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Specific Antibody Response to Oligomannosidic Epitopes in Crohn's Disease

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Elevated antibody levels against the yeast *Saccharomyces cerevisiae* have been reported in sera from patients with Crohn's disease and not with ulcerative colitis. The aim of the study was to identify the nature of the epitopes supporting this antibody response. Whole cells from different *S. cerevisiae* strains were selected in immunofluorescence assay for their ability to differentiate the antibody responses of patients with Crohn's disease and ulcerative colitis. Their cell wall phosphopeptidomannans were then tested as antigen in enzyme-linked immunosorbent assay (ELISA) against sera from 42 patients with Crohn's disease, 20 patients with ulcerative colitis, and 34 healthy controls. Graded chemical degradations were performed on the most reactive strain phosphopeptidomannan. The discriminating epitope was determined through gas-liquid chromatography-mass spectrometry. The greatest discrimination among patients with Crohn's disease, ulcerative colitis, and controls was obtained with Su1, a *S. cerevisiae* strain used in brewing of beer. ELISA directed against phosphopeptidomannan of this strain was 64% sensitive and 77% specific for discriminating Crohn's disease versus ulcerative colitis and 71% sensitive and 89% specific for Crohn's disease versus controls. Periodate oxidation and selective degradation demonstrated that the most important polysaccharide epitope was shared by both the acid-stable and the alkali-labile domains of the phosphopeptidomannan. The determination of oligomannose sequences of *S. cerevisiae* Su1 phosphopeptidomannans suggested that a mannotetraose, Man(1→3)Man(1→2)Man(1→2)Man, supported the serological response seen in Crohn's disease. Further identification of the immunogen eliciting this antibody response as a marker of the disease may help to understand its etiology.

Systemic antibodies to various dietary and bacterial antigens in patients with Crohn's disease (CD) and ulcerative colitis (UC) have been described (16). Using enzyme-linked immunosorbent assay (ELISA) which employs whole killed yeast cells as antigens, elevated anti-*Saccharomyces cerevisiae* immunoglobulin A (IgA) and IgG levels were reported in the sera of patients with CD and not in those from patients with UC (21). The IgA response had an important discriminating value between CD and UC (2, 11). A clinical study suggested that inclusion or exclusion of baker's yeast from the diet might influence the activity of CD (1). Finally, increased IgA anti-*S. cerevisiae* in healthy relatives of concordant pairs of twins with CD suggested that these antibodies could be a subclinical marker of genetic susceptibility in CD (19).

The nature of *S. cerevisiae* antigens supporting the specific antibody response in CD is still unknown. Comparable IgG antibody levels have been detected in CD regardless of the origin of the *S. cerevisiae* strains used (bakery, brewery), suggesting that these antigens may be common in food and drink (25). Other studies, including adsorption of patients' sera with *S. cerevisiae* strains, revealed a great antigenic heterogeneity (26). A 200-kDa *S. cerevisiae* glycoprotein whose reactivity was abolished by periodate oxydation was identified in Western blotting (immunoblotting), suggesting that the discriminating epitopes comprised polysaccharides (4). A commercial preparation of *S. cerevisiae* cell wall mannan (phosphopeptidomannan [PPM]) had the same ability as *S. cerevisiae* whole cells in detecting these antibody responses (19).

The yeast cell wall PPM is a water-soluble component that may be extracted by autoclaving. It comprises a complex of mannose residues linked to a protein backbone either by O-glycosidic or N-glycosidic linkages (Fig. 1) (28). The PPM has long been recognized as a major antigen in yeast cells. In *S. cerevisiae*, detailed immunochemical studies have identified the nature of the oligomannosides (chemically released sequences of mannose residues, characterized by the length of the mannosyl chain and the type of linkage) that correspond to the epitopes of antibodies raised in animals (10). However, although *S. cerevisiae* may pose a potential problem in patients with compromised immune function (5), the antibody response in humans against this commonly considered innocuous yeast PPM remains ignored, as compared with what is known about responses to PPM from *Candida albicans*, an important opportunistic yeast pathogen (37). Studies of the *C. albicans* PPM oligomannosidic repertoire (7, 13) reveal that oligomannosidic epitopes may be shared by a great number of different unrelated yeast molecules (34, 41, 42). The corresponding epitopes also occur over a wide variety of organisms, explaining immunological cross-reactivities among yeast species, yeast genera (10), filamentous fungi (14), viruses (27), bacteria (20, 30), and human glycoproteins (22). Having this in mind, the understanding of anti-*S. cerevisiae* antibodies in CD requires as a first step the structural identification of the oligomannosides which act as specific epitopes.

We thus embarked on a study which included the following

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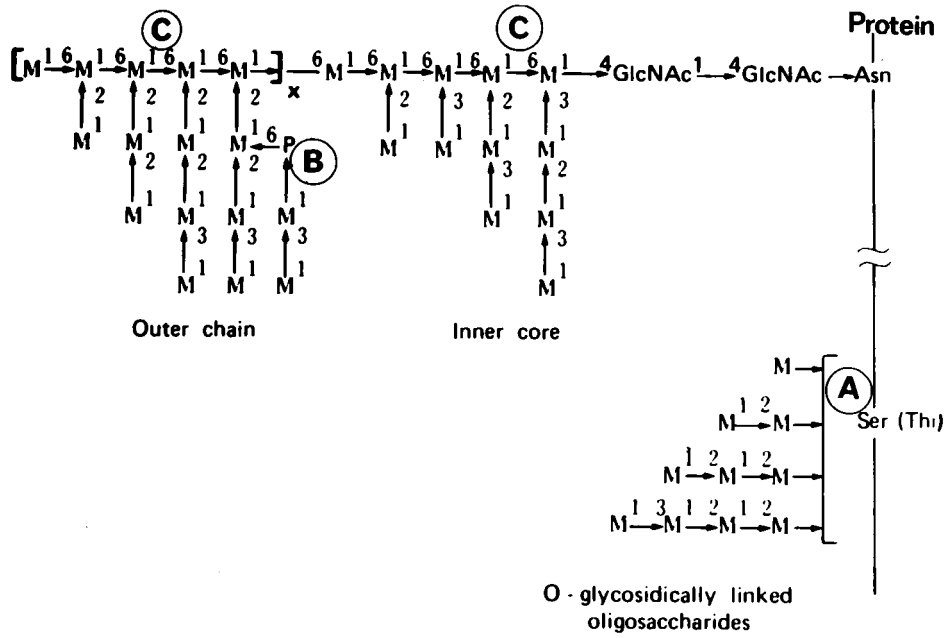

