INFECTION AND IMMUNITY, Oct. 1997, p. 4100–4107 Vol. 65, No. 10
0019-9567/97/$04.00+0

Biochemical Characterization of *Candida albicans* Epitopes That Can Elicit Protective and Nonprotective Antibodies

YONGMOON HAN,[1] TOSHIO KANBE,[2] ROBERT CHERNIAK,[3] AND JIM E. CUTLER[1]*

Department of Microbiology, Montana State University, Bozeman, Montana 59717-3520[1]; Laboratory of Medical Mycology, Research Institute for Disease Mechanism and Control, Nagoya University School of Medicine, Showa-ku, Nagoya 466, Japan[2]; and Department of Chemistry (LBCS), Georgia State University, Atlanta, Georgia 30303-3083[3]

Received 13 May 1997/Returned for modification 26 June 1997/Accepted 11 July 1997

We previously reported that the immunoglobulin M (IgM) monoclonal antibody (MAb) B6.1 protects mice against disseminated candidiasis, whereas the IgM MAb B6 does not. Both MAbs are specific for an adhesin fraction isolated from the cell surface of *Candida albicans*, but their epitope specificities differ. In the present study, we examined the surface locations of both epitopes and obtained structural information regarding the B6.1 epitope. Immunofluorescence confocal microscopic analysis of *C. albicans* yeast forms showed that epitope B6.1 is displayed rather homogeneously over the entire cell surface, whereas epitope B6 appears to have a patchy distribution. Both antibodies were essentially nonreactive with the surfaces of mycelial forms of the fungus, indicating that neither epitope is expressed on the surfaces of these forms. For isolation of the B6.1 epitope, the adhesin fraction consisting of cell surface phosphomannan was subjected to mildly acidic (10 mM HCl) hydrolysis and was fractionated into acid-labile and acid-stable portions by size exclusion chromatography. Antibody blocking experiments showed that the B6.1 epitope is an acid-labile moiety of the phosphomannan and that the B6 epitope is located in the acid-stable fraction. The B6 epitope appeared to be mannan because it was stable to heat (boiling) and protease treatments but was destroyed by α-mannosidase digestion. The B6.1 epitope eluted from the size exclusion column in two fractions. Mass spectroscopic analyses showed that one fraction contained material with the size of a mannotriose and that the other was a mixture of mannotriose- and mannotetraose-size substances. Dose response inhibition tests of the fractions indicated that the B6.1 epitope is associated with the mannotriose. Nuclear magnetic resonance (NMR) spectroscopic analysis of the epitope yielded data consistent with a β-(1→2)-linked mannotriose. The fine structure of the B6 epitope is under investigation. Information derived from these investigations will be useful both in understanding protective versus nonprotective antibody responses to *C. albicans* and in improving anti-*Candida* vaccine formulations.

Antibodies specific for *Candida albicans* may enhance host defense against candidiasis. A positive influence of antibody against disseminated disease was first suggested more than 30 years ago (24, 25), but confirmatory evidence required an additional 10 years (27). During the last 6 years, investigators have further characterized antibodies that protect against both disseminated disease (10, 11, 21, 23) and *Candida* vaginal infection (3, 10a, 28). Although most workers have focused on experimental animals, there is evidence to suggest that protective antibodies can be found and are operative in humans (21, 22, 28).

Antibodies against certain protein epitopes of *C. albicans* may be protective against disseminated candidiasis. The fungus apparently produces a 43-kDa immunosuppressive protein, and antibodies specific for the protein are protective (34). In other studies, patient recovery from disseminated candidiasis correlated with serum antibodies that reacted with both human and *Candida* heat shock protein (hsp) 90 (21, 22). These antibodies and monoclonal antibodies (MAbs) specific for the same antigen protected mice against the disease (23). The most common protective antibody specificity was to a LKVIRK conserved sequence in hsp 90; however, specificity for a DEPAGE epitope was also found. In rather striking results, Polonelli and coworkers reported that vaginal fluid from patients with *Candida* vaginitis had antibodies that were directly candidacidal (28). These antibodies had specificities similar to those of anti-idiotypic antibodies specific for the idiotope of anti-yeast killer toxin antibodies. The human antibodies may have arisen as a result of an immune response against the *Candida* killer toxin receptor (KTR), followed by an anti-idiotype response against anti-KTR antibodies. To our knowledge, there are only two other reports of antibodies that directly affect *C. albicans*. Fab fragments of a MAb specific for a hyphal cell wall moiety inhibited germ tube formation (2), and two other MAbs depressed *C. albicans* filamentation (29).

We have reported on the isolation of two MAbs, MAb B6.1 and MAb B6 (10). Both antibodies are of the immunoglobulin M (IgM) isotype, both agglutinate *C. albicans* yeast cells, and both are specific for cell wall mannan; however, MAb B6.1 enhances resistance against experimentally disseminated candidiasis, and MAb B6 does not. MAb B6.1 protects normal inbred mice, outbred mice, SCID mice (10), and mice with either short-term or protracted neutropenia (11). The antibody also protects mice against experimental *Candida* vaginal infection and can be used therapeutically in this animal model (10a).

Structural considerations of epitopes that lead to production of protective and nonprotective antibodies require an understanding of the cell surface phosphomannan (PM) of *C. albicans*. Suzuki and coworkers (18, 30, 33) have proposed a structural model of the PM complex of both A and B serotypes of *C. albicans*. Serotype B strains have an α-(1→6)-linked mannan backbone from which α-(1→2)-linked oligomannosyl side

* Corresponding author. Mailing address: Montana State University, Department of Microbiology, Lewis Hall 109, Bozeman, MT 59717. Phone: (406) 994-2373. Fax: (406) 994-4926. E-mail: umbjc@gemini.oscs.montana.edu.

chains of various lengths emanate. Linkages within the oligomannosyl side chains are primarily α-(1→2), but α-(1→3), and even further side branches of α-(1→6) may be found (33). Because the backbone and side chains are stable in the presence of boiling 10 mM HCl, this part of the PM is called acid stable (PM-AS). Depending on growth conditions, additional oligomannosyl chains are phosphodiester linked to the AS side chains. All of the mannose-mannose linkages in the phosphodiester-linked oligomannosyl chains are of the β-(1→2) type. Mildly acidic treatment (boiling in 10 mM HCl) causes hydrolysis of the phosphodiester bond, resulting in release of the β-(1→2)-linked chains. The β-(1→2)-linked chains are referred to as the acid-labile part of the PM (PM-AL). Serotype A strains are very similar, except that a few non-phosphodiester-linked β-(1→2)-mannose-mannose groups can be found in the PM-AS.

Herein, we show that both epitopes are part of the PM of *C. albicans* yeast cells. The B6.1 epitope is a mannotriose released by mildly acidic hydrolysis, and the B6 epitope is located in the AS part.

MATERIALS AND METHODS

Organisms and culture conditions. *C. albicans* A-9 (serotype B) was grown from frozen glycerol stocks as previously described (12, 16). The strain was grown at 37°C under constant aeration until stationary phase was obtained (24 h) in glucose-yeast extract-peptone broth (GYEP). The stationary-phase cells were serially transferred to fresh GYEP five times at 24-h intervals under the conditions described above. This procedure ensured that the resulting stationary-phase yeast cells had a hydrophilic cell surface (12). For some experiments, *C. albicans* CA-1 (serotype A) was used as before (20), and it was prepared in a manner similar to that used for strain A-9.

