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Serological and Cellular Immune Activity of Peptidoglucomannan Fractions of *Candida albicans* Cell Walls

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A two-stage extraction of isolated cell walls of *C. albicans* resulted in 45% solubilization into antigens of high molecular weight leaving a wall residue which also had antigenic properties. Ice-cold dilute alkali removed 25% of the defatted cell walls. The extract was nondialyzable, had a glucose-to-mannose ratio of 2:3 and an amino acid content of 7.32%, and was designated peptidoglucomannan (PGM). An additional 26% of the walls resistant to stage I were solubilized by sonic treatment yielding a fraction having a glucose-to-mannose ratio of 6:1, termed soluble mannoglucan (sMG). The residue after extraction and sonic treatment contained 10.9% mannose, which was the insoluble mannoglucan. The gel permeation behavior of PGM and sMG on BioGel A5M was similar; each contained two components, one estimated to exceed 5×10^6 molecular weight and a second smaller species. The soluble cell wall fractions were active in immunodiffusion and carried antigenic group specificity. Immunoelectrophoresis of PGM, sMG, and mannan revealed some heterogeneity. The insoluble mannoglucan had agglutinating activity. A distinctive immunodiffusion pattern of cell wall antigens was formed with the serum of a leukemic patient with candidiasis. All three cell wall antigens and mannan elicited delayed-type hypersensitivity as measured by skin-test and specific inhibition of macrophage migration. A dose of 25 μg of PGM was sufficient to inhibit 89.9% migration in the peritoneal exudates of guinea pigs immunized with cell walls, and 10 μg of PGM inhibited 91.7% migration in guinea pigs immunized with insoluble mannoglucan.

Previous immunological studies of *Candida albicans* (3, 30, 33) employed a different antigenic preparation for measuring antibody than for eliciting delayed-type hypersensitivity (DTH). A reliable precipitin reaction was obtained with the purified polysaccharide, mannan, whereas candidin was active in detecting cutaneous hypersensitivity. Mannans of known mannohexaose hapten configuration were extracted from whole cells by autoclaving and precipitation of their Cu^{2+} complexes (23, 31). Candidins are concentrated culture filtrates of 30-day-old cultures of as yet undefined chemical composition (6). The protein component of candidin has been generally regarded as the skin test-active material, but evidence has been accumulating that polysaccharides may be involved in some DTH reactions. A tuberculin carbohydrate fraction (4) and the type II pneumococcal polysaccharide (8) were found to induce skin reactivity.

The cell wall composition of *C. albicans* was studied (5, 16), and it was found that 0.5 N alkali for 1 h at room temperature was sufficient to solubilize 50 to 60% of defatted cell walls into nondialyzable components containing glucose, mannose, and protein. Lipids were extracted in the presence of acid at 50 C which increased the alkali susceptibility of the cell wall (16). One of these fractions, glucomannan protein II, was serologically active in a precipitin test (15). However, the role of cell wall antigens in cellular immune phenomena is the more compelling area for investigation, since T cells involved in DTH or protective behavior have the most direct access to materials at the surface of a pathogen. A multiplicity of cell wall antigens could be implicated in the cellular components of the immune response, and some sorting out is warranted.

An objective of the present work was to compare the humoral and cellular immune responses to different cell wall antigens. By first preparing clean cell walls, the participation of extraneous cytoplasmic proteins could be ruled out. To allow maximal preservation of antigenic determinants, cell wall fractionation procedures were as mild as possible, excluding heat or

acidic pH. In this respect, thermolabile agglutinins were previously reported in some *Candida* sp. (33). Both skin testing and in vitro macrophage migration inhibitory factor (MIF) assays were undertaken to determine the capacity of various cell wall fractions for inducing DTH reactions.

MATERIALS AND METHODS

C. albicans 526 (group B) and *C. albicans* 20 (group A) were selected on the basis of typical morphology and pathogenicity. They formed abundant chlamydospores on rice-Tween 80 medium and typical pseudomycelium on cornmeal agar and assimilated but did not ferment sucrose, and an intravenous challenge of 5×10^7 yeast cells of each strain was fatal for mice within 24 h. The test strains formed germ tubes in pooled human sera. Their antigenic groups were verified by agglutinin tests in group-specific antiserum (11).

Medium and conditions of growth. The yeast was grown in submerged liquid culture in 16-liter batches in glass carboys with forced aeration and constant stirring. The medium for growth was composed of the following: glucose, 2%; neopeptone, 1%; yeast extract, 0.05%; and potassium phosphate buffer, (pH 6.5), 5×10^{-3} M. Silicone emulsion antifoam (AF 72, General Electric) was added (2.5 ml to each 16-liter batch). The inoculum consisted of freeze-dried log-phase yeast to give a final concentration of 10^5/ml. Cultures were grown at 27 C until the glucose in the medium was exhausted, as monitored by the anthrone reaction, which occurred at between 44 and 50 h. Under these conditions a yield of 350 g (wet weight) of yeast cells corresponding to 114 g (dry weight) was obtained per carboy.

