

Biochemical and Immunological Characterization of MP65, a Major Mannoprotein Antigen of the Opportunistic Human Pathogen *Candida albicans*

MARIA J. GOMEZ,¹ BRUNO MARAS,² ALESSANDRA BARCA,¹ ROBERTO LA VALLE,¹
DONATELLA BARRA,² AND ANTONIO CASSONE^{1*}

Laboratory of Bacteriology and Medical Mycology, Istituto Superiore di Sanità,¹ and Department of Biochemical Sciences "A. Rossi Fanelli," University La Sapienza,² Rome, Italy

Received 9 August 1999/Returned for modification 21 September 1999/Accepted 11 November 1999

In the search of the antigenic determinants of a 65-kDa mannoprotein (MP65) of *Candida albicans*, tryptic fragments of immunoaffinity-purified MP65 preparations were tested for their ability to induce lymphoproliferation of human peripheral blood mononuclear cells (PBMC). Five major peptides (T1 to T5) were shown to induce a vigorous proliferation of PBMC from the majority of the eight healthy human subjects tested. With the use of synthetic peptides, critical amino acid sequences of the two most immunoactive (T1 and T2) peptides were determined. Similar to what was found for the MP65 molecule, no PBMC multiplication was induced by the antigenic peptides in cultures of naive cord blood cells. The amino acid sequence analysis of tryptic and chymotryptic peptides of MP65 demonstrated a substantial homology with the deduced sequences of two cell wall proteins of *Saccharomyces cerevisiae*, encoded by the genes *YRM305C* and *YGR279C*. However, the antigenic peptides were those showing the least similarity with the corresponding regions of the above proteins. In particular, the lymphoproliferation-inducing sequence of the T1 peptide scored only 20% identity with the homologous regions of *S. cerevisiae* proteins. Besides disclosing the amino acid sequence of MP65, this study provides an initial characterization of some of its antigenic determinants, as well as of synthetic peptides of potential use to detect specific immune responses against MP65, a major target of anticandidal cell-mediated immunity in humans.

Yeasts belonging to the genus *Candida* are major fungal pathogens for the immunocompromised host. *Candida albicans* is especially prevalent in subjects infected by the human immunodeficiency virus with severe functional and numerical deficits in CD4⁺ T lymphocytes, which are critical components of cell-mediated immunity (CMI) (24). Despite the increased awareness of the important role that CMI plays in the defense against this fungal pathogen (reviewed in references 1 and 38), very few CMI antigen targets for it have so far been identified. These include heat shock proteins, enolase, and a number of as yet uncharacterized mannoproteins, some with adhesin function (6, 12, 15, 27, 29, 30, 40).

We have long been studying a 65-kDa mannoprotein (designated MP65) which is present in both the structural and secretory mannoprotein material and which is recognized by T cells of peripheral blood of practically all healthy individuals (quasiuniversal antigen) (20, 42–44). In mice immunized with whole fungal cells or MP65-rich mannoprotein extract (MP-F2) (44), a vigorous lymphoproliferative response with a prevalent T-helper type 1 (Th1) cytokine pattern was elicited in vitro MP65-stimulated lymphomonocyte cultures (31). In addition, the MP-F2 extract was capable of inducing a moderate but significant degree of protection against a challenge with a highly virulent *C. albicans* strain in a model of murine disseminated candidiasis. This protection was significantly increased by coadministration of interleukin-12 (IL-12) or by treatment with antibodies against IL-10 (32, 33).

Thus, MP65 contains Th1-inducing and potentially protec-

tive T-cell epitopes, and its further biochemical and immunological characterization could be extremely useful for devising an immunotherapeutic or vaccination strategy. With this aim in mind, we have sequenced a large number of peptides obtained by enzymatic digestion of an immunoaffinity-purified antigen. This sequencing revealed that MP65 of *C. albicans*, while bearing substantial homology to the products of a *Saccharomyces cerevisiae* gene family encoding putative glucanase enzymes (10), also possesses rather distinctive antigenic determinants in the N-terminal region of the protein.

MATERIALS AND METHODS

Strains and culture conditions. *C. albicans* BP, serotype A, from the established stock collection of the Istituto Superiore di Sanità, was used throughout this study. Its origin and culture maintenance have been described elsewhere (44). It was grown in Winge broth (0.2% glucose, 0.3% yeast extract; Difco) or modified Lee's medium (28) buffered in 0.1 M phosphate buffer, pH 6.5, as specified for single experiments.

Sera and MAbs. 7H6 is a mouse immunoglobulin G2b (IgG2b) monoclonal antibody (MAb) specific for a peptide epitope of MP65. MAb 4H12 is a mouse IgG2a specific for the protein moiety of a 70-kDa mannoprotein of *C. albicans* (20). Both MAbs were prepared by fusion of the myeloma cell line X63-Ag8.653 with splenocytes of mice immunized with a secreted mannoprotein material from hyphal cell cultures of *C. albicans* and purified as described in detail elsewhere (20). Polyclonal anti-MP65 antibodies were raised in 2-month-old, female BALB/c mice (Charles River, Calco, Italy) by immunization with the purified MP65 coupled to concanavalin A (ConA; Sigma Chemical, St. Louis, Mo.)-agarose beads, as follows. Thirty micrograms (polysaccharide) of MP65 was incubated with 150 µg of ConA (12 mg of ConA per ml; Sigma) in 100 µl of buffer containing 10 mM Tris-HCl, 150 mM NaCl, 1 mM CaCl₂, and 1 mM MgCl₂ (pH 7.5) for 1 h at 25°C; the mixture was brought to 1 ml with double-distilled H₂O and emulsified into an equal volume of complete Freund's adjuvant. Two doses (200 µl) of this preparation were administered intraperitoneally to four previously pristitized mice (0.5 ml of pristane given up to 2 weeks before) at an 8-week interval. Five weeks later, mice received a third dose of 8 µg of the soluble MP65 in incomplete Freund's adjuvant. Ascites developed after the second or third injection, and ascites fluid was collected and tested in an enzyme-linked immunosorbent assay (20).

* Corresponding author. Mailing address: Department of Bacteriology and Medical Mycology, Istituto Superiore di Sanità, Viale Regina Elena, 299, 00161 Rome, Italy. Phone: 39-06-49387113. Fax: 39-06-49387112. E-mail: cassone@iss.it.

