INFECTION AND IMMUNITY, May 1977, p. 690-700
Vol. 16, No. 2

Printed in U.S.A.

Antigenic, Chemical, and Structural Properties of Cell Walls of *Histoplasma capsulatum* Yeast-Form Chemotypes 1 and 2 After Serial Enzymatic Hydrolysis

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Received for publication 19 October 1976

Cell walls of *Histoplasma capsulatum* yeast-form chemotypes 1 (*chem 1*) and 2 (*chem 2*) treated sequentially with several polysaccharolytic enzymes and Pronase yielded soluble, nondialyzable polysaccharides at each step, which were analyzed for monosaccharides, protein composition, and serological activity. Polysaccharide recovered after digestion of *chem 1* walls with β(1→3)-glucanase contained glucose > mannose > glucosamine > galactose. This fraction (*chem 1* βG_1) was analyzed by polyacrylamide gel electrophoresis and contained a component having an apparent molecular weight of 120,000. The *chem 1* βG_1 fraction was reactive in immunodiffusion (ID), producing an immune precipitate not identical to the H and M factors of histoplasmin. In a side-by-side ID comparison with extracts of *chem 2*, the *chem 1* βG_1 antigen contained an additional determinant not found in *chem 2* extracts when tested with goat antiserum to *H. capsulatum*. Therefore, the *chem 1* antigen gave preliminary ID evidence of antigenic group specificity. A chemical difference observed was the absence of glucosamine from *chem 2* polysaccharide. In complement fixation (CF) tests, 9 of 17 sera from human histoplasmosis patients reacted with *chem 1* βG_1, but some cross-reactivity with sera of patients with other systemic mycoses occurred. The immunoelectrophoretic patterns of *chem 1* wall-derived polysaccharides showed a marked shift in mobility after Pronase digestion, implying the presence of covalent peptides. The ultrastructural appearance and serological activity of intact walls and enzyme-resistant mural cores were also studied. The surface of the mural cores of both chemotypes was perforated and frayed. In shadow-cast preparations both fibrillar and globular areas persisted in the mural cores. The CF end point serum dilutions showed an increase after α- and β-glucanase extractions of *chem 2* walls and fourfold reduction after Pronase digestion. The mural cores of both chemotypes were still reactive in CF tests and retained some ability to bind fluorescent antibody. The *chem 1* mural core reacted with specific fluorescein-labeled *H. capsulatum* antiglobulins produced by adsorption with *Blastomyces dermatitidis*, thus indicating at least partial retention of *H. capsulatum*-specific factors. The presence of galactose, mannose, and glucose was detected in the mural cores as well as enriched levels of amino sugar, despite exposure to chitinase.

The ability of polysaccharolytic enzymes and a protease to effect 50 to 60% lysis of cell walls of *Histoplasma capsulatum* yeast forms was the subject of earlier work from this laboratory in which walls were hydrolyzed with a series of enzymes consisting of α(1→3)-glucanase, β(1→3)-glucanase, Pronase, and chitinase (23). In addition to monosaccharide and amino acid products, small amounts of nondialyzable polysaccharide were released during the procedure, which was termed serial enzymolysis. The two serotypes studied showed differences in enzyme susceptibility, which served to identify them with respect to chemotype. An objective of the present work was to assay polysaccharide released by enzymolysis from walls of both chemotypes for serological activity, monosaccharide composition, and protein content. The effect of enzymolysis on the antigenicity of the cell wall was followed by comparing the fluorescent-antibody (FA) and complement fixation (CF) reactions of the intact as well as the partially digested walls with the resistant residue, referred to below as the mural core. Furthermore,

the effect of serial enzymolysis was studied to determine whether alterations in the mural ultrastructure were associated with changes in FA and CF reactivities. The antigenicity of soluble polysaccharides and particulate glucanase-extracted cell walls was studied with human histoplasmosis case sera to determine whether such defined reagents might be of value in the serodiagnosis of this disease.

It is useful at the outset to summarize how the terms chemotype and serotype have been applied to cell wall components of *H. capsulatum*. Yeast-form chemotypes were first described by Domer (4), based in part on earlier work of Pine and Boone (22). These chemotypic categories received substantial support from the recent results of Kanetsuna et al. (11). The cell wall type designated chemotype 1 (*chem 1*) had an elevated chitin content, and $\alpha(1\rightarrow3)$-glucan was absent, whereas in *chem 2* a reduced level of chitin was observed, and $\alpha(1\rightarrow3)$-glucan occurred as a major mural component (4, 11). In the serial enzymolysis experiment (23), the walls of serotype 1,2,3 lacked $\alpha(1\rightarrow3)$-glucan and had a chitin content of 30.3%, which thus indicated the isolate was *chem 1*. In contrast, walls obtained from a strain bearing serotype 1,4 determinants were lysed to the extent of 27.3% by $\alpha(1\rightarrow3)$-glucanase and had a reduced chitin content (7.5%), fitting the criteria for *chem 2*.

The discovery of four serotypes among isolates of *H. capsulatum* was made on the basis of FA cross-staining and adsorption studies (12, 15). The significance of serotypes for the immunodiagnosis of histoplasmosis is that serotypic factors 1 and 4 are shared with *Blastomyces dermatitidis*, whereas factors 2 and 3 are specific for *H. capsulatum*. It would be advantageous to remove polysaccharide from the cell wall in a manner that would preserve the serotype determinants for subsequent isolation and chemical characterization.

After each stage of serial enzymolysis (23), portions of the cell walls resistant to lysis were withdrawn, and the soluble, nondialyzable material was also recovered. These fractions and the mural core were the starting materials for the analyses presented below.

