INFECTION AND IMMUNITY, Nov. 1990, p. 3537–3544
0019-9567/90/113537-08$02.00/0

Vol. 58, No. 11

Immunoreactivity of Neoglycolipids Constructed from Oligomannosidic Residues of the *Candida albicans* Cell Wall

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Received 24 April 1990/Accepted 3 August 1990

To establish a model to study the immunoreactivity of oligosaccharidic structures from the *Candida albicans* cell wall, we attempted to construct neoglycolipids with these residues by using oligomannosides released after mild acid hydrolysis of the phosphopeptidomannans isolated from yeast forms. From a mixture of manno-oligosaccharides ranging from mannobiose to mannononaose, the structure of a quantitatively major component (mannotriose) was determined to be Man (β1-2) Man (β1-2) Manα by ^{1}H nuclear magnetic resonance analysis. After coupling of the pool of oligosaccharides to a lipid (4-hexadecylaniline), the synthesized molecules were injected into mice and rats. Antibody responses were detected on enzyme-linked immunosorbent assay plates coated with either phosphopeptidomannans or neoglycolipids. The hybrid molecules exhibited both immunogenicity and antigenicity. The kinetics of antibody responses as well as immunofluorescence patterns observed on whole *C. albicans* cells strongly mimicked results from the immunization of animals with natural antigens. Construction of neoglycolipids could therefore provide an interesting approach to the study of specific oligosaccharides of *C. albicans* and their recognition by the host immune system.

The immunochemistry of the *Candida albicans* cell wall has been studied mostly for fundamental and diagnostic purposes (23). Early studies have demonstrated that major antigenic determinants reside in the phosphopeptidomannans (PPM), water-soluble matrix components of the cell wall (9, 30, 34). Oligomannosidic residues obtained by partial depolymerisation of the polysaccharidic moiety of the PPM have been identified as species- or serotype-specific epitopes, as evidenced by the use of polyclonal rabbit immunoglobulin G (IgG) rendered specific by adsorption (34). Recent refinements in methods for fractionation and structure determination have led to the description of a model for the polysaccharidic moiety of the PPM molecule linked to the peptidic moiety via chitobiosylaspartamido bonds. This highly branched structure, far more complex than previously expected (37), is composed of a main chain of mannopyranosyl residues linked by α(1-6) bonds, to which are attached oligosaccharidic side chains in which mannopyranosyl residues are linked through α(1-2), α(1-3), β(1-2), β(1-6), and phosphodiester bonds (8, 11, 15, 25, 31).

Generation of monoclonal antibodies (MAbs) against *C. albicans* cell walls has led to the production of mouse or rat immunoglobulins reacting with cell surface epitopes, some of which were shown to have similarities to the immunodominant epitopes identified by polyclonal monospecific antisera (4, 17, 23, 33). However, cytological analyses performed with MAbs have demonstrated an unexpectedly extreme antigenic variability of epitope expression among genera, species, strains, and even cells in a given culture (3, 7, 16, 20). Therefore, the apparent complexity of global chemical analyses of oligomannosides could reflect structural variabilities from one cell to another in a given batch and could not be related to the expression of all structures in a single cell. However, conventional immunochemical methods are poorly adapted to an extensive screening of all potential oligomannosidic epitopes. Large quantities of oligosaccharides are needed for inhibition reactions in the liquid phase (1, 8, 19, 27), and interpretation of results is impaired by the multivalent character of IgM (36), the predominant immunoglobulin isotype specific for oligosaccharidic epitopes in mice and rats used to produce MAbs (3, 4, 10, 17, 33).

In view of these problems, we attempted to develop a convenient model to determine the molecular basis of oligomannosidic recognition by experimental animal and human immune systems. Oligosaccharides were coupled to a lipid to form neoglycolipids (NGL) by a previously described method to study the antigenicity of oligosaccharides derived from human or bacterial glycoproteins (18, 28, 29, 32, 36). This work concerns oligosaccharides released from *C. albicans* PPM by mild acid hydrolysis (1, 24–26, 33). The immunogenicity of the hybrid molecules was studied by monitoring the humoral response to *C. albicans* antigens following injection of mice and rats with the NGL. The antigenicity of the pool as well as the antigenicity of a quantitatively major component (a mannotriose) was studied by using these NGL with mouse and rat antisera in a micro-enzyme-linked immunosorbent assay (micro-ELISA).