FIG. 1. Generalized structure of *S. cerevisiae* cell wall phosphopeptidomannan according to Nakajima and Ballou (28) and principle of chemical hydrolysis methods. Mannopyranosyl residues (M) organized in short chains of unramified oligomannosides are linked to the protein backbone by O-glycosidic linkages to serine (Ser) or threonine (Thr). The N-glycosidic part is linked to asparagine (Asn) of the protein and corresponds to a macromolecular edifice sustained by a linear backbone of α-1,6 linkages to which are attached side chains composed of α-1,2- and α-1,3-linked mannopyranosyl residues; short additional chains are branched to these side chains by phosphodiester bridges. O-linked oligomannosides are released from the phosphopeptidomannan by β elimination (A), a mild alkali hydrolysis method, and are named alkali-labile oligomannosides. Oligomannosides linked to side chains of the N-glycosidic part of the phosphopeptidomannan are released by mild acid hydrolysis (B), which breaks phosphodiester bridges, and are named acid-labile oligomannosides. Side chains of the N-glycosidic part of the PPM are released by acetolysis (C), a more drastic acid hydrolysis method which breaks α-1,6 linkages.

steps. First, several *S. cerevisiae* strains from different origins were screened for their ability to differentiate the serological response of patients with CD and UC. Thereafter, we extracted PPM (the major water-soluble surface antigens) from the most reactive strains. These PPM were then employed in an ELISA against sera from patients and controls. We then used periodate oxidation and selective degradations in additional ELISAs to assess in which domain of the PPM the serological reactivity resides. Finally, we characterized the chemical structure of those oligomannosides among which the important epitopes were.

MATERIALS AND METHODS

Patient sera. The first series of experiments (immunofluorescence tests) were conducted on sera from 30 patients with inflammatory bowel disease (IBD). Diagnosis was based on usual clinical, morphologic, and histologic data. There were 20 patients with CD (seven male, 13 female; mean age, 37 years). Crohn's disease involved both the small and large bowel in 15 patients, only the colon in 4 patients, and only the small bowel in 1 patient. The disease was active (ACD) in 10 patients (i.e., Crohn's disease activity index [CDAI] > 150) (3) and quiescent (QCD) in 10 patients (CDAI < 150). Ten patients with UC were studied (seven male, three female; mean age, 37 years). There were two cases of proctitis, five of left-sided colitis, and three of universal colitis. Nine had active UC, and the remaining one was quiescent.

The second series of experiments (ELISAs) was conducted with sera from 42 patients with CD (20 male, 22 female; mean age, 28 years). Twenty-one patients had ACD (10 male, 11 female; mean age, 28 years). Crohn's disease involved both the small and large bowel in 15 patients, only the colon in 4 patients, and the small bowel in 2 patients. At the time of the study, nine of them were receiving oral steroids, nine were taking 5-aminosalicylic acid, and the remaining three had no treatment. Twenty-one patients had QCD (10 male, 11 female; mean age, 28 years). Crohn's disease involved both the small and large bowel in eight patients, only the colon in eight patients, and only the small bowel in five patients. Ten of those patients were receiving steroids or immunosuppressive drugs, 10 were taking sulphasalazine or 5-aminosalicylic acid, and the last one had no treatment. Twenty patients with UC were studied for comparison (12 male, 8 female; mean age, 37 years). There were five with proctitis, nine with left-sided colitis, and six with universal colitis. Eleven had active UC, and nine were quiescent. The control group comprised 34 healthy blood donors.

Yeast strains and culture conditions. Six *S. cerevisiae* strains and one *C. albicans* strain were used in this study. The *S. cerevisiae* strains consisted of (i) four brewing strains: Su1 (for formerly *S. uvarum* 1), Su2, BM 151, and BM 156; (ii) one strain, frequently isolated as a contaminant in breweries and designated Sd (for formerly *S. diastaticus*) (these strains were kindly furnished by different brewing companies); and (iii) one strain used in a winery, CBS 1315. The *C. albicans* strain (serotype A), designated VW 32, is used for routine serological diagnosis of candidosis (35). Strains were maintained on Sabouraud dextrose agar (SDA).

Immunofluorescence assays (IFA). Antigens consisted of yeast cells grown on SDA for 24 h either at 37°C (*C. albicans*) or at 25°C (*S. cerevisiae* strains). 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.

IFA was performed as previously described (35). Serial dilutions of sera (20 μl) 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-human immunoglobulins (G, A, M) (Sanofi Diagnostics Pasteur, Marnes-la-Coquette, France) diluted 1/100 in PBS containing Evans blue (Candida Spot, Bio-Mérieux, France) at a dilution of 1/50,000 were incubated in each well. After five washes in PBS, reactions were read on a Leitz Orthomat fluorescence microscope. Results were expressed as the titer that corresponded to the reciprocal of the greatest serum dilution giving a strong fluorescence of all yeast cells.

ELISA. Antigens consisted of PPM extracted from yeast cells from cultures in bioreactors. *C. albicans* cells were grown and harvested as previously described (7). *S. cerevisiae* cells from strains Su1, Su2, and CBS 1315 were grown in a slightly different medium composed of 2 g of yeast extract per liter, 20 g of glucose per liter, 0.25 g of $MgSO_4$ per liter, and 2 ml of 28% NH_4OH per liter, with the pH adjusted to 4.5 with H_3PO_4. PPM were extracted by the method of Kocourek and Ballou (18) except that a single extraction in citrate buffer was done (7).

ELISA was performed as previously described (7). Plates were coated with 100 μl of PPM at a concentration of 1 μg/ml of sugars (determined by the phenol-sulfuric acid method) in sodium carbonate buffer (60 mM, pH 9.6) for 1 h at 37°C and overnight at 4°C in moist chambers and washed four times in TNT (50 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20, pH 7.5). Patient sera were diluted 1/8,000 and 1/1,000 in TNT respectively for *C. albicans* and *S. cerevisiae* antigens. Alkaline phosphatase-labeled goat anti-human immunoglobulin (G, A, M) (H

and L chains), (Zymed, Biosoft, Paris) was diluted 1/3,000 in TNT. A color reaction was rendered by using substrate biotrol EIA 405 (Laboratoires Biotrol, Paris, France) for alkaline phosphatase. Reading of the plates was done at 405 nm on an Immunotech (Luminy, France) automatic reader. Standardization of tests was achieved by using pools of patient sera strongly reacting with yeast mannans. These consisted of sera from patients with candidiasis when the *C. albicans* antigen was used (sérum positif titré, Candida Spot, bio-Mérieux, France) and sera from patients with Crohn's disease when *S. cerevisiae* antigens were used. The standards were aliquoted and stored at −20°C. Any set of experiments included a standard reactivity curve corresponding to optical density (OD) evolution when standards were allowed to react at dilutions ranging from 1/32,000 to 1/500 up to saturation. Reactivity of individual sera was expressed through the use of the ELIOT program (Immunotech, Luminy, France) as a percentage of the highest reactivity observed with the standard arbitrarily defined as 100%. When the same sera were tested after an interval of several days, this mode of expressing results appeared to minimize variations in the OD values.

