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Isolation and Characterization of Cell Surface Mutants of *Candida albicans*

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Mutant strains of *Candida albicans* were obtained by selecting for cells that escaped agglutination by a polyclonal antiserum raised against standard *C. albicans* serotype A isolate B311. Mutants were obtained from strains B311 and B792 and from four strains isolated from patients with acquired immunodeficiency syndrome. All 15 tested mutants retained characteristic sugar assimilation patterns. All but one of the mutants retained the ability to form germ tubes and chlamydospores. Two mutants from an acquired immunodeficiency syndrome-derived isolate were deficient in binding complement ligands iC3b and C3d, whereas another mutant was deficient in binding ligand iC3b but not C3d. The hyphae of these three mutants lacked antigens when examined by Western immunoblotting with monoclonal antibody Ca-A, which detects several glycoproteins, including C3d-binding proteins. One of the complement-binding-deficient mutants was tested for its ability to colonize the gastrointestinal tract of rabbits but did not differ from the wild-type parent in site or degree of colonization. The proton magnetic resonance spectra of bulk mannan carbohydrate extracted from tested mutants showed the loss of a signal characteristic of the mannosyl α-PO_4 linkage; each mutant also had a distinct pattern of other changes.

In the opportunistic pathogen *Candida albicans*, cell wall structures are critical determinants of pathogenesis, as they are in other organisms (21, 27, 28, 30). However, fungal cell wall architecture is not well defined, and the difficulty of genetics in this asexual diploid organism has hindered molecular and genetic analyses (39, 40). The cell wall is composed of glucan, chitin, and mannoprotein, of which the mannoprotein is the major antigen (7, 26). The spatial relationships between these three polymers are not known, and their roles in the physiology of the organism have not been tested.

Each of the two serotypes of *C. albicans*, A and B, has several antigenic determinants (24, 26, 35). These determinants reside in the cell wall mannoprotein, which contains mannosyl residues in α and β linkages as well as mannosyl phosphoryl diesters (15, 22, 26, 36). Mutants with altered mannoprotein have been occasionally isolated in screens for drug resistance or colony morphology mutants (4, 5, 13, 17, 29, 31, 32). However, the structural differences in the carbohydrate were clearly associated with a variety of other defects, and neither the genetic basis nor the physiological consequences of the mannan structural changes are known.

We therefore instituted a search for mannoprotein mutants to assess the physiological and structural effects. By using the procedures developed in the laboratory of Ballou and co-workers for the isolation of cell surface mutants of *Saccharomyces cerevisiae* (25), we isolated such mutants of *C. albicans*. In our experiments we relied upon serial enrichments to isolate mutants that were not agglutinated by polyclonal antiserum raised against a standard *C. albicans* strain. The enrichment provided a variety of mutants with potential use for characterization of the cell surface.

MATERIALS AND METHODS

Strains. *C. albicans* A3, A9, and A11 were isolated from the oral cavities of three patients with acquired immunodeficiency syndrome (AIDS). Strains A4 and A10 were esophageal isolates from two other AIDS patients. These strains were graciously provided by Philip Smith of the National Institute of Dental Research, Bethesda, Md. *C. albicans* reference strains B311 (serotype A) and B792 (serotype B) were described previously (12, 35).

Media and culture methods. The ability of isolates to utilize various carbohydrates for growth was assessed with the API-20C kit (Analytab Products, Plainview, N.Y.). Cultures were tested for their ability to form chlamydospores at room temperature in cornmeal agar medium (Difco Laboratories, Detroit, Mich.) supplemented with 1% (vol/vol) Tween 80. Cultures to be tested for their ability to form germ tubes were grown at room temperature in YEPD broth (10) to the stationary phase, and a sample was diluted 1:100 in prewarmed (37°C) germ tube medium (Quality Biological, Gaithersburg, Md.).

