiltration (YM 10 membrane) after dissociating soluble immune complexes with heat and EDTA. Because of this low sensitivity, serotype B antigenemia was not investigated further with these reagents.

Subsequently, we found that MAb CB6, which reacted with *C. albicans* serotype A and *C. tropicalis* mannans but not with *C. albicans* B, could function well as an indicator antibody (Table 5). The ability of CDE-344-peroxidase, CB6-peroxidase, or a combination of the two MAbs to detect antigenemia is shown in Table 5. In that experiment, two groups of four and five rabbits were immunosuppressed and infected with *C. albicans* 3181A or *C. tropicalis*, respectively. The group of rabbits infected with *C. albicans* A had higher concentrations of antigenemia than the *C. tropicalis*-infected group. The peroxidase-conjugated antibodies detected circulating antigens from *C. albicans* 3181 serotype A in the following decreasing order of sensitivity: MAb CB6 IgM > rabbit anti-*C. albicans* 20A cell walls IgG > MAbs CB6 plus CDE 344 > MAb CDE 344. The other two MAb-peroxidase conjugates tested, AC3 IgM and DC5 IgG, could not detect *C. albicans* type A antigen and detected *C. tropicalis*-induced antigenemia only modestly.

Immunofluorescence. In a panel of 71 *C. albicans* cultures (36 serotype A, 35 type B) typed with both the MAbs (AC3 and CB6) and antisera, discrepant results with 3 cultures were obtained. These three were serotype A strains which did not react with the MAb CB6 and thus lacked the A-specific factor. When retested with antiserum reagents, these isolates were again at variance with the MAbs. The level of agreement between the MAbs and antisera as typing reagents was 95.8%. Based on their typical yeastlike morphology and intense staining reactions, *C. albicans* 65 (serotype A) and *C. albicans* Lecocq (serotype B) were selected as positive reference cultures for use with immunofluorescence serotyping reagents. *Candida parapsilosis* B390 was used as the negative control. Because direct immunofluorescence affords greater speed and simplicity, the CB6 IgM MAb was purified and labeled with fluorescein. When a panel of 20 *C. albicans* isolates was screened (equal numbers of A and B serotypes), only type A isolates were stained. No discrepancies were observed between the results with the indirect and direct immunofluorescence methods.

Competitive inhibition between MAbs and monofactorial antisera. A panel of 13 *C. albicans* serotype A isolates was tested for agglutination by using monofactorial Iatron rabbit antisera specific for factor 6 or factor 13b. Anti-factor 6 globulins agglutinated 12 of the 13 *C. albicans* serotype A isolates, whereas anti-factor 13b agglutinated 8 of the 14 *C. albicans* serotype B cultures.

As expected on the basis of the serotyping patterns, MAbs CB6 and AC3 reacted differently when tested in an inhibition EIA in which monofactorial rabbit anti-*Candida* spp. sera (Iatron) were used as probes. First, it was necessary to verify the binding of Iatron factors 1, 4, 5, 6, and 13b to blastoconidia (Fig. 2). Based on this experiment, a 40-fold dilution of these factors and of the positive inhibition controls was used in the inhibition experiments because the Iatron reagents capable of binding were then present at

TABLE 5. Comparison of rabbit anti-*C. albicans* 20A cell walls IgG and MAbs against *C. albicans* and *C. tropicalis* mannan for the ability to detect antigenemia in infected rabbits by double-antibody sandwich EIA

Infecting strain	Rabbit no.	CFU/g of kidney[a]	Indicator Antibody(s) (ng/ml ± SD)[b]			
			Rabbit anti-CW IgG[c]	CB6	CDE 344 + CB6	CDE 344
C. albicans 3181A	1	1×10^7	48 ± 8.7	74.8 ± 17.2	38.7 ± 7.8	35.1 ± 13.0
	2	1.9×10^7	91 ± 10.5	176 ± 29.6	77.9 ± 4.2	62.3 ± 3.8
	3	1.2×10^7	77 ± 4.6	133 ± 27.9	41.1 ± 7.4	28.9 ± 0.6
	4	5×10^7	128 ± 15.4	252 ± 42.7	113 ± 18.2	83.8 ± 2.1
C. tropicalis	1–5	4.1×10^7	13.4 ± 4.2	13.1 ± 8.2	6.1 ± 5.0	9.3 ± 2.0

[a] Fresh weight; mean of right and left kidneys.