ELISA performed after Periodate oxidation. In order to define the nature of epitopes involved in serum reactivity, we performed a periodate oxidation which denatures the PPM polysaccharide components (45). Prior to ELISA, each well of the PPM-coated microtiter plates was treated with 100 μl of 0.1% $HIPO_4$ in 0.1 M acetate buffer, pH 5.5, for 1.5 h at room temperature. Controls consisted of wells treated with 0.1 M acetate buffer–HCl, pH 5.5. For this set of experiments, results were expressed as OD.

ELISA performed after acid and alkali treatment of the *S. cerevisiae* Su1 PPM. The PPM extracted from *S. cerevisiae* Su1 was subjected to two different chemical treatments as illustrated in Fig. 1. For mild acid hydrolysis (Fig. 1B) (7), 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 component remaining after this procedure, corresponding to the acid-stable fraction of the PPM (AcS PPM), was separated from the acid-labile oligomannosides released by two successive precipitations in 80% ethanol and lyophilized. For elimination of O-linked oligomannosides (Fig. 1A) (13), the PPM was dissolved in 100 mM NaOH and incubated for 24 h at 25°C. The component remaining after this procedure and corresponding to the alkali-stable fraction of the PPM (AlS PPM) was separated from the O-linked released oligomannosides and lyophilized as described above. Acid-stable and alkali-stable fractions of the *S. cerevisiae* Su1 PPM were coated on microtiter plates as previously described.

Chemical analysis of *S. cerevisiae* Su1, Su2, and CBS 1315 PPM. Total carbohydrate and protein compositions were determined respectively by the phenol sulfuric acid (6) and Markwell (23) methods. PPM was acetolyzed by the method of Hamada et al. (12). The 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 (100:100:1), and heated for 13 h at 40°C on a rotary shaker. The reaction was stopped by pouring the solution onto ice, and the mixture was neutralized with sodium bicarbonate. The released oligosaccharides 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. The released oligomannosides were separated on a BioGel P2 (Bio-Rad) column (1.0 by 120 cm). The column was eluted with distilled water at a flow rate of 4 ml/h at room temperature; 0.6 ml fractions were collected and analyzed for their sugar content by the phenol-sulfuric acid method. Composition of each fraction was checked by thin-layer chromatography. In order to determine the structure of *S. cerevisiae* Su1 mannobiose to mannotetraose, a gas-liquid chromatography-mass spectrometry study was performed after reduction, methylation, methanolysis, and acetylation of the oligomannosides by the method of Fournet et al. (8).

Statistical analysis. The significance of differences between antibody levels in patients and control groups was determined by Wilcoxon's sign rank. For ELISAs, receiver operating characteristic (ROC) curves, obtained by plotting sensitivity against specificity, were used to determine cutoff value giving the best compromise between sensitivity and specificity.

RESULTS

Immunofluorescence assay. Strong differences in levels of serum reactivity were observed among 6 different strains (Fig. 2). The lowest level of reactivity was observed for *S. cerevisiae* Sd; whereas the most reactive strain was *S. cerevisiae* BM 156. This overall reactivity was independent of the strain's ability to discriminate between ACD, QCD and UC. The best discrimination between CD and UC patients (either ACD or QCD) was obtained by using *S. cerevisiae* strain Su1 (Table 1).

ELISA responses against phosphopeptidomannans extracted from *S. cerevisiae* Su1, Su2, CBS 1315 and *C. albicans* VW32 strains. Sera from 96 different patients were reacted in an ELISA with PPM extracted from the most discriminating strain, i.e., *S. cerevisiae* Su1 (Fig. 3). Despite a pronounced heterogeneity in responses within CD patients, all of the highest titers were observed in CD patients, and there was low reactivity in controls and moderate reactivity in UC patients. No significant differences were observed for *C. albicans* reactivity levels among all groups. Anti-*S. cerevisiae* Su1, Su2, and CBS 1315 responses were significantly higher in CD than in UC patients and controls (Table 2), with no significant differences occurring between patients with active and quiescent disease (data not shown). No correlations between ELISA titers and clinical parameters were found. Cutoff values for the ELISA test giving the best compromise between sensitivity and specificity are shown in Table 2.

Shared immunoreactivity between *S. cerevisiae* Su1 and other PPM is shown in Fig. 4. Correlation was almost perfect

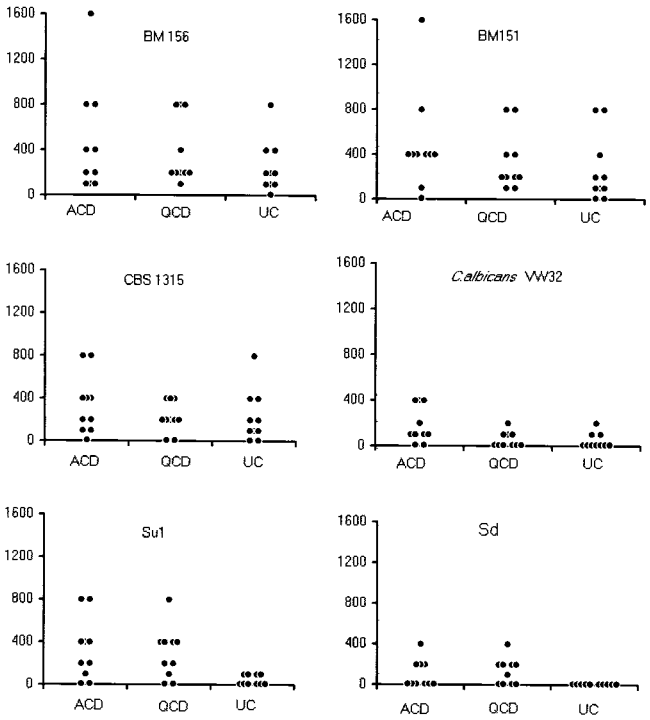

FIG. 2. Immunofluorescence assay titers observed on the different yeast strains reacting with the three categories of human sera. BM 156, BM 151, CBS 1315, Su1, Sd: *S. cerevisiae* strains. *C. albicans*: VW32 strain $n = 10$ each for ACD, QCD, and UC.