Preparation of cell walls. Yeast was centrifuged ($9,000 \times g$, 15 min), washed three times with demineralized water, and diluted so that a 1:1,000 dilution gave an optical density at 550 nm (OD_{550}) of 0.325, corresponding to a packed-cell-to-water ratio of 1:2. Batches consisting of 35 ml of this suspension and 48 g of glass beads (0.45 to 0.5 mm diameter) were disrupted in the Braun cell homogenizer (model MSK, Bronwill Scientific, Rochester, N.Y.), cooled by a constant stream of liquid CO_2. The optimal conditions for complete breakage were determined by monitoring the turbidity of the homogenate (14) by phase-contrast microscopy and the Gram stain. An initial cycle of 3 min of homogenization followed by a second pass of 1 min was sufficient for greater than 90% disruption. Electron microscopy showed that cell walls retained an ellipsoid shape but were cracked open. The purified cell wall fraction was obtained from disrupted cells by the method of N. S. Orenstein (Ph.D. thesis, Rutgers University, New Brunswick, N.J., 1971). The temperature for all steps was 0 to 4 C. Cells from the first cycle of disruption were washed twice with water and three times with 0.1% sodium dodecyl sulfate (SDS) (Eastman) in 10% sucrose. The upper layer consisting of walls was homogenized for an additional minute and centrifuged ($4,000 \times g$, 10 min), and the ensuing pellet was washed three times in 0.1% SDS in 10% sucrose ($5,500 \times g$, 10 min). Walls were suspended in 0.1% SDS in 10% sucrose and placed in an ice bath for 18 h to solubilize any traces of remaining membrane. Walls then were washed three times in 0.1% SDS in 10% sucrose, three times in 1% NaCl in 10% sucrose, to aid in removing the SDS, six times in 1% NaCl, and ten times in demineralized water. During these steps non-homogeneous areas of the pellet were removed. The purified walls were lyophilized and stored at −40 C. The yield of cell walls on a dry weight basis was 10%.

Fractionation of purified cell walls. For lipid extraction, cell walls (1 g) of each strain were moistened with 3 ml of water, combined with 100 ml of chloroform:methanol (2:1, vol/vol), and extracted at 26 C for 7 h under N_2 with one change of solvent. The walls were recovered after filtration over a sintered glass filter, and the last traces of solvent were removed in vacuo. Defatted cell walls then were treated with ice-cold dilute alkali (stage I). Cell walls (500 mg) and ice-cold 0.5 N NaOH (150 ml) were combined in a plastic, stoppered bottle, immersed in an ice bath, and stirred constantly for 24 h. Steps taken to minimize oxidation were the use of freshly prepared and degassed alkali and the absence of an air space above the suspension being extracted. The suspension then was centrifuged ($12,000 \times g$, 10 min), and the residue was washed once with ice-cold 0.5 N NaOH and four times with ice-cold water. The supernatant fluid and washings were neutralized with glacial acetic acid and concentrated in a flash evaporator at 30 C. The concentrated extract was dialyzed for 24 h versus running demineralized water (5 liters/h) and lyophilized. The washed residue was lyophilized and extracted by sonic treatment in cold dilute alkali (stage II). The cell walls resistant to stage I (300 mg) were sonically treated in cold 0.5 N NaOH for 3 min with a Branson 20 kilocycle sonifier (model S-75) at a setting of 8, with the chamber jacketed and cooled by a stream of methanol at −6 C. Under these conditions the contents remained below 10 C. The sonically treated suspension was centrifuged at $17,000 \times g$ for 10 min, and the residue was washed once with ice-cold 0.5 N NaOH and four times with water. The supernatant fluid and washings were neutralized with acetic acid, concentrated at reduced pressure, dialyzed versus running demineralized water for 24 h, and lyophilized. Mannan was extracted from whole cells of *C. albicans* 20A by the method of Peat et al. (23).

Chemical analyses. Total N was determined by the micro-Kjeldahl method, and total P was determined with ammonium molybdate and hydrazine sulfate (13). Neutral sugars present in acid hydrolysates were converted to their alditol acetate derivatives prior to gas-liquid chromatography. Samples containing 1 mg were combined with 0.5 ml of 2 N trifluoroacetic acid in a hydrolysis tube, sealed under N_2, and hydrolyzed at 100 C. The sonic extract was heated for 2 h while the sonic residue and ice-cold alkali extract received 4.5 h of heat treatment. The acid was removed in vacuo and chased with three portions of water. Alditol acetates were prepared by the method of Shaw and Moss (29) modified so that 0.2 ml of pyridine:acetic anhydride (2:1, vol/vol) was added and reacted at 80 C for 2 h. Separation was

achieved on a column of ECNSSM in a Perkin-Elmer model 9000 gas chromatograph with ribitol as the internal standard. The amount of each sugar recovered was quantitated by digital integration.

The amino acids present in a cell wall fraction were measured by automated detection on a column of Bio-Rad Aminex A-6 after acid hydrolysis of a 5-mg sample (6 N HCl, 100 C, 20 h, in vacuo) and removal of the acid in a rotary evapomix (Buchler).

For permeation chromatography, BioGel A5M (Bio-Rad), a 6% agarose gel, was employed. The column, 1.2 by 75 cm, was eluted with a solution of NaCl (0.05 M), Triton X-100 (Rohm & Haas Co.) (0.25%), adjusted to pH 7 with potassium phosphate buffer (3.5×10^{-4} M). Carbohydrate in the resulting fractions was determined with phenol and sulfuric acid (12).