MP65 purification. The MP65 was affinity purified from the material spontaneously released from *C. albicans* mycelial cultures, as previously reported (20). Briefly, the fungus was grown in Lee's medium with 1 μ g of tunicamycin/ml for 24 h at 37°C. The culture supernatant was concentrated and dialyzed by ultrafiltration (Diaflow Ultrafilter YM10; Amicon Corp., Danvers, Mass.) and passed through two sequential affinity columns which were prepared by covalently coupling MAb 7H6 or MAb 4H12 to protein A-Sepharose CL4B (Pharmacia) with dimethylpimelimidate (Sigma). The first (MAb 4H12) column was used to minimize nonspecific binding of material from the mycelial secretion to the MP65-specific MAb 7H6 column. MP65 was eluted from the second (MAb 7H6) column with 100 mM triethylamine, pH 11.5, neutralized with 2 M Tris, dialyzed against double-distilled H₂O, and kept at -20°C. The purified antigen was substantially free from other mannoproteins or proteins, as assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), silver staining, ConA detection, and immunoblotting with MAbs or polyclonal antibodies directed against other components of the mycelial secretion (16).

Total polysaccharide and protein compositions of MP65 were determined by the phenol-sulfuric acid method (18) and the Bio-Rad (Hercules, Calif.) protein assay, respectively.

SDS-PAGE and Western blotting (immunoblotting). Samples were analyzed by SDS-PAGE with 5 to 15 or 5 to 20% gradient acrylamide slab gels with a 3.5% acrylamide stacking gel (25, 27). After electrophoresis, samples were either stained with the Bio-Rad silver stain kit or transferred electrophoretically onto nitrocellulose (0.1- or 0.2- μ m pore size) or polyvinylidene difluoride (PVDF) membranes (Immobilon-P; Millipore Corp., Bedford, Mass.) for detection with ConA, MAbs, or polyclonal antibodies or for amino acid sequencing, as previously described (7, 20).

Enzyme treatments. For limited proteolysis, 10 μ g of denatured MP65 (0.1% SDS, 100°C, 5 min) was resuspended in 50 mM ammonium bicarbonate, pH 8.0, and digested with trypsin (Sigma Chemical Co.) (enzyme/substrate ratio, 1:10 or 1:50) or chymotrypsin (enzyme/substrate ratio, 1:100) for 1 h at 37°C. For extensive proteolysis with trypsin or chymotrypsin, 400 μ g of heat-denatured (100°C, 5 min) MP65 in 50 mM Tris-HCl buffer, pH 8.0, was treated with the corresponding enzyme (enzyme/substrate ratio, 1:10) for 2 to 5 h at 37°C. Cleavage with endoproteinase Asp-N (Boehringer GmbH, Mannheim, Germany) (enzyme/substrate ratio, 1:50) was carried out (under the same conditions) in the presence of 8% acetonitrile. The extent of digestion was assessed by SDS-PAGE and silver staining.

High-performance liquid chromatography (HPLC) separation. The peptide mixtures obtained following the enzymatic digestions with trypsin (T peptides) or chymotrypsin (CH peptides) were purified using the Beckman Gold chromatography system on a macroporous reversed-phase column (Acquapore RP-300; 4.6 by 250 mm, 7 μ m; Brownlee Labs, Foster City, Calif.). Peptides were eluted with a linear gradient of 0 to 70% acetonitrile in 0.2% (vol/vol) trifluoroacetic acid, at a flow rate of 1.0 ml/min. The elution was monitored using a diode array detector (Beckman; model 168) at 220 and 280 nm. Peptides represented by peaks were collected, lyophilized, and finally resuspended in 200 μ l of Dulbecco's phosphate-buffered saline (PBS; Oxoid).

Amino acid sequencing. The peptides obtained by limited proteolysis were separated in a 15 to 20% acrylamide gradient gel and transferred onto PVDF membranes. After being stained with 0.1% Coomassie blue in 50% methanol and destained with 50% methanol, visualized bands were excised and subjected to automated Edman degradation on a gas phase sequencer (model AB 476A; Perkin-Elmer, Foster City, Calif.) (15). HPLC-purified peptides were spotted (20 μ l) in PVDF membranes before sequencing. In some cases, the peptides were covalently linked to the membrane (Sequelon AA membranes, Millipore) to avoid its detachment throughout sequencing cycles (31).

Synthetic peptides. Synthetic peptides were purchased from Tana Laboratories, Houston, Tex.). They were provided as >80% pure and were further assessed for purity by HPLC amino acid analysis and sequencing, as described above. Because of their high hydrophobicity, they were dissolved in 5% dimethylsulfoxide (DMSO; Sigma) at a concentration of 1 mg/20 μ l and then diluted to the desired concentration in RPMI 1640 medium. DMSO controls demonstrated that the concentrations used for peptide solubilization did not affect peripheral blood mononuclear cell (PBMC) proliferation.

Lymphocyte proliferation assay. Lymphoproliferation assays were performed as previously reported (2, 3, 44). Briefly, PBMC from venous blood samples of healthy human donors were isolated by centrifugation on a density gradient (Lymphoprep; Nyegaard, Oslo, Norway) and cultured at 37°C under a 5% CO₂ atmosphere in RPMI 1640 medium-5% pooled AB human serum (Sigma) in 96-microwell trays (10⁶ cells per ml, 200 μ l per well) in the presence of various doses of the stimulant and controls, as indicated in each single experiment. Peptides isolated by HPLC were resuspended in Dulbecco's PBS, heat inactivated (80°C, 10 min) as specified before, filtered, and used at 1:100 or 1:1,000 (final dilution). In some experiments, antigen-unresponsive PBMC from umbilical cord blood were used as responder cells. Cell proliferation was measured on day 7 of the culture by [³H]thymidine incorporation and expressed as counts per minute per 2 $\times$ 10⁵ cells (mean $\pm$ standard deviation of triplicate wells) and by stimulation index (SI; test counts per minute divided by control counts per minute for the PBMC culture without stimulant). SI > 3 was considered to indicate a positive lymphoproliferation.

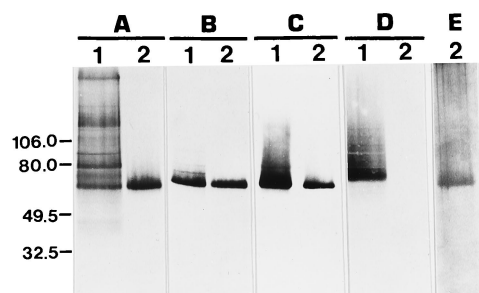


FIG. 1. Chemical and immunochemical detection of immunoaffinity-purified MP65. (A) ConA stain; (B to D) immunoreactivity with MAb 7H6 (B), anti-MP65 mouse serum (C), and MAb 4H12 (D); (E) silver stain. Lanes 1, secretory mannoprotein material from tunicamycin-treated mycelial cultures of *C. albicans* used as starting material for the immunoaffinity purification of MP65, for which 2 μ g of polysaccharide material was reacted with ConA and 10 μ g was reacted with each antibody preparation; lanes 2, 50 ng of purified MP65 was reacted with ConA, 100 ng was reacted with antibodies, and 1 μ g was reacted with silver. Molecular mass markers (left) are in kilodaltons.