Anti-*C. albicans* MAbs B6.1 and B6. MAbs B6.1 and B6 were isolated as previously described (1, 5, 10). Both MAbs are IgM and agglutinate *C. albicans* (10). The MAbs were produced commercially (Montana ImmunoTech, Inc., Bozeman, Mont.) in serum-free medium (HB 101 Liquid Kit; Irvine Scientific, Santa Ana, Calif.), concentrated by ammonium sulfate precipitation, and exhaustively dialyzed against Dulbecco's phosphate-buffered saline (DPBS) (Sigma Chemical Co., St. Louis, Mo.). The precipitated antibodies were suspended to the desired concentration in the same buffer. The signal strengths of protein bands that developed following sodium dodecyl sulfate-polyacrylamide gel electrophoresis profiles showed that antibody accounted for more than 80% of the protein in the preparations. The concentration of each antibody was approximately 3.5 mg/ml (as determined by absorbance at 280 nm), which produced slide agglutinin titers against latex beads coated with PM (10) of 1,280 and 640 for MAb B6.1 and MAb B6, respectively.

Epifluorescence microscopy. *C. albicans* hydrophilic stationary-phase yeast cells were fixed by suspending the washed cells in 3% formalin–25 mM phosphate buffer (pH 7.2) for 2 h at 20 to 25°C. For some experiments, stationary-phase yeast cells were suspended in distilled water, and the suspension was incubated for 4 h at 37°C. The starved cells, which were primed for germination, were suspended at 5×10^5 yeast cells per ml of Dulbecco's modified Eagle's medium–10% fetal bovine serum and incubated for 3 h at 37°C to allow germination. The resulting germ tubes were also formalin fixed. The formalin-fixed yeast cells or germ tubes were washed four times in phosphate buffer (PB) and blocked in 3% bovine serum albumin (BSA) in phosphate buffer containing 0.15 M NaCl (PBS) for 30 min at 20 to 25°C. The cells were washed three times in PBS containing 1% BSA (BSA-PBS) and suspended in 4 μg of MAb B6.1 or MAb B6 (diluted in 1 ml of BSA-PBS), and the suspension was stored overnight at 4 to 6°C. The yeast cells were then washed three times in BSA-PBS and suspended in fluorescein isothiocyanate-conjugated anti-mouse IgM goat serum (μ chain specific, diluted 1:50 in BSA-PBS; Cappel Research Products, Durham, N.C.) for 4 h at 20 to 25°C and washed in BSA-PBS. The cells were observed by use of an epifluorescence microscope (BH2-RFK; Olympus Optical Co., Ltd., Tokyo, Japan) and photographed (Tmax ASA 400 film; Kodak).

Confocal microscopy. *C. albicans* hydrophilic yeast-form cells were washed four times in cold (5 to 8°C) deionized water and suspended in cold DPBS at 2×10^6 cells/ml. The yeast cells in 1 ml of the cell suspension were pelleted by centrifugation and suspended in 1 ml of MAb B6.1 or MAb B6. MAb B6.1 was tested over a range of 3.5 to 70 μg of MAb/ml, and MAb B6 was tested over a broader range of 3.5 to 3,500 μg of MAb/ml. The tube containing the suspension was plunged into ice and incubated for 30 min. The cells were washed three times, each time in 1 ml of cold DPBS, and suspended in goat anti-mouse IgM (μ-chain specific; Sigma; diluted 1:100 in DPBS), and the suspension was incubated in an ice bath for 30 min. The yeast cells were washed in cold DPBS three times, suspended in Cy 3-conjugated rabbit anti-goat IgG (Jackson ImmunoResearch Lab., Inc., West Grove, Pa.), incubated in an ice bath for 30 min, washed as described above, and suspended in 100 μl of cold DPBS. Optical sections of the cells were examined by confocal microscopy (Nikon DVC-250 scanning confocal argon-krypton laser [488-nm-excitation wavelength] microscope, 60× oil immersion lens; NA 1.4, as obtained from Bio-Rad, Hercules, Calif.]).

PM extraction and acid hydrolysis. Live *C. albicans* serotype B and serotype A yeast cells grown as described above were treated with 2-mercaptoethanol (2-ME) to yield a 2-ME extract as described previously (6, 16). The extracts from both serotypes were further fractionated on a concanavalin A affinity column and eluted with α-methyl-D-mannopyranoside in the manner described previously (15, 16) to yield a fraction from each serotype that was rich in PM. The PM fraction isolated from the serotype B strain is the same as the adhesin complex that we described previously (16). The PM fraction was hydrolyzed by boiling in 10 mM HCl for 1 h, neutralized, and applied to a size exclusion column consisting of either Bio-Gel P-2 (400 mesh; Bio-Rad Laboratory, Richmond, Calif.) as previously described (15, 18, 20, 31) or Toyopearl HW 40S (Toyo Soda Manufacturing Company Ltd., Tokyo, Japan) as previously described (15, 32). Both kinds of columns have similar size exclusion characteristics and allow for separation of the PM into AS and AL components. The PM-AS material eluted in the void volume. The PM-AL components were retained by the columns and separated by the numbers of mannose units in each oligomannosyl side chain released as a result of the acid hydrolysis. All fractions were eluted with deionized water, and the presence of material was monitored by phenol-sulfuric acid detection of hexoses (8). Each of the fractions was tested for reactivity with MAbs B6.1 and B6 as follows.

Agglutination, flow cytometry, and dot blots. (i) Agglutination. The various fractions eluted with deionized water from the P-2 column fractions were tested for their abilities to react with the MAbs. The fractions were lyophilized, and solutions of 2, 20, 200, and 2,000 μg of each fraction per ml were prepared. The highest concentration (2,000 μg/ml) was prepared in deionized water. The various dilutions of this preparation were made in DPBS containing 1% BSA. Stock PM solutions were prepared at 4 mg/ml in deionized water. The stock was diluted in DPBS containing 1% BSA to obtain a working solution of 200 μg of PM/ml. MAb B6.1 and MAb B6 were attached to latex beads by use of a procedure and reagents described previously (20). BSA was also attached to latex by the same procedure and was used as negative control beads. As before, the beads were suspended in DPBS plus 1% BSA and 0.02% sodium azide.

Each column fraction was assessed for its ability to either directly agglutinate antibody-coated beads or to inhibit agglutination of antibody-coated beads and PM. Ten microliters of each fraction at the various concentrations was mixed either with an equal volume of antibody-coated latex beads or with BSA-coated beads as a control. The beads were observed for direct agglutination. When agglutination did not occur, 10 μl of the PM preparation was added and mixed, and the beads were observed for inhibition of agglutination. As a positive control for agglutination, PM was mixed with antibody-coated beads in the absence of column fractions. PM did not cause agglutination of BSA-coated beads.