Antiserum. To prepare antibody suitable for selection of mutants, a New Zealand White rabbit was immunized over the course of 1 year by repeated intravenous injections of 10^8 Formalin-killed blastoconidia of *C. albicans* B311 (serotype A). Immunoglobulin G (IgG) was purified from the serum of this rabbit by sequential caprylic acid precipitation (34) and batch ion exchange (33) to remove non-IgG proteins. The resulting IgG preparation, designated JM-1, agglutinated both serotypes of *C. albicans*. The titer was 2,048 against

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strain A9 (serotype B) by standard methodology (described below) for agglutination. Agglutination of strain A9 was abolished by preincubation (for 20 min at 30°C) of 30 μg of B311 mannoprotein with JM-1 antibody (5 μl in 4 ml of phosphate-buffered saline [0.01 M sodium phosphate–0.14 M NaCl, pH 7.4]).

Selection of mutants. Cultures (1 ml) were grown overnight at room temperature in YEPD on a Rollordrum (New Brunswick Scientific Co., Inc., Edison, N.J.). The cultures were centrifuged, and the cells were washed with 4 ml of phosphate-buffered saline. To select nonagglutinating mutants, cells (approximately 10^8 from each clone) were suspended in phosphate-buffered saline (4 ml), and antibody preparation JM-1 (5 μl) was added. These suspensions, contained in polystyrene tubes (12 by 75 mm), were mixed by repeated inversion (six inversions per min) at 37°C for 30 to 60 min. Agglutinated cells, present as macroscopic clumps, were separated from nonagglutinated cells by gentle centrifugation in a Damon/IEC (Needham Heights, Mass.) model PR-J centrifuge, which was allowed to reach 1,000 rpm (150 × *g*) and then immediately turned off and allowed to coast to a halt. The pellet was discarded, and the cells in the supernatant were collected, washed, suspended in 1 ml of YEPD, and grown overnight. Cells from this culture (10^8) were then treated with antibody in the second round of enrichment.

In a typical first round of selection of a clone, 1×10^8 cells were subjected to agglutination and 8×10^6 cells were retained. Serial rounds of growth and selection for nonagglutinating mutants were continued with each culture until that culture appeared to be enriched for nonagglutinating cells.

Iodination of antiserum and binding to cells. Polyclonal IgG preparation JM-1 was labeled with ^{125}I as described by Markwell (20). Nonimmune IgG and IgM preparations were labeled in parallel. To study binding of iodinated IgG to the wild type and mutants, cells (1.2×10^8) were suspended in 2.5 ml of phosphate-buffered saline containing radiolabeled immunoglobulin and 1 mg of bovine serum albumin for 30 min and then pelleted by centrifugation. The amount of radioactivity that sedimented with cells was determined with a Minimax gamma spectrometer model 5550 (United Technology-Packard, Downers Grove, Ill.).

Assays for iC3b and C3d receptors. The strain A9 and variants V1 through V10 derived from it were grown to induce conversion to their hyphal forms as previously described (6, 18). To assay for iC3b and C3d receptor activities, hyphal particles (50 μl) were added to antibody-sensitized sheep erythrocytes (EA) (50 μl) coated with either the iC3b (EAiC3b) or C3d (EAC3d) ligand, generated as previously described (6, 18). Receptor activity was measured by determining the percentage of *C. albicans* particles of each strain exhibiting a rosetting of EAiC3b or EAC3d (6, 18). One hundred hyphal particles were counted for each determination.

Preparation of hyphal extracts. Four strains (A9, A9V1, A9V2, A9V4), chosen because of their rosetting pattern, were transferred to fresh Sabouraud dextrose agar slants and incubated at room temperature (25°C) for 48 h. Cells were recovered from the slants, washed, transferred to 4 ml of Lee medium at a concentration of approximately 10^5 to 10^7 cells per ml, and grown to the stationary phase for 20 h at 24°C (16). Yeast cells were harvested, washed and suspended in 200 ml of fresh prewarmed (37°C) Lee medium. The mycelial form was induced by cultivation of each cell suspension on a New Brunswick Gyrotory shaker at 150 rpm at 37°C for 18 h. The cells in each culture were then harvested by centrifugation, washed twice with 0.86% NaCl, resuspended in sterile distilled water containing 1 mM phenylmethylsulfonyl fluoride (Sigma Chemical Co., St. Louis, Mo.), and disrupted by shaking with 0.45-mm glass beads in a Braun MSK cell homogenizer (B. Braun, San Mateo, Calif.) cooled with liquid CO_2. The homogenate was centrifuged at 10,000 × *g* for 20 min at 4°C and at 40,000 × *g* for 1 h and membrane filtered, and the clear supernatant was lyophilized. The protein content of each preparation was determined by the method of Lowry et al. (19).