RESULTS

MP65 purification. The MP65 was purified from protein-enriched secretory mannoprotein from hyphal cells of *C. albicans* by double sequential immunoaffinity chromatography. To this end, two MAbs, one of which (MAb 4H12) adsorbed an abundant 70-kDa molecule and other irrelevant mannoproteins of the secretory material and the other of which (MAb 7H6) bound specifically the MP65 constituent, were used. As shown in Fig. 1, the purified antigen intensely reacted with specific reagents and was substantially free from other mannoproteins or proteins, as assessed by silver staining, measurement of ConA reactivity, and immunoblotting with MAb 4H12 directed against the 70-kDa mannoprotein-rich material. This purification procedure was reproduced with similar efficiencies for all four batches of MP65 used throughout our experiments. The immunoaffinity-purified MP65 did not contain *Limulus* amoebocyte lysate-detectable lipopolysaccharide (data not shown).

MP65 protease digestion and PBMC proliferation. To identify enzymatic treatments useful to generate MP65 peptides with functional antigenic determinants, several preparations of the purified molecule were subjected to enzymatic digestion with trypsin or chymotrypsin or Asp-N endoproteinase and tested in a lymphoproliferation assay. Only the peptide mixture generated by trypsin cleavage had preserved the capacity of inducing a strong proliferation of PBMC from MP65-responsive donors in vitro (data not shown). Therefore, tryptic digestions of four different batches of MP65 were carried out, and the resulting main peptides obtained were separated by reversed-phase HPLC. The chromatographic profiles obtained in the four digestions were similar, but some minor differences in the number (from 40 to 45) or in the height of the main peptide peaks were seen. Figure 2 is a representative chromatogram of a trypsin digest of one of the four immunoaffinity-purified MP65 preparations.

PBMC from eight unrelated healthy blood donors were tested with all the HPLC-separated peptides, five of which (T1 to T5, at a dilution of 1:100) were found to induce proliferation of PBMC from at least one of the donors. At the above dilution, two peptides (T1 and T2) stimulated cell proliferation in all (T1) or the majority (T2) of PBMC donors, with proliferation indices falling in the same range as those achievable with MP65 and the polyclonal stimulant IL-2 (data not shown; see also below). No cell proliferation was induced by control buff-

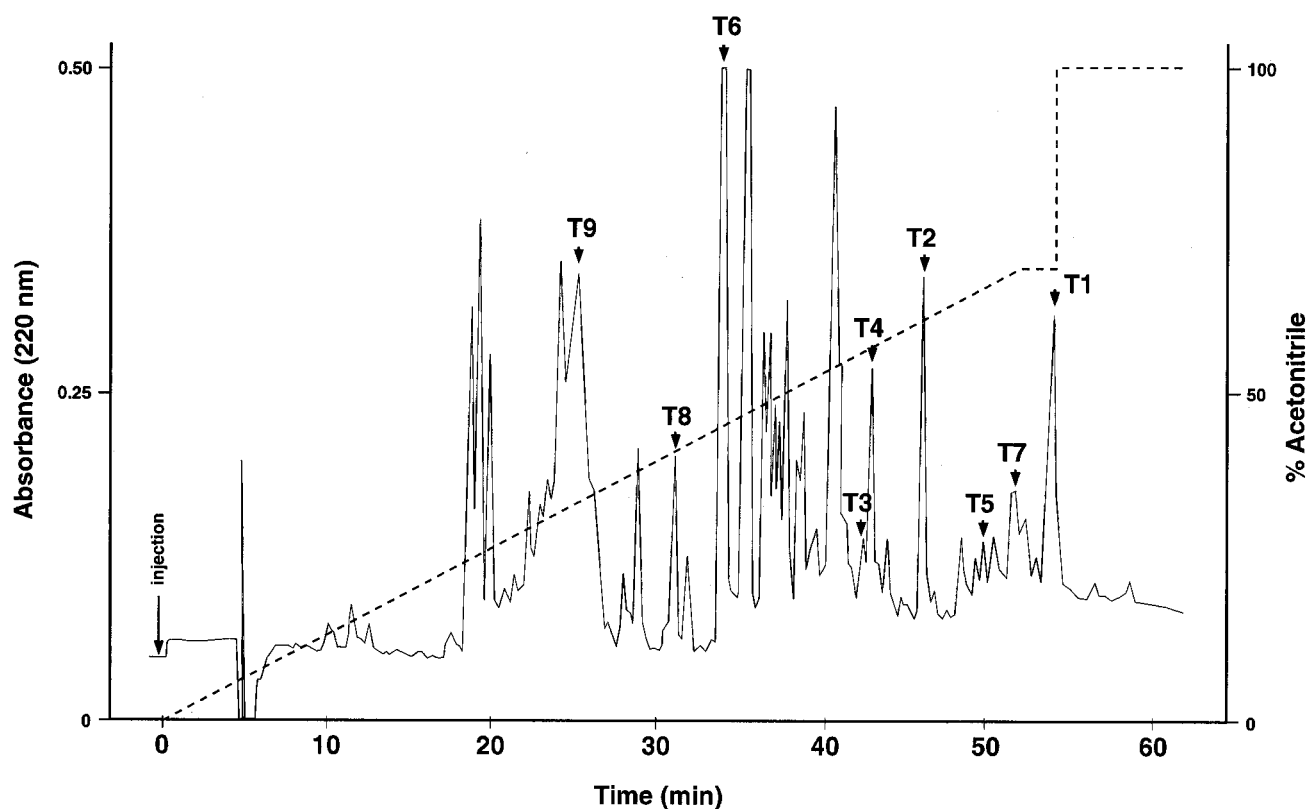


FIG. 2. HPLC separation of MP65 peptides obtained by complete proteolysis with trypsin. The chromatogram is one representative of four independent preparations. Arrows, peptides analyzed for amino acid sequence (T1 to T9); dashed line, gradient concentration.

ers. On this basis, all the subsequent experiments of PBMC stimulation by antigenic MP65 fragments and synthetic peptides were carried out with the peptides T1 and T2 (see below).

