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Mapping of *Candida albicans* Oligomannosidic Epitopes by Using Monoclonal Antibodies

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Six monoclonal antibodies (MAbs) from various laboratory sources (EB-CA1, EB-CA2, H5, AF1, C6, and 5B2), reacting with the polysaccharidic moieties of *Candida albicans* mannoproteins, were used for epitope mapping by an enzyme-linked immunosorbent assay (ELISA) with neoglycolipids and by Western blotting (immunoblotting) of a *C. albicans* germ tube extract. The ELISA involved neoglycolipids constructed from three families of oligomannosides released by sequential depolymerization of *C. albicans* phosphopeptidomannan by acid hydrolysis (NGLH), β-elimination (NGLO), and acetolysis (NGLA). All of the MAbs exhibited low reactivities against NGLO. MAbs EB-CA1, EB-CA2, and H5 reacted mainly against NGLA, and MAbs C6 and AF1 recognized mainly NGLH, whereas MAb 5B2 reacted with both families of neoantigens. When this method was compared with Western blotting, strong reactivity to NGLA was associated with the presence of epitopes shared by high-molecular-weight mannoproteins, whereas strong reactivity to NGLH was associated with a reactivity to a family of 14- to 18-kDa antigens. The reactivity of MAb 5B2 was associated with both high-molecular-weight mannoproteins and the 14- to 18-kDa antigens. In relation to the present knowledge about the structure of the *C. albicans* phosphopeptidomannan oligomannosidic repertoire, these results provide preliminary data concerning the molecular basis of the recognition of mannopyranosyl sequences by MAbs and their distribution among *C. albicans* mannoproteins.

During the last decade, numerous monoclonal antibodies (MAbs) have been generated against *Candida albicans* proteins (36) or mannoproteins (2, 8, 9, 26, 38) for both fundamental and applied purposes. Studies performed with these MAbs confirmed the existence of components preferentially expressed on the mycelial cell wall (8) and the great variability in the expression of *C. albicans* cell surface determinants according to strains used (4, 10, 15, 19), length of incubation (1, 3, 10), and growth media (1, 5, 10). Analysis of the specificities of these MAbs has been performed essentially by Western blotting (immunoblotting) on various strains grown on different media and on antigens extracted by numerous methods, such as zymolyase digestion (8), heat extraction (9, 29), or mechanical disruption (28). It therefore appeared difficult to compare the specificities of MAbs for mannoproteins on the basis of the results described in the literature. For MAbs directed against the saccharidic moieties of mannoproteins, the problem is even more complicated, since they usually react with a large number of bands (33), including high-molecular-weight polydisperse material (32, 39).

However, analysis of the specificities of such MAbs as established on mannoproteins can also be focused on the nature of the oligomannosidic epitopes recognized. In order to perform these studies, oligomannosides can be released by depolymerization of the phosphopeptidomannan (PPM) of *C. albicans* and coated onto enzyme-linked immunosorbent assay (ELISA) plates through the construction of neoglycolipids (NGL) (12, 13, 18).

We used this alternative method together with Western blotting to determine the preferential reactivities of six MAbs, originating from different laboratories, against the three oligomannoside families released by sequential depolymerization of *C. albicans* PPM. This was undertaken with the objective of defining a relationship between the reactivities observed with mannoproteins by Western blotting and those observed with oligomannoside families, some of which are becoming well characterized chemically (34).

MATERIALS AND METHODS

Yeast strain and growth conditions. *C. albicans* strain VW.32 (serotype A) was used throughout this study for antigen preparation. Cultures were maintained routinely on Sabouraud dextrose agar. For germ tube formation, yeast cells were grown for 24 h at 28°C on Sabouraud agar and then allowed to germinate on RPMI medium at 37°C for 4 h. Under these conditions, at least 95% of the blastoconidia were converted to germ tubes.