MATERIALS AND METHODS

Antigens. Soluble, nondialyzable products of serial enzymatic hydrolysis obtained at each stage of extraction of yeast-form cell walls of *H. capsulatum* A811 (serotype 1,2,3; *chem 1*) and of *H. capsulatum* 105 (serotype 1,4; *chem 2*) and the corresponding mural cores (23) were analyzed. Fractions were designated according to the notation shown in Table 1. The cultures were obtained from the Mycology Division, Center for Disease Control, Atlanta, Ga.

Chemical analyses. Volatile derivatives were prepared and analyzed in a Perkin-Elmer 990 gas chromatograph to determine monosaccharide ratios present in cell fractions. For neutral sugars, vials containing 1-mg samples received 0.5 ml of 2 N trifluoracetic acid and then were sealed under N_2 and hydrolyzed at 100°C. Soluble fractions were heated for 2 h, whereas insoluble samples required 4 h; then acid was removed in vacuo over soda lime, and the corresponding alditols were produced by addition of $NaBH_4$. Conditions for the reduction and preparation of *n*-butyl boronic acid ester derivatives were those of Eisenberg (5). A column (3 m in length and 4 mm in diameter) of 3% OV17 (50% phenyl-substituted phenylmethyl silicone) and coated on Gas-Chrom Q was programmed from 150 to 200°C; detection was by flame ionization, quantitation was by a discontinuous integrator, and the internal standard was fucose. Amino sugar not liberated by chitinase was determined by the Elson-Morgan reaction (2) after acid hydrolysis (100°C, 6 N HCl for 3, 6 or 18 h). Methods for determination of total protein, carbohydrate, N, and P were those used previously (23, 24).

Serology. Eight 9-month-old female goats were used for the production of antisera. Two were injected intravenously once a week with 5×10^7 live 6-day-old yeast-form cells of *H. capsulatum* serotype 1,2,3; two others received 5×10^7 live 6-day-old yeast-form cells of *H. capsulatum* serotype 1,4 once a week. Two were injected intravenously twice a week with 1 mg of yeast-form cell walls (23) of *H. capsulatum* serotype 1,2,3, and two received 1 mg of cell walls of *H. capsulatum* serotype 1,4 twice a week. The live cells and cell walls used for immunization were suspended in 0.85% NaCl. The presence of CF antibodies and precipitins reactive in immunodiffusion (ID) tests with histoplasmin was monitored weekly (8, 13, 14), and a plateau in CF titer of 1:256 was reached in 8 to 9 weeks. The human sera used came from patients with diagnoses of fungus disease; the sera were supplied by physicians and the diagnoses were supported by positive serological findings in both CF and ID tests. In addition, Zell McGee supplied serum of one patient with histoplasmosis seen at the Vanderbilt University Hospital.

For immunoelectrophoresis (IEP), 100-μg samples were placed into wells in layers (5 by 7.6 cm) of 1% agarose, operated at 9 V/cm for 2 h in borate buffer (pH 8.6; ionic strength, 0.025), and developed at 9°C overnight. In the direct FA tests, fluorescein-labeled rabbit antiglobulins to whole formaldehyde-killed yeast-form cells of *H. capsulatum* serotype

TABLE 1. *Notation for designating soluble nondialyzable fractions extracted from H. capsulatum chem 1 and chem 2 walls during serial enzymatic hydrolysis*

Code	Stage of extraction and contact time (h)
βG_1	$\beta(1\rightarrow3)$-glucanase, 5
βG_2	$\beta(1\rightarrow3)$-glucanase, 24
αG_1	$\alpha(1\rightarrow3)$-glucanase, 6
αG_2	$\alpha(1\rightarrow3)$-glucanase, 24
Pr	Pronase, 10

1,2,3,4 were used. Fluorescein-conjugated globulins of normal rabbits served as the control, and, in addition, a conjugate specific for factors 2 and 3 was prepared by adsorption of labeled antiglobulins to factors 1, 2, and 3 with whole yeast-form cells of *B. dermatitidis* (12, 15). The methods used for carrying out the direct FA examinations were those of Kaufman and Kaplan (15). A Leitz SM microscope with a cardioid dark-field condenser was used as well as a Leitz 250 lamp housing equipped with an Osram HBO-200 mercury vapor lamp. A BG12 primary filter and a GG9 barrier filter were used. Photography of immunofluorescence was recorded on Kodak Plus-X film developed in Kodak D76.

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) was performed on one antigenic fraction, *chem 1* BG_1, and on the $\beta(1\rightarrow3)$-glucanase enzyme with which it was extracted from the cell wall. The conditions for electrophoresis in phosphate buffer and 8 M urea were those of Obijeski et al. (21).

Electron microscopy. For ultramicrotomy, lyophilized cell wall pellets were stained with 2% aqueous potassium permanganate (20) and embedded in Epon 812 (19). Sections were cut on a Sorvall MT-2B ultramicrotome, poststained with lead citrate (25), and viewed in a Philips EM 30V electron microscope. Negative staining of wall components was accomplished with saturated aqueous uranyl acetate or 2% phosphotungstic acid. Other preparations were shadowed with platinum-paladium.