TABLE 1. Significance (*P*) of differences between antibody levels against various yeast strains in patients and control groups as determined by the nonparametric Wilcoxon rank order test

Antigen	*P*			
	UC ($n = 10$) vs:			QCD vs ACD
	All CD ($n = 20$)	QCD ($n = 10$)	ACD ($n = 10$)	
BM156	NS[a]	NS	NS	NS
BM151	NS	NS	NS	NS
CBS 1315	NS	NS	NS	NS
VW32	NS	0.024	NS	0.04
Su1	0.0022	0.0082	0.008	NS
Sd	0.0088	0.03	0.005	NS

[a] NS, not significant.

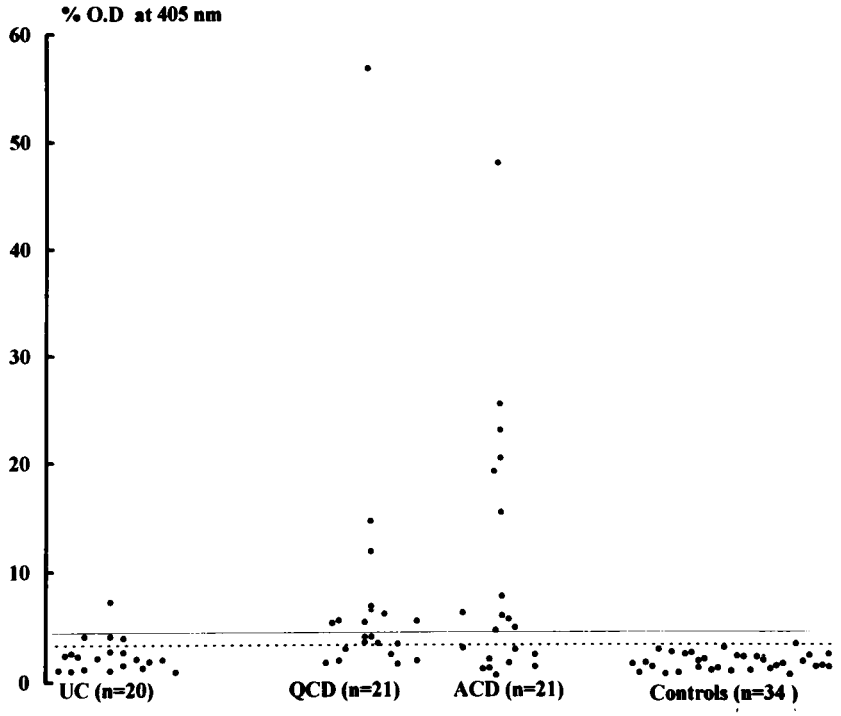

FIG. 3. Individual serum antibody responses against *S. cerevisiae* Su1 phosphopeptidomannan as determined by ELISA. Results are expressed as percentages of a standard to make allowance for day-to-day variability of the ELISA. For examples of corresponding OD values observed in one set of experiments, see Fig. 5. Dotted line indicates the cutoff value of 3.09%, which gave the best compromise between sensitivity and specificity for CD identification, as determined following the establishment of ROC curves (Table 2). As a matter of comparison, the continuous line has been drawn from three standard deviations above the mean of control responses (this corresponds to a sensitivity of 57.14% and a specificity of 80%).

with *S. cerevisiae* Su2, with a slope of about 45 degrees, suggesting that the antigen supporting cross-reactivities is present to the same extent in both strains (Fig. 4a). A greater dispersion of values and a lesser slope was observed with *S. cerevisiae* CBS 1315 PPM, revealing qualitative and quantitative differences in antigen recognition by patient sera (Fig. 4b). Finally, no correlation at all was observed with *C. albicans* PPM (Fig. 4c).

Analysis of the role of PPM polysaccharides as antigenic determinants through the use of periodate oxidation. Variations in the levels of antibody response against *S. cerevisiae* Su1 PPM before and after periodate oxidation are given in Fig. 5. Periodate oxidation resulted in a decrease of antibody reactivity for all sera of patients with CD. This decrease was dramatic for patients having high initial antibody levels whereas lower anti-*S. cerevisiae* Su1 PPM antibody levels were affected to a lesser degree by this procedure. In contrast, periodate oxidation resulted in an increased ELISA signal for UC patients. Few variations were observed in controls.

TABLE 2. Ability of ELISA antigens to discriminate between serum antibodies associated with clinical forms of inflammatory bowel diseases and control sera

Antigen	CD vs UC		CD vs controls	
	Significance[a]	Cutoff[b]	Significance	Cutoff
Su1	0.0019	3.09 [64,77]	0.0001	2.59 [71,89]
Su2	0.0017	2.85 [66,89]	0.0001	2.95 [64,83]
CBS 1315	0.013	2.5 [59,71]	0.0001	2.50 [59,89]
VW 32	NS		0.03	1.04 [76,53]

[a] Level of significance as determined following application of the Wilcoxon test. NS, not significant.

[b] Cutoff values of ELISA (in percentages of the standard), following application of ROC curves [sensitivity, specificity].

Antibody response in ELISA against *S. cerevisiae* Su1 PPM and its acid (AcS PPM) and alkali (AlS PPM) stable fractions. Results obtained when the sera from patients were allowed to react with the three antigens are shown in Fig. 6. The effect of chemical treatment was more obvious for sera from CD patients with high antibody levels against PPM. The reactivity of sera from other patients was less affected by these procedures. Mild acid treatment resulted in an increase of antibody reactivity in 21% of patients with CD. The overall reactivity of CD patient sera for the acid-stable fraction remained significantly different from the reactivity of other groups (CD versus UC, $P = 0.0005$ for AcS PPM and 0.0001 for PPM; CD versus controls, $P = 0.0001$ for AcS PPM and $P = 0.0001$ for PPM). By contrast, cleavage of O-glycosidic linkages of the PPM and subsequent removal of alkali-labile oligomannosides resulted in a systematic decrease of CD patient antibody responses, leading to a less significant discrimination between CD and UC patients ($P = 0.005$). The differences between UC and controls remained unchanged from that revealed by the intact PPM.