The antigenic nature of the peptide-induced lymphoproliferative response, such as that previously shown for MP65 (20, 44), was confirmed by the inability of the relevant preparation to stimulate lymphoproliferation in cultures of mitogen-re-

sponsive, naive lymphocytes from the human umbilical cord blood (data not shown). Table 1 shows the actual lymphoproliferative responses of all eight donors whose PBMC were stimulated in vitro with peptides T1 and T2. Peptide T6 served as the negative control.

Amino acid sequences of antigenic and nonantigenic peptides. The amino acid sequences of the MP65 peptides were

TABLE 1. Lymphoproliferation induced by MP65 and some of the internal peptides separated from MP65 by HPLC of human PBMC cultures from adult healthy donors or umbilical cord blood

Stimulator	Concentration ^b	Lymphoproliferation ^a for PBMC donor:							
		1	2	3	4	5	6	7	8
None		2.0 ± 0.4	0.6 ± 0.1	0.5 ± 0.1	0.7 ± 0.1	0.5 ± 0.05	0.6 ± 0.2	0.2 ± 0.1	1.2 ± 0.3
IL-2	100 U/ml	40.7 ± 6.1	21.0 ± 3.3	25.5 ± 3.8	22.4 ± 3.7	ND ^c	23.2 ± 2.1	ND	59.1 ± 3.0
MP65	1 µg/ml	30.7 ± 2.4	25.2 ± 1.7	6.3 ± 0.6	23.8 ± 1.8	5.2 ± 0.5	21.9 ± 1.2	16.1 ± 1.3	30.6 ± 7.2
	0.1 µg/ml	16.5 ± 1.0	11.0 ± 0.8	4.1 ± 0.5	10.9 ± 0.8	4.5 ± 0.6	11.5 ± 1.6	10.4 ± 0.9	5.6 ± 3.3
T1	1:100	12.0 ± 1.8	9.7 ± 0.3	7.5 ± 0.3	10.4 ± 0.7	8.0 ± 0.4	6.8 ± 0.1	7.9 ± 5.0	10.5 ± 2.3
	1:1,000	1.5 ± 0.1	0.5 ± 0.1	0.2 ± 0.1	1.0 ± 0.3	0.9 ± 0.7	0.5 ± 0.1	0.5 ± 0.1	2.2 ± 2.8
T2	1:100	16.5 ± 3.1	ND	ND	19.3 ± 3.9	10.7 ± 1.6	18.7 ± 0.7	10.7 ± 8.6	3.0 ± 0.3
	1:1,000	ND	ND	ND	11.0 ± 2.5	3.0 ± 0.6	7.0 ± 1.4	3.0 ± 2.6	0.7 ± 0.2
T6	1:25	ND	ND	ND	ND	ND	ND	0.5 ± 0.05	1.4 ± 0.6
	1:100	1.7 ± 0.1	0.5 ± 0.01	0.3 ± 0.2	0.6 ± 0.1	0.3 ± 0.1	0.5 ± 0.1	0.3 ± 0.01	1.0 ± 0.4
	1:1,000	1.8 ± 0.3	0.5 ± 0.01	0.4 ± 0.2	0.5 ± 0.1	0.3 ± 0.1	0.6 ± 0.1	0.4 ± 0.1	0.2 ± 0.1
Buffer			ND	ND	ND	ND	ND	1.0 ± 0.2	1.4 ± 0.6
	1:100	0.6 ± 0.4	0.8 ± 0.1	1.3 ± 0.2	ND	ND	ND	0.9 ± 0.4	ND

^a Evaluated as [³H]thymidine incorporation and expressed as counts per minute (10³) (mean ± standard deviation).

^b For MP65 internal peptides, fractions obtained by HPLC separation of 400 µg of trypsin-digested MP65 were resuspended in 200 µl of PBS and tested at the indicated final dilutions.

^c ND, not done.

TABLE 2. N-terminal amino acid sequences of T-cell proliferation-inductive and -noninductive (control) peptides

Peptide	Amino acid sequence ^b
T1.....	IFAGIFDVSSITSGIESLAEAVK
T2.....	SSSQIASEIAQLSGFNIVR
T3.....	LYGVDXX
T4.....	XYGWDDIYTVSIGNELVNAGXA
T5.....	Blocked ^a
T6.....	YWGIYSN
T7.....	XXXGXXAILFTAFNDLWK
T8.....	XITYSPY
T9.....	AVHVVX

^a Chemical block at the N-terminal amino acid; no sequence could be determined.

^b X, unidentified amino acid.

determined following total digestion with trypsin or chymotrypsin. In particular, the complete amino acid sequence of peptide T1 was determined after a further cleavage with chymotrypsin, HPLC separation, sequencing of the resulting peptides, and comparison with the amino acid composition of the whole, initial peptide from trypsin digestion. None of the main peptides examined had overlapping amino acid sequences, indicating that they were indeed distinct MP65 fragments (Table 2). The sequence of the T9 peptide corresponded to the previously reported N-terminal sequence (20).

For the amino acid homology search, the peptides above and other MP65 fragments obtained by partial or complete cleavage with trypsin or chymotrypsin were sequenced and screened with database protein sequences. Following limited digestion with trypsin or chymotrypsin, several fragments ranging in apparent molecular mass from around 28 to less than 14 kDa were obtained (Fig. 3). Only fragments of trypsin digestion with molecular masses of 28, 25, and 15 kDa (T28, T25, and T15, respectively) as well as one from chymotrypsin digestion with a molecular mass of 26 kDa (CH26) were detected after electrophoretic transfer to a PVDF membrane and Coomassie blue staining. None of these large fragments reacted with ConA in Western blotting showing that they were minimally, if at all, glycosylated, and none was recognized by Mab 7H6 (Fig. 3). As shown in Table 3, the amino acid sequences of the 28- and 25-kDa tryptic fragments were similar and overlapped with the sequence of the 26-kDa chymotryptic fragment. Other overlappings with peptides generated by extensive proteolysis were seen; in particular, the 15- and 28-kDa tryptic fragments clearly contained the T1 and T2 peptides, respectively (compare with Table 2).

A search of protein databases revealed a high degree of similarity between the sequences of most of the MP65 peptides and the deduced amino acid sequences of two proteins encoded by the *S. cerevisiae* genes *YRM305C* and *YGR279C*. Figure 4 shows the sequences of these *S. cerevisiae* gene products aligned with the putative corresponding peptides of *Candida* MP65. The previously reported N terminus sequence of MP65 is also shown. Based on sequence comparison, the homology between the sequences of the MP65 peptides and the deduced amino acid sequences encoded by the *S. cerevisiae* genes is particularly high (up to 70% identity) in several regions of the C-terminal moiety of the protein. However, the identity score was much lower in the MP65 antigenic peptides, in particular for the T1 peptide (compare sequences of amino acids 195 to 217 of Fig. 4).