RESULTS

Chemical composition of walls, mural cores, and soluble fractions released during serial enzymolysis. Since glucose, mannose, galactose, and glucosamine were known to be components of the *H. capsulatum* cell wall (4, 6, 11, 16, 22), the proportion of each of these monosaccharides in the products of serial enzymolysis was determined in order to assess their possible roles as antigenic determinants (Table 2). Although the enzyme-resistant mural core of *chem 1* comprised only 18.7% of the starting wall weight (23), the initial and final proportions of glucose, mannose, and galactose remained similar (Table 2). The level of amino sugar, initially 29.8%, was elevated to 34.9% in the core, despite extensive chitinase treatment. In the instance of *chem 2*, the core as compared with the intact wall was poorer in glucose content, but richer in amino sugar. The soluble, nondialyzable polysaccharide released at each stage of serial enzymolysis amounted to a cumulative total of 12.1% of *chem 1* and 9.3% of the *chem 2* wall (23). These fractions contained galactose, mannose and a predominant amount of glucose (Table 2). In a fraction that was found to be antigenic *chem 1* βG_1, the ratio of mannose to glucose to galactose was 6.6:13.3:1. Because of the differences seen in IEP patterns of mural antigens (see Fig. 2), which are explained in detail below, it was noteworthy that the monosaccharide distribution found in *chem 1* βG_1 recovered after 5 h of $\beta(1\rightarrow3)$-glucanase digestion differed from that found in *chem 1* βG_2 recovered after 24 h of glucanase digestion. In interpreting the protein content data shown in Table 2, it is important to note that the total protein in soluble extracts was the sum of protein indigenous to the cell wall and enzyme protein that was used to effect serial enzymolysis. In Table 2, the contribution of extraneous enzyme proteins was

TABLE 2. *Monosaccharide and protein composition of H. capsulatum yeast-form cell walls, mural cores, and soluble, nondialyzable polysaccharide products of serial enzymolysis*

Cell wall or fraction	Dry wt yield (mg)	% of dry wt[a]				
		Man	Glc	Gal	GlcN	Protein
chem 1						
Cell walls	400	3.3[b]	39.7[b]	1.1[b]	29.8[c]	14.5[d]
Mural core	59.5	5.8	39.6	1.7	34.9	9.3[d]
βG_1	28.8	17.8	35.8	2.7	5.4	11.0[e]
βG_2	14.6	31.9	33.5	4.1	7.9	6.5[e]
Pr	3.7	3.8	11.5	9.5	1.7	13.4
chem 2						
Cell walls	500	2.9	72.5	1.3	16.8	9.4
Mural core	51.5	3.5	60.3	1.3	25.4	2.1
αG_1	14.0	5.3	27.2	6.6	0	8.4
αG_2	11.8	10.5	29.8	3.3	0	8.6
βG_1	9.4	25.5	18.6	5.8	Trace	0.9

[a] Abbreviations: Man, mannose; Glc, glucose; Gal, galactose; GlcN, glucosamine.
[b] Gas chromatography as *n*-butyl boronic acid esters.
[c] Elson-Morgan test.
[d] Nessler test.
[e] Folin phenol.

subtracted to give indigenous protein values. In addition to neutral sugars, soluble fractions derived from *chem 1* walls contained glucosamine, which was totally released after 3 hr of hydrolysis, but not detected in fractions of *chem 2* hydrolyzed for 3, 6, and 18 h. Elemental analysis of the *H. capsulatum* cell walls and the mural core for total P showed an increase in the core of *chem 1* (intact wall = 0.04% P; core = 0.14% P); however, the result obtained with the *chem 2* core was inconclusive.

ID and IEP. The reactions of wall-derived soluble fractions of both chemotypes with serum from a goat injected with live yeast-form cells and with serum from a human patient with histoplasmosis are shown in Fig. 1 and give some evidence of serotype differences. Serum of a goat immunized with *chem 1* yeast-form cells reacted with *chem 2* extracts, and in addition to this common factor the serum formed an immune precipitate that was related only to *chem 1* antigens (Fig. 1B). The occurrence of type-specific factors in extracts *chem 1* βG_1 and βG_2 was preliminary evidence that serotype markers up to now recognized only with FA could be detected by use of material extracted from isolated cell walls. The relationship of *chem 1* βG antigens and those present in histoplasmin is shown in Fig. 1A. Lines of intersection between the H antigen of histoplasmin (8, 9) and *chem 1* βG components were detected, indicating that cell wall-derived antigens prepared in this manner did not contain H or M factors.

The IEP patterns obtained in the reaction of *chem 1* antigens and goat antiserum are shown in Fig. 2. Each fraction was heterogeneous, and there were differences among βG_1, βG_2, and Pr antigens in the amounts and mobilities of the components. The first fraction released from the *chem 1* walls, βG_1, contained only one major anodic antigen, whereas an additional weakly cathodic component occurred in βG_2. The pattern caused by the Pr extract was different from that due to βG_1, βG_2 due to an additional strongly cathodic component. This shift in mobility after digestion with Pronase may have altered the size and net charge of the cell wall antigen. A human histoplasmosis serum that contained precipitins reactive with *H. capsulatum* H and M antigens (8, 9) was employed in IEP to compare the mobility of these factors with that of cell wall-derived antigens. Histoplasmin components migrated toward the anode in compact zones, and, on the basis of the ID pattern and electrophoretic mobility, it was concluded that the cell wall antigens were not identical to the H or M factors.

CF. Sera from human patients with histoplasmosis, blastomycosis, and coccidioidomycosis (Table 3) were screened in CF tests with polysaccharide extracts *chem 1* βG_1 and βG_2. The soluble extracts were less sensitive (9/17, 8/14) as antigens than were particulate glucanase-extracted cell walls (14/17), and the Pr extract was not reactive. The ability of *chem 1* walls to react in the CF test with goat antiserum and with serum from a human with histoplasmosis was not diminished by serial enzymolysis. After α(1→3)-glucanase treatment, *chem 2* walls were more reactive in the CF test, but Pronase treatment led to a fourfold decrease in end point serum dilution (Table 4) with human serum, but not with goat serum.