[b] Concentration with respect to a standard curve of *C. tropicalis* mannan in serum. All data are triplicates and the mean concentrations from three daily runs (nine replicates).

[c] Anti-cell walls *C. albicans* 20A IgG also served as capture IgG for all variations in the experiment.

saturating concentrations. It was noted that factor 6 bound only to *C. albicans* serotype A and to *C. tropicalis*, factor 13b bound only to *C. albicans* B, and factor 5 bound to a reduced extent compared with factors 1 and 4.

The binding of CB6 to *C. albicans* A blastoconidia and to *C. tropicalis* mannan was inhibited by Iatron factors 1, 4, and 6 (Table 6), but CB6 was incapable of binding, over a wide range of concentrations, to *C. parapsilosis* blastoconidia that carry factors 1, 5, 13, and 13b. Moreover, CB6 could not bind to *C. albicans* B blastoconidia which express factor 4 in addition to factors 1, 5, and 13b. Concerning the binding specificity of MAb AC3, there was good inhibition by factors 1 and 4. But AC3 does not bind to *C. parapsilosis* which contains factor 1 but lacks factor 4. Thus, AC3 appears to specify the factor 4 epitope. Factor 5 antisera did not inhibit the binding of these MAbs to any of the mannans or blastoconidia that were tested.

DISCUSSION

The first objective, to produce MAbs against *C. tropicalis* mannan, was readily accomplished because cell walls were a good immunogen for mice. The murine antimannan response was predominantly IgM, but a rare hybridoma secreting IgG was selected even though no antigen-specific IgG was detected in the mouse serum. In contrast, rabbits and humans produce antimannan IgG (13, 14). In humans, immunoglobulin responses to mannan are T cell dependent (20). The genetic control and T-cell dependence of the murine response to mannan are worth investigating. We found that two inbred mouse strains were weaker responders to mannan than outbred mice.

Having produced MAbs against mannan, we determined whether any of them was capable of detecting antigenemia by using, as a source of antigen, serum from infected rabbits. Antigenemia resulting from infection with *C. albicans* serotype A or *C. tropicalis* was detected with peroxidase-conjugated CB6 IgM, an MAb that reacts with mannan of *C. albicans* A and *C. tropicalis* but not with that of *C. albicans* B. The epitope responsible for serotype A specificity is conserved in the circulating antigen during the infection.

The use of peroxidase-conjugated CB6 IgM in the double-antibody sandwich EIA to detect *C. albicans* A antigenemia provided some increased sensitivity compared with the rabbit anti-*C. albicans* A cell wall-IgG conjugate. The sensitivity of the assay is important because antigenemia is present in the low nanogram-per-milliliter range (8) and is degraded to mannose (7). The sandwich EIA with CB6 IgM as the enzyme-labeled conjugate could be used to detect antigenemia caused by *C. albicans* serotype A, *Candida guilliermondii*, *C. tropicalis*, and *Torulopsis glabrata* but not antigenemia resulting from *C. albicans* serotype B (*Candida stellatoidea*), *C. parapsilosis*, or *Candida pseudotropicalis* (A. Stenderup, H. Schønheyder, E. Reiss, and H. Brincker, submitted for publication).

Only one of the MAbs, CB6, was capable of acting as the capture antibody in the double-antibody sandwich EIA with antigenemic rabbit serum. When mannan was spiked into serum, all of the MAbs were able capture antibodies. This lack of avidity for the antigen produced in vivo underscores the effect that selective pressure of the host exerts on the composition of the antigens that circulate during infection.

Another aspect that became evident was that antigenemia concentrations varied between serotypes and between closely related species (i.e., *C. albicans* serotype A and *C. tropicalis*). The concentration of antigenemia in *C. albicans* A-infected rabbits was much higher than in rabbits infected with *C. tropicalis*, a difference that either was peculiar to the strain of *C. tropicalis* or resulted from the use of anti-*C. albicans* IgG as the capture antibody.