(ii) Flow cytometry. Formalin-killed yeast form cells were washed in 25 mM PB (pH 7.2) and suspended in 3% BSA in PB containing 0.15 M NaCl (PBS) for 30 min at 20 to 25°C. MAb B6.1 or MAb B6 was added to give 4 μg of the respective antibodies/ml of cell suspension along with 100 or 500 μg of the AL fractions per ml. The mixtures were incubated overnight at 4 to 6°C. The cells were washed, suspended in fluorescein isothiocyanate-conjugated anti-mouse IgM antibody (μ-chain specific; diluted 1:50 in 1% BSA-PBS; Cappel Research Products) for 4 h at 20 to 25°C, and washed. The forward scatter, side scatter, and fluorescence intensities of the yeast cells were analyzed in a flow cytometer (argon laser at 488 nm; EPICS XL; Coulter Corporation, Miami, Fla.).

(iii) Dot blots. PMs from the serotype B and serotype A strains were prepared at 10 mg of the respective PMs per ml of distilled water. Multiple 1-μl spots of each were placed onto nitrocellulose (NC) sheets (Cellulosenitrat E BA 85; Schleicher & Schuell, Dassel, Germany) and allowed to air dry. Each sheet was blocked by saturation in 3% skim milk in 20 mM Tris-HCl (pH 7.5) containing 0.5 N NaCl (TBS) and by incubation for 30 min at 20 to 25°C. The sheets were transferred to TBS containing 1% skim milk with either MAb B6.1 or MAb B6 (at 4 μg of each antibody/ml) plus either the appropriate PM at 250 μg/ml or the appropriate PM-AS fraction at 250 μg/ml. As a positive control for dot development, some sheets were treated as described above, but without the addition of PM or PM-AS. All sheets were incubated overnight at 4 to 6°C, washed in TBS containing 1% skim milk, and incubated with horseradish peroxidase (HRP)-conjugated anti-mouse IgM (μ-chain specific, diluted 1:1,000 in TBS containing 1% skim milk; Cappel) for 4 h at 20 to 25°C. After washing in TBS, the sample sheets were incubated with a mixture of 4-chloro-1-naphthol and H_2O_2 for 20 min at 20 to 25°C.

The presence of mannans on NC sheets was tested by reactivity with concanavalin A. Sample sheets were soaked in TBS containing 3% BSA for 30 min and then incubated with concanavalin A conjugated to HRP (4 μg/ml, diluted in TBS containing 1% BSA; Honen Co., Tokyo, Japan) for 60 min at 20 to 25°C. After washing in TBS, the sheets were developed as described above for HRP.

Treatment of the PM-AS fraction. For each of the following treatments, a stock solution of PM-AS was prepared by solubilizing the fraction in deionized water to a concentration of 30 mg of PM-AS/ml.

(i) Mannosidase digestion. PM-AS was diluted in 0.1 M acetate buffer (pH 4.6) to a concentration of 2 mg/ml. Five units of α-mannosidase was added to 300 μl of the solubilized PM-AS. Two different α-mannosidase preparations were

tested (from almonds [Sigma M-1266] and from jack beans [Sigma M-7257]). PM-AS in acetate buffer without addition of the enzyme was used as the control. The various mixtures were incubated at room temperature for 30 min under constant gentle shaking. Each sample was tested for its respective ability to cause agglutination of either latex beads coated with MAb B6 or latex beads coated with MAb B6.1. Latex beads were coated with antibody as previously described (20).

(ii) Protease digestion. PM-AS was diluted in DPBS to a concentration of 4 mg/ml. Ten units of protease (from *Streptomyces griseus*; Calbiochem-Behring, La Jolla, Calif.) was added to 300 μl of the diluted PM-AS. PM-AS in DPBS without addition of the enzyme was used as the control. The mixture was incubated at room temperature (23 to 24°C) for 1 h under constant gentle shaking. The remaining procedures were as described above for the mannosidase digestion.

MS. The molecular sizes of selected size exclusion column fractions were determined by electrospray-mass spectroscopy (MS) (VG TRIO-2) and fast-atom bombardment (FAB)-MS (VG 70E-HF). Polyethylene glycol was used for calibration of electrospray-MS. Reference sugars were raffinose (Sigma) and stachyose (Sigma) for tri- and tetrasaccharides, respectively. Ten microliters of each test sample at 2 mg/ml was mixed with 50 μl of solvent (methanol-water-acetic acid at 50:50:2), and 0.5 μl of the mixture was analyzed. For FAB-MS, glycerol was used as the matrix and the samples were bombarded with xenon atoms accelerated at 8 KeV and 1-mA primary current. Ten microliters of each test sample at 2 mg/ml was mixed with 2 μl of glycerol, and this 2 μl was added to the probe tip.

NMR spectroscopy. Fraction III (0.8 mg) was exchanged with 99.96% D_2O with intermediate lyophilization. The sample was dissolved in 0.7 ml of 99.96% D_2O and transferred to a 5-mm nuclear magnetic resonance (NMR) tube (Wilmad 535-PP). All NMR experiments were recorded at 30°C on a Varian Unity-Plus 600 spectrometer equipped with a 5-mm triple-resonance probe (^1H, ^{13}C, and ^{15}N). A gradient heteronuclear single-quantum correlation (G-HSQC) experiment was done without ^{13}C decoupling (7). The proton spectral width was 3,000 Hz, and the carbon spectral width was 10,000 Hz; $2 \times 256 \times 4096$ datum points were recorded. Squared gradient pulses of 2.0 and 0.5 ms in duration with gradient strengths of 18.75 and −18.75 (N type)/18.75 (P type) G/cm were used. Quadrature detection in the t_1 dimension (^{13}C) was achieved by combing the N- and P-type data (7). Each t_1 increment consisted of 170 scans. The data were processed off-line with FELIX 95.0 software (BIOSYM/Molecular Simulations, San Diego, Calif.) on a Silicon Graphics Indy workstation. A Lorentzian-to-Gaussian weighting function (lb = −0.5, gb = 0.05) was applied in the t_2 domain (^1H dimension), and a squared cosine-bell function was applied in the t_1 domain. Zero filling was applied in both dimensions. ^1H and ^{13}C chemical shifts were measured relative to acetone (2.217 and 31.98 ppm, respectively).

RESULTS

Cell surface distribution of the B6.1 and B6 epitopes. Confocal microscopic examination of stationary-phase yeast cells stained with MAbs B6.1 and B6 revealed a difference in the respective epitope cell surface distribution (Fig. 1). The B6.1 epitope tended to be expressed uniformly over the entire surface of yeast cells, but the B6 epitope had a more patchy distribution. Figure 1 shows the results of cells stained with either antibody at a concentration of 70 μg/ml. The patchy distribution of the B6 epitope did not change even at the highest concentration (3,500 μg/ml) of antibody (data not shown). Epifluorescence examination of hyphae stained with either MAb indicated that epitopes B6.1 and B6 were poorly expressed on the surface of this form of the fungus (data not shown). The confocal results imply that MAb B6.1 recognizes an epitope different from the one recognized by MAb B6.