FA staining of intact and enzymatically treated cell walls. Intact cell walls of the *chem 1* strain belonging to serotype 1,2,3 were stained with conjugated *H. capsulatum* antibodies to a 1,2,3,4 serotype. After β(1→3)-glucanase treatment, staining was a little brighter, but Pronase treatment of β(1→3)-glucanase-extracted *chem 1* walls decreased the immunofluorescence. The mural core still stained, but the intensity of fluorescence was low. The *chem 1* core also stained with specific conjugated *H. capsulatum* antibodies produced by adsorption with the yeast form of *B. dermatitidis*, which indicated the persistence of an *H. capsulatum*-specific factor. *chem 2* walls after α(1→3)-glucanase treatment stained more brightly than untreated walls, which was unexpected since α(1→3)-glucanase removed 27% of the wall dry weight. A decline in staining occurred after Pronase and chitinase extractions, but the *chem 2* core still was capable of reacting with labeled *H. capsulatum* antibodies.

SDS-PAGE of a cell wall-derived antigen. Since significant N was associated with the polysaccharide antigenic fractions, it was of interest to examine this preparation to estimate its molecular size and the number of separable protein or glycoprotein components (Fig. 3). A pattern of four proteins was observed in the *chem 1* βG_1 fraction, three of which coincided in mobility with the ones seen in a concentrated sample of β(1→3)-glucanase. A line nearest the origin did not occur in the enzyme sample and was presumed to be the antigen. A comparison of the mobility of this line with standard-size marker proteins (β-galactosidase and phosphorylase *a*) indicated a molecular weight of 120,000. Carbohydrate moieties were implied in the case of all four lines that reacted positively with the periodic acid-Schiff reagents. Toward the distal portion of the antigen-containing gel, a diffuse staining area was observed, which may also represent a cell wall component.

Electron microscopy. When viewed in nega-

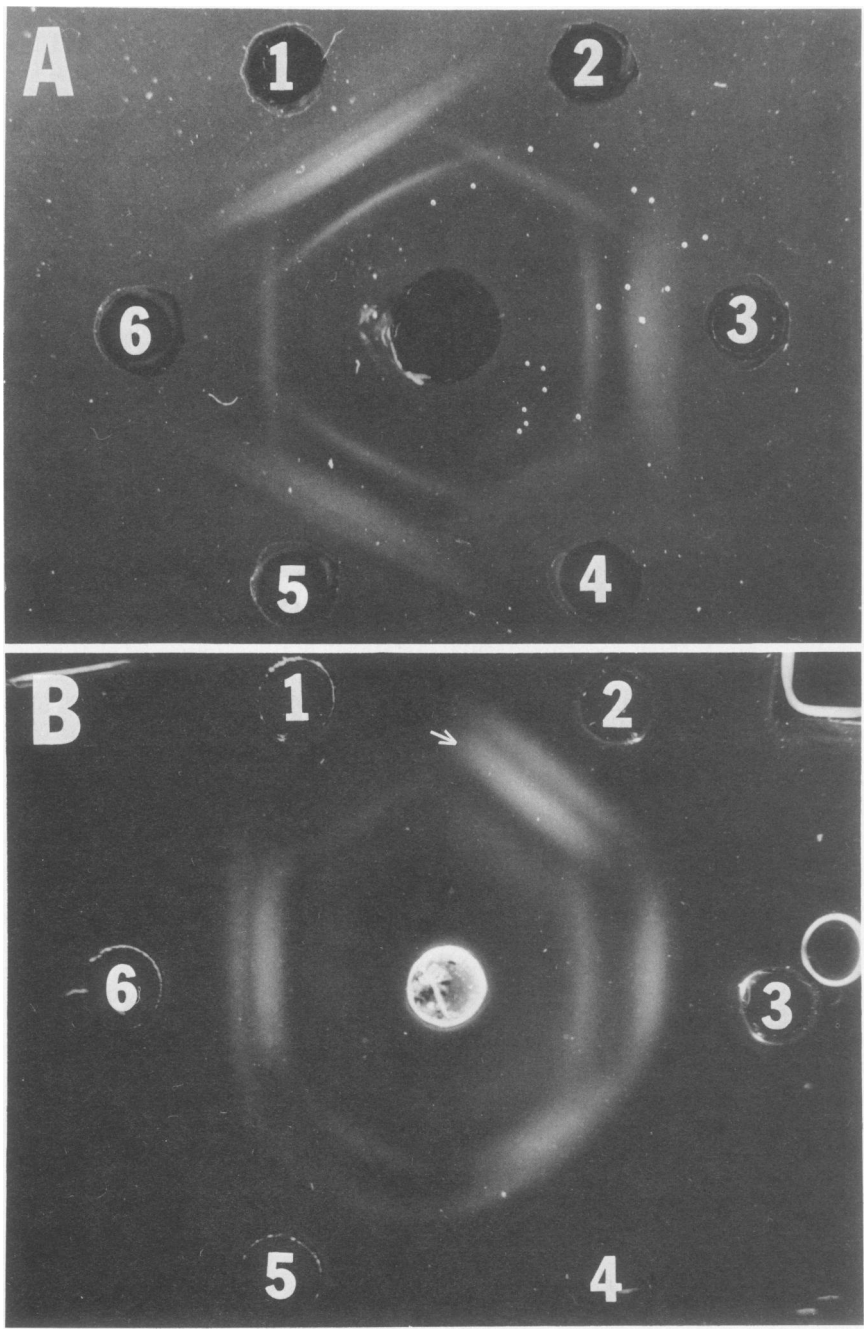

FIG. 1. *ID patterns of H. capsulatum cell wall-derived antigens versus sera of human and goat origin. (A) Center well: human serum from a patient with histoplasmosis; outer wells: 1, 3, and 5, histoplasmin; 2, chem 1 βG_1; 4 and 6, chem 1 βG_2. (B) Center well: serum of goat immunized with live chem 1 yeast-form cells; 1, chem 2 αG_1; 2, chem 1 βG_1; 3, chem 2 αG_2; 4 and 6, chem 1 βG_2; 5, chem 2 βG_1. Arrow refers to immune precipitate present in chem 1 and absent from chem 2 antigens in adjacent wells.*