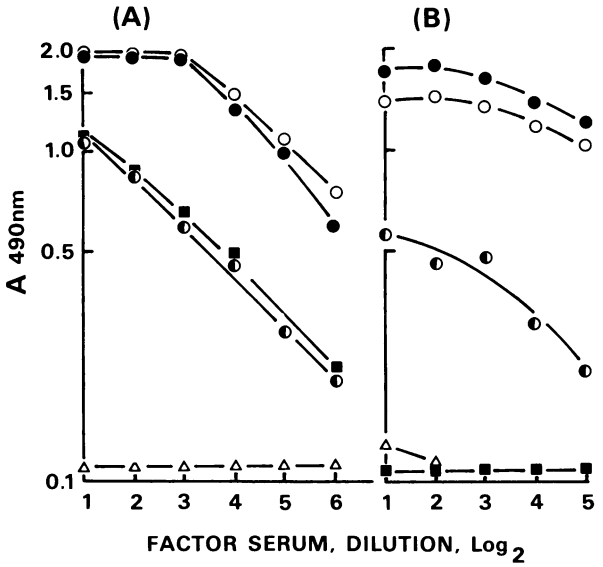

FIG. 2. Results of indirect EIA showing binding of monofactorial rabbit antisera (Iatron) to *C. albicans* serotype A (strain 65) blastoconidia (A) and *C. albicans* serotype B (strain Lecocq) (B). Factor antisera: 1, ●; 4, ○; 5, ◐; 6, ■; 13b, △. Horizontal axis units are $\log_2$ dilutions of factor sera beginning at 1/160.

The detection of serotype B mannanemia was disappointingly low and led to another project that was reported separately (21). That research indicated that successful detection of type B antigenemia depends on the use of a serotype-specific conjugate. Previously, we showed that whereas the mannan of serotype B is susceptible to enzymolysis by exo-α-mannan hydrolase of *Arthrobacter* sp. GJM, the mannan of serotype A is resistant to this enzyme (22). Structural analyses of *C. albicans* mannan do not account for such resistance, but Zhang and Ballou (29) suggested that terminal β-linked nonreducing mannosyl residues occur in the otherwise α-linked mannan from serotype A of *C. albicans*.

The ability of MAbs reactive against mannan to detect antigenemia resulting from infection with *C. albicans* A and *C. tropicalis* but not from infection with *C. albicans* B provided additional proof of the nature of the circulating antigen. We propose that the circulating antigen is mannan based on its heat stability and recognition by an MAb that binds to the surface mannan of *C. albicans* A and *C. tropicalis*. These findings are consistent with our earlier results in the rabbit candidiasis model (7) showing that antigenemia declines in inverse proportion to the production of antimannan IgG and to the appearance in the serum of increased mannose concentrations. The exact nature of the circulating antigen, or antigens, remains to be determined, but they first need to be isolated and purified. Fischer et al. (9) stated that an antigen circulating in children with chronic mucocutaneous candidasis was mannan, but definite proof, such as compositional analysis, was not presented.

After the MAbs were screened to detect antigenemia, the binding specificities of CB6 and AC3 were assessed with respect to the Tsuchiya et al. (27) scheme of *Candida* species surface antigens. Thus, according to this scheme, *C.*

TABLE 6. Relation of rabbit monospecific (Iatron) factors 1, 4, 6, and 13b to MAbs against *C. tropicalis* measured by EIA inhibition

Antigen[a]	Peroxidase-labeled MAb[b]	% Inhibition (mean ± SD)[c] by potential inhibitor[d]					
		Iatron factor				MAb control	
		1	4	6	13b	AC3	CB6
C. albicans A blastoconidia (strain 65)	AC3	29.9 ± 11.8	30.7 ± 14.6	3.3 ± 2.5	1.1 ± 0.4	90.3 ± 1.0	13.8 ± 1.0
	CB6	46.5 ± 13.6	52.6 ± 8.5	21.0 ± 6.6	1.1 ± 2.2	79.0 ± 11.2	78.1 ± 0.6
C. tropicalis mannan	AC3	15.0 ± 4.5	36.0 ± 6.1	0	0	94.0 ± 1.0	24.0 ± 2.0
	CB6	52.0 ± 8.0	87.4 ± 6.0	17.0 ± 4.3	1.0 ± 2.0	26.2 ± 4.0	86.4 ± 1.0
C. albicans B blastoconidia (strain Q16)	AC3	33.3 ± 7.0	25.0 ± 3.0	8.4 ± 5.0	7.0 ± 2.0	90.0 ± 8.2	3.0 ± 3.4
	CB6	NA[e]					
C. albicans B mannan	AC3	21.6 ± 2.4	11.2 ± 2.4	1.4 ± 0.4	1.6 ± 2.7	95.0 ± 1.2	0.3 ± 0.6