MATERIALS AND METHODS

Strain and growth conditions. We used a cloned strain of *C. albicans* serotype A designated VW.32. This strain was selected because of its high reactivity against sera from patients suffering from candidiasis (21). The reactivity of this strain against polyclonal antibodies (22) and MAbs from

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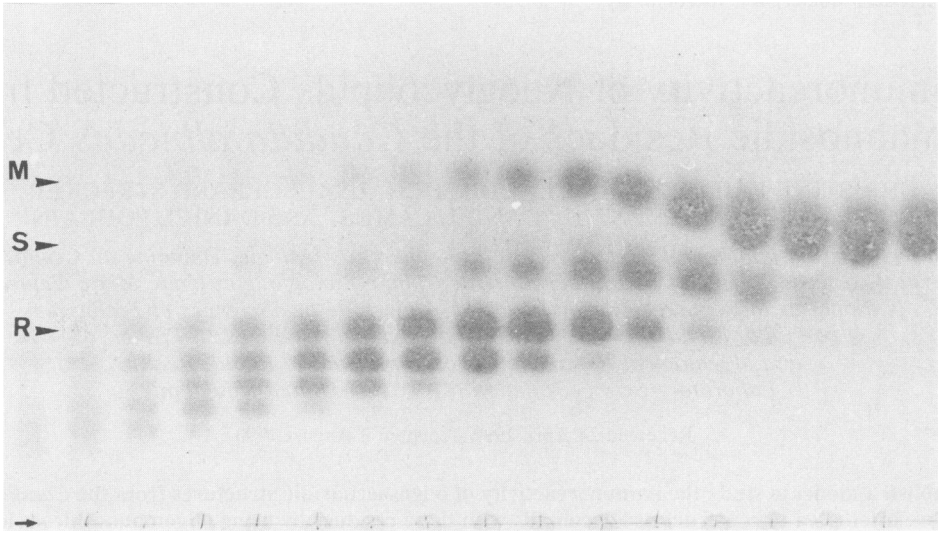

FIG. 1. Thin-layer chromatogram of oligosaccharides released by mild acid hydrolysis. Five microliters of each fraction collected after desorption from the Bio-Gel P4 column (1.6 by 40 cm) was spotted. Standards: M, mannose; S, saccharose; R, raffinose. Arrow, Point of application.

immunized (6) or infected (10) animals has been previously characterized.

Cells were grown in a 50-liter bioreactor (Setric, Toulouse, France) as follows. A 4-liter inoculum (5×10^7 cells per ml) was added to 25 liters of fresh medium containing yeast extract (2 g/liter), glucose (20 g/liter), $MgSO_4$ (0.25 g/liter), and NH_4OH (2 ml/liter). The pH was adjusted to 4.5 with H_3PO_4. Cells were grown under batch conditions (28°C; pH 4.5 [NH_4OH]; agitation, 150 rpm; aeration, 150 to 450 liters/h; defoaming agent, Rhodorsil [Prolabo, Paris, France], 2% in water). When all the glucose had been taken up (Clinitest; Miles S.A., Paris, France), an incremental nutrient feed was applied with 5 liters of medium containing yeast extract (15 g/liter), glucose (150 g/liter), and $MgSO_4$ (1.5 g/liter), the pH being adjusted to 4.5 with H_3PO_4. Two hours after the end of the feeding, yeasts were harvested by centrifugation on a CEPA LE centrifuge (Bioblock, Strasbourg, France) and washed twice with water to remove the yeast extract.

Production of PPM. PPM were extracted by the method of Kocourek and Ballou (14), except that a single extraction in citrate buffer was done.

Acid hydrolysis of PPM. PPM (1 g) were dissolved in 50 ml of 0.1 M HCl, and the solution was heated at 100°C for 30 min. After being cooled under cold tap water, the solution was neutralized with NaOH and lyophilized. The oligosaccharides and the modified PPM were separated on a Bio-Gel P4 column (40 by 1.6 cm; Biorad, Watford, England). The different fractions were pooled and lyophilized.

Preparations of NGL. The procedure for the preparation of NGL was adapted from that described by Tang et al. (32). 4-Hexadecylaniline (200 mg; Aldrich Chemical Co., Milwaukee, Wis.) was added to 500 μl of a solution containing 5 mg of sodium cyanoborohydride, 3.5 ml of methanol, and 400 μl of acetic acid. The solution was heated (80°C), and 20 mg of oligosaccharides solubilized in 30 μl of water was added. After 90 min at 80°C, the solution was cooled, mixed with chloroform-water (1:1, vol/vol), and centrifuged. The lower phase was discarded, and the upper phase was dried, dissolved in methanol (2 ml), and centrifuged. The pellet, containing the uncoupled oligosaccharides, was discarded.

Analytical methods. (i) TLC. Thin-layer chromatography (TLC) was done as follows. Oligosaccharides were separated on Silicagel 60-precoated plates (Kieselgel 60; Merck, Darmstadt, Federal Republic of Germany) with *n*-butanol–acetic acid–water (20:10:10, vol/vol/vol) as the solvent. Sugars were stained with a solution of orcinol (2 g/liter) in 20% sulfuric acid and heated for 10 min at 105°C.