tively stained preparations, the enzyme-resistant mural cores of both chemotypes were thinned out and had pocked, cratered regions, in contrast to the smooth, denser appearance of the untreated walls (Fig. 4). No clear-cut differences were observed between chemotypes. There were subtle differences in texture; for example, the negatively stained *chem 2* mural core had a very prominent pocked surface. Whereas the *chem 2* wall was shattered by a Braun homogenizer, *chem 1* walls had a greater tendency to maintain an elliptical shape after mechanical disruption. Shadow-casting made globular patches and underlying fibrils visible in both walls and mural cores (Fig. 5). In *chem 1* walls, globular patches seemed more prevalent than in *chem 2* walls, and a coarser network of fibrils occurred in *chem 1*. Mural cores of both chemotypes showed the persistence of globular patches coexisting with fibrils. The fibrils in the *chem 1* mural core were more clearly delineated and thicker than those of *chem 2*.

DISCUSSION

The process of unraveling the antigenic structure of *H. capsulatum* cell walls was aided by serial enzymolysis of two strains that differed in serotype and chemotype. It was possible to recover relatively small amounts of soluble polysaccharide fragments from the enzyme digest that were antigenically reactive with goat antiserum to *H. capsulatum* and sera from humans with histoplasmosis. The insoluble wall residue after enzymolysis, i.e., the mural core, also bore antigenic determinants detected in CF and FA tests. Of the soluble extracts, the soluble polysaccharide extracted from the cell wall of *chem 1* at the $\beta(1\rightarrow3)$-glucanase stage (*chem 1* βG_1) was antigenically significant since it was not identical to H or M factors of histoplasmin and appeared to contain a sero-

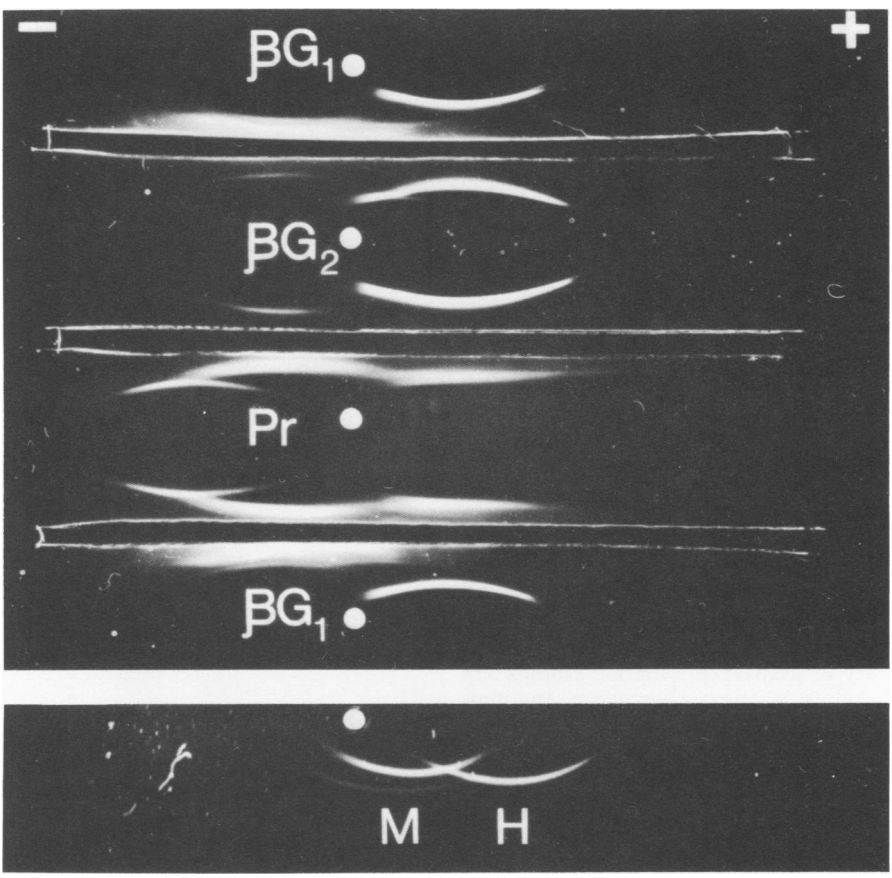

FIG. 2. *IEP of chem 1 antigens (wells). Serum of a goat was immunized with live chem 1 yeast-form cells (troughs). H and M factors of histoplasmin are shown with respect to a rabbit antiserum (trough).*

TABLE 3. *Reactivity in the CF test of solubilized antigens and cell wall residues of H. capsulatum after β(1→3)-glucanase treatment versus sera of patients with histoplasmosis and other mycotic infections*

Source of serum with respect to disease of patient	Antigen of *chem 1*		
	Soluble[a]		Insoluble
	βG_1	βG_2	GECW[b]
Histoplasmosis	9/17[c]	8/14	14/17
Blastomycosis	2/4	2/4	4/4
Coccidioidomycosis	1/5	2/4	3/5

[a] A 5-μg dose.
[b] GECW, β(1→3)-glucanase-extracted cell wall residue (1.6-μg dose).
[c] Number of positive reactors among total sera tested.