Polyacrylamide gel electrophoresis and immunoblotting. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and immunoblotting were performed as described previously (18) with the following modifications: purified IgG of monoclonal antibody Ca-A (1:1,000 dilution) was used instead of whole ascites fluid as a primary antibody, and protein A-alkaline phosphatase conjugate (Sigma) was used as the secondary reagent. The polyacrylamide concentration was 10%, and 5 to 10 μg of protein was loaded into each well.

Colonization of rabbit gastrointestinal tract. *C. albicans* A9 and A9-V4 were grown overnight in Sabouraud dextrose broth at 37°C, washed, and suspended in sterile saline solution at a density of 5×10^7 CFU/ml. A 1.5-ml inoculum was administered by oral gavage daily for 7 days.

Pathogen-free female New Zealand White rabbits (Hazelton, Rockville, Md.) weighing 2 to 3 kg were surgically fitted with silastic central venous catheters as previously described (37). During the course of inoculation, the rabbits received dexamethasone (1 mg/kg per day) to suppress cellular immunity. Gentamicin and vancomycin were administered in drinking water throughout the experiment to suppress endogenous bacterial flora. Blood and stool samples were cultured daily to monitor the course of the infection.

Rabbits were euthanized 3 weeks after the initial inoculation. The lumen and walls of eight sections of the gastrointestinal tracts were quantitatively cultured. The lumenal surface of 5.0-cm sections of esophagus, duodenum, proximal jejunum, midjejunum, ileum, appendix, and rectum were gently lavaged with 10 ml of Hanks balanced salt solution per tissue. Each washing was quantitatively cultured by plating 0.1 ml of undiluted and serially 10-fold diluted samples on Sabouraud dextrose agar. The washed wall of each segment was quantitatively cultured after sectioning and 10 min of homogenization in a sterile polyethylene bag (38). Blood, lung, kidney, liver, and spleen were also cultured. Rabbits not inoculated with *C. albicans* showed no infection with *C. albicans*.

Preparation of mannoprotein for structural studies. *C. albicans* wild-type and mutant cultures were each grown in YEPD medium (500 ml for each strain) at 37°C for 40 h with shaking. Microscopic examination revealed no hyphae or pseudohyphae. The cultures were centrifuged, and the cells were washed twice with water. Mannoprotein was extracted by autoclaving and purified by two rounds of precipitation with Fehling solution at 0°C (14, 23). For nuclear magnetic resonance (NMR) analysis, 5 to 10 mg of mannoprotein was digested with 50 μg of *Streptomyces griseus* protease (Sigma) at 48°C overnight in 0.01 M sodium phosphate buffer (pH 7.4). The digested samples were lyophilized and redissolved twice from 99.8% D_2O (Aldrich Chemical Co., Milwaukee, Wis.) and once from 99.96% D_2O before final redissolution in 99.96% D_2O and analysis. The spectra were recorded on a JEOL FX 400 spectrometer at 25°C. Peak

assignments were made by comparison to standard spectra of oligosaccharide fragments (3, 8, 11). Composition analyses of B311 and B311V1 mannan carbohydrate were carried out by hydrolysis and high-pressure liquid chromatography at the Complex Carbohydrates Research Center, Athens, Ga. Other mannans were hydrolyzed in 1 M trifluoroacetic acid at 120°C for 1 h, and monosaccharides were separated by thin-layer chromatography on silica gel in isopropanol-ethyl acetate-water (7:1:2). The chromatograms were visualized with alkaline silver reagent (1).