Chemical analysis of *S. cerevisiae* mannans used as antigens. Chemical analysis of the PPM from *S. cerevisiae* Su1, Su2, and CBS 1315 revealed similar compositions of proteins, polysaccharides, and phosphorus (Table 3). Acetolysis, followed by Biogel P2 gel filtration chromatography led to the definition of strain-specific oligomannosides patterns, as well as definition of the ratio of each oligomannoside (Table 3). For *S. cerevisiae* Su1, the linkage types of mannobiose, mannotrioses (which corresponded to two isomers), and the mannotetraose are shown in Table 3. The peak corresponding to *S.*

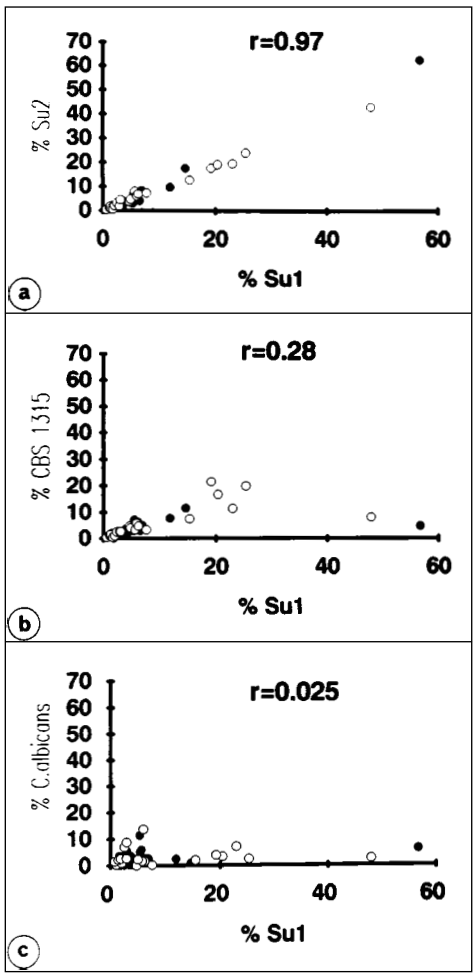

FIG. 4. Correlations between CD patients' serum reactivities in ELISAs against phosphopeptidomannan from *S. cerevisiae* Su1 and phosphopeptidomannan from *S. cerevisiae* Su2, CBS 1345, and *C. albicans* VW32. ●, ACD (n = 21); ○, QCD (n = 21).

cerevisiae Su1 mannotetraose Man(1→3)Man(1→2)Man(1→2)Man was shared by *S. cerevisiae* Su2 but not by the less discriminating strain CBS 1315.

DISCUSSION

Evidence has been gathered of an antibody response against *S. cerevisiae* antigen in patients with CD, but not in patients with UC (1, 2, 4, 11, 19, 21, 25, 26). No explanation has been found to the complex heterogeneity in both patient responses and *S. cerevisiae* strain antigenicity (26). In this study we attempted to define that component of the yeast that represented the best candidate(s) as epitope(s) supporting antibody response in CD.

A preliminary screening of sera from patients with CD, UC (either chronic or acute), and controls was performed by IFA. Highly variable levels of human antibodies against yeast surface antigens were detected, depending on the species and the strains. Surface antigens of the endosaprophytic yeast *C. albicans* did not discriminate between CD and UC patients. By contrast, some *S. cerevisiae* strains (present in various diets) led to discrimination between patients and controls. When PPM of the most discriminative *S. cerevisiae* strain, Su1; the related brewing strain designated Su2; a wine-making strain, *S. cerevisiae* CBS 1315; and *C. albicans* VW32 were tested by ELISA against a large panel of human sera, they mimicked the ability of the respective intact yeast cell surface antigens to detect antibodies associated with IBD. PPM from *S. cerevisiae* Su1 contained the most discriminating epitopes. The expression of these epitopes was less important or absent in *S. cerevisiae* CBS 1315 and *C. albicans* VW32. Periodate oxidation selectively denatures the polysaccharide components of the glycoproteins (45). This oxidation resulted in a disappearance of serological reactivity against *S. cerevisiae* Su1 PPM in CD patients, demonstrating that the discriminating epitopes were in the polysaccharide and not in the protein moiety.

There are several questions regarding mannose-induced antibody responses in CD. Despite the good sensitivity and specificity of ELISA testing with *S. cerevisiae* Su1 PPM, some CD patient sera failed to react. All patients with high anti-*S. cerevisiae* antibody levels had CD, but the reverse was not true; all CD patients did not have anti-*S. cerevisiae* antibody. Differences in anti-*S. cerevisiae* antibody levels were not related to steroid therapy or anatomical location or duration of the disease.

Different hypotheses concerning the origin of these antibodies could be proposed. They must take into account the widespread distribution of oligomannosides, for which the yeasts represent a privileged model, since they express a complex repertoire whose components may be shared by many unrelated molecules present in a wide variety of organisms (10, 20, 22, 30). The first hypothesis to consider is that anti-*S. cerevisiae* antibodies may originate from immunization by yeasts. In this instance, both dietary yeasts and yeast species that are natural inhabitants of the gastrointestinal tract must be considered immunogen candidates. An increased intestinal permeability in CD might lead to an increased exposure of yeast antigens to immune reactive cells (15, 43, 46, 47). The role of dietary yeast has been explored (1, 2, 4, 11, 19, 21, 25, 26) without definitive conclusions. By contrast, investigations concerning an antigenic stimulation from yeasts that colonize the digestive tract have been limited. The yeast species most frequently isolated as human commensals are *C. albicans* and *C. glabrata* (31). And at least for *C. albicans*, an intense colonization of the gut has been shown to generate a systemic antibody response (32). Absence of specific serological reactivity of CD patients against *C. albicans* has been documented and confirmed several times, including in the present work. However, strains used as antigens for these studies belong to *C. albicans* serotype A, which accounts only for 60 to 80% of human *C. albicans* isolates (31). The other isolates belong to serotype B, which differs from serotype A by several phenotypic characteristics, including PPM antigenicity (39). It is worth noting that McKenzie et al. (26) have already reported that *C. albicans* serotype B absorbed antibodies to more *S. cerevisiae* strains than did serotype A. As evidence has been obtained that *C. albicans* surface antigens are highly variable in relation to growth conditions (33), no definitive conclusions about the role of yeast antigens as immunogens in Crohn's disease can be drawn without testing *C. albicans* serotype B reactivity and that of *C. glabrata*. Interestingly, the mannan from this species cross-reacted with *S. cerevisiae* serotype Ia and Ib mannans (10).