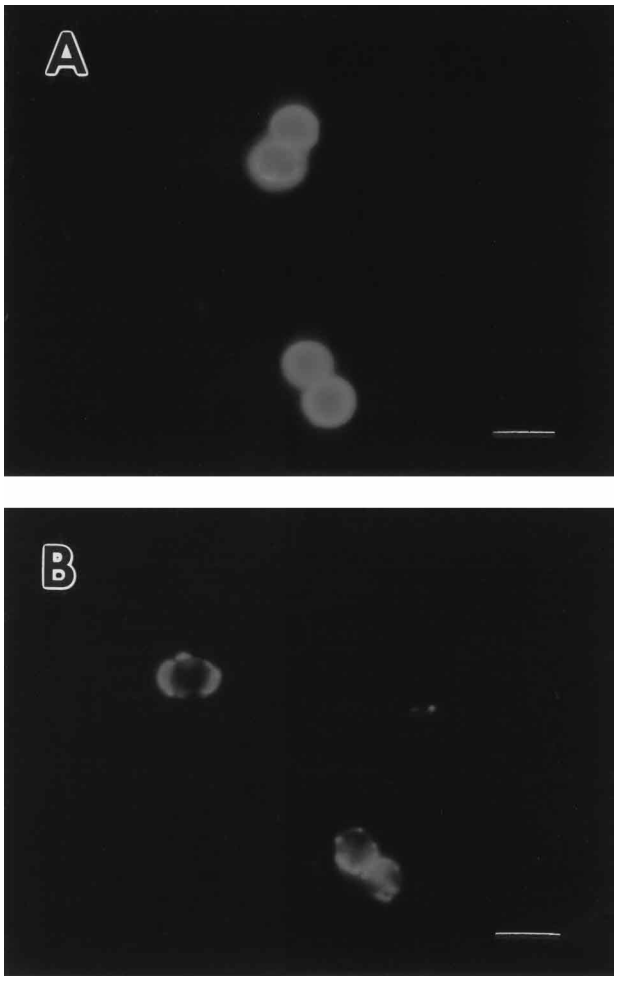

FIG. 1. Surface distribution of the B6.1 and B6 epitopes as revealed by confocal immunofluorescence microscopy. Hydrophilic stationary-phase yeast cells (strain A-9) were reacted with either MAb B6.1 (A) or MAb B6 (B), washed, and counterreacted with fluorescently labeled anti-mouse IgM. Note that the B6.1 epitope is located over the entire cell surface, whereas the B6 epitope has a patchy distribution. Bar, 10 μM.

Acid hydrolysis of PM resulted in separation of the B6.1 and B6 epitopes. PM-AS and PM-AL fractions obtained from acid hydrolysis of PM were separated by size exclusion chromatography (Fig. 2). The PM-AS fraction eluted in the void volume, and the AL oligomannosyl chains fractionated on the basis of mannan chain length. Dot blot analysis of NC showed that the B6 epitope was associated with the PM-AS part of the PM and that the B6.1 epitope was not (Fig. 3). That is, the PM-AS inhibited the reactivity of MAb B6 with PM blotted onto the NC, but reactivity of MAb B6.1 was not affected by premixing the MAb with PM-AS. In control experiments, PM blocked the reactivity of both MAbs with blotted PM. These results were confirmed by the finding that MAb B6-coated latex beads agglutinated in the presence of PM-AS but that PM-AS did not agglutinate latex beads coated with MAb B6.1 (data not shown). The mannan nature of the B6 epitope was confirmed by the finding that MAb B6-coated latex beads did not agglutinate in the presence of PM-AS pretreated with α-mannosidase but that agglutination was unaffected by pretreatment with protease. The following results confirm that the B6.1 epitope is located in the PM-AL part of the PM.

Isolation of the B6.1 epitope. The AL fractions were collected as fractions I to VII as indicated in Fig. 2. Each fraction was tested for the B6.1 epitope by the following three methods: inhibition of PM-induced agglutination of MAb B6.1-coated latex beads, inhibition of antibody reactivity with whole yeast cells (flow cytometric analysis), and inhibition of dot blot reactivity of MAb B6.1 with PM on NC. Only fractions III and IV inhibited agglutination of MAb B6.1-coated latex beads (Table 1). The flow cytometric results agreed with the above findings (Fig. 4). When the cells were reacted only with the anti-mouse IgM fluoresceinated secondary antibody, the fluorescence intensity of the cells was low. The average intensity was high when the cells were reacted with either MAb B6.1 or MAb B6 prior to mixing with the secondary antibody (B6.1/cells or B6/

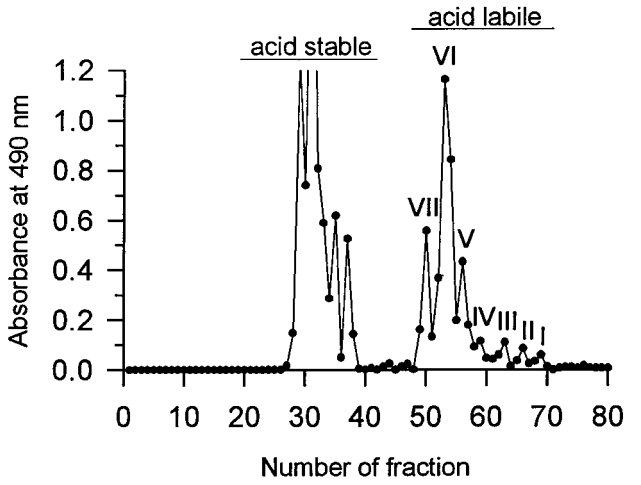

FIG. 2. Fractionation of the PM-AL and PM-AS components by size exclusion chromatography. The PM was hydrolyzed by treatment with 10 mM HCl, and the various fractions were separated by passage over either P-2 or Toyo Pearl size exclusion columns. Both columns gave similar profiles, and the P-2 results are shown here. The mildly acidic treatment degraded the PM into AS and AL oligomannosyl residues as previously described by the Suzuki group (30, 31). The column resolved four AS fractions near the void volume and seven oligosaccharide AL peaks (fractions I to VII). All of the AS fractions were pooled and are referred to as the PM-AS. Each of the individual AL peaks was collected separately.

cells). When fraction II was added to either antibody and mixed with the yeast cells, the fluorescence intensity was not altered (B6.1/cells/FII or B6/cells/FII). However, when fractions III and IV were added, the reactivity of MAb B6 with the yeast cells was markedly reduced (B6.1/cells/FIII or FIV), but these fractions did not interfere with the reactivity of MAb B6 with the yeast cells (B6/cells/FIII or FIV). In addition to these findings, only fractions III and IV inhibited the reactivity of MAb B6.1 with the PM blotted onto NC (data not shown). These results give strong evidence that the B6.1 epitope is contained in the PM-AL fractions III and IV but that the B6 epitope is not in any of the AL fractions.