For cell wall PPM extraction, cells were grown at 28°C in bioreactors in yeast extract medium containing glucose as previously described (13). Under these conditions, the cellular population consisted of at least 95% yeast cells.

MAbs. MAbs from various origins were chosen according to their abilities to react with *C. albicans* saccharidic determinants. The MAbs chosen were, first, MAbs whose production and characteristics have been described previously in the literature: MAb 5B2 from our laboratory (6, 19) and MAbs AF1 (9), C6 (2, 3), and EB-CA1 (17), kindly provided by A. Cassone (Istituto Superiore de Sanita, Roma, Italy), J. E. Cutler (Montana State University, Bozeman), and D. Stynen (Sanofi/Diagnostics Pasteur, Genk, Belgium), respectively. The other MAbs were MAb H5, a mouse immunoglobulin M (IgM) generated against *C. albicans* heat-killed yeast cells kindly provided by R. Guinet (Institut Pasteur, Lyon, France) and MAb EB-CA2, a rat IgG generated against *C. albicans* killed yeast cells and kindly provided by

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D. Stynen. MAb 3D9, a mouse IgM (27), kindly provided by R. Robert and J. M. Senet (Faculté de Pharmacie, Angers, France), was included in this study as a control, since it reacts with a *C. albicans* peptidic epitope (26a).

Antigens. (i) PPM. The PPM was extracted from the *C. albicans* cell wall by heating in 20 mM sodium citrate (pH 7) for 90 min at 125°C according to the method of Kocourek and Ballou (23), with modifications (13).

(ii) NGL. NGL were constructed from the different families of oligomannosides obtained after sequential depolymerization of the PPM (13).

The first step of this depolymerization consisted of mild acid hydrolysis: PPM was dissolved in 10 mM HCl and heated for 30 min at 100°C. After cooling, the solution was neutralized with 100 mM NaOH and lyophilized. The oligomannosides released through this procedure of acid hydrolysis, designated oligomannosides H, were separated from the depleted PPM by two successive precipitations in 80% ethanol at room temperature and were lyophilized.

The second step was a β-elimination: the depleted PPM was dissolved in 100 mM NaOH and incubated for 24 h at 25°C. The solution was neutralized with 1 N acetic acid and lyophilized. These oligomannosides released by the breakage of *O* glycosidic bonds, designated oligomannosides O, were separated from the depleted PPM by ethanol precipitation as described above and were lyophilized.

The last step was mild acetolysis: the depleted PPM was dissolved in 10 ml of formamide, mixed with 50 ml of acetic anhydride-pyridine (vol/vol), and heated for 13 h at 30°C on a rotary shaker. This solution was then poured into water and centrifuged at 1,500 × *g* for 15 min. The pellet was dried, suspended in 50 ml of acetic anhydride-acetic acid-sulfuric acid (20:20:1, vol/vol/vol), and heated for 13 h at 40°C on a rotary shaker. The reaction was stopped by pouring the solution onto ice and was neutralized with sodium hydrogenocarbonate. The released oligosaccharides A were extracted with chloroform, incubated for 15 min at room temperature in 10 ml of methanol adjusted to pH 9 with sodium methoxide, and centrifuged for 15 min at 1,500 × *g*. The pellet was then washed twice in methanol and dissolved in water.

In order to establish the fingerprinting of the oligomannosidic families, 50 mg of each family was applied to a Bio-Gel P-2 (Bio-Rad, Richmond, Calif.) column (1.8 by 140 cm). The column was eluted with distilled water at a flow rate of 8 ml/h at room temperature, and fractions of 1.5 ml were collected. The carbohydrate concentration in each fraction was determined by the phenolsulfuric method of Dubois et al. (11). The degree of polymerization of the separated oligomannosides was then checked by thin-layer chromatography on silica gel 60-precoated plates (Kieselgel 60; Merck, Darmstadt, Germany) with *n*-butanol–acetic acid–water (20:10:10, vol/vol/vol) as a solvent. Mannose, saccharose, and raffinose were used as standards, and sugars were stained with the orcinol-sulfuric acid reagent.