RESULTS

Selection of mutants. Antigenic variants were selected on the supposition that they would not agglutinate, or would agglutinate weakly, when exposed to antibody raised against *C. albicans* B311. Clones of each strain were selected by successive enrichment for nonagglutinable cells. Antigenic mutants were obtained from most tested strains of *C. albicans* under the standard protocol, including B311 (serotype A), B792 (serotype B), and AIDS-derived strain A9 (serotype B); 9 to 13 rounds of antibody selection were sufficient to derive clonal variants from each strain. Enrichments of UV-mutagenized cultures of strain A9 yielded mutants after as few as seven rounds of enrichment. Enrichments were also attempted with four other AIDS-derived strains: A3, A4, A10, and A11. Strains A3, A10, and A11 yielded clonal variants within 13 rounds of enrichment. Therefore, six of seven tested strains yielded mutants by the above protocol.

Fifteen mutants were chosen for further study: 10 derived in separate enrichments from independent clones of A9 and 1 each from B311, B792, A3, A10, and A11. Since only one clone was selected from each enrichment, the mutants were assumed to be independent. This assumption was supported by the variety of phenotypes observed in the mutants of strain A9 (see below). All parent strains and all mutants (except one) were capable of germ tube and chlamydospore formation under standard conditions and had growth rates similar to those of the parent strains. The exceptional mutant (A9V7) differed from the others in three respects: it did not form germ tubes under conditions that supported germ tube formation at high frequency in the parent strain A9, it did not form chlamydospores, and it grew with a doubling time longer than that of the parent strain (82 versus 66 min in YEPD at 37°C). The pattern of sugar utilization by A9V7 was identical to that of parent A9, i.e., typical of *C. albicans*. The mutations appeared to be stable, since all mutants retained the nonagglutination phenotype in subcultures carried throughout the study (up to 1 year).

Decreased binding of iodinated immune IgG. It was expected that parent strain A9 would bind detectable amounts of IgG in the preparation (JM-1) used to select mutants. This was tested by radiolabeling preparation JM-1 with ^{125}I and exposing cells to it under standard conditions. Ten clones of wild-type strain A9, from which mutants A9V1 through A9V10 had been independently selected, bound similar amounts of radiolabel (Fig. 1). In contrast, the mutants isolated from these wild-type clones showed variable binding (Fig. 1), but all bound less radiolabel than the parents ($P \leq 0.002$ by a paired Student t test; $n = 10$). Binding of nonimmune IgG (and IgM) was negligible in these experiments: less than 6% of the added label was bound.

Mannan carbohydrate structural studies. Because mannan carbohydrate is the major antigen of *C. albicans*, we sought evidence that an alteration in carbohydrate structure was associated with the agglutination-negative phenotype. Purified mannoproteins contained mannose (>98%) and glucose (<2%) as the only detectable saccharides after hydrolysis. The proton NMR spectra of the regions characteristic of the anomeric protons (Fig. 2) showed major peaks at 4.95, 5.08, and 5.325 ppm. These are characteristic of mannosyl β1→2, mannosyl α1→3, and mannosyl α1→2 protons, respectively (3, 8, 11). We chose to study strain B311 and agglutination-negative mutant B311V1. B311 is a standard (serotype A) strain that has been studied repeatedly (12, 14), and mutant B311V1 differed from the parent strain in peak intensity at δ = 4.89 (mannosyl α1→6), 4.95 (mannosyl β1→2), 5.17 (assignment not known), 5.28 (mannosyl α1→2), 5.41 (perhaps mannosyl β1→PO_4), and 5.58 (mannosyl α1→PO_4) ppm. It appeared that B311V1 had no α-phosphoryl mannoside but had substantially more of the 5.41-ppm species than did the parent strain.