Serological procedures. Hyperimmune serum directed against the test strains was obtained from male New Zealand albino rabbits. The schedule of immunization was such that 1.5 mg (dry weight) of cell walls was injected intravenously three times weekly for a month. Agglutinin titers of 1:1,280 were considered acceptable. Agglutinin tests contained a total volume of 0.5 ml per tube, each tube receiving 75 μg of cells or cell walls. Immunodiffusion in agar used antigens at 1 mg/ml concentration, each well receiving 35 μliters. Immunoelectrophoresis was carried out in a Gelman apparatus in borate buffer ($Na_2B_2O_7$, 0.02 M, pH 9.2) at 8.5 mA per frame for 1.5 h.

Skin testing. Guinea pigs, NIH strain 13 N, were sensitized with 2.5 mg of the test antigen (cell walls, group A or insoluble mannoglucan, group A) in incomplete or complete Freund adjuvant given intradermally in the nuchal area. *Mycobacterium tuberculosis* var. *hominis* (2.5 mg) was present in the complete adjuvant. Animals were skin tested only with the homologous antigen for cutaneous basophil hypersensitivity (Jones-Mote reactivity, 5 days) and later at 21 days for classic reactivity to various antigens, including the purified protein derivative of tuberculin (Connaught, Toronto) and streptokinase-streptodornase (Lederle). Skin test results were expressed as the mean square radius of induration from three animals.

MIF assay. The direct method (7) was employed with a few modifications. The tissue culture medium was RPMI 1640 containing 25 mM *N*-2-hydroxymethylpiperazine-*N'*-2'-ethanesulfonic acid buffer (Grand Island Biological Co.) and supplemented with 0.03% L-glutamine just prior to use. Sterile mineral oil (Drakeol, 20 ml) was instilled into the peritoneal cavity 28 days after sensitization. After 3 more days the animals were anaesthetized with ether and exsanguinated by cardiac puncture. Peritoneal exudates pooled from three animals were exposed to isotonic NH_4Cl at 3 C to lyse red blood cells (25), followed by three washes in Hanks balanced salts solution. The system was constituted in Sykes-Moore chambers (Bellco), and measurement of migrating tufts was accomplished by photography and planimetry. For each antigen concentration results were expressed as the mean of three chambers containing two capillaries each. The control containing no antigen comprised four chambers each with two capillaries.

RESULTS

Extraction of glucomannan complexes from cell walls. The lipid content of isolated cell walls was low; only 5 mg of lipid per g of dry walls was found in the chloroform-methanol extract, indicating that SDS treatment had removed virtually all membrane from the preparation. The release of glucomannan by ice-cold dilute alkali from defatted cell walls resulted in a 20% reduction in turbidity after 24 h. This solubilization is referred to as stage I of the extraction procedure (Table 1). As a control for the stage I extraction, walls were pretreated with acidified ethanol-ether (1:1 [vol/vol] + 1% concentration of HCl) by refluxing for 1 h under N_2. It was previously found (16) that the susceptibility of cell walls to dilute alkali depended on the removal of bound lipids with acidified alcohol-ether. Cell walls (100 mg) pretreated in this way were solubilized to the extent of 30% by ice-cold dilute alkali compared with 27.1% solubilization, omitting the acidified alcohol-ether step. It was concluded that no significant benefit was obtained by acid and heating under these conditions and that prolonged refluxing might be deleterious to the preservation of antigens. A stage II extraction was developed in order to obtain greater solubilization. Sonic treatment of β-eliminated cell walls in either cold dilute alkali or dimethyl sulfoxide (ME_2SO) (1) was attempted. Aqueous ME_2SO (ME_2SO:water = 9:1, vol/vol) was capable of solubilizing 34% of the stage I-extracted cell walls after 4 min of sonic treatment. Pretreatment with Cleland reagent to reduce disulfide bridges did not enhance solubilization. Although the ME_2SO extract retained antigenicity as measured by immunodiffusion, it was not considered the solvent of choice due to the difficulty of removing ME_2SO from the extract.

TABLE 1. *Comparison of cell wall extraction of Candida albicans test strains 20 group A and 526 group B*

Determination	20 A[a]		526 B[a]	
	I[b]	II[b]	I[b]	II[b]
Starting wt[c]	500	204	500	303
Extract[c]	123	54.5	126	79.5
Residue[c]	344	141	358	195
Extracted[d]	24.6	26.7	25.2	26.2
Residue[d]	68.8	69.3	71.6	64.4
Recovery[d]	93.5	96	96.8	90.6

[a] Test strain, group.
[b] Stage of extraction.
[c] Yield, dry weight, in milligrams.
[d] Percentage.

The method adopted for stage II extraction was sonic treatment for 3 min in cold dilute alkali. Table 1 is a comparison of the effects of both stages on defatted walls of groups A and B. Between 24 and 25% of the cell walls were removed in stage I, and an additional 26 to 27% of the resistant walls were solubilized by sonic treatment in cold dilute alkali. These amounts represent nondialyzable material recovered from the extracts by lyophilization. The same amount, 44.9%, was extractable from the group A and group B strains.