As observed in Table 1, fraction III was more efficient at blocking agglutination than was fraction IV. Also, note that a

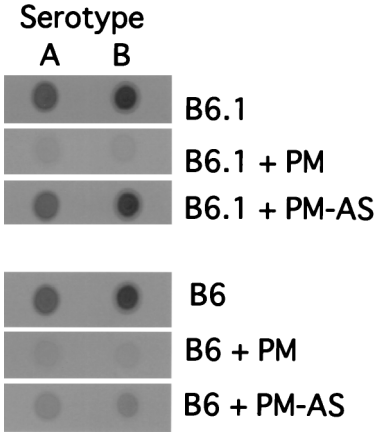

FIG. 3. The B6 epitope is in the PM-AS part of the PM, but the B6.1 epitope is not. By dot blot analysis, the soluble PM blocked binding of MAb B6.1 to PM, whereas soluble PM-AS did not. Either soluble PM or soluble PM-AS blocked binding of MAb B6 to PM attached to NC. Identical results were observed when target PM was obtained from either serotype strain of *C. albicans.*

TABLE 1. Abilities of P-2 column fractions III and IV to inhibit PM-induced agglutination of MAb B6.1-coated latex beads[a]

Fraction(s)	Result with the following fraction concns (μg/ml) used for inhibition:			
	2,000	200	20	2
I, II, and V-VII	NI	NI	NI	NI
III	I	I	I	NI
IV	I	I	NI	NI
III and IV[b]	I	I	I	NI
All[c]	I	I	NI	NI

[a] Each fraction (10 μl), at the indicated concentrations, was individually mixed with 10 μl of latex beads coated with MAb B6.1 and observed for direct agglutination of the beads. When tested without antibody, none of these fractions agglutinated the beads. A 10-μl volume of PM at 200 μg/ml was added, mixed, and observed for agglutination. NI, agglutination not inhibited; I, agglutination inhibited.

[b] Ten microliters of fraction III and 10 μl of fraction IV were used in this case.

[c] Equal amounts of each fraction were mixed, and 10 μl of the mixture was used in this experiment.

mixture of fractions III and IV did not improve the inhibition activity. These observations suggest that the MAb B6.1 epitope is primarily in fraction III and that fraction IV either is an epitope with lower affinity for the antibody or is composed of a mixture of the epitope plus an additional molecular species.

Molecular species in fractions III and IV. Electrospray-MS revealed that fraction III contained a predominant molecular species with the exact size of a mannotriose. Fraction IV was comprised of a mixture of two molecular species with the size of a mannotriose and a mannotetraose. The reference sugars raffinose (trimer) and stachyose (tetramer) were matched exactly to the masses of the respective molecular species found in the two test fractions (Fig. 5). We concluded that the relatively low epitope activity associated with fraction IV was due to contamination of that fraction with fraction III. Fraction III was chosen for further molecular analysis.

Monosaccharide linkages of the mannotriose (B6.1 epitope) in fraction III. Two sets of three chemical shifts were detected in the anomeric region of the spectrum; they are labeled 1 through 6 in Fig. 6 (top trace). The three most intense resonances, which were labeled 1, 5, and 6 in Fig. 6, correspond to the anomeric protons of the mannotriose that has the α configuration at its reducing terminus, (5)mannopyranosyl-β-(1→2)-(6)mannopyranosyl-β-(1→2)-1(1)mannopyranosyl-α. The second set of resonances, labeled 2, 3, and 4, correspond to the anomeric protons of the mannotriose that has the β configuration at its reducing terminus, (3)mannopyranosyl-β-(1→2)-(4)mannopyranosyl-β-(1→2)-(2)mannopyranosyl-β. The two forms of the mannotriose are in equilibrium, but the majority is of the α isomer. The configurations of the anomeric linkages (1-6) were determined by the G-HSQC two-dimensional (2D) experiment recorded without ^{13}C decoupling. Each anomeric proton is split into a doublet due to coupling to ^{13}C (Fig. 6, contour plot). The magnitude of the coupling constant ($^{1}J_{CH}$) is attributed to the anomeric configuration. A coupling constant of ~170 Hz is characteristic of the α configuration, and a coupling constant of ~160 Hz is characteristic of the β configuration. The anomeric configuration assigned in Fig. 6 confirms that the B6.1 epitope is identical to the β-(1→2)-mannotriose described by Shibata et al. (30).

DISCUSSION

The B6.1 and B6 epitopes represent an ideal study set for gaining insights into *C. albicans* surface structures that lead to

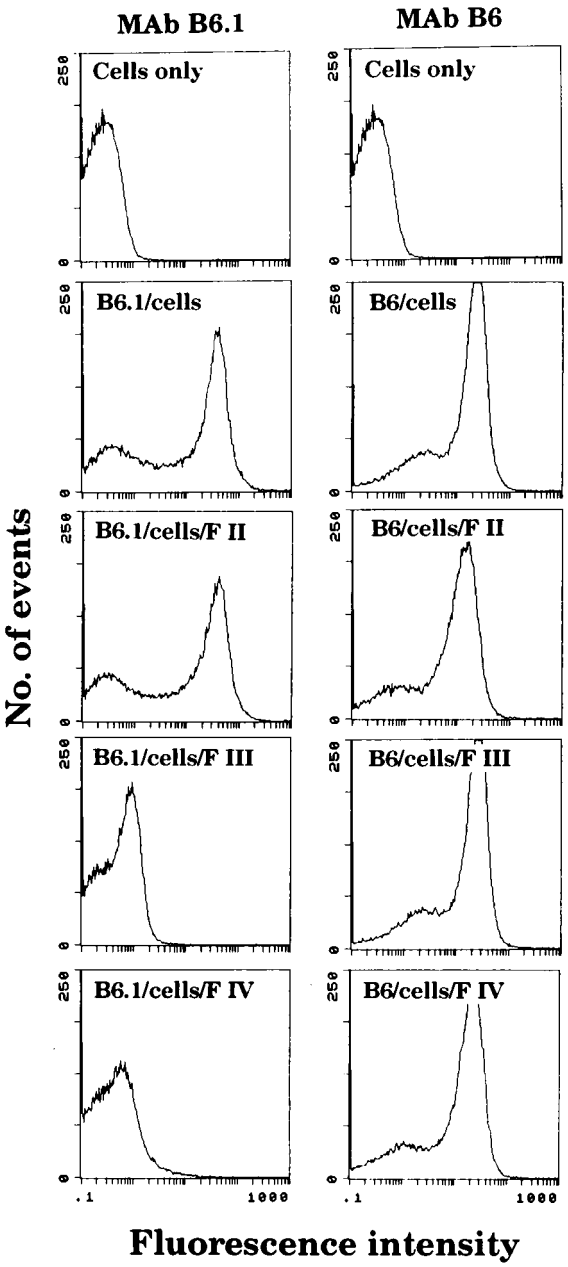

FIG. 4. Identification of the fraction(s) containing the B6.1 epitope. PM-AL fractions I to VII were tested for the B6.1 epitope by antibody-blocking experiments. The ability of MAb B6.1 to bind to yeast cells in the presence of the various fractions was assessed by flow cytometry. Fractions III and IV caused a shift in fluorescence intensity from right to left, indicating inhibition of antibody binding to yeast cells. Neither fraction II (shown) nor any of the other PM-AL fractions (not shown) caused a shift in fluorescence. None of the PM-AL fractions affected MAb B6 binding to yeast cells.

production of protective and nonprotective antibodies. A fundamental finding of these investigations is the surface distribution of the epitopes. Confocal images showed that the B6.1 epitope, whose specific antibody is protective, is distributed rather uniformly across the surfaces of stationary-phase yeast cells. Conversely, the B6 epitope has a more patchy distribution. The B6 pattern was not due to limiting concentrations of the antibody, since the image results were stable over the wide range of MAb B6 concentrations tested. It is noteworthy that fluorescence microscopy revealed undetectable expression of the B6.1 epitope on filamentous forms of *C. albicans*; however, hyphal production is widely held to be a virulence characteristic of this fungus (4).