TABLE 4. *End point serum dilutions in the CF test, using H. capsulatum chem 2 cell walls and particulate fractions resistant to serial enzymolysis*

Cell wall antigen[a]	Anti-*H. capsulatum* serum titer^{-1}	
	Human	Goat[b]
Pretreatment walls	32	64
α(1→3)-glucanase resistant	64	64
β(1→3)-glucanase resistant	128	64
Pronase resistant	32	128
Mural core	32	64

[a] Antigen dose, 0.8 μg.
[b] Anti-*chem 2*.

type-specific component revealed by ID. In a side-by-side ID comparison with extracts of the *chem 2* (1,4 serotype) isolate, the *chem 1* βG_1 antigen contained an additional determinant not found in *chem 2* extracts when analyzed with goat antiserum produced against the *chem 1* strain. There have been no previous demonstrations of serotype specificity in precipitin tests with antigens of *H. capsulatum*. The initial description of the 1,4 serotype of *H. capsulatum* gives the geographic distribution as being mainly in Central America. It was also found in the United States, but less frequently than serotype 1,2,3 (12). Further studies are needed to relate serotype, chemotype, and mating type (18) to the origin of the isolate, whether from soil or from the human host.

The *chem 1* βG_1 and βG_2 extracts were separated into a maximum of three antigenic components by IEP, and the sugar composition varied with increased time of digestion with β(1→3)-glucanase. Several human sera that were reactive in CF tests with whole yeast-form *H. capsulatum* cells and in ID tests with histoplasmin were screened for reactions with *chem 1* βG_1, βG_2, and β(1→3)-glucanase-extracted *chem 1* walls. The results indicated that 57% of the panel reacted with βG_1, 60% with βG_2, and 82% with the particulate antigen. Cross-reactivity was most frequent in the case of the particulate fraction and less evident with *chem 1* βG_1. This screening of human sera was preliminary in nature. A thorough evaluation of these and other *H. capsulatum* antigens with a panel of serial serum specimens from humans with acute, disseminated, and chronic histoplasmosis is warranted to see whether the sequence of precipitins directed against different purified histoplasmal components correlates with the clinical status of the patient.

The presence of glucose, galactose, mannose, glucosamine, and 11.0% protein was detected in the *chem 1* βG_1 extract, and the structural relationship of the sugars present is being

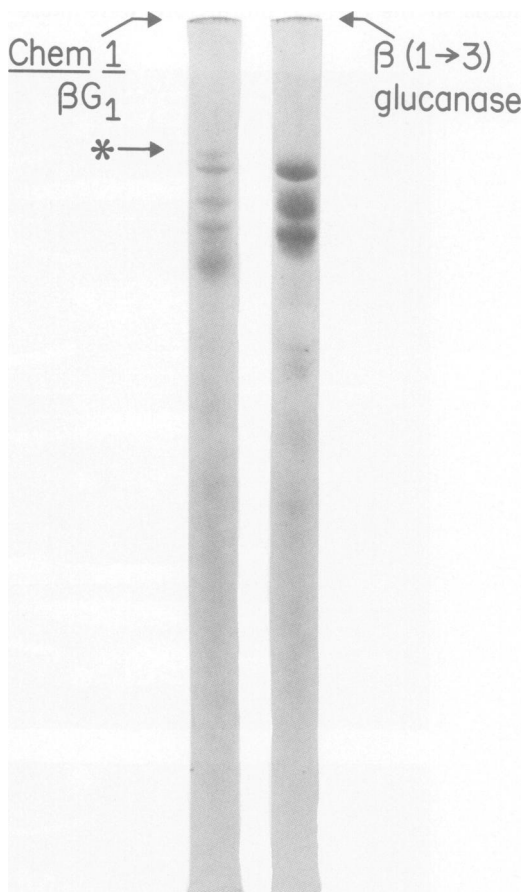

FIG. 3. *SDS-PAGE of chem 1 βG_1 antigenic fraction of H. capsulatum cell walls (400 μg) and concentrated sample of β(1→3)-glucanase (100 μg). Asterisk shows presumed location of antigen component. Coomassie blue G stain.*