Hexose content and chromatographic behavior of the two water-soluble and one insoluble fraction. The results of gas chromatography of the hexoses obtained from the three fractions and other compositional analyses are shown in Table 2. The amino acid composition of peptidoglucomannan is shown in Table 3. On the basis of the amino acid content and glucose-to-mannose ratio, the stage I extract was designated peptidoglucomannan (PGM). The sonic extract was termed soluble mannoglucan (sMG), and the sonic residue was designated insoluble mannoglucan (iMG). The prefix "peptido-" was not applied to the sMG and iMG fractions since amino acids were not determined; however, it is probable that these fractions have a peptide moiety. It is noteworthy that the extraction procedure does not remove all mannose from the cell wall and that the glucan residue contains 10.9% mannose. Mannan prepared from autoclaved whole cells by the Cu^{2+} precipitation method of Peat and Whalen (23) contained no glucose detectable by gas chromatography. Gel permeation chromatography on BioGel A5M was performed as a means of estimating the molecular size distribution of each cell wall extract and mannan. The elution profiles are shown in Fig. 1, 2, and 3. Triton X-100 was included in the eluting solution to assist in maintaining the solubility of the antigens. The cell wall antigens PGM and sMG

TABLE 2. *Quantitative analysis of monosaccharides by gas chromatography, and total N and P in cell wall extracts obtained from C. albicans 20A*

Extract	Component	% Extract (dry wt)	G/M[a]
Ice-cold alkali (peptidoglucomannan)	Glucose	31.2	
	Mannose	41.7	
	Total hexose	72.9	2:3
	N	1.47	
	P	0.05	
Sonic extract (soluble mannoglucan)	Glucose	69.3	
	Mannose	10.9	
	Total hexose	80.2	6:1
	N	0.94	
	P	0	
Sonic residue (insoluble mannoglucan)	Glucose	89.9	
	Mannose	10.9	
	Total hexose	100.8	9:1
	N	0.85	
	P	0	

[a] Glucose/mannose.

TABLE 3. *Amino acid composition of peptidoglucomannan of C. albicans 20A*[a]

Amino acid	μmol/100 mg
Lys	0.85
His	0.79
Arg	0.79
Asp	6.18
Thr	11.2
Ser	4.18
Glu	6.42
Pro	4.84
Gly	5.33
Ala	6.36
Val	6.06
Ile	2.67
Leu	2.00
Tyr	2.36
Phe	0.97
Glucosamine	1.3

[a] Total protein recovered as amino acid was 7.32% of PGM dry weight.

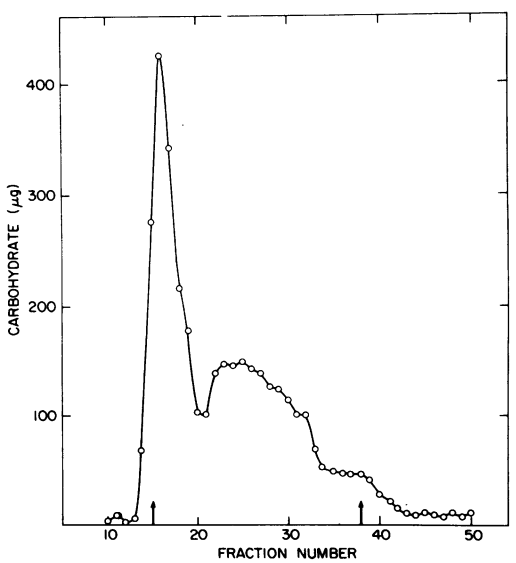

FIG. 1. *Elution profile of C. albicans 20A PGM on BioGel A5M. Fraction volume, 2.1 ml; marker at fraction 15 is blue dextran 2000, at fraction 38 is cytochrome c. Detection was with phenol-sulfuric acid.*

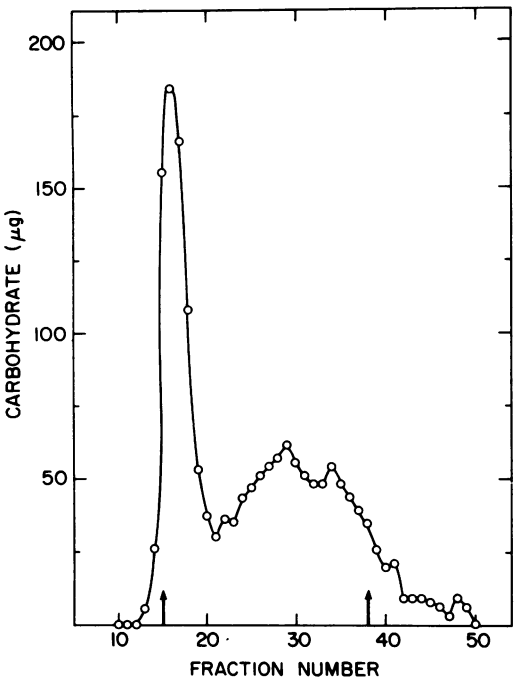

FIG. 2. *Elution profile of sMG on BioGel A5M. Conditions were identical with Fig. 1.*