The three oligomannosidic pools, H, O, and A, were coupled with 4-hexadecylaniline to form NGL according to a previously described mole-to-mole binding (13) to form NGLH, NGLO, and NGLA, respectively. Efficiency of the binding was routinely checked by thin-layer chromatography. After migration, separated NGL were visualized under UV light or after orcinol-sulfuric acid staining (13).

Whole-cell extract. For Western blotting, whole-cell antigens were obtained by an alkali extraction method under reducing conditions described by Yaffe and Schatz (40), with slight modifications. Briefly, a pellet containing approximately 5×10^8 washed germ tubes was incubated in 1.85 M NaOH–5% 2-mercaptoethanol for 15 min on ice. Extracted proteins were then precipitated with the cell pellet by incubation with an equal volume of 50% trichloracetic acid for 15 min on ice. The pellet was washed twice with 1 M Tris (pH 11.5) and then boiled for 5 min in 62.5 mM Tris-HCl (pH 6.8)–2% sodium dodecyl sulfate (SDS)–10% sucrose. After the removal of insoluble material, the protein concentration was adjusted to 4 mg/ml, and samples were stored at −30°C until they were used. Before electrophoresis, 5% (vol/vol) 2-mercaptoethanol was added to the sample before it was heated at 100°C for 5 min.

ELISA. PPM and NGL were coated onto microELISA plates (Bioblock; Nunc, Strasbourg, France). Each well received 100 μl of a solution of antigen containing 0.1 μg of carbohydrate in 60 mM sodium carbonate buffer (pH 9.6). After washes of the wells in TNT (50 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20 [pH 7.5]), MAbs diluted in the same buffer were incubated in each well. When tested against NGL, MAb concentrations were chosen in order to obtain an optical density greater than 0.4 with at least one family of neoantigens. After washes of the wells with TNT, 100 μl of immunopurified rabbit or goat anti-rat IgG or IgM or anti-mouse IgM peroxidase-labeled antibodies (Zymed; Biosoft, Paris, France), diluted 1/500, were incubated in each well. After washes of the wells with TNT, each well received 100 μl of the enzymatic substrate containing 0.1% 1,2-phenylenediamine–0.05% H_2O_2. After 15 or 30 min of incubation (when PPM or NGL, respectively, was used as the antigen), the reaction was stopped by the addition of 50 μl of 4 N H_2SO_4. Plates were scanned at 490 nm on a microELISA reader.

SDS-PAGE and Western blotting. SDS-polyacrylamide gel electrophoresis (PAGE) was carried out for 18 h at a constant current of 4 mA per gel on a 5 to 15% (wt/vol) acrylamide gel slab (14 by 14 cm), as previously described by Laemmli (25), in a vertical apparatus (Hoefer Scientific Instruments, San Francisco, Calif.).

The electrophoretic transfer to nitrocellulose sheets was performed at 200 mA for 1 h with a semidry blotter (Biometra, Göttingen, Germany), and transferred proteins were stained with Ponceau S to evaluate transfer efficiency. Proteins on blots were also stained with China ink diluted 1/10,000 in 10 mM phosphate buffer–150 mM NaCl–0.3% Tween 20 (pH 7.2).

For the identification of mannoproteins, strips cut in the nitrocellulose sheet were blocked with 5% bovine serum albumin in buffer A (50 mM Tris-HCl, 150 mM NaCl [pH 7.5]) and incubated with 1 μg of concanavalin A (ConA)-peroxidase per ml in buffer B (buffer A plus 0.05% Tween 20) for 1 h at 37°C. Strips were then developed in buffer B–0.05% diaminobenzidine–0.1% H_2O_2.