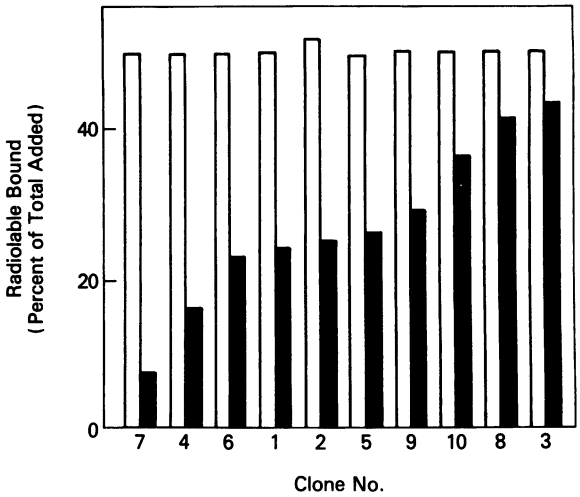

FIG. 1. Binding of ^{125}I-labeled polyclonal antibody by wild-type clones and mutant clones. The amount of radiolabeled material bound to 1.2×10^8 cells is expressed as a percentage of the total label added. For the JM-1 preparation (specific activity, 10^6 cpm/μg), 10^5 cpm were added. The amount of label bound by each mutant (■) is shown next to the amount of label bound by the wild-type strain A9 clone from which this mutant was selected (□).

The spectra of strain A9 and its variants showed similar changes. All tested mutants appeared to lack the mannosyl α1→PO_4 (5.58 ppm) and mannosyl α1→6 (4.89 ppm) resonances and had a variety of other changes as well. Mutant A9V1 had a particularly simple spectrum and appeared to lack at least six peaks that were present in the wild-type mannan. It therefore appeared that the selection had resulted in diverse and complex changes in structure of the mannan carbohydrate.

Binding of complement fragments. Wild-type *C. albicans* A9 hyphae bound complement ligands iC3b and C3d when exposed to ligand-coated erythrocytes. That result was expected in view of previous demonstrations that other *C. albicans* strains bound these fragments (6). The binding is specific for iC3b and C3d, since neither uncoated erythrocytes nor erythrocytes coated with complement components before C3b conversion are rosetted (6). Among the 10 mutants isolated from strain A9, 3 were defective in binding either iC3b, C3d, or both (Table 1). In four rosetting experiments considerable variation occurred, particularly in experiments with mutant A9V1. Mutant A9V2 exhibited reduced binding to iC3b but was similar to the wild type in the extent of C3d binding. On the other hand, mutant A9V4

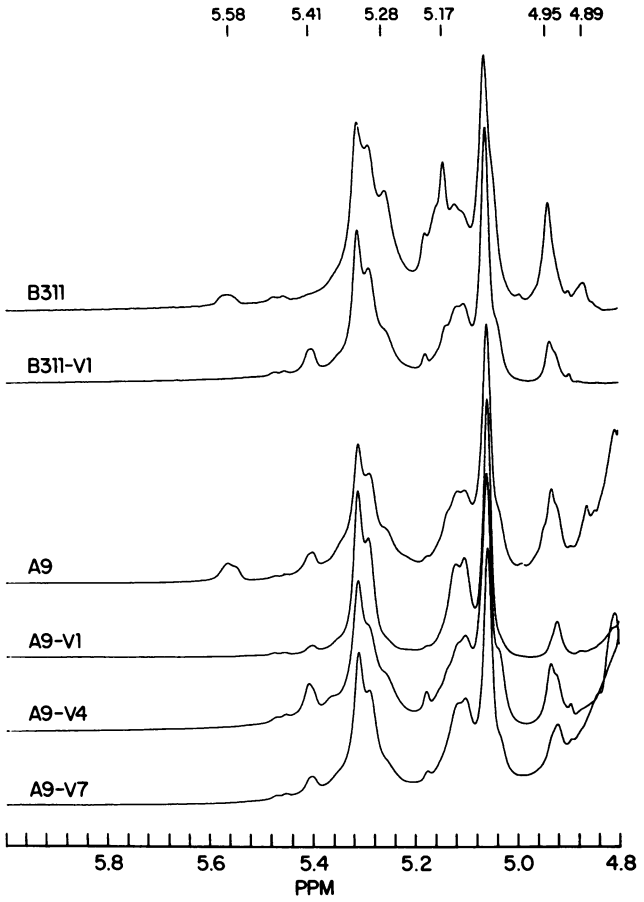

FIG. 2. Proton magnetic resonance spectra of the anomeric proton regions on wild-type and mutant mannan preparations.

lacked C3d-binding activity; its ability to bind iC3b was variable albeit reduced in comparison with that of the wild type.