NGL were separated on Silicagel 60 F_{254}-precoated plates (DC Alufolien 60 F_{254} Kieselgel; Merck) with chloroform–methanol–water (60:35:4, vol/vol/vol) as the solvent. Lipids and NGL were detected in short-wave UV light (254 nm), and sugars were stained with the orcinol-sulfuric acid reagent.

(ii) Gel filtration chromatography. Oligosaccharides were applied to a Bio-Gel P4 column (1.6 by 40 cm or 1.8 by 140 cm). The column was eluted with distilled water at a flow rate of 8 ml/h at room temperature. Fractions of 1.2 ml were collected and analysed by TLC and by the phenol-sulfuric acid method.

(iii) 400-MHz ^{1}H NMR analysis. The solution of oligosaccharides was exchanged several times in D_2O with intermediate lyophilization. Nuclear magnetic resonance (NMR) spectral analysis of the compounds in D_2O (99.95%; Commissariat à l'Energie Atomique, Gif sur Yvette, France) was carried out on a Bruker AM-400 WB spectrometer operating in the Fourier transform mode at a probe temperature of 300 K. Chemical shifts are given relative to sodium 2,2-dimethyl 2-silapentane 5-sulfonate (indirectly to acetone in D_2O: δ = 2.225 ppm). Resolution enhancement of the ^{1}H NMR spectra was achieved by Lorentzian-to-Gaussian transformation. Interpretation of the ^{1}H NMR spectrum was done as described by Cohen and Ballou (5) and Kobayashi et al. (11).

Immunological methods. (i) Animal immunizations. Three-month-old BALB/c mice (Iffa Credo, L'arbresle, France) and 9-month-old Lou rats (CNRS, Gif sur Yvette, France) were injected with NGL (100 μg of carbohydrate [determined by the phenol-sulfuric acid method] per injection in 400 μl of physiological saline for rats and 200 μl for mice). The first injection was made by a subcutaneous route after mixing with Freund complete adjuvant (300 μl for rats and 150 μl for mice). Animals then received intraperitoneal