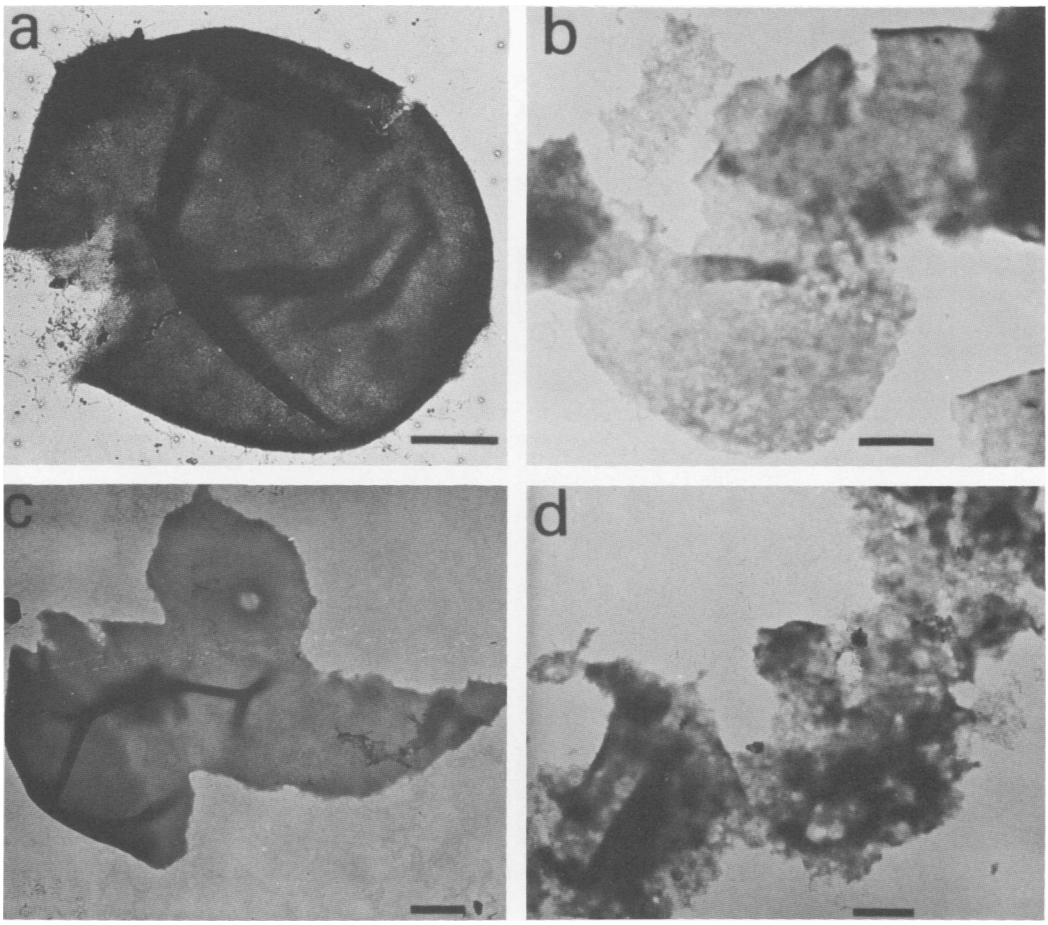

FIG. 4. *Negatively stained cell walls (a and c) and mural cores (b and d) of H. capsulatum chem 1 (a and b) and chem 2 (c and d). Marker = 1 μm.*

sought. The molecular size estimate of 120,000 obtained by SDS-PAGE in the instance of *chem 1* βG_1 should assist in the choice of conditions for further separations. Every soluble antigenic fraction and the enzyme-resistant mural cores contained galactose and mannose in addition to glucose. Galactomannan has been described as an antigen of *H. capsulatum* (1, 24), and in the present study *chem 1* walls were found to be richer in it than those of *chem 2*. It appeared that this heteroglycan may contribute to the antigenicity of all the fractions described above. Previous methods for extracting galactomannan with dilute alkali may not conserve peptide bridges, particularly in view of the well-documented alkaline cleavage of *O*-glycosidic linkages to hydroxylamino acids of the peptide moiety of glycoproteins (7). For these reasons it is important to characterize the structure of the fractions *chem 1* βG_1 and βG_2 to determine whether covalent bonds link glucose oligosaccharides to galactomannan and peptide components. Material extracted at the Pronase stage was found to be antigenic in IEP. The apparent lack of CF activity may have been due to traces of Pronase sufficient to inactivate complement components, making it advisable in the future to try immobilized Pronase. A minimal role of the peptide moiety of *H. capsulatum* cell walls in the antigenic structure was inferred from the observations that Pronase-resistant walls were capable of binding FA and that the soluble fraction of *chem 1* released at the Pronase stage of digestion was resolved into three components in IEP. The only evidence of an antigenic role for the peptide portion of the walls studied was the fourfold reduction of CF end point serum dilution of walls of *chem 2* after Pronase digestion. Although the soluble cell wall-derived antigens were referred to as polysaccharide, considerable protein was associated with them (Table 2). The existence of