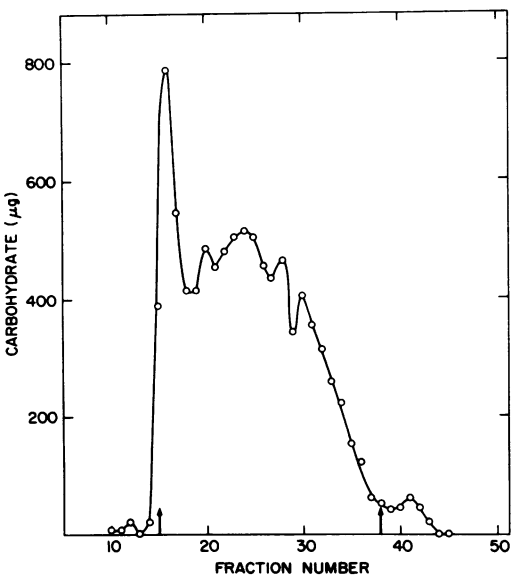

FIG. 3. *Elution profile of mannan on BioGel A5M. Conditions were identical with Fig. 1.*

dissolved in water or saline at 1 mg/ml concentration at 25 C, whereas mannan entered solution without difficulty at 30 mg/ml. The portion of each fraction excluded in the void volume and that permeating the gel matrix is shown in Table 4. The elution profiles of PGM and sMG were similar; each contained two components, one estimated to exceed 5×10^6 molecular weight and a second species apparently larger than the size marker cytochrome *c* (molecular weight 12,800). Under the same conditions mannan extracted from whole cells was less highly polymerized.

Serological activity of cell wall fractions. The agglutinin titer of hyperimmune rabbit serum against whole *C. albicans* cells was 1:1,280. The titer obtained with isolated cell walls was 1:160, and insoluble mannoglucan gave a 1:80 titer.

Precipitin bands in immunodiffusion were observed with rabbit antisera and with serum obtained from a leukemic patient with candidiasis being treated at the National Institutes of Health Clinical Center (Fig. 4). When antigens of group B were paired with the corresponding group A antigens (Fig. 4A), group specificity was seen to occur in mannan, PGM, and sMG. Usually, each antigen produced two precipitates. The serum of some rabbits produced the pattern of Fig. 4C in which the sMG antigens formed a distinct precipitate close to the center well. Absorption of antiserum to group A with group B cells resulted in removal of all group B precipitins (Fig. 4D).

Immunoelectrophoretic patterns of the soluble cell wall antigens are shown in Fig. 5. The patterns obtained with PGM, sMG, and mannan indicated that the bulk of the antigenic material migrated only slightly; however, a gull-wing element was seen indicating the presence of polar components carrying antigens.

Ability of Candida cell wall extracts to elicit delayed-type hypersensitivity. The ability of *Candida* cell wall extracts to elicit delayed-type hypersensitivity was studied by skin testing and the direct MIF test. Strain 13/N guinea pigs were sensitized by the injection of cell walls incorporated in complete or incomplete Freund adjuvant (Table 5). Another group

TABLE 4. *Gel permeation chromatography on Biogel A5M*

Sample	Excluded		Retained		Total (mg)
	mg	%	mg	%	
PGM[a]	1.6	44.5	2.0	55.5	3.6
sMG[a]	0.76	45.1	0.934	55.9	1.7
Mannan[b] ..	1.53	23.9	4.85	76.1	6.37

[a] Fractions excluded, 14 to 19; fractions retained, 20 to 50.

[b] Fractions excluded, 14 to 18; fractions retained, 19 to 45.

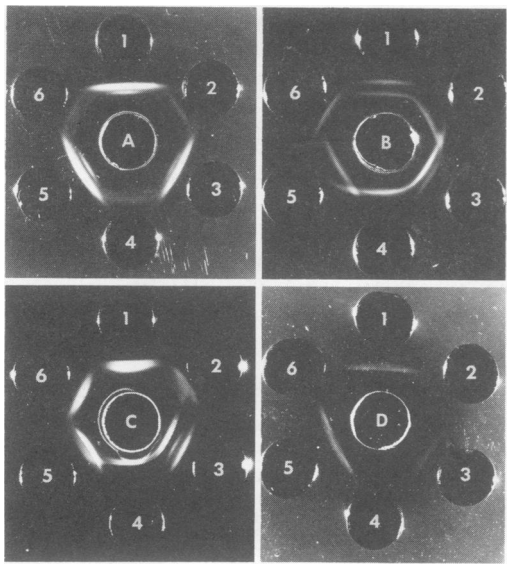

FIG. 4. *Immunodiffusion of cell wall antigens of C. albicans. Outer wells contain antigens (1 mg/ml): (1) PGM 20A; (2) mannan 526B; (3) sMG 20A; (4) sMG 526B; (5) mannan 20A; (6) PGM 526B; Center wells contain antisera: (A) C. albicans group A; (B) leukemic patient with candidiasis; (C) C. albicans group B; (D) C. albicans group A absorbed with whole cells of group B.*

of guinea pigs was sensitized with the iMG fraction of cell walls (Table 6). Responses after 5 to 6 days (Jones-Mote reactivity) to homologous antigens were observed in animals sensitized with cell walls but minimal in guinea pigs sensitized to iMG. The group which received cell walls and incomplete adjuvant showed a possible immediate-type sensitivity with questionable cellular-type reactivity, since reactions were obtained to 100 μg but not to 10 μg.