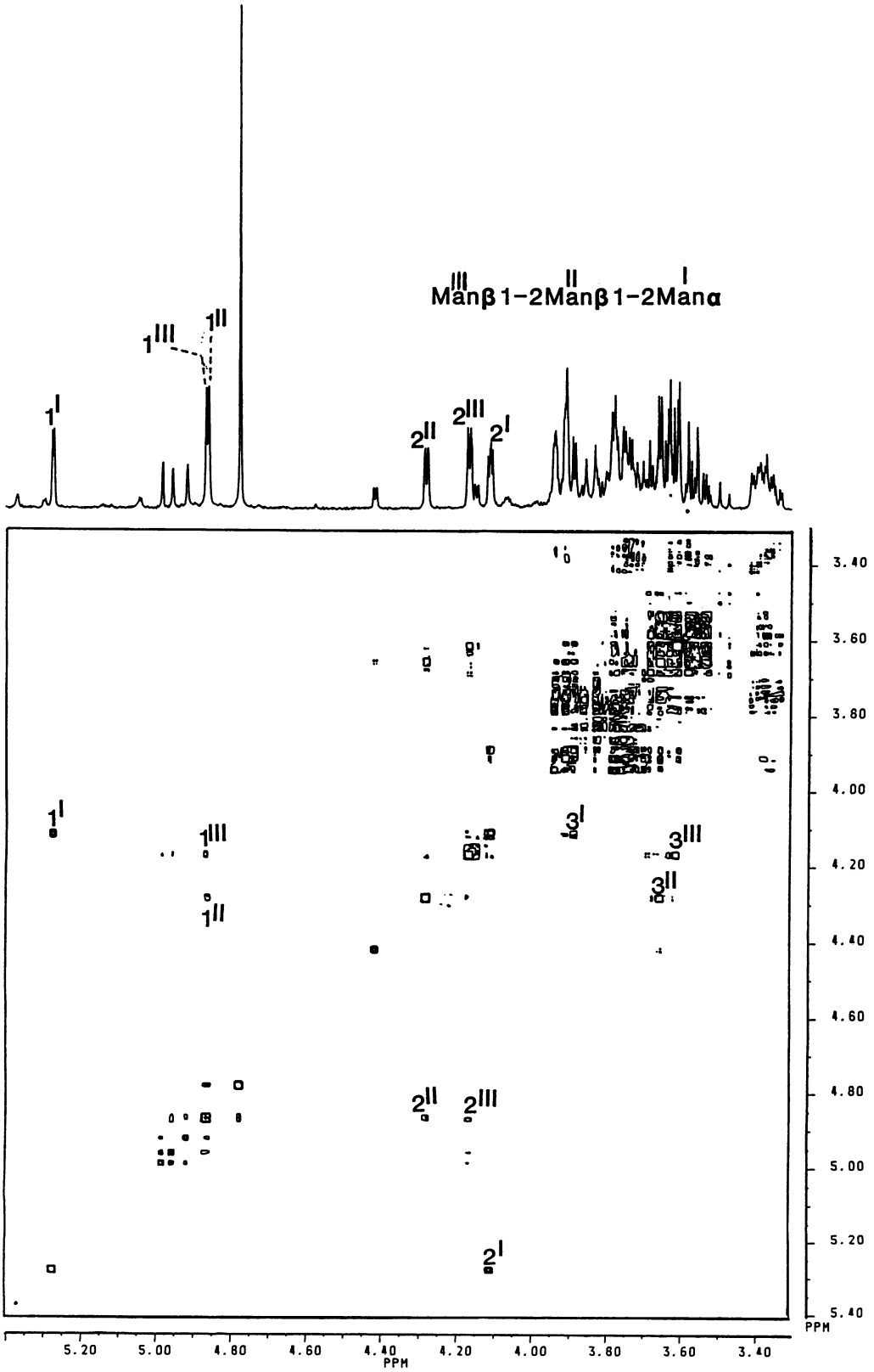

FIG. 2. 400-MHz partial ^1H NMR double-relayed correlation spectroscopy spectrum of mannotriose released by mild acid hydrolysis. Arabic and roman numerals refer to the H-1, H-2, and H-3 atoms of the Man I, Man II, and Man III residues.

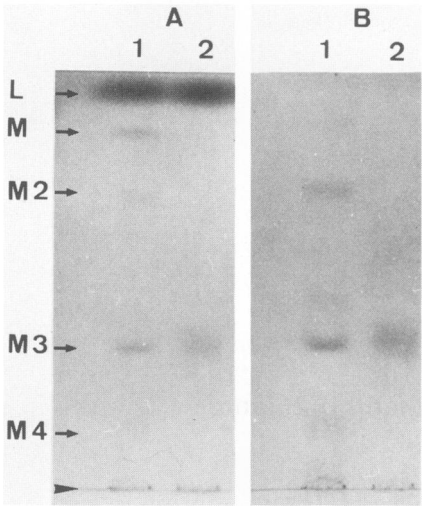

FIG. 3. Thin-layer chromatograms of NGL. Lanes: 1, NGL constructed from the pool of oligosaccharides released by mild acid hydrolysis; 2, NGL constructed with mannotriose isolated by gel filtration chromatography. (A) NGL revealed by fluorescence. (B) Same chromatogram as in panel A but stained with orcinol reagent. L, Uncoupled lipid; M, M2, M3, and M4, NGL constructed, respectively, from mannose, mannobiose, mannotriose, and mannotetraose. Arrows, Points of application.

injections of NGL plus Freund incomplete adjuvant at days 7, 14, 21, and 35. Animals were bled before immunization and systematically 6 days after each injection for the mice and after the third and following injections for the rats to monitor the kinetics of antibody responses.

(ii) Micro-ELISA. PPM or NGL were coated onto 96-well polystyrene plates (Nunc, Polylabo, Strasbourg, France). Each well received 200 μl of a solution of antigen (5 μg of carbohydrate per ml [determined by the phenol-sulfuric acid method]) in sodium carbonate buffer (60 mM, pH 9.6). Plates were incubated for 1 h at 37°C and overnight at 4°C and washed five times in TNT (50 mM Tris, 150 mM NaCl, 0.05% Tween 20; pH adjusted to 7.5 with HCl); the last wash was allowed to stand for 10 min. The plates were stored at −20°C.

Serum (100 μl) diluted 1/100 in TNT was incubated in each well for 1 h at 37°C. After four washes in TNT, 100 μl of peroxidase-labeled antibody (immunopurified rabbit or goat anti-rat or anti-mouse IgM or IgG or IgG plus IgM conjugates [Zymed, Paris, France]) diluted in TNT (1/500) was incubated in each well for 1 h at 37°C. After five washes in TNT, each well received 100 μl of enzymatic substrate containing 1 mg of 1,2-phenylenediamine (Merck-Clevenot S.A., Nogent sur Marne, France) per ml in TNT and 0.5 μl of hydrogen peroxide per ml. After 30 min of incubation at room temperature in the dark, the reaction was stopped by the addition of 50 μl of 4 M H_2SO_4. Plates were scanned at 490 nm on a micro-ELISA reader.

(iii) IFA. Immunofluorescence assays (IFA) were done with germ tubes grown for 4 h in RPMI 1640 (Boehringer, Mannheim, Federal Republic of Germany) at 37°C or with mycelium grown for 24 h under the same conditions. Cells were washed twice in phosphate-buffered saline (PBS) and suspended to a concentration of 5×10^6 cells per ml. Cell suspensions (20 μl) were placed in each well of IFA slides and allowed to dry at room temperature. Dried microscope slides were individually packed and stored at −20°C for up to 3 months.