Identification of T1 and T2 as putatively immunodominant MP65 sequences. Since all (for T1) or most (for T2) of the

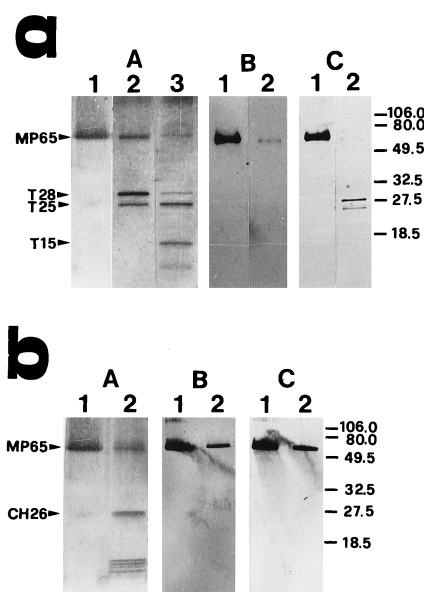


FIG. 3. Analysis by SDS-PAGE and Western blotting of the MP65 fragments obtained by limited cleavage with trypsin (a) or chymotrypsin (b). Samples of MP65 (lanes 1 of both panels) were digested at an enzyme/substrate ratio of 1:50 or 1:10 for trypsin (a, lanes 2 and 3, respectively) or 1:100 for chymotrypsin (b, lanes 2). Protein fragments were separated in 15 to 20% acrylamide gels and visualized with silver stain (A) or with ConA (B) (both panels) or anti-MP65 mouse serum (a, C) or Mab 7H6 (b, C) after electrophoretic transfer to PVDF membranes. Peptide fragments analyzed for amino acid sequence were CH26, T28, T25, and T15. Molecular mass markers (right) are in kilodaltons.

subjects tested responded to the T1 and T2 peptides, we focused our attention on these two peptides as highly antigenic, putatively immunodominant determinants of MP65 (see above). To this end, the whole T1 and T2 peptides and two partially overlapping sequences (14 to 16 amino acids) of each of the two peptides were synthesized by using the sequence information derived from complete and incomplete MP65 enzymatic digestion (Tables 2 and 3) and used to stimulate in vitro PBMC from MP65- and natural T1- and T2-responsive donors. The whole T1 and T2 synthetic peptides were extremely hydrophobic and could hardly be dissolved at sufficient concentrations in PBS to be tested in lymphoproliferation assays. As shown in Table 4, the synthetic peptides possessing the last six and four C terminus amino acids of T1 and T2 peptides, respectively, induced lymphoproliferation in all four donors

TABLE 3. Amino acid sequences of major MP65 fragments from limited trypsin or chymotrypsin digestion or complete chymotrypsin digestion

Treatment	Peptide	Amino acid sequence ^b
Limited proteolysis		
Trypsin	T28	GIIYSPYSDNGGCKSSSQIASEIAQL
	T25	GMYSYPY ^a
	T15	TSXQKIFAGIFDVSSITXGIECLA
Chymotrypsin	CH26	XPYSDNGXXXXX
Complete proteolysis with chymotrypsin	CH1	TVSIGNEL
	CH2	TGPVVSVDTF
	CH3	ITETGWPS

^a The sequencing was arrested after the seventh amino acid.

^b X, unidentified amino acid.

TABLE 5. Comparison between the deduced amino acid compositions of the *S. cerevisiae* mature proteins encoded by *YRM305C* and *YGR279C* and that of *C. albicans* MP65^a

Amino acid residue	YRM305C		YGR279C		MP65 ^b (%)
	No. of residues	%	No. of residues	%	
Asp/Asn	38 (22/16)	10.6	39 (19/20)	11.0	9.8
Thr	38	10.6	29	8.2	6.8 ^c
Ser	61	16.7	53	14.9	15.1 ^c
Glu/Gln	33 (17/16)	9.2	34 (17/17)	9.6	3.9
Pro	9	2.5	10	2.8	3.9
Gly	28	7.8	24	6.7	13.6
Ala	37	10.3	48	13.5	12.0
Cys	5	1.4	5	1.4	ND ^d
Val	38	10.6	36	10.1	11.3
Met	1	0.3	1	0.3	0.0
Ile	15	4.2	15	4.2	5.2
Leu	15	4.2	12	3.4	4.0
Tyr	16	4.4	15	4.2	3.2
Phe	6	1.7	8	2.3	2.3
His	2	0.6	1	0.3	Traces
Lys	14	3.9	16	4.5	4.5
Arg		0.6	4	1.1	4.5
Trp		1.4	6	1.7	ND

^a See reference 20. For YRM305C, YGR279C, and MP65, the following values, respectively, were found: amino acid number, 360, 356, and unknown; pI, 4.54, 4.72, and 4.1; molecular mass by SDS-PAGE, 66, 66, and 65 kDa; molecular weight of the protein moiety, 40,448, 40,158, and unknown.

^b The amino acid analysis was performed with affinity-purified MP65 subjected to SDS-PAGE and electrophoretic transfer to PVDF membranes. The absolute numbers of amino acids in the whole molecule could not be determined since the molecular weight of the protein moiety remains unknown.

^c No correction for the destructive loss of Ser and Thr during hydrolysis was made.

^d ND, not determined.

glycosylation site was also identified in both *S. cerevisiae* deduced amino acid sequences, but no such a site was present in any of the MP65 sequenced peptides.

DISCUSSION

In this paper, a 65-kDa mannoprotein (MP65), a major antigen target of anti-*Candida* CMI, has been dissected into a number of PBMC-stimulatory peptide fragments. At least two peptides have been found to contain major antigenic determinants of the fungus. They were seen to belong to a protein closely homologous to *S. cerevisiae* cell wall mannoproteins, designated by Cappellaro et al. (10) Scw4p and Scw10p and apparently encoded by the yeast genes *YRM305C* and *YGR279C* (20).