Animals sensitized with whole cell walls in complete adjuvant responded at 21 days to soluble antigens in the following order of decreasing response: PGM > mannan > sMG. Immediate-type reactions were evident after intradermal challenge with PGM but not with mannan. At the 10-μg level no response was elicited by sMG. The PGM antigen elicited strong 24-h reactions in this group, as did mannan. At 48 h the reaction to PGM persisted but that towards mannan had faded substantially. The sMG and iMG antigens elicited weaker skin reactions than either PGM or mannan. Guinea pigs sensitized by the insoluble mannoglucan responded well only to an eliciting dose of 100 μg of iMG with further suggestion of Arthus-type activity in a mild early response and an equal response when sensitization was made with incomplete adjuvant. No skin test activity was elicited by these antigens in a normal nonimmunized control group.

Macrophage MIF assay. Peritoneal exudates of guinea pigs sensitized with cell walls or with insoluble mannoglucan were tested (Tables 7 and 8; Fig. 6). Referring to the MIF activity in animals sensitized with cell walls, it is seen that 25 μg of antigen per ml is sufficient to induce a near-maximal response with the PGM, sMG, and mannan; of these, the sMG induced a slightly lower response. No toxicity nor nonspecific inhibition of migration was found when the antigens were tested with exudates from normal, nonimmunized guinea pigs. No reactivity was demonstrable at the lower antigen concentrations in a control group immunized with complete Freund adjuvant in the absence of *Candida* antigen. The cells of animals immunized with iMG were also capable of responding to PGM, sMG, and mannan, although the percentage of inhibition in the presence of mannan was low compared to that obtained with PGM. The iMG at 1 μg/ml elicited a MIF response of 55.3% inhibition.

DISCUSSION

Solubilization of 45% of isolated and defatted cell walls occurred with ice-cold dilute alkali as the extracting solvent. The soluble, nondialyza-

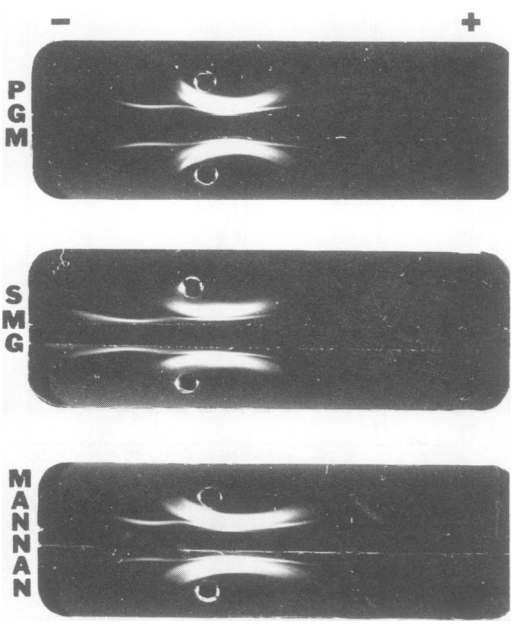

FIG. 5. *Immunoelectrophoresis of C. albicans 20A cell wall antigens and mannan in borate buffer. Wells contained antigens at 5 mg/ml. Troughs contained antiserum against cell walls.*

TABLE 5. *Cutaneous hypersensitivity elicited by C. albicans cell wall fractions in guinea pigs immunized with cell walls in Freund adjuvant*

Antigen	Dose (μg)	Adjuvant[a]	Days post-immunization	Induration ($\bar{r}^2$)[b] (mm²)		
				4 h	24 h	48 h
Cell walls	10	C	5	—[c]	11	18
	100			7	27	29
	10	I		—	6	—
	100			6	30	11
	10	C	20	6	14	9
	100			20	38	30
	10	I		6	14	7
	100			14	28	22
Peptido-glucomannan	10	C	20	—	23	18
	100			14	64	60
	10	I		—	20	12
	100			25	56	49
Soluble mannoglucan	10	C	20	—	—	—
	100			—	17	16
	10	I	20	—	—	—
	100			—	10	9
Insoluble mannoglucan	100	C	20	7	11	12
	100	I		6	9	9
Mannan	100	C	20	—	48	17
	100	I		—	42	16
PPD	10	C	20	—	28	9
		I		—	—	—

[a] Abbreviations: C, complete Freund adjuvant; I, incomplete Freund adjuvant.
[b] $\bar{r}^2$, Mean square radius of induration.
[c] –, Negative.

ble peptidoglucomannan (glucose/mannose = 2:3), and soluble mannoglucan (glucose/mannose = 6:1) bore good antigenic determinants as manifested in precipitin and cellular immune assays. A reaction which could explain the release of the PGM antigen under such mild conditions is β-elimination effected by cleavage of *O*-glycosidic linkages to serine and threonine of the peptide moiety. The β-elimination reaction was shown to occur in the fractionation of glycopeptides of *Saccharomyces* (27) and *C. albicans* (18), as well as in bovine submaxillary mucin (10). After amino analysis of the PGM fraction it was found that threonine and glutamic acid were most prevalent. These amino acids have been implicated in glycopeptide linkages as reviewed by Phaff (24). The term "peptido-" instead of protein was applied to PGM because the amino acid content was low (7.3%), and sulfur amino acids were absent or below the limit of detection (0.03 μmol). This nomenclature is in accord with the description by Lloyd (21, 22) of an antigenic peptido galactomannan from *Cladosporium* and a peptido rhamnomannan from *Sporothrix*.