A second hypothesis is that yeast mannan oligomannosides could correspond to epitopes shared by other microorganisms. On the basis of structural similarities (20, 29, 36), such cross-reactivities could exist between polysaccharide moieties of yeast PPM and *Mycobacterium* lipoarabinomannans (LAM). To explore this hypothesis, all sera tested against yeast PPM

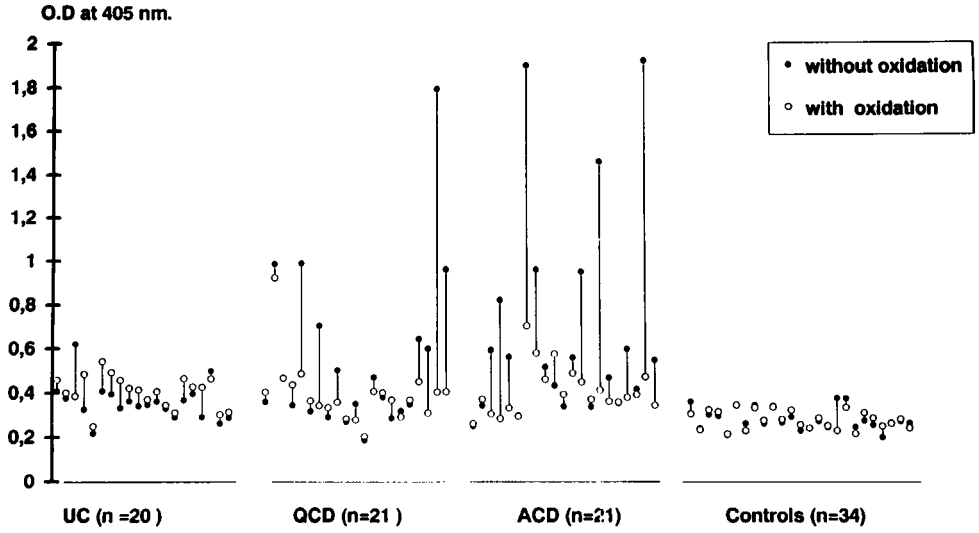

FIG. 5. ELISA detection of antibody responses against *S. cerevisiae* Su1 PPM before (●) and after (○) periodate oxidation in patients with ACD or QCD, patients with UC, and blood donors (controls).

have been tested by ELISA against LAM from *Mycobacterium bovis* as a representative mannosylated antigen of *Mycobacterium* species. A double-blind collaborative study with Puzo's group (36) has not revealed any correlation between antibody levels against both antigens (data not shown). This experiment therefore suggests that the presently evidenced anti-*S. cerevisiae* antibody did not originate from an immunization resulting from the presence of mycobacteria that have been implicated as a potential etiologic agent of CD (40, 44).

The ubiquitous character of mannose residue sequences raised a third hypothesis: some *S. cerevisiae* oligomannosides could share structural homologies with oligomannosides expressed on human glycoconjugates (16) as autoantigens or neoautoantigens.

Whatever the mechanism, complexity and variability of both the yeast oligomannoside repertoire and its recognition by the human immune system require a precise identification of the reactive oligomannosides. Partial depolymerization of the *S. cerevisiae* Su1 PPM by mild acid hydrolysis (Fig. 1B) has demonstrated that the discriminating epitopes were not located in PPM acid-labile components, since the acid-stable domain of the PPM was able to differentiate patients with CD and UC as well as the intact PPM. By contrast, removing alkali-labile O-linked oligomannosides (Fig. 1A), although preserving the differentiation between CD and UC, decreased the level of reactivity (0.005 versus 0.0001). This suggests the presence of discriminating epitopes in both the alkali-stable and the alkali-labile domains of the PPM polysaccharide moiety.

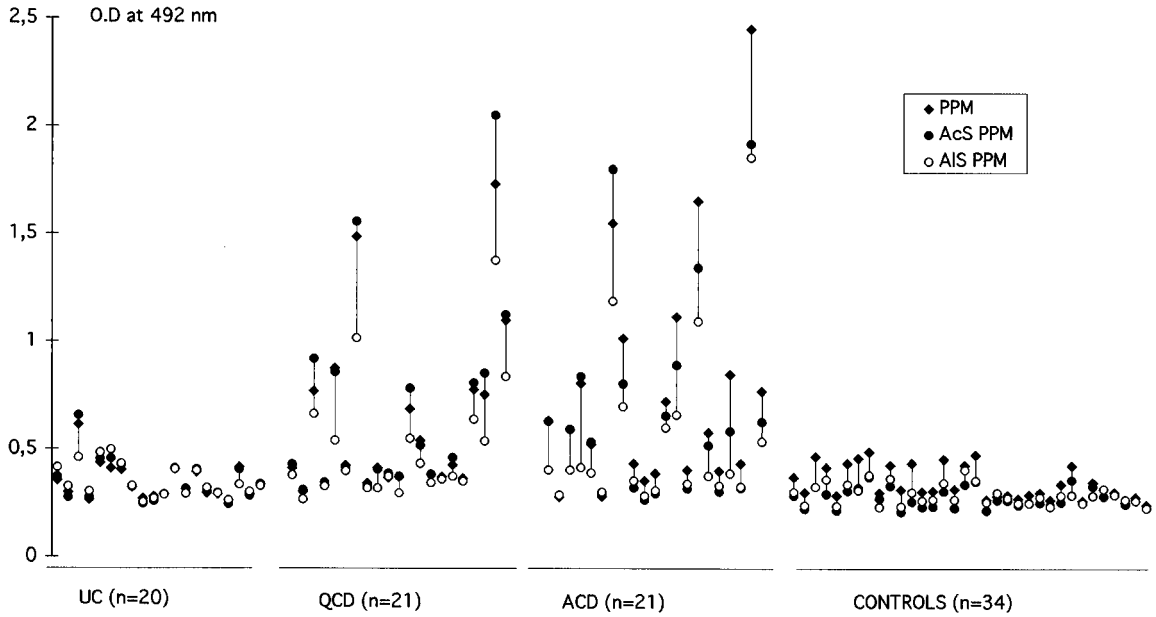

FIG. 6. ELISA detection of antibody responses in sera from different groups of patients against *S. cerevisiae* Su1 PPM, Su1 PPM AcS PPM, and Su1 PPM AlS PPM.

TABLE 3. Chemical composition and acetolysis-released oligomannosides from the PPMs of three *S. cerevisiae* strains

Parameter	Result for:		
	Su1	Su2	CBS 1315
% Proteins	5.2	4.4	5.1
% Phosphorus	0.16	0.10	0.15
% Carbohydrate	68	74	78
Mannose[a]	27	20	26
Mannobiose	34 [Man (1-2) Man][b]	36	36
Mannotriose	27 [Man (1-2) Man (1-2) Man], [Man (1-3) Man (1-2) Man]	30	38
Mannotetraose	10 [Man (1-3) Man (1-2) Man (1-2) Man]	12	
Mannopentaose	2	2	

[a] Oligomannosides and mannose were released from PPM by acetolysis and separated by gel filtration on a Bio-Gel P2 column. Composition of each fraction was checked by thin-layer chromatography, and their respective percentages were determined by the phenol-sulfuric method.

[b] Structure of oligomannosides released from Su1 as determined by mass spectroscopy of methylated and acetylated residues.