Monoclonal antibodies can be used to identify *C. albicans* complement-binding proteins (6, 9). The molecular basis of loss of C3d binding was determined with monoclonal antibody Ca-A, which recognizes C3d-binding proteins of 60 to 62 and 70 kilodaltons in hyphal extracts of *C. albicans* 4918 (6, 18). This antibody detected multiple antigens in immunoblots of extracts of strains A9, A9V1, A9V2, and A9V4 (Fig. 3). Mutants A9V1 and A9V2, which bind iC3b poorly, lacked an antigen of 55 to 60 kilodaltons. Mutant A9V4, which binds iC3b but not C3d, had wild-type levels of this antigen but had reduced intensity in the region of 31 and 95 kilodaltons.

TABLE 1. Binding of iC3b and C3d to wild-type strain A9 and derived mutants[a]

Strain	% of *Candida* particles bound to antibody-sensitized sheep erythrocytes coated with:	
	iC3b	C3d
A9 (wild type)	89, 89, 87, 86	64, 62, 62, 56
A9V1 (mutant)	12, 90, 15, 45	54, 28, 34, 7
A9V2 (mutant)	12, 12, 18, 15	63, 30, 62, 60
A9V4 (mutant)	52, 18, 32, 12	0, 0, 0, 4

[a] Six other mutants (V3, V5, V6, V8, V9, V10) were indistinguishable from the wild type in preliminary rosetting experiments and were not studied further. Mutant A9V7 displayed greatly reduced binding of both ligands but was not included because it did not form hyphae.

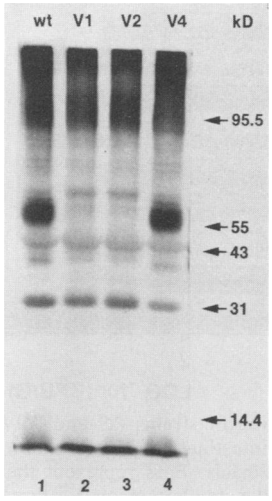

FIG. 3. Western blot analysis of *C. albicans* A9 (wild type) and mutants A9V1, A9V2, and A9V4 with monoclonal antibody Ca-A. Whole-cell extracts of each strain (identified at the top of the gel) were electrophoresed, transferred to nitrocellulose, and blotted with Ca-A.

Colonization of gastrointestinal tract. Because strain A9V4 was severely affected in complement binding and had an altered mannan carbohydrate structure, we tested its ability to colonize the gastrointestinal tract in a rabbit model. The colonization abilities of A9V4 and the parent strain A9 were equivalent, with the highest concentration of *C. albicans* in the esophageal wall, where levels of about 10^6 CFU/g were found (Fig. 4). This level was significantly above the colonization levels in all other tissues ($P \leq 0.05$) by Student's t test. Results were similar when the nonadhering cells were cultured (lavages), with no significant difference between colonization of A9 and A9V4. There was no evidence of disseminated candidiasis in blood, lung, liver, spleen, or kidney.

DISCUSSION

We isolated a number of *C. albicans* cell surface mutants. Since agglutination of *C. albicans* blastoconidia by the polyclonal antiserum was much reduced by preincubation of purified *C. albicans* mannan with the antiserum, cells escaping agglutination would lack mannan determinants recognized by the agglutinating antibody. The enrichment was successful, since in all tested cases the mutants had altered mannan, and all but one strain had normal growth, nutritional, and developmental characteristics. These mutants contrast with previously reported mannan carbohydrate mutants, which had pleiotropic defects (4, 5, 13, 17, 29, 31, 32). By enriching from independent clones, we isolated strains with a variety of cell surface alterations, indicated by changes in antibody-binding capacity, altered structure of the mannan carbohydrate, and altered binding of components of the complement system. Although we cannot determine how many genes are represented by the reported mutants, the independence of the mutants and the variety of phenotypes argue for mutations in several loci.