The presence of glucan in the extracts is consistent with previous observations of yeast cell walls (16, 18, 19). A study of cell wall biosynthesis in *Saccharomyces* (28) has shown that cells pulsed with [U-^{14}C]glucose incorporated the label as glucose into a mannan glycopeptide of the cell wall, which was extracted, as well as into insoluble glucan. Since data based on co-extraction are insufficient criteria for covalent bonds between glucan and mannan polymers, further structural analysis is required to demonstrate linkages that may occur in the intact wall. After cell walls of *C. albicans* were treated with ethylenediamine for 3 days at 37 C (18), the extract was separated on Sephadex G75 into a mannan peptide of 5.5×10^4 molecular weight and a low-molecular-weight glucan peptide.

The antigenic reactivity of the two soluble and one insoluble cell wall fractions was compared in parallel with mannan prepared from whole cells by autoclaving for 2 h and Cu^{2+} precipitation. Much of the immunochemical work on *Candida* has been done on mannan prepared in this way (20, 26). *Candida* mannan is composed of an α(1 → 6) linked backbone with side chains consisting of α(1 → 2) and α(1 → 3) linked oligosaccharides (9). Group specificity was shown to be conferred by the mannohexaose hapten which was isolated as an acetolysis product by Suzuki and Sunayama (31). The

TABLE 6. *Cutaneous hypersensitivity in Guinea pigs immunized with the insoluble mannoglucan fraction of C. albicans cell walls in Freund adjuvant*

Insoluble mannoglucan (μg)	Adjuvant[a]	Days post-immunization	Induration ($\bar{r}^2$)[b] (mm²)		
			4 h	24 h	48 h
10	C	5	—	[c] —	—
100			6	11	—
10	I		—	—	—
100			—	10	—
10	C	21	—	12	7
100			9	51	33
10	I		—	14	11
100			10	53	32

[a] Abbreviations: C, complete Freund adjuvant; I, incomplete Freund adjuvant.
[b] $\bar{r}^2$, Mean square radius of induration.
[c] –, Negative.

TABLE 7. *Macrophage migration inhibitory factor assay of C. albicans 20A cell wall antigens*

Immunizing antigen	Test antigen		Inhibition migration (% at 24 h)
	Composition[a]	Concn (μg/ml)	
Cell walls	PGM	25	89.9
		100	89.9
	sMG	25	84.4
		100	88.7
	iMG	50	79.2
		150	84.4
	Mannan	25	86.9
		100	87.0
	PPD	25	91.4
	SKSD	50 U	10.0
Insoluble mannoglucan	PGM	10	91.7
		100	94.1
	sMG	10	82.3
		100	89.4
	Mannan	10	51.1
		100	90.0
	iMG	1	55.3
		5	67.6
		25	72.2
		100	77.6
	PPD	25	91.8
	SKSD	50 U	(25.7 stimulation)

[a] Abbreviations: PGM, peptidoglucomannan; sMG, soluble mannoglucan; iMG, insoluble mannoglucan; PPD, purified protein derivative; SKSD, streptokinase-streptodornase.

soluble cell wall antigens PGM and sMG differed from mannan chemically, in solubility properties, and in gel permeation characteristics. The PGM extract accounted for 25% of the cell wall dry weight, compared with 19.5% due to sMG. Both fractions contained glucose, whereas mannan contained no glucose detectable by gas chromatography. The PGM and sMG cell wall fractions were soluble at 1 mg/ml in saline at 25 C, whereas mannan entered solution readily at 30 mg/ml. Gel permeation chromatography on 6% agarose indicated that PGM and sMG were excluded in the column void volume to the extent of 55% and, thus, had a higher degree of polymerization than mannan, which was 24% excluded.

The insoluble mannoglucan residue after stage I and stage II extraction contained 89.1% glucose. The structure of *C. albicans* glucan has been studied (2, 34) and was determined to be a highly branched polysaccharide with $\beta(1 \rightarrow 6)$ the predominant configuration (73%) and a significant number of $\beta(1 \rightarrow 3)$ linkages.

The antigenic groups of *C. albicans* were first recognized by Hasenclever and Mitchell (11), by the agglutination of group A cells with antiserum absorbed with group B cells. Subsequently, Summers et al. (30) showed that group specificity resided in the mannan. In the present work, group specificity was demonstrated in both the PGM and sMG antigens as well as in mannan. In the serum of a leukemic patient with candidiasis, two bands were seen with the PGM of group A but only one with the PGM of group B. The mannan of group A gave two precipitins, whereas the mannoglucan antigens showed a particularly prominent band with the leukemic serum. No group differences were seen between the sMG of group A and sMG of group B, but there was a spur formed between group A mannan and sMG of group B.

After both stage I and stage II extraction, the iMG residue had a measurable ability to be agglutinated by rabbit antiserum. Either this activity is due to the mannan present (10.9%) or possibly is indicative of the unmasking of an insoluble glucan antigen.