Specificity of antioligosaccharide antibodies depends on the mannosyl chain length (9), the anomeric configuration (10, 29, 34, 39, 42), and the position of linkage (17). Thus it could be speculated that, within the oligomannosidic repertoire of this strain, the best candidate for an epitope discriminating between CD and UC is the mannotetraose. Mannotetraose was present in both Su1 and Su2 but not in the less discriminating CBS 1315 strain; this mannotetraose, known as *S. cerevisiae* serotype Ib (10), also occurred in the PPM alkali-labile domain (Fig. 1).

In conclusion, we demonstrated that an antibody response against a sequence of mannose residues was specifically associated with CD. Experiments with purified oligomannosides converted into neoglycolipids (7) are now in progress to confirm the nature of the discriminating epitope and to generate specific monoclonal antibodies. As far as they are representative of the human polyclonal response, these probes may then be useful from a clinical point of view to help in the diagnosis of CD versus UC, for epidemiological studies as a subclinical marker of CD, possibly in relation to intestinal permeability (24), and in a more fundamental way in defining the nature of the immunogen and the role of its oligomannosides as signals for the immune system (38).

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REFERENCES

1. **Barclay, G. R., H. McKenzie, J. Pennington, D. Parratt, and C. R. Pennington.** 1992. The effect of dietary yeast on the activity of stable chronic Crohn's disease. Scand. J. Gastroenterol. **27:**196–200.
2. **Barnes, R. M. R., S. Allan, C. H. Taylor Robinson, R. Finn, and P. M. Johnson.** 1990. Serum antibodies reactive with *Saccharomyces cerevisiae* in inflammatory bowel disease: is IgA a marker for Crohn's disease? Int. Arch. Allergy. Appl. Immunol. **92:**9–15.
3. **Best, W. R., J. M. Becktel, J. W. Singleton, and F. Kern.** 1976. Development of a Crohn's disease activity index. Gastroenterology **70:**439–444.
4. **Bridget, T., A. Susan, and R. M. R. Barnes.** 1991. Identification of a 200 kDa glycoprotein antigen of *Saccharomyces cerevisiae*. Immunol. Lett. **28:**181–186.
5. **Byron, J. K., K. V. Clemons, J. H. McCusker, R. W. Davis, and D. A. Stevens.** 1995. Pathogenicity of *Saccharomyces cerevisiae* in complement factor five-deficient mice. Infect. Immun. **63:**478–485.
6. **Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Reber, and F. Smith.** 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. **28:**350–356.
7. **Faille, C., D. W. R. Mackenzie, J. C. Michalski, and D. Poulain.** 1992. Evaluation of an enzyme immunoassay using neoglycolipids constructed from *Candida albicans* oligomannosides to define specificity of anti-mannan antibodies. Eur. J. Clin. Microbiol. Infect. Dis. **11:**438–446.
8. **Fournet, B., G. Strecker, Y. Leroy, and J. Montreuil.** 1981. Gas liquid chromatography and mass spectrometry of methylated and acetylated methyl glycosides. Application to the structural analysis of glycoprotein glycans. Anal. Biochem. **116:**489–502.
9. **Frosch, M., I. Görgen, G. J. Boulnois, K. N. Timmis, and D. Bitter-Suermann.** 1985. NZB mouse system for production of monoclonal antibodies to weak bacterial antigens: isolation of an IgG antibody to the polysaccharide capsules of Escherichia coli K1 and group B meningococci. Proc. Natl. Acad. Sci. USA **82:**1194–1198.
10. **Fukazawa, Y.** 1989. Antigenic structure of *Candida albicans*: immunochemical basis of the serologic specificity of the mannans in yeast, p. 37–62. *In* E. Kurstak (ed.), Immunology of fungal diseases. Marcel Dekker Inc., New York.
11. **Giaffer, M. H., A. Clark, and C. D. Holdsworth.** 1992. Antibody to *Saccharomyces cerevisiae* in patients with Crohn's disease and their possible pathogenic importance. Gut **33:**1071–1075.
12. **Hamada, T., F. Noda, and K. Hayashi.** 1984. Structure of cell wall and extracellular mannans from *Saccharomyces rouxii* and their relationship to a high concentration of NaCl in the growth medium. Appl. Environ. Microbiol. **48:**708–712.
13. **Hayette, M. P., G. Strecker, C. Faille, D. Dive, D. Camus, D. W. R. Mackenzie, and D. Poulain.** 1992. Presence of human antibodies reacting with *Candida albicans* O-linked oligomannosides revealed by using an enzyme-linked immunosorbent assay and neoglycolipids. J. Clin. Microbiol. **30:**411–417.
14. **Hearn, V. M., E. V. Wilson, J. P. Latge, and D. W. R. Mackenzie.** 1990. Immunochemical studies of *Aspergillus fumigatus* mycelial antigens by polyacrylamide gel electrophoresis and western blotting techniques. J. Gen. Microbiol. **136:**1525–1535.
15. **Hollander, D., C. M. Valdheim, E. Brettholz, G. M. Petersen, T. Delahunty, and J. I. Rotter.** 1986. Increased intestinal permeability in patients with Crohn's disease and their healthy relatives—a possible etiologic factor. Ann. Intern. Med. **105:**883–885.
16. **Jewell, D. P., and J. A. Snook.** 1990. Immunology of ulcerative colitis and Crohn's disease, p. 127–146. *In* R. N. Allan, M. B. R. Keighley, J. Alexander-Williams, and C. Hawkins (ed.). Flammatory bowel diseases. Churchill Livingstone, Edinburgh.
17. **Kikuchi, S., A. Ohinata, Y. Tsumuraya, Y. Hashimoto, Y. Kaneko, and H. Matsushima.** 1993. Production and characterization of antibodies to the β-(1→6)-galactotetraosyl group and their interaction with arabinogalactan-proteins. Planta **190:**525–535.
18. **Kocourek, J., and C. E. Ballou.** 1969. Method for fingerprinting yeast cell wall mannans. J. Bacteriol. **100:**1175–1181.
19. **Lindberg, E., K.-E. Magnusson, C. Tysk, and G. Järnerot.** 1992. Antibody (IgG, IgA, and IgM) to baker's yeast (*Saccharomyces cerevisiae*), yeast mannan, ovalbumin, gliadin, and betalactoglobulin in monozygotic twins with inflammatory bowel disease. Gut **33:**909–913.
20. **Maekawa, S., and T. Ohara.** 1966. On the existence of cross reactivity antigens between *Candida* and *Mycobacterium*. Jpn. J. Microbiol. **10:**1–11.
21. **Main, J., H. McKenzie, G. R. Yeaman, M. A. Kerr, D. Robson, C. R. Pennington, and D. Parrat.** 1988. Antibody to *Saccharomyces cerevisiae* (baker's yeast) in Crohn's disease. Br. Med. J. **297:**1105–1106.
22. **Manning-Zweerink, M., C. S. Maloney, T. G. Mithell, and H. Weston.** 1986. Immunoblot analyses of *Candida albicans*-associated antigens and antibodies in human sera. J. Clin. Microbiol. **23:**46–52.
23. **Markwell, M., S. M. Haas, L. L. Bieber, and N. E. Tolbert.** 1978. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. Anal. Biochem. **87:**206–210.
24. **May, G. R., L. R. Sutherland, and J. B. Meddings.** 1994. Is small intestinal permeability really increased in relatives of patients with Crohn's disease? Gastroenterology **104:**1627–1632.
25. **McKenzie, H., J. Main, C. R. Pennington, and D. Parrat.** 1990. Antibody to select strains of *Saccharomyces cerevisiae* (baker's and brewer's yeast) and *Candida albicans* in Crohn's disease. Gut **31:**536–538.
26. **McKenzie, H., D. Parrat, J. Main, and C. R. Pennington.** 1992. Antigenic heterogeneity of strains of *Saccharomyces cerevisiae* and *Candida albicans* recognised by serum antibodies from patients with Crohn's disease. FEMS Microbiol. Immunol. **89:**219–224.
27. **Müller, W. E., M. Bachmann, B. E. Weiler, H. C. Schröder, G. Uhlenbruck,**