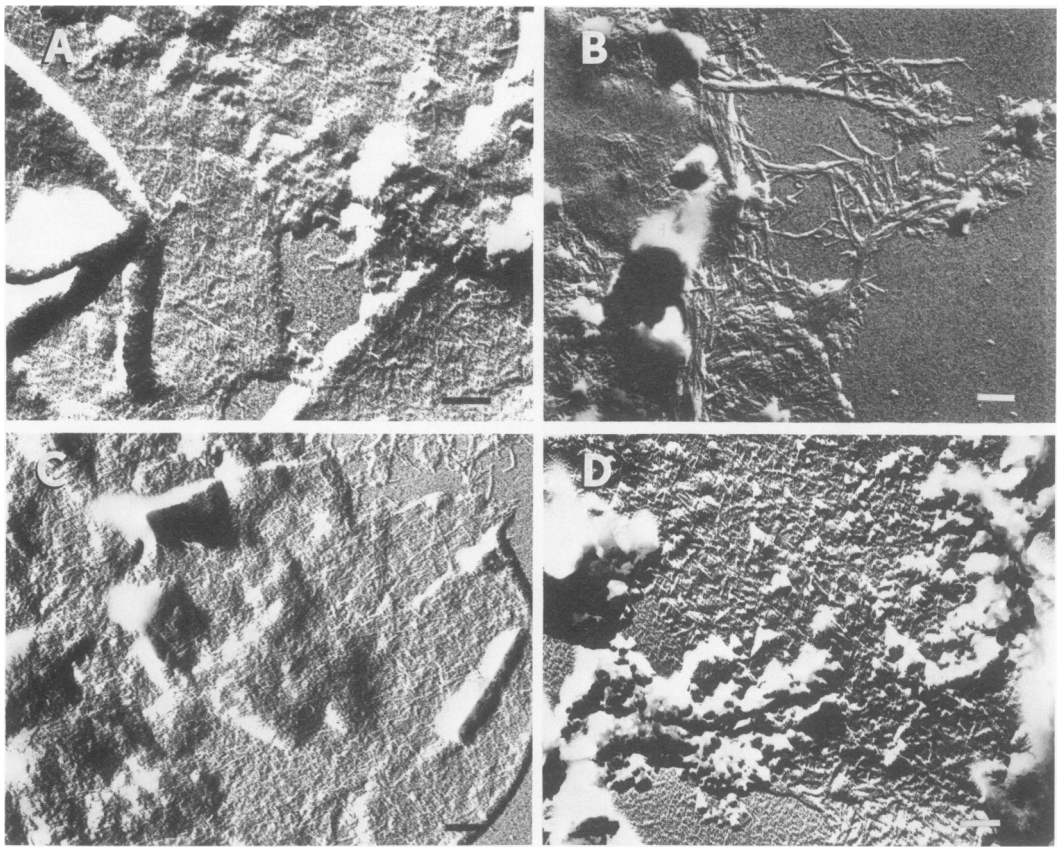

FIG. 5. *Shadow-cast cell walls (A and C) and mural cores (B and D) of H. capsulatum chem 1 (A and B) and chem 2 (C and D). Marker = 0.25 μm.*

peptidopolysaccharides was given credence by the staining reactions of the *chem 1* βG_1 extract during SDS-PAGE.

The occurrence of glucosamine in extracts of *chem 1*, but not of *chem 2*, led to the speculation that this sugar may have a role as a serotype-specific hapten. A previous study also implicated glucosamine as a significant component of ethylenediamine extracts of *H. capsulatum* cell walls (6). In the present work, the complete liberation of glucosamine during short-term acid hydrolysis from soluble fractions suggested that the sugar occurred in a terminal position. A large segment of the mural core was composed of amino sugar apparently resistant to chitinase. Extraction of $\alpha(1\rightarrow3)$-glucan to the extent of 27.3% of the *chem 2* mural dry weight (23) did not result in any detectable decrease in FA and CF reactions, and thus it was not possible to ascribe an antigenic role to $\alpha(1\rightarrow3)$-glucan by this indirect means.

The enzyme-resistant mural cores that retained some antigenicity merit further study to define what other specific enzyme-mediated changes would result in additional depolymerization and loss of FA and CF activities. In both *chem 1* and *chem 2* cores there was an enrichment in galactose, mannose, and especially amino sugar, despite prolonged chitinase digestion. Among the linkages to be considered as resistant to the serial enzymolysis procedure are those of the chitosan variety and also phosphodiesters. In an analogous situation the mannan core of bakers' yeast resistant to exo-α-mannanase was found to be enriched in phosphodiesters (3). In the *chem 1* mural core there was a 3.5-fold increase in total P as compared with the walls before enzymolysis.

chem 1 and *chem 2* walls were monitored at each stage of serial enzymolysis by CF, FA, and electron microscopy. Although *chem 1* walls lost 61.5% of the initial dry weight as monomer and polysaccharide as a result of enzyme treatment (3), there was no evident decrease in CF activity in the mural core. After $\beta(1\rightarrow3)$-glucanase extraction, the immunofluorescence of

chem 1 walls was brighter than that of intact walls. It was hard to reconcile this observation with the extraction at the β(1→3)-glucanase stage of fragments that were antigenic in CF and ID tests. One possible explanation was that the digestion of β(1→3)-glucan exposed more surface area for binding antibody and compensated for the loss of antigen through solubilization. Electron microscopic observation supported this view since a loosening of fibrils was observed in enzymatically treated walls. The residual immunofluorescence persisted in the *chem 1* mural core stained with anti-*H. capsulatum* globulins adsorbed with *B. dermatitidis* yeast-form cells. This immunofluorescence indicated that determinants responsible for the markers Kaufman and Blumer (12) designated as 2,3 were at least partly retained. In the case of *chem 1* walls, an increase in CF reactivity was seen after extraction with β(1→3)-glucanase and Pronase. Since this effect was observed with sera from both an immunized goat and a human histoplasmosis case, it was given some credence. It is not known whether this increased CF activity was due to an unmasking of different antigenic determinants or the result of smaller particle size that left more surface area for antibody binding.

The conclusion drawn from electron microscopy of the *H. capsulatum* wall was that it is made up of a combination of tightly woven fine fibrils covered with a discontinuous or patchy layer of cottony, globular elements. Enzymolysis eroded craters in the wall matrix and removed some globular material, but the fibrils persisted even after chitinase treatment. Unlike the effect in bakers' yeast (17), β(1→3)-glucanase did not remove the globular patches. In contrast to the walls of *H. farciminosum*, two size classes of fibrils were not detected in the *H. capsulatum* walls of either chemotype (26). In *Neurospora crassa* walls both amorphous and fibrillar regions occurred, but β(1→3)-glucanase and Pronase treatment resulted in an open net of coarse fibers that was not seen in the *H. capsulatum* walls (10).

Electron microscopy was a valuable adjunct to the serological monitoring of enzymolysis. Although the cell wall carbohydrate composition of the two chemotypes differed, the surface texture offered only minor evidence in *chem 1* walls of more globular patches and a coarser underlying layer. Negative staining and shadow-casting gave different information, and both techniques were required since globular patches were visible only in shadow-cast preparations, whereas negative staining revealed cratering in the mural cores. The persistence of globular patches and fibrils in the mural cores coincided with the occurrence of galactose, mannose, and glucosamine. It is tempting to speculate that the globular material is galactomannan adhering to residual chitin fibrils.

ACKNOWLEDGMENTS

We would like to thank J. F. Obijeski for conducting the gel electrophoresis studies and Maxine Clark for assistance with CF tests.

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