[a] Antigen concentrations: mannans, 1 μg/ml; blastoconidia, 1/1,024 dilutions of a 1:1 suspension in PBS.
[b] 32,000-fold dilution of 2.5-mg/ml stock solutions.
[c] Percent inhibition as means of triplicates from three experiments (nine replicates).
[d] Fortyfold dilutions.
[e] NA, Not applicable; CB6 does not bind to serotype B.

tropicalis and *C. albicans* A contain factors 1, 4, 5, 6, and, rarely, 13b, *C. albicans* B has the 1, 4, 5, and 13b phenotype, and *C. parapsilosis* shares factors 1, 5, 13, and 13b. Observations from the inhibition EIAs were surprising because a single MAb was inhibited by more than one monofactorial antiserum. Neither MAb was capable of binding to *C. parapsilosis*, and CB6 did not bind to *C. albicans* B. An explanation consistent with our inhibition experiments is that CB6 identified the same epitope as Iatron factor 6, even though the inhibition was a relatively low 17 to 21%. The binding of factors 1 and 4 either occluded the site of factor 6 or indicated that the monofactorial sera have traces of factor 6 activity. With respect to AC3, inhibition was positive in the presence of factors 1 and 4, but AC3 did not bind to *C. parapsilosis*, which carries factor 1 but lacks factor 4. Thus AC3 appears to specify the factor 4 epitope. Alternatively, these MAbs may identify epitopes proximal to but different from the ones described by the Iatron reagents. Miyakawa et al. (16) described two MAbs against *C. albicans*. On the basis of agglutinin activity and immunofluorescence, they assigned their specificities to factors 6 and 5 of the scheme described in reference 27. Clearly, well-defined oligosaccharides are needed as probes to pursue this line of research.

Using fluorescence-labeled MAbs as probes was helpful in further demonstrating that *C. albicans* isolates can be divided into two serological groups, serotypes A and B, and that type specificity resides in mannan, an α-linked branched homopolymer of mannose linked to protein (4). Furthermore, *C. tropicalis* is closely related to *C. albicans* A, and serologically *C. albicans* B and *C. stellatoidea* are either closely related or identical. Tsuchiya et al. (27) described a third serotype, C, with major agglutinin factors 6 and 7, but the difficulty in producing a C typing reagent led them to include serotype C with type A. Although the original work by Summers et al. (25) contended that serotype A contains all the determinants present in type B and, in addition, the A-specific determinant, Okubo et al. (18) showed that serotype B mannan can be fractionated on the basis of *O*-phosphonomannan content. One subfraction was serologically specific for type B and did not react with type A mannan. MAbs that react with the mannan of serotype B and not with serotype A of *C. albicans* have not yet been produced.

In this work, only 3 discrepant *C. albicans* isolates of 71 tested by immunofluorescence occurred when monoclonal typing reagents were used as compared with antiserum reagents. The three discrepant isolates typed conventionally as serotype A but did not stain with MAb CB6, our A-specific reagent. This discrepancy might arise if conventional typing sera contain traces of serotype B-reactive antibodies. Otherwise, serotype A may not be determined by a single epitope. In that respect, our inhibition experiments showed positive but modest competition between the MAb CB6 and monofactorial anti-factor 6 (24, 27) specific for *C. albicans* A. We cannot exclude the possibility that our three discrepant yeast isolates that did not stain with CB6 are serotype C. The facile production of MAbs that bind selectively to mannan and to blastoconidia of serotype A appears to confirm the assortment of *C. albicans* into at least two distinct serotypes. The production of a large panel of MAbs will probably reveal other distinctions within the *C. albicans-C. tropicalis* group.

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