Animal sera (20 μl) diluted in PBS were incubated in each well for 1 h at 37°C. After four washes in PBS, 20 μl of fluorescein isothiocyanate-conjugated rabbit anti-rat or anti-mouse immunoglobulins (Zymed) diluted 1/40 in PBS containing Evans blue at a concentration of 2×10^{-5} was incubated in each well. After five washes in PBS, reactions were read on a Leitz Orthomat fluorescence microscope.

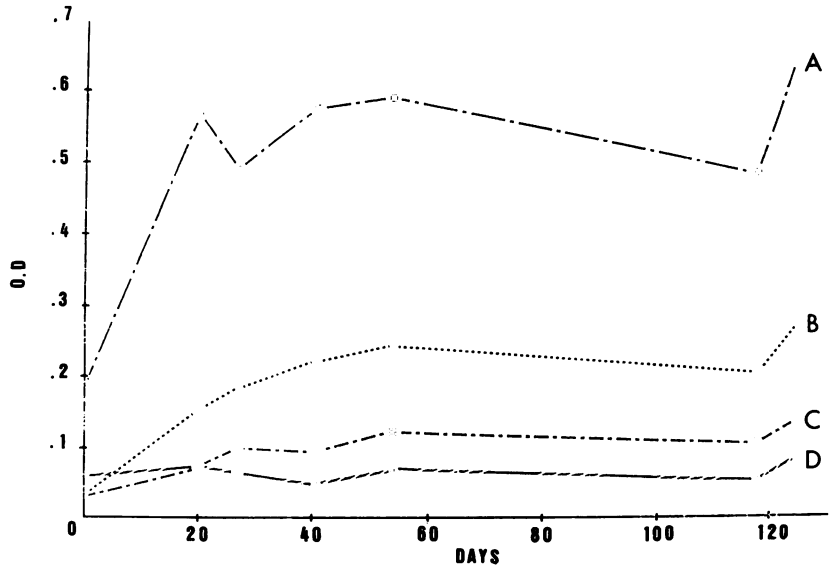

FIG. 4. Kinetic analysis of the humoral IgG and IgM responses of a mouse immunized with NGL constructed from oligomannosides released by mild acid hydrolysis from *C. albicans* PPM. The results shown are from the micro-ELISA with sera reacted with PPM or homologous neoantigens. O.D., Optical density at 490 nm. (A) IgM response against PPM. (B) IgM response against NGL. (C) IgG response against PPM. (D) IgG response against NGL.

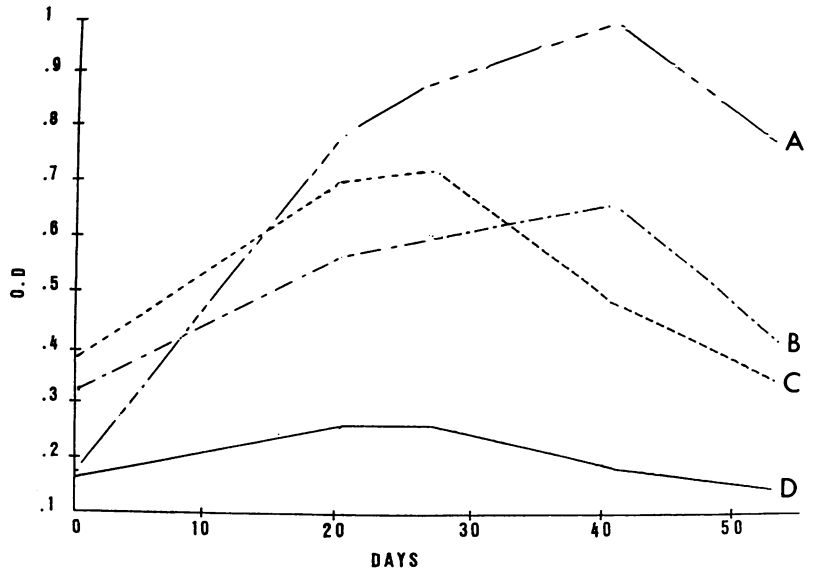

FIG. 5. Kinetic analysis of the humoral IgG and IgM responses of a rat immunized with NGL constructed from oligomannosides released by mild acid hydrolysis from *C. albicans* PPM. The results shown are from the micro-ELISA with sera reacted with PPM or homologous neoantigens. O.D., optical density at 490 nm. (A) IgG response against PPM. (B) IgG response against NGL. (C) IgM response against PPM. (D) IgM response against NGL.