To our knowledge, MP65 is the first cell wall mannoprotein antigen of *C. albicans* that has been partially dissected into its antigenic determinants after molecular characterization and sequencing. There are several other mannoproteins of this fungus which approximate the molecular weight of the MP65 constituent, and some of them have been, in part, characterized at a molecular level. In general, they have usually been studied for adhesion to plastic or the cell surface or as receptors for soluble factors of host immunity rather than as CMI targets (4, 5, 8, 9, 19, 45, 46). A few of them have also been shown to be recognized as antigens. For instance, the C3d-binding protein (a doublet of 55 to 60 kDa and with a pI around 4) was antigenically expressed and it induced in vitro proliferation of splenocytes from *Candida*-infected mice (8). However, no precise identification of its antigenic determinants has been made, and the protein remains quite distinct

from the MP65 antigen of *C. albicans*. Lack of biochemical characterization and sequence data for other MP-rich antigenic preparations of *C. albicans* precludes any sensible structural and functional comparison with our antigenic constituent (16, 17, 35).

Besides the remarkable technical difficulties in purifying (and sequencing) individual mannoproteins from polydispersed, highly glycosylated material, as previously discussed (11, 34), the paucity of data on the protein constituent of mannoproteins is also due to the fact that most research has been focused on saccharide determinants of the antibody response for serotyping and diagnostic purposes (39, 41). Consequently, a wealth of knowledge has been accumulated on α - and β -linked oligomannosides involved as B-cell epitopes, one of which has recently been shown to induce antibody-mediated protective immunity (21, 22). In contrast, the protein moieties of most mannoproteins remain rather mysterious, despite previous indirect evidence that their epitopes may constitute the target of such responses as delayed type hypersensitivity in vivo, ex vivo lymphoproliferation, and Th1 cytokine production (2, 3, 16, 17, 32), all critical elements of anti-*Candida* immunity (36, 38).

Amino acid sequence analysis of MP65 fragments obtained from partial or complete trypsin and chymotrypsin digestion revealed a substantial homology of this protein to products of a family of *S. cerevisiae* open reading frames (ORF), mostly *YRM305C* and *YGR279C*, recently identified by Cappellaro et al. (10) and shown to belong to a family of cell wall glucanases or transglycosidases. Among the sequenced MP65 peptides, those having the strongest homology to the deduced *S. cerevisiae* amino acid sequences were present in the C-terminal half of the molecule. The N-terminal amino acid sequences of fragments T28 and T25 and, mostly, the T1 fragment from the complete trypsin digestion showed the lowest degree of sequence homology with the *S. cerevisiae* gene products. All these sequences apparently belonged to the N-terminal half of the molecule. The two *S. cerevisiae* gene products show a high degree of potential O-glycosidic bonds, serine and threonine accounting for more than 20% of all amino acids. A long stretch of serine residues in positions 106 to 130 also characterizes the products of the two *S. cerevisiae* ORF. This region should be therefore considered as putatively involved in most of the MP65 O glycosylation. This hypothesis is supported by the observation that MP65 peptides obtained by limited proteolysis (T28, T25, T15, and CH26), while matching the corresponding *S. cerevisiae* regions (amino acid positions 146 and 144 of *YRM305C* and *YGR279C*, respectively) did not react with ConA in Western blotting (Fig. 3), nor did antigenic peptides T1 to T5 react with this lectin (as indicated in Fig. 2).

Other close resemblances between MP65 and the Scw10p and Scw4p of *S. cerevisiae* concern the nature of their cell walls and of their secretory mannoproteins and their release from the cell wall by reducing agents (10, 44). Although one site of potential N glycosylation (NXS or NXT) is present in both Scw10p and Scw4p, no such sites were identified in the putatively corresponding positions in any of the sequenced MP65 peptides. Previous studies showed that α -mannosidase and β -elimination, but not endoglycosidase, treatments affected the molecular mass of MP65 (20, 45). Moreover, the purified molecule was not reactive with any MAb recognizing common α - or β -mannan epitopes of a large variety of *Candida* mannoproteins (M. J. Gomez and A. Cassone, unpublished observations). It is possible that N glycosylation sites reside in other nonsequenced MP65 regions or that the protein is not N glycosylated, as happens for other cell wall mannoproteins (23).

Since MP65 is particularly abundant in the secretory man-

noprotein material from the hyphal cells of *C. albicans* (44), its apparent homology with enzymes of the glucan metabolism would suggest a major function of it during mycelial growth, when the level of β -glucanase activity is high and dramatic rearrangements of cell wall structure and cell surface antigen modulations occur (14). However, no direct data on the biochemical function of MP65 are presently available.

A main purpose of the present study was the dissection of MP65 into its main antigenic determinants recognized by human PBMC. A number of tryptic or chymotryptic fragments were indeed separated by HPLC, their amino acid sequences were largely determined, and their abilities to induce PBMC proliferation in a number of subjects were tested. A more extensive testing with a high number of subjects and with various exact (molar) concentrations of each peptide could not be done as the amounts of peptide recovered from HPLC were low and the amount of MP65 itself achievable on a laboratory scale was not sufficiently high for complete chemical characterization of its trypsin digests. Moreover, the methods used could not guarantee that the antigenic peptides identified were totally sequenced, so as to exactly define their concentrations. Nonetheless, initial experiments with synthetic peptides provided direct evidence that at least two peptides (T1 and T2) contained genuine antigenic determinants and helped to more closely define the T1 and T2 regions representing or containing the epitope sequences. Interestingly, upon alignment of their sequences with the corresponding sequences of *S. cerevisiae* cell wall proteins, both peptides appeared to be localized in that portion of the molecule showing the least homology with the corresponding region of the yeast proteins. In fact, the immunoactive T1b peptide showed 80% sequence diversity compared to the corresponding *S. cerevisiae* region, whereas for the T2b peptide the diversity was >40%. The distinctiveness of what putatively contains major T-cell epitopes of MP65 might explain why mannoprotein extracts of *S. cerevisiae*, also containing MP65-like molecules, are substantially devoid of lymphoproliferation induction ability (A. Cassone, M. J. Gomex, and A. Torosantucci, unpublished data). In addition, the Scw10p and Scw4p sequences did not contain the lymphoproliferation-relevant epitope recognized by MA b 7H6 (20) that has recently been identified close to the N-terminal end of MP65 (C. Bromuro, R. La Valle, and A. Cassone, unpublished data).

It was previously shown that the MP65-containing MP-F2 fraction is recognized by human PBMC as a classical antigen rather than as a mitogen or a superantigen because its recognition requires antigen-presenting cells, is blocked by antibodies against class II histocompatibility antigens, and does not occur in naive cord-blood lymphocytes (2, 3, 26, 45). Although more studies with T-cell lines or clones are required for a full definition of the antigenic properties of MP65 peptides, including their recognition by T-cell epitopes, we have confirmed here that both T1 and T2 peptides are unable to stimulate naive cord blood lymphocytes to proliferate, thus possibly behaving as true antigens. A point of interest is also the low level or absence of glycosylation of these peptides, as shown by their inability to react with ConA. This suggests that the MP65 molecule is not uniformly glycosylated and that highly antigenic determinants of the human anti-*Candida* response have been selected among the least-glycosylated molecular regions of it. The immunoactivity of the synthetic peptides T1b and T2b supports this interpretation.