Both of the epitopes are part of the PM complex on the fungal cell wall surface. The results obtained from acid hydrolysis indicate that the B6.1 epitope is phosphodiester linked to the PM complex and that the B6 epitope is associated with the AS part. This conclusion is in accord with the PM structure of *C. albicans* proposed by Suzuki and coworkers to which allusion was made in the introduction. The extent of mannosylation correlates directly with the length of fibrils that emanate from the fungal cell surface (13), and production of the β-(1→2)-linked oligomannosyl side chains is influenced by environmental factors such as pH (17, 19).

One interpretation of the confocal microscopic and acid hydrolysis findings is that the PM phosphodiester-linked AL components, which are represented by the B6.1 epitope, are located on the distal ends of the *Candida* cell wall microfibrils. The AS part, containing the B6 epitope, is more proximal to the cell surface. The AL components may partially block reactivity with MAb B6, resulting in an apparent patchy distribution of the B6 epitope. Alternatively, the B6.1 epitope may simply be produced in greater abundance than the B6 epitope. However, this explanation implies either that there are several B6.1 epitopes per PM or that the B6.1 epitope is associated with cell wall mannosylated proteins that do not conform to the PM model as proposed by the Suzuki group. Both of these possible explanations are under investigation.

The B6.1 epitope was separated from the B6 epitope by size exclusion chromatography. The separation profile was typical for fractionation patterns of yeast oligomannosyl residues as reported by others (19, 26, 32). The B6.1 epitope, which was detected by its ability to inhibit MAb B6.1 reactivity with PM, eluted primarily as a single fraction from the column. The material in this fraction was determined by MS to be a mannotriose. The B6.1 epitope was also found in approximately equal amounts with a mannotetraose in the fraction that eluted immediately after the mannotriose-containing fraction. The fraction containing the mannotriose-mannotetraose mixture had less inhibitory activity than the mannotriose fraction (Table 1), which led us to the conclusion that the B6.1 epitope is the mannotriose. NMR spectroscopy of the mannotriose produced chemical shifts in the 1D proton spectrum that matched the NMR data for the β-1,2-linked mannotriose characterized by Shibata et al. (30). These data indicated that the three mannoses are covalently bound in a linear configuration by β-(1→2) linkages. According to the PM structure as proposed by Suzuki and coworkers, β-(1→2) linkages are found only in the AL part of the PM in serotype B strains of *C. albicans*. Furthermore, the structure of the B6.1 epitope is the same as that of the M3 fraction described by Shibata et al. (30).

Both we and others have described additional interesting biologic functions associated with β-(1→2)-linked oligomannosyl residues in the cell wall of *C. albicans*. A β-(1→2) mannotetraose is partly responsible for specific adherence of *Candida* hydrophilic yeast cells to mouse splenic marginal zone macrophages (20) and adherence to mouse peritoneal macrophages (9). The AS part of the PM complex also accounts for adherence of yeast cells to marginal zone macrophages in the spleen (15); however, a structural definition of this adhesin site is still under investigation. β-(1→2)-linked oligomannosides may stimulate mouse macrophages to release tumor necrosis factor alpha, but this activity correlates somewhat with the number of mannose units in the oligomannoside (14). The mannotriose does not stimulate release of tumor necrosis fac-

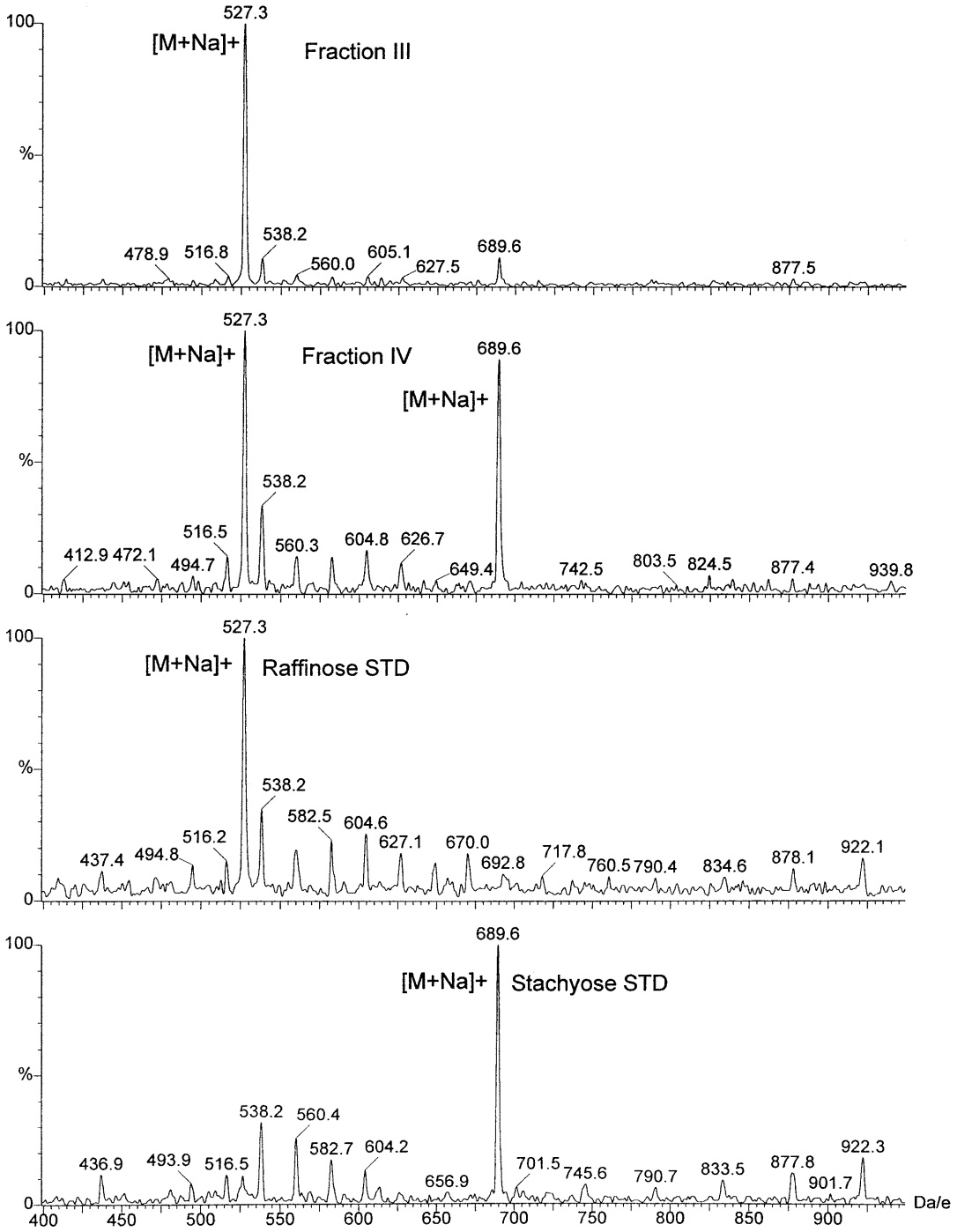

FIG. 5. Molecular masses of components found in PM-AL fractions III and IV. Fraction III contained primarily a component that exactly matched the 527.3 molecular mass of the triose raffinose. This value was derived from the mass of three hexoses (180 × 3) minus the mass of two water molecules (18 × 2) per triose plus the mass of one sodium molecule (23) per triose. Fraction IV contained primarily two components, one with a mass equal to that of raffinose (a triose) and the second with a mass that matched that of stachyose (a tetraose).

tor alpha, which is a favorable characteristic when vaccine formulations designed to elicit an antibody response against the B6.1 epitope are considered.