The mutations resulted in changes in the structure of the mannoprotein. Ten mutants isolated from strain A9 bound less radiolabeled immune IgG than did parallel samples of strain A9. These experiments also indicated that the mutants varied among themselves in this respect, suggesting that

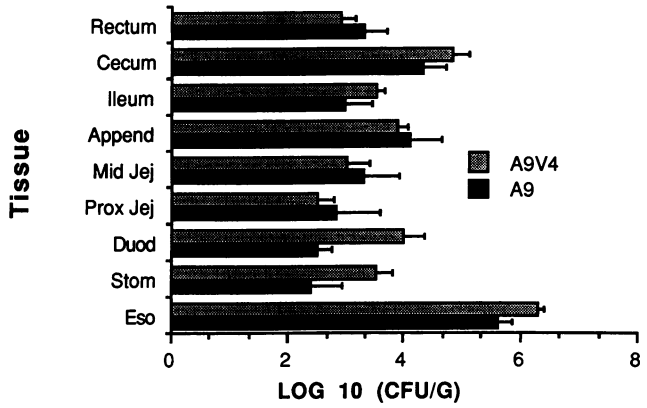

FIG. 4. Colonization of strains A9 and A9V4 in different segments of the gastrointestinal tract in a rabbit model of chronic gastrointestinal candidiasis. Bars represent the mean values, and error bars represent the standard errors for three rabbits per group. Abbreviations: Append, appendix; Mid Jej, middle portion of the jejunum; Prox Jej, proximal portion of the jejunum; Duod, duodenum; Stom, stomach; Eso, esophagus.

they differed in terms of the kind or extent of alteration in cell wall structure or in cell surface exposure of the antigenic determinants. This heterogeneity was confirmed by analyses of the carbohydrate. Four tested mutants showed diverse changes in the NMR pattern of the carbohydrate, implying differences in prevalence of several mannosyl linkages. The common loss of signals characteristic of mannosyl α-phosphoryl and mannosyl α1→6 linkages implies that the antibody selected against determinants that were linked to the mannoprotein through such bonds. The antigenicity of similar mannosyl α-phosphoryl bonds is well documented in *S. cerevisiae* (2).

The antigenic and structural differences in the mutants can be related to functional alterations of the cell surface, since several of the mutants exhibited defective binding for the complement ligands, iC3b and/or C3d. Mutants either lacked one activity (A9V2 had reduced iC3b binding) or both activities (A9V4 had reduced iC3b binding and negligible C3d binding) or had variable activity (A9V1).

The diversity of the mutations was also demonstrated in Western immunoblots with monoclonal antibody Ca-A, which we have used previously to identify C3d-binding proteins from *C. albicans*. The three tested mutants showed two different patterns. Strains A9V1 and A9V2, which have reduced binding of iC3b, lacked a 60-kilodalton antigen. Strain A9V4, which showed variable binding of iC3b and no binding of C3d, had reduced levels of high-molecular-weight antigens. Preliminary (unpublished) evidence points to a polysaccharide epitope for Ca-A.

As a further functional test, mutant strain A9V4 was tested in a rabbit model of chronic gastrointestinal candidiasis. This strain had very low antibody binding, no binding of C3d, reduced iC3b binding, and the characteristic loss of the proton resonances corresponding to the mannosyl α-phosphoryl and mannosyl α1→6 bonds. Nevertheless, it colonized all sites in the rabbit gastrointestinal tract with an efficiency similar to that of the parent strain. This result implies that the immunodominant determinants in *C. albicans* are not directly involved in colonization ability in this model.

In summary, we successfully selected antigenic mutants of *C. albicans* with a range of phenotypes and chemotypes. The phenotypes of the mutants have allowed us to begin to correlate mannoprotein structure with several aspects of the mammal-*C. albicans* interaction.

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