For the analysis of MAb specificities, strips were blocked with 5% nonfat dry milk in buffer A for 30 min at 37°C and then incubated for 1 h at 37°C with MAbs at the appropriate dilutions in buffer B and then with the peroxidase-labeled antibodies described above diluted in buffer B (1/750).

RESULTS

Fingerprinting of oligomannoside families. Elution profiles on a Bio-Gel P-2 column showed different patterns for the three oligomannoside families (Fig. 1). The pool of oligomannosides H contained mannose to mannodecaose, with predominant amounts of mannotriose and mannotetraose (Fig. 1A). Oligomannosides O extended from mannose to manno-

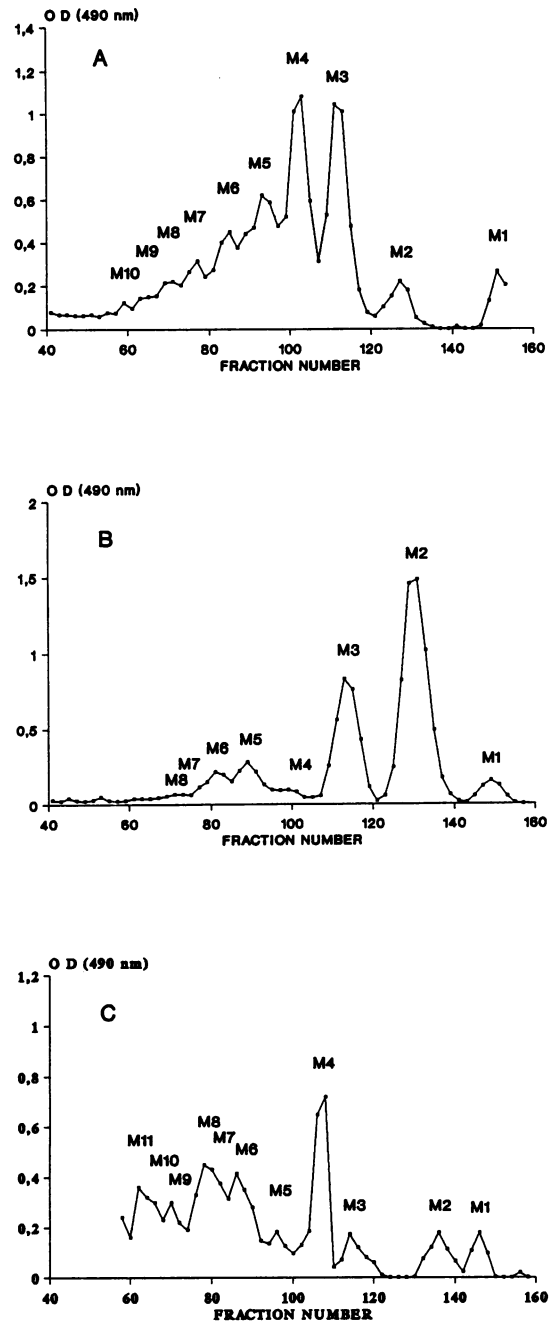

FIG. 1. Gel filtration of oligomannosides H (A), oligomannosides O (B), and oligomannosides A (C) on a column of Bio-Gel P-2 (1.8 by 140 cm). M1 to M11 refer to the degrees of polymerization of oligomannosides in chromatographic fractions checked by thin-layer chromatography.

hexaose, with mannobiose and mannotriose being the quantitatively major components (Fig. 1B). Mannose to mannoundecaose was found in the oligomannoside A family (Fig. 1C); mannotetraose was the predominant residue of this pool, in which oligomannosides with a degree of polymerization greater than six appeared to be more abundant than they were in the oligomannoside H family.