T. Shinoda, H. Shimizu, and H. Ushijima. 1991. Antibodies against defined carbohydrate structures of *Candida albicans* protect H9 cells against infection with human immunodeficiency virus-1 *in vitro*. J. Acquired Immune Defic. Syndr. **4:**694–703.

28. **Nakajima, T., and C. B. Ballou.** 1974. Structure of the linkage region between the polysaccharide and protein parts of *Saccharomyces cerevisiae* mannan. J. Biol. Chem. **249:**7685–7694.
29. **Nishikawa, A., T. Sekine, R. Ikeda, T. Shinoda, and Y. Fukazawa.** 1990. Reassessment of antigenic determinant of *Saccharomyces cerevisiae* serotype Ia. Microbiol. Immunol. **34:**825–840.
30. **Nnalue, N. A., A. Weintraub, and A. A. Lindberg.** 1991. Properties of a rat monoclonal antibody reactive with both the mannan of *Candida* species and the O-antigen 6,7 polysaccharide of serogroup C_1 salmonellae. Infect. Immun. **59:**229–233.
31. **Odds, F. C.** 1988. *Candida* and candidosis. A review and bibliography, 2nd ed. Baillière Tindall, London.
32. **Odds, F. C., and E. G. V. Evans.** 1980. Distribution of pathogenic yeasts and humoral antibodies to *Candida* among hospitalized patients. J. Clin. Pathol. **33:**750–756.
33. **Okawa, Y., Y. Takahata, M. Kawamata, M. Miyauchi, N. Shibata, H. Kobayashi, and S. Suzuki.** 1994. Temperature-dependent change of serological specificity of *Candida albicans* NIH A-207 cultured in yeast extract-added Sabouraud liquid medium: disappearance of surface antigenic factors 4, 5 and 6 at high temperature. FEBS Lett. **345:**167–171.
34. **Poulain, D., C. Faille, C. Delaunoy, P. M. Jacquinot, P. A. Trinel, and D. Camus.** 1993. Probable presence of β(1-2)-linked oligomannosides that act as human immunoglobulin G3 epitopes and are distributed over a *Candida albicans* 14- to 18-kilodalton antigen. Infect. Immun. **61:**1164–1166.
35. **Poulain, D., J. Fruit, L. Fournier, E. Dei Cas, and A. Vernes.** 1986. Diagnosis of systemic candidiasis: application of Co-counterimmunoelectrophoresis. Eur. J. Clin. Microbiol. **23:**46–52.
36. **Puzo, G.** 1990. The carbohydrate- and lipid-containing cell wall of mycobacteria, phenolic glycolipids: structure and immunological properties. Microbiology **17:**305–327.
37. **Reiss, E.** 1986. Molecular immunology of mycotic and actinomycotic infections. Elsevier Science Publishers, New York.
38. **Ryan, C. A.** 1994. Oligosaccharide signals: from plant defense to parasite offense. Proc. Natl. Acad. Sci. USA **91:**1–2.
39. **Shibata, N., T. Ichikawa, M. Tojo, N. Takahashi, N. Ito, Y. Okubo, and S. Suzuki.** 1985. Immunochemical study on the mannans of *Candida albicans* NIH A-207, NIH-B792, and J-1012 strains prepared by fractional precipitation with cetyltrimethylammonium bromide. Arch. Biochem. Biophys. **243:** 338–348.
40. **Stainsby, K. J., J. R. Lowes, R. N. Allan, and J. P. Ibbotson.** 1993. Antibodies to *Mycobacterium paratuberculosis* and nine species of environmental mycobacteria in Crohn's disease and control subjects. Gut **34:**371–374.
41. **Trinel, P. A., M. Borg-Von-Zepelin, G. Lepage, T. Jouault, D. W. R. Mackenzie, and D. Poulain.** 1993. Isolation and preliminary characterization of 14- to 18-kilodalton *Candida albicans* antigen as a phospholipomannan containing β-1,2-linked oligomannosides. Infect. Immun. **61:**4398–4405.
42. **Trinel, P. A., C. Faille, P. M. Jacquinot, J. C. Caillez, and D. Poulain.** 1992. Mapping of *Candida albicans* oligomannosidic epitopes by using monoclonal antibodies. Infect. Immun. **60:**3845–3851.
43. **Ukabam, S. O., J. R. Clamp, and B. T. Cooper.** 1983. Abnormal small intestinal permeability to sugars in patients with Crohn's disease of the terminal ileum and colon. Digestion **27:**70–74.
44. **Wayne, L. G., D. Hollander, B. Anderson, H. A. Sramek, C. M. Vadheim, and J. I. Rotter.** 1992. Immunoglobulin A (IgA) and IgG serum antibodies to mycobacterial antigens in Crohn's disease patients and their relatives. J. Clin. Microbiol. **30:**2013–2018.
45. **Woodward, M. P., W. W. Young, and R. A. Bloodgrood.** 1995. Detection of monoclonal antibodies specific for carbohydrate epitopes using periodate oxidation. J. Immunol. Methods **78:**143–153.
46. **Yacyshyn, B. R., and J. B. Meddings.** 1995. CD45RO expression on circulating CD19+ B cells in Crohn's disease correlates with intestinal permeability. Gastroenterology **108:**132–137.
47. **Young, C. A., A. Sonnenberg, and E. A. Burns.** 1994. Lymphocyte proliferation responses to Baker's yeast in Crohn's disease. Digestion **55:**40–43.