RESULTS

Cultivation of yeast-form cells and PPM extraction. Growth of cells for 48 h in a large-capacity bioreactor under fed-batch conditions with a semisynthetic medium resulted in a yield of 250 g (dry weight) of yeast-form cells. From this, approximately 10 g of PPM extract was collected (4%).

Analysis of oligosaccharides released by mild acid hydrolysis. TLC of oligosaccharides released by mild acid hydrolysis revealed more than nine different fractions (Fig. 1). The quantitatively major components ranged from mannose to mannotetraose. Two isomers could be identified for mannobiose. Gel filtration chromatography (column measuring 1.8 by 140 cm) resulted in the separation of these major components. The structure of mannotriose, for which large quantities of oligosaccharide were obtained, was established.

^{1}H NMR analysis of mannotriose. The ^{1}H NMR spectrum of mannotriose indicated the presence of three major anomeric protons at δ = 5.276 ppm (αMan), 4.868 ppm (βMan), and 4.861 (βMan) (Fig. 2). A correlation spectroscopy experiment showed the H-1 atom of αMan I to be correlated with an H-2 atom at 4.112 ppm, proving its C-2 substitution. For the two βMan residues, namely, βMan II and βMan III, the correlation spectroscopy experiment furnished correlations among the coupled values 4.868 and 4.166 ppm and 4.861 and 4.281 ppm. Consequently, the βMan II residue (δ H-2 = 4.281 ppm) is C-2 substituted, while the βMan III residue, located at the nonreducing terminal position, possesses a normal H-2 chemical shift at 4.166 ppm. Therefore, the structure of mannotriose was established to be Man (β1-2) Man (β1-2) Manα.

Analysis of NGL. The mixture of NGL and the NGL constructed with the isolated mannotriose could be analyzed by TLC. These compounds could be visualized either in UV light according to their lipid moiety (Fig. 3A) or after orcinol staining according to their carbohydrate moiety (Fig. 3B). The isolated mannotriose yielded a single spot (Fig. 3A and B, lanes 2). For the mixture of NGL, the solvent system used allowed the separation of oligosaccharides composed of up to four residues (mannotetraose).

Humoral responses against PPM of animals immunized with NGL as analyzed by micro-ELISA. When successive samples of sera from immunized mice and rats were tested against *C. albicans* PPM, an antibody response gradually appeared, demonstrating that the NGL were immunogenic. Figures 4 and 5 show representative examples of results obtained with mice and rats, respectively. Isotype determinations showed that the detectable response in mice was essentially of the IgM type. Rats produced both types of immunoglobulins, the stronger signal being observed for IgG, the peak of synthesis of which followed the IgM one.

Humoral responses against homologous neoantigen of animals immunized with NGL as analyzed by micro-ELISA. When the antibody responses were evaluated against the homologous NGL, both animal species had curves of specific IgG and IgM evolution similar to those observed with PPM (Fig. 4 and 5), demonstrating the antigenicity of the NGL coated on micro-ELISA plates. When purified mannotriose coupled to 4-hexadecylaniline was used as an antigen, testing of animal sera before and after immunization with the pool of NGL resulted in the appearance of antibodies reacting with this peculiar neoantigen (data not shown).

Humoral responses against *C. albicans* growth forms of animals immunized with NGL as analyzed by IFA. When sequential samples of sera from immunized animals were reacted with blastoconidia, young germ tubes, or mycelial forms of *C. albicans*, reactivity gradually appeared. However, all reactions, whatever the origin of the antiserum, revealed the extreme heterogeneity of surface antigen expression. Examples are shown in Fig. 6. Variability in blastoconidial surface antigens was obvious (Fig. 6A). The same striking differences in reactivity were observed for three germ tubes originating from the same mother blastoconidium (Fig. 6B) or three successive segments of the same mycelium (Fig. 6C). Whatever the reactivity of the mycelial