Reactivities of MAbs against PPM and the three families of NGL by ELISA. All of the MAbs except MAb 3D9, which was used as a control, exhibited strong reactivities when tested against *C. albicans* PPM (Fig. 2). When NGL were used as antigens, all of the MAbs exhibited low reactivities against NGLO (Fig. 3). However, strong differences in the patterns of MAb reactivities against NGLA and NGLH were observed (Fig. 3). The first pattern occurred with MAbs EB-CA1, EB-CA2, and H5, which exhibited stronger reactivities against NGLA. The second pattern occurred with MAbs C6 and AF1, which showed strong reactivities against NGLH. The third pattern was displayed by the last MAb, 5B2, which recognized both NGL families equally. MAb 3D9 was unreactive, no matter which neoantigen was in use.

Western blot analysis. Staining of proteins with China ink (Fig. 4, lane 1) revealed essentially low-molecular-weight proteins from 14,000 to 60,000. Treatment of the blot with ConA-peroxidase (Fig. 4, lane 2) resulted, in contrast, in continuous staining of high-molecular-mass material which behaved as a smear from the top of the gel to an apparent molecular mass of 80 kDa. From this area to a 17-kDa component, ConA-reactive material corresponded to 20 to 30 bands which were more or less narrow and well resolved. The staining intensity of the material decreased with its molecular mass. No staining has been observed for material with a molecular weight lower than 16,000.

Treatment of the blot by the panel of MAbs (Fig. 4, lanes 3 to 8) resulted in the staining of bands revealed by ConA, except for a part of the 14- to 18-kDa material, according to three different patterns.

Pattern 1 corresponded to MAbs EB-CA1, EB-CA2, and H5, which reacted with mannoproteins in the same way as ConA, i.e., they stained the material in the smear and, to a lesser extent, the lower-molecular-weight mannoproteins. However, the ability of EB-CA1 to react with mannoproteins with high molecular weights was greater than those of EB-CA2 and H5, despite the fact that the antibody concentrations used, evaluated by dot blots with the same conjugates (data not shown), were similar. Moreover, the reactivity of H5 with mannoproteins with molecular weights lower than 80,000 was very faint when compared to those of EB-CA1 and EB-CA2.

Pattern 2 corresponded to MAbs AF1 and C6, which reacted with low-molecular-mass components. The reactivity of MAb C6 allowed the recognition of three bands of 15, 16, and 17 kDa. These bands were partially and faintly stained by ConA. MAb AF1 also first revealed these bands, which afterwards quickly disappeared during revelation in a homogeneous complex whose molecular mass extended from 14 to 18.5 kDa. MAb AF1 also stained faintly, even at high concentrations, two smears with approximate molecular masses of 78 to 290 and 440 to 660 kDa.

Pattern 3 was represented by MAb 5B2, which reacted strongly both with high-molecular-weight mannoproteins, such as MAbs producing pattern 1, and with antigens from the 14- to 18-kDa family, such as MAbs producing pattern 2.

DISCUSSION

MAbs reacting with *C. albicans* polysaccharidic determinants have up to now contributed to significant advances in the knowledge of *C. albicans* cell biology and pathogenicity. They have allowed the confirmation of *C. albicans* cell surface antigenic variability from cell to cell (10, 15, 19) or from different strains (4, 10, 15) or according to the growth phase and growth conditions (1, 3, 5, 10). They have been applied to the study of the cellular processing of molecules bearing epitopes (32) and have allowed the identification of