As discussed elsewhere (11, 12), MP65 is recognized by almost all healthy subjects that have so far been tested in our and other laboratories (overall totaling several hundred subjects). The T1 and T2 peptides studied here also induced

lymphoproliferation in the great majority of the subjects examined, although the number of subjects was rather small. A mixture of well-defined MP65 peptides or a recombinant MP65 could therefore constitute a much more reliable reagent than those crude antigenic fractions used as recall antigens to assess the CMI response under various conditions, including the loss and recovery of CD4⁺ T-cell function in human immunodeficiency virus-positive subjects under therapy (13, 37). Crude antigenic fractions are indeed difficult to standardize and often produce unreliable results. The peptide sequences described here along with the recognized homology with the *S. cerevisiae* cell wall mannoproteins (10) will also facilitate the molecular cloning and the expression of a recombinant protein, a necessary step for a full evaluation of the immunogenicity and protective values of this immunodominant antigen of *C. albicans*.

ACKNOWLEDGMENTS

This work was supported by the National AIDS Project, Istituto Superiore di Sanità, Contract no. 50B/B.

The authors are grateful to Anna Botzios and Francesca Girolamo for help in the preparation of the manuscript.

REFERENCES

- Ashman, R. B., and J. M. Papadimitriou. 1995. Production and function of cytokines in natural and acquired immunity to *Candida albicans* infection. *Microbiol. Rev.* 59:664–672.
- Ausiello, C. M., G. C. Spagnoli, M. Boccanera, I. Casalnuovo, F. Malavasi, C. U. Casciani, and A. Cassone. 1986. Proliferation of human peripheral blood mononuclear cells induced by *Candida albicans* and its cell wall fractions. *J. Med. Microbiol.* 22:195–202.
- Ausiello, C. M., F. Urbani, S. Gessani, G. C. Spagnoli, M. J. Gomez, and A. Cassone. 1993. Cytokine gene expression in human peripheral blood mononuclear cells stimulated by mannoprotein constituents from *Candida albicans*. *Infect. Immun.* 61:4105–4111.
- Bendel, C. M., J. St. Sauver, S. Carlson, and K. Hostetter. 1995. Epithelial adhesion in yeast species: correlation with surface expression of the integrin analog. *J. Infect. Dis.* 171:1660–1663.
- Bouchara, J. P., G. Tronchin, V. Annaix, R. Robert, and J. M. Senet. 1990. Laminin receptors on *Candida albicans* germ tubes. *Infect. Immun.* 58:48–54.
- Bromuro, C., R. La Valle, S. Sandini, F. Urbani, C. M. Ausiello, L. Morelli, C. Fè d'Ostiani, L. Romani, and A. Cassone. 1998. A 70-kilodalton recombinant heat shock protein of *Candida albicans* is highly immunogenic and enhances systemic murine candidiasis. *Infect. Immun.* 66:2154–2162.
- Burnette, N. 1981. "Western blotting": electrophoretic transfer of protein from dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Anal. Biochem.* 112:195–203.
- Calderone, R., E. Enache, T. Eskandri, E. Wadsworth, and J. Sturtevant. 1997. Adherence of *Candida albicans* to mammalian cells in vitro: nutritional influences, p. 73–83. In S. Suzuki and M. Suzuki (ed.), *Fungal cells in biodefense mechanism*. Saikon Publishing Co., Ltd., Tokyo, Japan.
- Calderone, R. A., L. Linehan, E. Wadsworth, and L. Sandberg. 1988. Identification of C3d receptors on *Candida albicans*. *Infect. Immun.* 56:252–258.
- Cappellaro, C., V. Marsa, and W. Tanner. 1998. New potential cell wall glucanases of *Saccharomyces cerevisiae* and their involvement in mating. *J. Bacteriol.* 180:5030–5037.
- Cassone, A. 1997. Mannoproteins of *Candida albicans* in host-parasite relationship, p. 147–154. In S. Suzuki and M. Suzuki (ed.), *Fungal cells in biodefense mechanism*. Saikon Publishing Co., Ltd., Tokyo, Japan.
- Cassone, A., F. De Bernardis, C. M. Ausiello, M. J. Gomez, M. Boccanera, R. La Valle, and A. Torosantucci. 1998. Immunogenic and protective *Candida albicans* constituents. *Res. Immunol.* 149:289–298.
- Cauda, R., E. Tacconelli, M. Tumbarello, G. Morace, F. De Bernardis, A. Torosantucci, and A. Cassone. 1999. Role of protease inhibitors in preventing recurrent oral candidosis in patients with HIV infections: a prospective case-control study. *J. Acquir. Immune Defic. Syndr. Hum. Retrovirol.* 21:20–25.
- De Bernardis, F., A. Molinari, M. Boccanera, A. Stringaro, R. Robert, J. M. Senet, G. Arancia, and A. Cassone. 1994. Modulation of cell surface-associated mannoprotein antigen expression in experimental candidal vaginitis. *Infect. Immun.* 62:509–519.
- Deepe, G. S., Jr. 1997. Prospects for the development of fungal vaccines. *Clin. Microbiol. Rev.* 10:585–596.
- Domer, J. E. 1989. *Candida* cell wall mannan: a polysaccharide with diverse immunologic properties. *Crit. Rev. Immunol.* 17:33–51.