The results presented here are insufficient to explain why MAb B6.1 is protective, but MAb B6 is not. Nonetheless, these studies show that the cell wall displays epitopes that may or may not lead to production of protective antibodies. We hypothesize that an individual exposed to whole fungal cells will make antibodies against the various cell wall determinants but that the antibody response will not necessarily be protective. Thus, the mere finding of *Candida*-specific precipitins or agglutinins in the sera of patients with candidiasis is not sufficient evidence to argue against a role of antibodies in host defense against this disease.

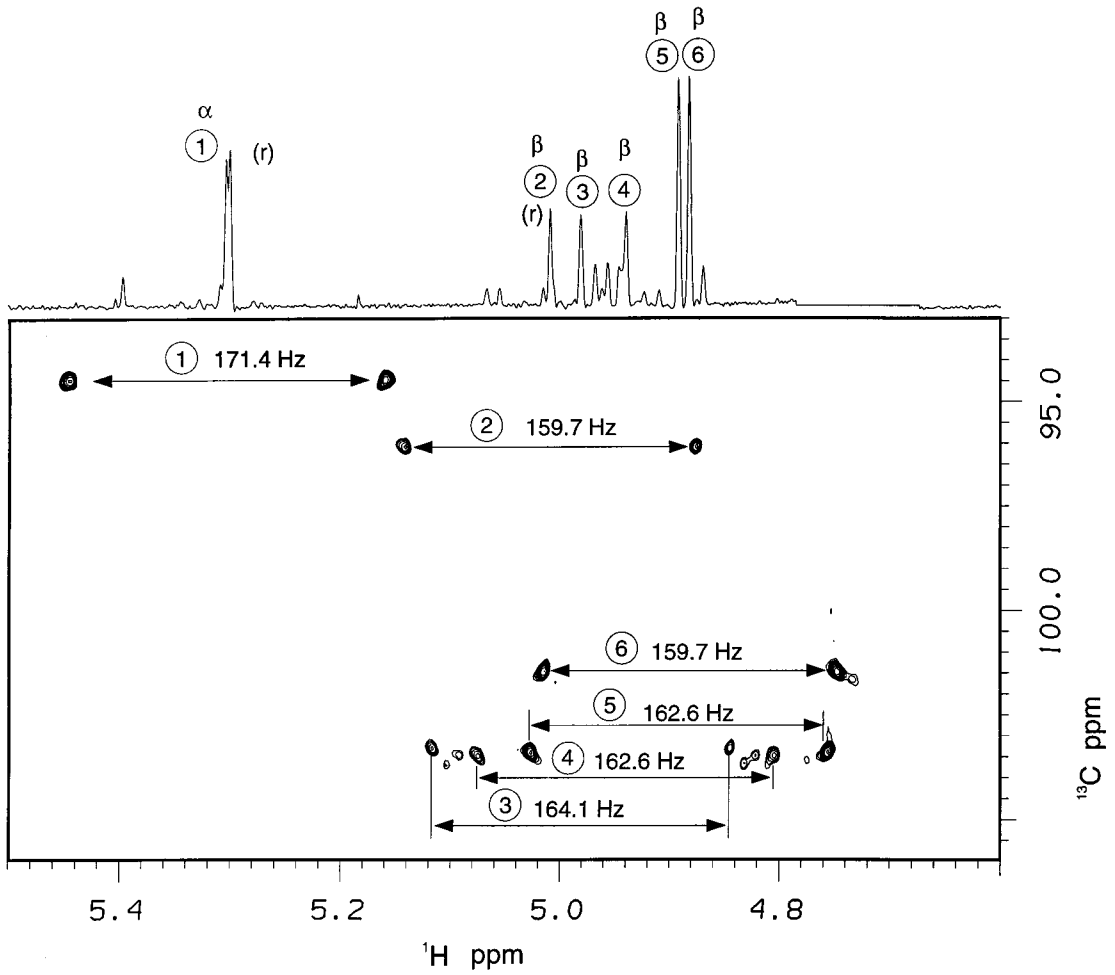

FIG. 6. Structural determination of the B6.1 epitope. Top trace is the anomeric region of the 1D proton spectrum. The bottom trace is the contour plot of the 2D (^{1}H and ^{13}C) G-HSQC data acquired without ^{13}C decoupling at 600 MHz and 30°C. The magnitudes of the $^1J_{CH}$ couplings are noted.

ACKNOWLEDGMENTS

We gratefully acknowledge The Can Caesar-Ton That, who helped us with the confocal microscopic analyses, and Joe Sears, who assisted with MS work.

This work was supported by individual (RO1) and program project (PO1) grants from the National Institutes of Health (5 RO1 AI24912 and 1 PO1 AI37194 [for J.E.C.] and RO1 AI31769 [for R.C.]) and by a grant-in-aid (no. 09670278) for Scientific Research from the Ministry of Education, Science, and Culture, Japan, and by a grant from the Kurozumi Medical Foundation (for T.K.).

REFERENCES

1. **Brawner, D. L., and J. E. Cutler.** 1984. Variability in expression of a cell surface determinant on *Candida albicans* as evidenced by an agglutinating monoclonal antibody. Infect. Immun. **43:**966–972.
2. **Casanova, M., J. P. Martinez, and W. L. Chaffin.** 1990. Fab fragments from a monoclonal antibody against a germ tube mannoprotein block the yeast-to-mycelium transition in *Candida albicans*. Infect. Immun. **58:**3810–3812.
3. **Cassone, A., M. Boccanera, D. Adriani, G. Santoni, and F. De Bernardis.** 1995. Rats clearing a vaginal infection by *Candida albicans* acquire specific, antibody-mediated resistance to vaginal reinfection. Infect. Immun. **63:**2619–2624.
4. **Cutler, J. E.** 1991. Putative virulence factors of *Candida albicans*. Annu. Rev. Microbiol. **45:**187–218.
5. **Cutler, J. E., Y. Han, and R. K. Li.** 1994. Production of monoclonal antibodies against glycan adhesins of *Candida albicans*, p. 197–206. *In* B. Maresca and G. S. Kobayashi (ed.), Molecular biology of pathogenic fungi: a laboratory manual. Telos Press, New York, N.Y.
6. **Cutler, J. E., R. K. Li, T. Kanbe, and M. H. Riesselman.** 1994. Isolation and characterization of glycan adhesin molecules on *Candida albicans*, p. 167–176. *In* B. Maresca and G. S. Kobayashi (ed.), Molecular biology of pathogenic fungi: a laboratory manual. Telos Press, New York, N.Y.
7. **Davis, A. L., E. D. Keeler, E. D. Laue, and D. Moskau.** 1992. Experiments for recording pure-absorption heteronuclear correlation spectra using pulsed filed gradients. J. Magn. Reson. **98:**207–216.
8. **Dubois, M., K. A. Gillis, J. K. Hamilton, P. A. Rebers, and F. Smith.** 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. **28:**350–356.
9. **Fradin, C., T. Jouault, A. Mallet, J. M. Mallet, D. Camus, P. Sinay, and D. Poulain.** 1996. β-1,2-linked oligomannosides inhibit *Candida albicans* binding to murine macrophage. J. Leukocyte Biol. **60:**81–87.
10. **Han, Y., and J. E. Cutler.** 1995. Antibody response that protects against disseminated candidiasis. Infect. Immun. **63:**2714–2719.