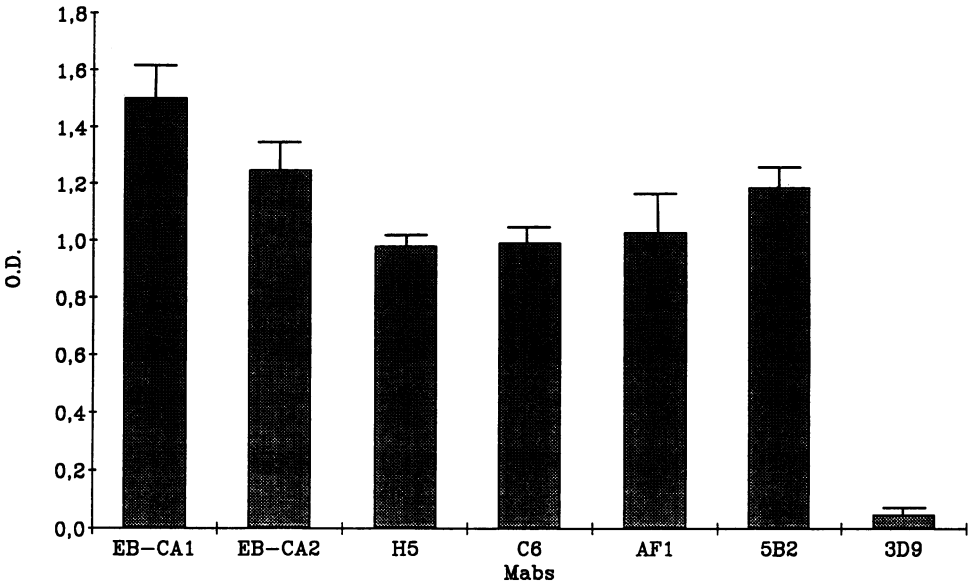

FIG. 2. MAb reactivities on *C. albicans* cell wall PPM as analyzed by ELISA. MAbs and antibody dilutions were as follows: EB-CA1, 1/100; EB-CA2, 1/200; H5, 1/200; C6, 1/50; AF1, 1/200; 5B2, 1/50; and 3D9, 1/50. OD, optical density at 490 nm. Means and standard deviations were calculated from three different series of reactions.

molecules acting as receptors (26) or immunomodulators (39). They have been used for antigen detection in sera from patients suffering from candidosis (16). However, firm conclusions about the fundamental significance of these data were impaired by the difficulties encountered in the identification of epitopes and epitopes bearing molecules (antigens) reacting with these MAbs, which were mainly (i) the absence of immunochemical screening methods allowing the determination of the sugar sequences corresponding to the epitope and (ii) the expression of the same epitope on numerous glycoproteins (28, 32, 33). However, according to a method recently developed, the construction of NGL from oligomannosides could provide a way to explore the specificities of the recognition of oligomannosides by MAbs (12, 13, 18). The aim of this study, therefore, was to compare the mapping of MAb epitopes by both ELISA with NGL constructed from oligomannosides released from *C. albicans* mannan and by Western blotting. For this purpose, we used a panel of MAbs from various sources, most of which have already been described in the literature in relation to their fundamental and/or diagnostic purposes and for which preliminary characterizations of the epitopes have shown their probable saccharidic nature (2, 9, 19).