17. Domer, J. E., S. Li, Y. Wang, and S. Lee. 1997. Immunoregulatory studies with *Candida albicans* mannan, p. 161–168. In S. Suzuki and M. Suzuki (ed.), *Fungal cells in biodefense mechanism*. Saikon Publishing Co., Ltd., Tokyo, Japan.
18. Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith. 1956. Colorimetric method for determination of sugars and related substances. *Anal. Chem.* **28**:350–356.
19. Gilmore, B. J., E. M. Retsinas, J. S. Lorenz, and M. K. Hostetter. 1988. A C3d receptor on *Candida albicans*: structures, function and correlates for pathogenicity. *J. Infect. Dis.* **157**:38–41.
20. Gomez, M. J., A. Torosantucci, S. Arancia, B. Maras, L. Parisi, and A. Cassone. 1996. Purification and biochemical characterization of a 65-kilodalton mannoprotein (MP65), a main target of anti-*Candida* cell-mediated immune responses in humans. *Infect. Immun.* **64**:2577–2584.
21. Han, Y., and J. E. Cutler. 1995. Antibody response that protects against disseminated candidiasis. *Infect. Immun.* **63**:2714–2719.
22. Han, Y., T. Kanbe, R. Cherniak, and J. E. Cutler. 1997. Biochemical characterization of *Candida albicans* epitopes that can elicit protective and non-protective antibodies. *Infect. Immun.* **65**:4100–4107.
23. Kapteyn, J. C., H. Van Den Ende, and F. M. Klis. 1999. The contribution of cell wall proteins to the organization of the yeast cell wall. *Biochim. Biophys. Acta* **1426**:373–383.
24. Klein, R. S., C. A. Harris, C. B. Small, B. Moll, M. Lesser, and G. H. Friedland. 1984. Oral candidiasis in high-risk patients as the initial manifestation of the acquired immunodeficiency syndrome. *N. Engl. J. Med.* **311**:354–358.
25. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**:680–685.
26. La Sala, A., F. Urbani, A. Torosantucci, A. Cassone, and C. M. Ausiello. 1996. Mannoproteins from *Candida albicans* elicit a Th-type1 cytokine profile in human *Candida* specific long-term T cell cultures. *J. Biol. Regul. Homeost. Agents* **10**:8–12.
27. La Valle, R., C. Bromuro, L. Ranucci, H. M. Muller, A. Crisanti, and A. Cassone. 1995. Molecular cloning and expression of 70kDa heat shock protein of *Candida albicans*. *Infect. Immun.* **63**:4039–4045.
28. Lee, K. L., H. R. Buckley, and C. C. Campbell. 1975. An amino acid liquid synthetic medium for the development of mycelial and yeast forms of *Candida albicans*. *Sabouraudia* **13**:148–153.
29. Mathews, R., and J. Burnie. 1992. The role of hsp90 in fungal infection. *Immunol. Today* **13**:345–348.
30. Mathews, R., D. Smith, J. Midgley, J. Burnie, I. Clark, M. Conolly, and B. Gazzard. 1988. *Candida* and AIDS: evidence for protective antibody. *Lancet* **ii**:263–265.
31. Matsudaira, P. 1990. Limited N-terminal sequence analysis. *Methods Enzymol.* **182**:602–613.
32. Mencacci, A., A. Torosantucci, R. Spaccapelo, L. Romani, F. Bistoni, and A. Cassone. 1994. A mannoprotein constituent of *Candida albicans* that elicits different levels of delayed-type hypersensitivity, cytokine production, and anticandidal protection in mice. *Infect. Immun.* **62**:5353–5360.
33. Mencacci, A., R. Spaccapelo, G. Del Sero, K. H. Essle, A. Cassone, F. Bistoni, and L. Romani. 1996. CD4⁺ T-helper cell responses in mice with low-level *Candida albicans* infection. *Infect. Immun.* **64**:4907–4914.
34. Nelson, R. D., N. Shibata, P. R. Podzorski, and M. J. Herron. 1991. *Candida* mannan: chemistry, suppression of cell-mediated immunity, and possible mechanisms of action. *Clin. Microbiol. Rev.* **4**:1–19.
35. Nelson, R. D. 1997. Effects of *Candida albicans* mannans and mannan catabolites on human cell-mediated immune function *in vitro*, p. 169–173. In S. Suzuki and M. Suzuki (ed.), *Fungal cells in biodefense mechanism*. Saikon Publishing Co., Ltd., Tokyo, Japan.
36. Puccetti, P., L. Romani, and F. Bistoni. 1995. A T_H-1-T_H-2-like switch in candidiasis: new perspectives for therapy. *Trends Microbiol.* **3**:237–240.
37. Quinti, I., C. Palma, B. C. Guerra, M. J. Gomez, A. Torosantucci, and A. Cassone. 1991. Proliferative and cytotoxic responses to mannoproteins of *Candida albicans* by peripheral blood lymphocytes of HIV-infected subjects. *Clin. Exp. Immunol.* **85**:485–492.
38. Romani, L., and D. H. Howard. 1995. Mechanisms of resistance to fungal infection. *Curr. Opin. Immunol.* **7**:517–523.
39. Shibata, N., M. Arai, E. Haga, T. Kikuchi, M. Najima, T. Satoh, H. Kobayashi, and S. Suzuki. 1992. Structural identification of an epitope of antigenic factor 5 in mannan of *Candida albicans*. *Infect. Immun.* **60**:4100–4110.
40. Sundstrom, P., J. Jensen, and E. Balish. 1994. Humoral and cellular immune response to enolase after alimentary tract colonization or intravenous immunization with *Candida albicans*. *J. Infect. Dis.* **170**:390–395.
41. Suzuki, S. 1997. Structural investigation of mannans of medically relevant *Candida* species. Determination of chemical structures of antigenic factors 1, 4, 5, 6, 9 and 13b, p. 1–15. In S. Suzuki and M. Suzuki (ed.), *Fungal cells in biodefense mechanism*. Saikon Publishing Co., Ltd., Tokyo, Japan.
42. Torosantucci, A., C. Palma, M. Boccanera, C. M. Ausiello, G. C. Spagnoli, and A. Cassone. 1990. Lymphoproliferation and cytotoxic responses of human peripheral blood mononuclear cells to mannoprotein constituents of *Candida albicans*. *J. Gen. Microbiol.* **136**:664–672.
43. Torosantucci, A., M. J. Gomez, C. Bromuro, J. Casalnuovo, and A. Cassone. 1991. Biochemical and antigenic characterization of mannoprotein constituents released from yeast and mycelial form of *Candida albicans*. *J. Med. Vet. Mycol.* **29**:361–372.
44. Torosantucci, A., C. Bromuro, M. J. Gomez, C. M. Ausiello, F. Urbani, and A. Cassone. 1993. Identification of a 65kDa mannoprotein as a main target of human cell-mediated immune response to *Candida albicans*. *J. Infect. Dis.* **168**:427–435.
45. Tronchin, G., J. P. Bouchara, V. Annaix, R. Robert, and J. M. Senet. 1991. Fungal cell adhesion molecules in *Candida albicans*. *Eur. J. Epidemiol.* **7**:23–33.
46. Yu, L., K. K. Lee, K. Ens, P. C. Doig, M. R. Carpenter, W. Staddon, R. S. Hodges, W. Paranchych, and R. Irvin. 1994. Partial characterization of a *Candida albicans* fimbrial adhesin. *Infect. Immun.* **62**:2834–2842.