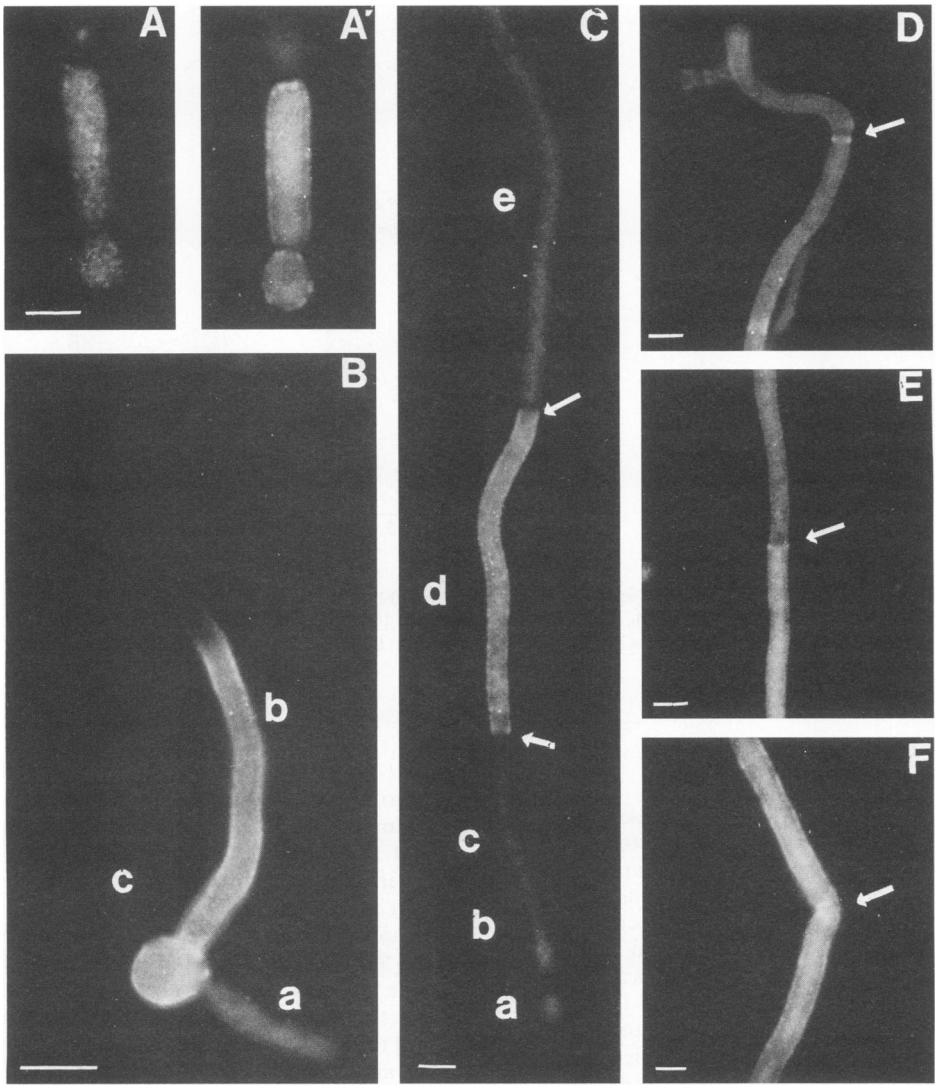

FIG. 6. Representative examples of humoral responses of mice and rats immunized with NGL (day 40 of immunization), analyzed by IFA, against *C. albicans* whole cells from different growth forms. A, C, E, and F, Rat serum diluted 1/400; B and D, mouse serum diluted 1/40. (A) Variability in cell surface reactivity among two blastoconidia and an intermediate pseudohypha. (A') Same microscopic field as in panel A, except that the change in the focalization reveals the existence of antigenic patches at the cell surface. (B) Variability in surface reactivity of three germ tubes (a, b, and c) derived from the same blastoconidium. (C) Variability in cell surface reactivity among successive segments of the same mycelium (b, c, d, and e) originating from a blastoconidium (a). Arrows, Septa. (D, E, and F) Septa are indicated by arrows. Bars, 10 μm.

elements, labeling in the region of the septa was a constant feature (Fig. 6C, D, E, and F).

DISCUSSION

Oligomannosidic structures obtained by depolymerization of the PPM extracted from the *C. albicans* cell wall have been described as immunodominant epitopes representing the molecular basis of serological specificity (1, 17, 23, 31, 33, 34) and the presumptive basis of antigenic variability (2, 3, 7, 16). The chemical literature provides more and more complex schemes for the organization of these structures, leading to the existence of dozens of potential antigenic determinants (8, 11, 15, 24–26, 31). We limited our investigation to a mixture of acid-labile phosphate-bound oligosaccharides which were released following mild hydrolysis and which have been the subject of recent extensive studies (24–26, 33). We observed, by TLC analysis of the different fractions obtained after gel filtration chromatography, more than nine components. Previous mild acid hydrolysis of *C. albicans* PPM from strains NIH B-792 and NIH A-207 yielded, respectively, seven and six oligomannosides when cells were grown as yeast forms, with a molar ratio different from that observed in our experiments (24, 26). These differences could be related to hydrolytic conditions, which were more drastic in our experiments (100 mM HCl instead of 10 mM HCl) and which could have led to the breakage of oligosaccharidic linkages. This would explain why mannobiose was seen to be composed of different isomers. However, these discrepancies could also be related to differences in culture conditions, PPM extraction procedure, or individual strain variability. However, global analysis of linkage types existing within this pool of oligosaccharides has revealed the

exclusive presence of β linkages among these oligosaccharides. These results are consistent with the original findings by Shibata et al. (25), who demonstrated that manno-oligosaccharides which were degraded in three *C. albicans* strains upon treatment with 10 mM HCl consisted solely of β-1,2-linked D-mannopyranose units. Similarly, results from the ^{1}H NMR analysis of a quantitatively major component of the pool, mannotriose, led to the determination of a structure which has already been described (11, 24). β(1-2) linkages through D-mannopyranosyl residues are specific neither for *C. albicans* among the species of the genus *Candida* (33) nor for the genus *Candida* itself (12, 13). However, antibodies against these structures could be of great value for antigen detection tests applied to the serodiagnosis of candidiasis, since β(1-2) linkages, in contrast to α linkages, which are found in a great variety of human glycoproteins, have never been described in mammals.