When tested by ELISA, all of these MAbs indeed exhib-

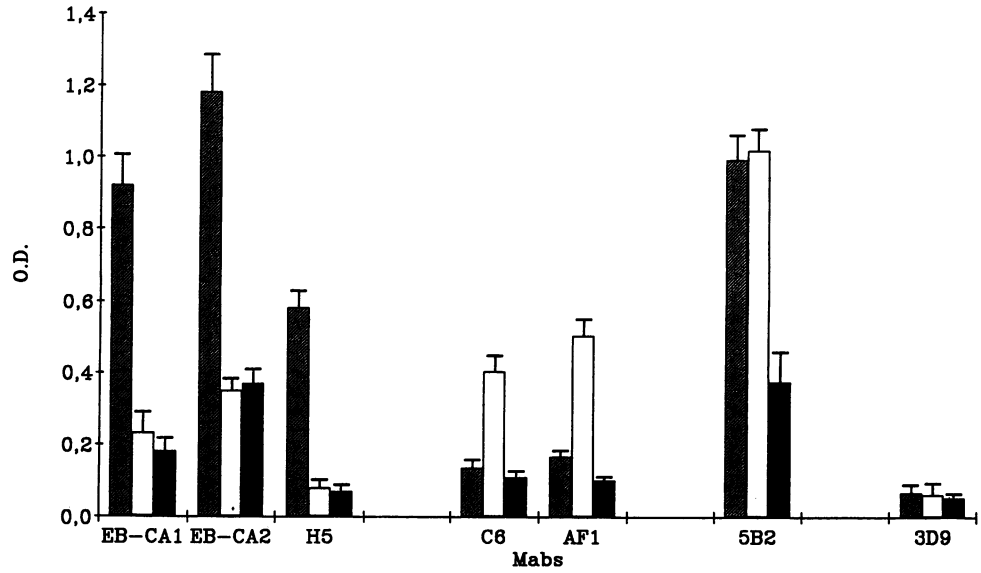

FIG. 3. MAb reactivities on NGLA (▨), NGLH (□), and NGLO (■). MAbs and antibody dilutions were as follows: EB-CA1, 1/500; EB-CA2, 1/100; H5, 1/200; C6, 1/100; AF1, 1/200; 5B2, 1/200; and 3D9, 1/50. OD, optical density at 490 nm. Means and standard deviations were calculated from three different series of reactions.

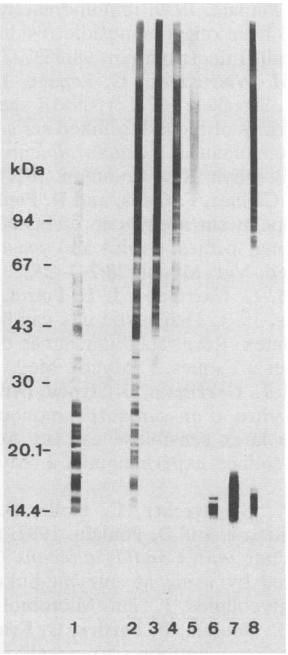

FIG. 4. Western blots of SDS-PAGE (5 to 15% polyacrylamide) loaded with an extract of *C. albicans* strain VW.32 germ tubes obtained by treatment with alkali under reducing conditions. Nitrocellulose strips were stained with China ink (lane 1), ConA (1 μg/ml) (lane 2), and MAb EB-CA1 at an antibody dilution of 1/100 (lane 3), EB-CA2 at dilution 1/80 (lane 4), H5 at dilution 1/50 (lane 5), C6 at dilution 1/50 (lane 6), AF1 at dilution 1/600 (lane 7), and 5B2 at dilution 1/100 (lane 8).

ited strong reactivities against PPM extracted from our *C. albicans* strain. However, different patterns of MAb reactivity were found on ELISA plates coated with NGL constructed from the three families of oligomannosidic structures released by sequential depolymerization of the same PPM. NGL constructed from oligomannosides O released after β-elimination were weakly reactive to all of the MAbs. These structures, which exhibit an inhibitory effect on human lymphocytic proliferation (30), are composed of one to more than eight mannosyl residues (Fig. 1B) from which the two major components (mannobiose and mannotriose) (18, 30) have been previously demonstrated to be bound mainly through α(1-2) linkages (18). The reactivities of the MAbs tested were, however, found to be associated mainly with other oligomannosidic families, either H or A or both. MAbs EB-CA1, EB-CA2, and H5 reacted preferentially against NGLA. Oligomannosides A, released after mild acetolysis, have been described as being composed of 1 to more than 11 mannosyl residues (12) (Fig. 1C) and linked mainly through α(1-2) and α(1-3) bonds (22, 24, 34, 35) and to a lesser extent through β(1-2) bonds (21, 34). They are known to contain mainly *C. albicans* antigenic determinants (20, 21). MAbs C6 and AF1 reacted preferentially against NGLH. Oligomannosides H have been described for *C. albicans* serotype A (34) and serotype B (35) strains but not for the closely related species *Candida stellatoidea* (37). A decrease in their degree of polymerization was also demonstrated during the yeast-mycelium transition of *C. albicans* strains (34, 35). Structural analyses have previously shown that mannosyl residues from mannobiose to mannoheptaose are bound through β(1-2) linkages (14, 22, 34, 35). Among the largest components of this pool (12), ^{1}H nuclear magnetic resonance analysis could show only the presence of β-mannosyl residues (11a). According to these data, it therefore seems that MAbs C6 and AF1 would react preferentially against β(1-2)-linked oligomannosides, reactivities which have already been described in the literature about MAbs generated against *C. albicans* (38).