10a. **Han, Y., and J. E. Cutler.** Unpublished data.

11. **Han, Y., and J. E. Cutler.** 1997. Assessment of a mouse model of neutropenia and the effect of an anti-candidiasis monoclonal antibody in these animals. J. Infect. Dis. **175:**1169–1175.
12. **Hazen, K. C., D. L. Brawner, M. H. Riesselman, M. A. Jutila, and J. E. Cutler.** 1991. Differential adherence between hydrophobic and hydrophilic yeast cells of *Candida albicans*. Infect. Immun. **59:**907–912.
13. **Hazen, K. C., and B. W. Hazen.** 1992. Hydrophobic surface protein masking by the opportunistic fungal pathogen *Candida albicans*. Infect. Immun. **60:**1499–1508.
14. **Jouault, T., G. LePage, A. Bernigaud, P.-A. Trinel, C. Fradin, J.-M. Wieruszeski, G. Strecker, and D. Poulain.** 1995. β-1,2-linked oligomannosides from *Candida albicans* act as signals for tumor necrosis factor alpha production. Infect. Immun. **63:**2378–2381.
15. **Kanbe, T., and J. E. Cutler.** 1994. Evidence for adhesin activity in the

acid-stable moiety of the phosphomannoprotein complex of *Candida albicans*. Infect. Immun. **62:**1662–1668.
16. **Kanbe, T., Y. Han, B. Redgrave, M. H. Riesselman, and J. E. Cutler.** 1993. Evidence that mannans of *Candida albicans* are responsible for adherence of yeast forms to spleen and lymph node tissue. Infect. Immun. **61:**2578–2584.
17. **Kobayashi, H., P. Giummelly, S. Takahashi, M. Ishida, J. Sato, M. Takaku, Y. Nishidate, N. Shibata, Y. Okawa, and S. Suzuki.** 1991. *Candida albicans* serotype A strains grow in yeast extract-added Sabouraud liquid medium at pH 2.0, elaborating mannans without beta-1,2 linkage and phosphate group. Biochem. Biophys. Res. Commun. **175:**1003–1009.
18. **Kobayashi, H., N. Shibata, M. Nakada, S. Chaki, K. Mizugami, Y. Ohkubo, and S. Suzuki.** 1990. Structural study of cell wall phosphomannan of *Candida albicans* NIH B-792 (serotype B) strain, with special reference to ^{1}H and ^{13}C NMO analyses of acid-labile oligomannosyl residues. Arch. Biochem. Biophys. **278:**195–204.
19. **Kobayashi, H., S.-I. Takahashi, N. Shibata, M. Miyauchi, M. Ishida, J. Sato, K. Maeda, and S. Suzuki.** 1994. Structural modification of cell wall mannans of *Candida albicans* serotype A strains grown in yeast extract-Sabouraud liquid medium under acidic conditions. Infect. Immun. **62:**968–973.
20. **Li, R.-K., and J. E. Cutler.** 1993. Chemical definition of an epitope/adhesin molecule on *Candida albicans*. J. Biol. Chem. **268:**18293–18299.
21. **Matthews, R., and J. Burnie.** 1996. Antibodies against *Candida*: potential therapeutics? Trends Microbiol. **4:**354–358.
22. **Matthews, R., J. P. Burnie, and W. Lee.** 1991. The application of epitope mapping in the development of a new serological test for systemic candidosis. J. Immunol. Methods **143:**73–79.
23. **Matthews, R., S. Hodgetts, and J. Burnie.** 1995. Preliminary assessment of a human recombinant antibody fragment to hsp90 in murine invasive candidiasis. J. Infect. Dis. **171:**1668–1671.
24. **Mourad, S., and L. Friedman.** 1961. Active immunization of mice against *Candida albicans*. Proc. Soc. Exp. Biol. Med. **106:**570–572.
25. **Mourad, S., and L. Friedman.** 1968. Passive immunization of mice against *Candida albicans*. Sabouraudia **6:**103–105.
26. **Nakajima, T., and C. Ballou.** 1974. Characterization of the carbohydrate fragments obtained from *Saccharomyces cerevisiae* mannan by alkaline degradation. J. Biol. Chem. **249:**7679–7684.
27. **Pearsall, N. N., B. L. Adams, and R. Bunni.** 1978. Immunologic responses to *Candida albicans*. III. Effects of passive transfer of lymphoid cells or serum on murine candidiasis. J. Immunol. **120:**1176–1180.
28. **Polonelli, L., F. De Bernardis, S. Conti, M. Boccanera, W. Magliani, M. Gerloni, C. Cantelli, and A. Cassone.** 1996. Human natural yeast killer toxin-like candidacidal antibodies. J. Immunol. **156:**1880–1885.
29. **San Millan, R., P. A. Ezkurra, G. Quindós, R. Robert, J. M. Senet, and J. Pontón.** 1996. Effect of monoclonal antibodies directed against *Candida albicans* cell wall antigens on the adhesion of the fungus to polystyrene. Microbiology **142:**2271–2277.
30. **Shibata, N., K. Hisamichi, T. Kikuchi, H. Kobayashi, Y. Okawa, and S. Suzuki.** 1992. Sequential nuclear magnetic resonance assignment of β-1,2-linked mannooligosaccharides isolated from the phosophomannan of the pathogenic yeast *Candida albicans* NIH B-792 strain. Biochemistry **31:**5680–5686.
31. **Shibata, N., K. Hisamichi, H. Kobayashi, and S. Suzuki.** 1993. Complete assignment of 1H and 13C nuclear magnetic resonance chemical shifts of β-1,2-linked mannooligosaccharides isolated from the phosphomannan of the pathogenic yeast *Candida albicans* NIH B-792 strain. Arch. Biochem. Biophys. **302:**113–117.
32. **Shibata, N., T. Ichikawa, M. Tojo, M. Takahashi, N. Ito, Y. Okubo, and S. Suzuki.** 1985. Immunochemical study on the mannans of *Candida albicans* NIH A-207, NIH B-792, and J-1012 strains prepared by fractional precipitation with cetyltrimethylammonium bromide. Arch. Biochem. Biophys. **243:** 338–348.
33. **Shibata, N., K. Ikuta, T. Imai, Y. Satoh, R. Satoh, A. Suzuki, C. Kojima, H. Kobayashi, K. Hisamichi, and S. Suzuki.** 1995. Existence of branched side chains in the cell wall mannan of pathogenic yeast, *Candida albicans*. Structure-antigenicity relationship between the cell wall mannans of *Candida albicans* and *Candida parapsilosis*. J. Biol. Chem. **270:**1113–1122.
34. **Tavares, D., P. Ferreira, M. Vilanova, A. Videira, and C. Arala.** 1995. Immunoprotection against systemic candidiasis in mice. Int. Immunol. **7:**785–796.

Editor: T. R. Kozel