In the present study, NGL were constructed to promote both immunogenicity and antigenicity of these oligomannosides. Such an approach has been previously developed to produce antibodies against oligosaccharides from bacterial cell walls (28) or oligosaccharides isolated from different, human glycoproteins (29, 32, 36). However, the present use of 4-hexadecylaniline offers several advantages, since the binding of oligosaccharides to lipids is realized in a short time with a one-step procedure; the alkylaniline derivatives may also be easily detected by UV light. With each mole of oligosaccharide being quantitatively converted to the corresponding NGL, TLC therefore provides an easy analysis of the different compounds which could be further used for immunological studies.

To determine how sequences of sugar units from the oligosaccharidic pool could correspond to potential epitopes, we studied the ability of the corresponding NGL to trigger the immune system; we used them to immunize mice and rats and analyzed the humoral response to natural *C. albicans* antigens. Kinetic analysis of animal humoral responses against PPM detected by micro-ELISA and by IFA (serial dilutions) demonstrated that a humoral response occurred. With both antibody detection methods, the antibody response was much easier to detect in rats than in mice. Similar differences between humoral responses in both animal species have been observed following immunization with either *C. albicans* whole cells or *C. albicans* cell wall extracts (35). Isotype determination showed that the specific immunoglobulins synthesized by mice following immunization with NGL consisted almost exclusively of IgM. In contrast, rats exhibited classical profiles of antibody evolution curves, with a peak of IgM synthesis preceding the peak of IgG synthesis. Therefore, the immune response against PPM induced when NGL were used as neoantigens had characteristics similar to those of immunization with natural antigens. As determined by IFA, whatever the animal species, immunization resulted in the appearance of heterogeneity in the reactivity against the *C. albicans* cell surface. This heterogeneity was shared by yeast and mycelial forms. It seems, therefore, that oligosaccharidic epitopes bound via phosphodiester bridges to the PPM molecule are not homogeneously distributed on the *C. albicans* cell surface. Once again, these results strongly correlate with the results of an immunocytological analysis performed with antibodies generated against *C. albicans* natural antigens. This correlation is particularly evident when one considers recent studies conducted with MAbs supposedly reactive with *C. albicans* oligosaccharides. In every instance, great heterogeneity in epitope surface expression was reported (3, 16, 20).

The use of NGL as antigens coated on micro-ELISA plates to study the humoral responses of immunized animals resulted in curves of specific IgM and IgG similar to those observed with PPM. These results demonstrated both the antigenicity of NGL under the technical conditions used and their ability to mimic the natural antigens from which they were prepared. When the NGL constructed from purified mannotriose was used as an antigen, immunization resulted in the appearance of antibodies reacting with this particular component of the NGL pool. These results demonstrate the ability of NGL constructs to determine the immunoreactivity of a given sequence of sugars.

These preliminary results concerning the immunoreactivity of NGL constructed from a group of oligomannosidic residues of PPM molecules from *C. albicans* demonstrated that at least some of the oligosaccharides in the pool were bound to the lipid molecule in a spatial relationship leading to an interaction with host molecules similar to the interaction which occurred when the original PPM were used. Production of a bank of *C. albicans* NGL could therefore provide a system for elucidating the relationships between oligosaccharide structure and the host. This approach could also be valuable in defining the molecular basis of *C. albicans* antigenic variability.

ACKNOWLEDGMENTS

This work was supported by the Institut National de la Santé et de la Recherche Médicale, the Centre National de la Recherche Scientifique (Unité Mixte 111), and Bayer Pharma, Sens, France. C.F. is the recipient of a Bayer postdoctoral fellowship. We also thank the Conseil Régional du Nord-Pas-de-Calais, the Centre National de la Recherche Scientifique, the Ministère de la Recherche et de la Technologie, the Ministère de l'Education Nationale, the Fondation pour la Recherche Médicale, and the Université des Sciences et Techniques de Lille, Flandres-Artois, for their contributions in the acquisition of the 400-MHz NMR apparatus.

We thank G. Lepage for valuable technical assistance, J. M. Wieruszeski for recording the NMR spectra, and the Institut National de la Recherche Agronomique (Station de Technologie Alimentaire) for providing facilities for the use of bioreactors.

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