The last MAb, 5B2, exhibited a strong reactivity against both NGLA and NGLH. Although a broad spectrum of oligomannosidic structures recognized by MAb 5B2 cannot be ruled out, the reacting structure may consist of mannopyranosyl units bound through β(1-2) linkages present both within the oligomannoside H pool (14) and at the nonreducing end of some oligomannosides A (21).

For Western blot analysis, we used, as a reference, a new method which gives rich, highly standardized antigenic profiles from germ tubes. The application of this procedure led to three main findings: (i) the demonstration of the ability of all of the MAbs tested to react with different mannoproteins stained by ConA, (ii) the fact that a large number of mannoproteins shared epitopes recognized by different MAbs (this is particularly true for polydisperse, high-molecular-weight components stained by MAbs EB-CA1, EB-CA2, H5, and 5B2 and, to a lesser extent, for mannoproteins with molecular weights lower than 80,000 which were more resolved but decreasingly stained by these MAbs), and (iii) the identification of a family of low-molecular-weight components sharing epitopes recognized by MAbs AF1, C6, and 5B2. A comparison of these data to those described in the literature could only involve the antigens reacting with MAbs AF1 and 5B2, which have been previously characterized by Western blotting. Both of them have been shown to react with mannoproteins with high molecular weights and, to a lesser extent, with mannoproteins with lower molecular weights (32, 39). Similarly, *C. albicans* low-molecular-weight components which may be identical to those supporting cross-reactivities between MAbs AF1, C6, and 5B2, on the basis of their relative molecular masses, have already been described by other groups (7, 28, 31). However, it is difficult to establish identity or relatedness between these low-molecular-weight components, since they could correspond to different native molecules or precursors or subunits of higher-molecular-weight proteins released by the extraction methods.

The most important information in this work resulted from the use of two different approaches for the analysis of saccharidic structures reacting with MAbs. The first one, which involved oligomannosides converted in NGL, represented a first step in the determination of epitopes. The second one, which involved a whole-cell extract, allowed the mapping of the corresponding oligomannosides on cell wall and cytoplasmic mannoproteins. Unexpected differences in the reactivities of MAbs against NGL and mannoproteins which correlated with each other were observed. Under these conditions, reactivity with oligomannosides H, composed essentially of β(1-2)-Man, was associated with the recognition of a family of antigens of 14 to 18 kDa, whereas reactivity with oligomannosides A, composed mainly of α-Man residues, was associated with high-molecular-weight mannoproteins. The absence of reactivity of MAbs EB-CA1, EB-CA2, and H5 against the 14- to 18-kDa family of antigens, as well as the absence of reactivity of MAb C6 and the low reactivity of MAb AF1 with high-molecular-weight mannoproteins, is more difficult to explain with the presumptive presence of β(1-2)-linked mannopyranosyl residues on such molecules (38). The influence of the growth conditions

used for each antigen preparation on these observations must be ruled out, since the same recognition patterns have been obtained with these MAbs on Western blots performed from the cell batch used for PPM extractions (data not shown). However, the reactivity of MAb 5B2, which concerned both families of oligomannosides and both families of antigens, confirmed the results obtained with more discriminating MAbs. The purification and the entire characterization of the *C. albicans* 14- to 18-kDa antigens will give further answers to this question, as will the application of the same approach to other MAbs reacting with *C. albicans* oligomannosides.

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