Candida infections are common among patients with severe defects in cellular immunity. Kirkpatrick et al. (17) reviewed the immune status of patients with chronic mucocutaneous candidiasis; all of those studied had demonstrable anti-*Candida* agglutinins and precipitins;

TABLE 8. *MIF assay control experiments employing normal nonimmunized guinea pigs and those receiving complete Freund adjuvant and no antigen*

Antigen[a]	Dose (μg)	Normal nonimmunized migration		Complete Freund migration	
		%	Inhibition (%)	%	Inhibition (%)
PGM	10	120		88.4	11.6
	25	97.6	2.4	94.4	5.6
	100	125		91.6	8.4
sMG	10	99.6	0.4	97.5	2.5
	25	137		79.6	20.4
	100	100		70.9	29.1
Mannan	10	169		86.6	14.4
	25	87	12.9	94.4	5.6
	100	136		83.2	17.8
iMG	10	132		83.2	17.8
	25	97.2	2.8	62.8	37.2
	100	67.7	32.3	65.3	34.7
SKSD	50 U	129		81.8	18.2
PPD	25	85.5	14.5	10.8	89.2

[a] Abbreviations same as in footnote *a*, Table 7.

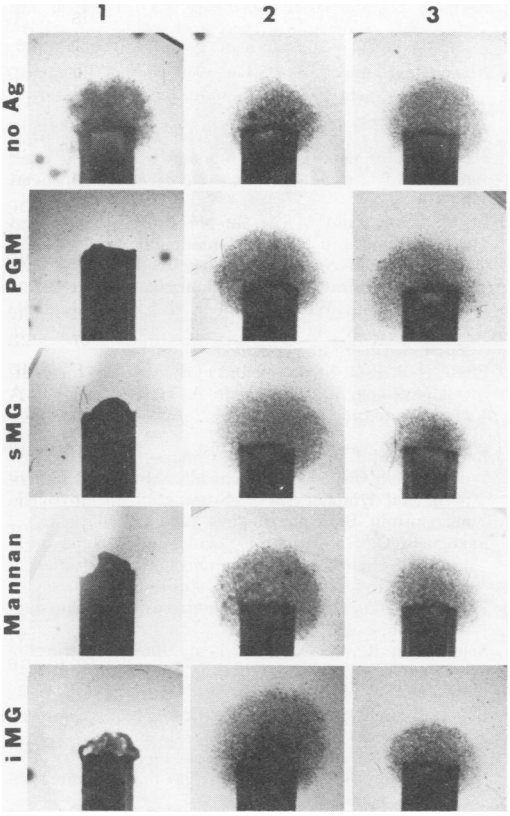

FIG. 6. *Direct MIF assay of C. albicans 20A cell wall fractions and mannan. Migration in the presence of cells obtained from guinea pigs immunized with (1) cell walls in complete Freund adjuvant (except row labeled iMG, immunization was with insoluble mannoglucan); (2) normal nonimmunized controls; (3) complete Freund adjuvant and no added antigen. The antigenic dose in the chambers shown was 25 μg/ml, except iMG was 10 μg/ml.*

however, circulating antibody was not protective. Skin tests and in vitro cellular immune responses of such individuals with *Candida* are often negative, although positive skin tests with other microbial antigens are sometimes found (17). Thus, the loss of immunological function in such cases may be a specific lack of antigen-reactive cells for antigens of *Candida*. In view of this possibility, the delineation of the antigenic structure of *Candida* is important to find out if there are only certain determinants uniquely involved in this failure of cellular recognition and others which are still capable of inducing reactions. The cell wall glucomannan antigens containing a small protein moiety and mannan (0.9% N) were potent in eliciting both cutaneous delayed-type hypersensitivity and in vitro MIF activity in the guinea pig. It is not known if the peptide moiety in the cell wall components of *Candida* is obligatory for eliciting DTH. There are very few documented examples of polysaccharides apparently devoid of peptide which are active with respect to cellular immunity (4, 8). The PGM antigen elicited the most marked and durable cutaneous response, to a 10-μg challenge dose, both at 5 and 21 days after sensitization. Mannan was also effective as a skin test antigen, with less tendency to a 5- to 6-h onset of erythema. The sMG was relatively weak in skin tests in contrast to its marked precipitin reaction, and the cutaneous response to particulate iMG was low.

The PGM and sMG fractions elicited a near-maximal MIF response at the 25 μg/ml concentration in cells of guinea pigs sensitized with *Candida* cell walls. Based on this experience, in the next experiment involving animals sensitized with the iMG fraction, a lower dose of antigen, 10 μg/ml, was found equally potent. The antigens were not toxic, and some of them actually stimulated macrophage migration in normal nonimmunized controls. No significant MIF activity to *Candida* antigens was attributable to the mycobacteria of Freund adjuvant. The PGM antigen appeared to be a slightly more active elicitor of MIF than mannan. Although the sMG antigen gave a relatively weak skin test, its performance in the MIF assay was comparable to mannan. In guinea pigs sensitized with the iMG fraction, mannan was not as active as PGM based on a dose of 10 μg of mannan resulting in a 51% inhibition of migration as compared to 91.7%. This difference may be due to the absence of glucose in the mannan, or possibly to another kind of linkage present in mannan remaining bound to the insoluble glucan of the cell wall.

The soluble cell wall antigens, PGM and sMG, are fractions of known carbohydrate and N content, free of cytoplasm, and prepared under mild conditions excluding acidic pH and heat. Antigenic group specificity was found in the PGM and sMG fractions and both gave strong precipitin reactions. The antigens' potency in cutaneous hypersensitivity and in the MIF assay renders them suitable for immunocompetence testing and for further studies of antigenic structure related to cellular immunity.

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