JOURNAL OF BACTERIOLOGY, June, 1966
Vol. 91, No. 6
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Antigenic Properties of the Cell Wall and Other Fractions of the Yeast Form of *Histoplasma capsulatum*

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Received for publication 11 December 1965

ABSTRACT

PINE, LEO (Communicable Disease Center, Atlanta, Ga.), CLARENCE J. BOONE, AND DAVE McLAUGHLIN. Antigenic properties of the cell wall and other fractions of the yeast form of *Histoplasma capsulatum*. J. Bacteriol. **91**:2158–2168. 1966.—Yeast-form cells of *Histoplasma capsulatum* were fractionated in an attempt to obtain complement-fixing antigens with greater specificity than those of the currently used whole yeast form. No specific fraction was isolated. Rupture of the whole cell produced soluble and insoluble highly antigenic fractions. The soluble fractions rapidly lost antigenicity. Marked reduction of activity of some particulate fractions was also observed. The purified cell wall, stripped of its protein components, was antigenic, relatively stable, and demonstrated low cross-reactivity with heterologous sera. All fractions cross-reacted with sera from cases of North American blastomycosis to a greater degree than did the whole yeast-form antigen of *H. capsulatum*. The production of certain cellular fractions varied with the strains used. In complement-fixation tests, insignificant differences in specificity and antigenicity were found with whole-cell antigens from 13 *H. capsulatum* strains as well as with purified cell wall fractions prepared from 2 of these strains.

Histoplasmin and whole yeast-form *Histoplasma capsulatum* cells are two antigens commonly used in complement-fixation (CF) tests for histoplasmosis. Both antigens, though useful, demonstrate cross-reactivity with sera from patients having blastomycosis and coccidioidomycosis (3, 4). A specific antigen would be of great value for the diagnostic laboratory.

Labzoffsky, Fischer, and Hamvas (10) attempted to obtain such an antigen by fractionation of pyridine-killed yeast-form cells. These workers isolated eight antigenic fractions, of which seven were soluble. All eight fractions were tested against hyperimmune sera obtained from rabbits immunized with viable or killed yeast-form antigens of *H. capsulatum* and *Blastomyces dermatitidis*, and mycelial elements of *Coccidioides immitis*. Three of the eight *H. capsulatum* fractions isolated (IV, VI, and IX) reacted only with the *H. capsulatum* antisera. Other workers, however, tested fractions such as crude cell walls (21), the soluble material released by sonic treatment (23), or whole-cell antigens killed with phenol, formaldehyde, or Merthiolate (Lindberg, Ph.D. Thesis, The Univ. Michigan, Ann Arbor, 1950). Markowitz (11), on the other hand, extensively studied the soluble antigens released during growth by strains of *H. capsulatum*, and made considerable progress in the separation and characterization of these antigens. However, no attempt to obtain specific fractions was reported. With the exception of the work by Labzoffsky et al. (10), no preparation has been described as being more specific than the whole yeast-form cell.

In addition to studying the effect of media and chemical treatment on the relative antigenicity and specificity of different yeast-form strains of *H. capsulatum*, we also were concerned with the isolation and the immunological and chemical characteristics of antigens obtained by previously described methods or by the physico-chemical procedures used here. Of these antigens, particular emphasis was placed upon the characteristics of purified cell walls.

MATERIALS AND METHODS

Cultures. *H. capsulatum* strains A811, L261, 28, 69, 1073, 6571, 6602, 6617, 6619, 6621, 6622, 6623,

6624, and F851 were used. These cultures have been described previously (17, 22, 23).

Media and cultivation. Four media were used: blood-glucose-cystine (BGC) medium of Campbell (2), Brain Heart Infusion (BHI) agar or broth (Difco), casein hydrolysate cysteine starch (CCS) medium of Pine and Drouhet (15), and a synthetic medium (Syn) in which the casein hydrolysate of the CCS medium was replaced by L-aspartic and L-glutamic acids. Broth cultures were grown at 37 C in 250-ml Erlenmeyer flasks with 50 ml of medium, or in 50-ml flasks with 10 ml of medium. They were shaken on a rotary shaker at 120 cycles per min. Agar cultures were prepared in 32-oz prescription bottles that were layered on the flat side with 75 ml of agar medium. Each bottle was inoculated with cells from a single 48- to 72-hr culture grown on an agar slant of CCS medium. The bottles and flasks were incubated for 7 days at 37 C, and then stored at 5 C until the cells were harvested.

Cell treatments. Cells were collected by centrifugation or removed from the surface of the agar with the rotating wire procedure previously described (14). They were washed three times in 0.001 M phosphate buffer (*p*H 8.0), resuspended in the *p*H 8.0 buffer to two to three times their packed volume, and then treated according to the procedures given for specific experiments, or sonically treated with a Branson Systems 100-w, 20 kc sonifier at the no. 8 amperage setting. The container for the cells was cooled by a fast-running jet of ice-salt water mixture for periods of 1 to 2 min between alternate 1-min periods of sonic treatment. The temperature was maintained at between 3 and 10 C.

After sonic treatment, the cell residues were treated as described in the specific experiments. In other experiments, cell residues were treated with pepsin or trypsin as described by Cummins and Harris (6). Protein determinations, by use of optical density (OD) measurements at 280 mμ at various time intervals, showed that the breakdown of the cell protein of strain F851 with trypsin was complete at 24 hr; however, all others still showed continuing release of amino acids at approximately the initial rate. All enzymatic reactions were nevertheless terminated at 30 hr by centrifugation and triple washing of the cell residue with distilled water.

After protein digestion, the cell residues were hydrolyzed with sulfuric acid as described by Cummins and Harris (6), and analyzed for the following substances as described. Reducing sugars were determined by the method of Park and Johnson (13); polysaccharides by the anthrone procedure of Scott and Melvin (24); total hexoses and polysaccharides by the cystein-sulfuric acid reaction of Dische (7); hexosamines by the Elson-Morgan reaction as modified by Belcher, Nutten, and Sambrook (1); *n*-acetyl hexosamine by the method of Reissig, Strominger, and Leloir (19); methyl pentose by the method of Dische and Shettles (8); amino acids by the method of Troll and Cannan (26); and protein by the method of Sutherland et al. (25), with the Folin-Ciocalteau phenol reagent.

Whole yeast-form cells of strain F851 were extracted with various solvents as follows. Suspensions (1 ml) of washed, viable, yeast-form cells were placed in 0.5% formalin, formamide, 90% phenol solution, glacial acetic acid, pyridine, or toluene, and were incubated for 24 hr at 5 C. Toluene suspensions were shaken vigorously at various times during the incubation period. In addition, 5 ml of a heavy suspension of cells was squirted with force into 100 ml of ice-cold acetone, incubated 24 hr at 5 C, filtered, washed three times with ether, air-dried, and resuspended in distilled water. Another portion of the cell suspension was placed in 50% urea solution and incubated 4 days at 5 C. All suspensions were then washed at least five times to remove all traces of solvent, and resuspended in distilled water. Merthiolate was added to the cell suspension to give a final preservative dilution of 1:10,000, and the suspensions were stored at 5 C.

Chromatography. Sephadex G-50 (medium) columns (22 by 300 mm) were prepared by use of 0.001 M phosphate buffer (*p*H 8.0). The solvent was passed through the column at a rate of 16 to 18 drops per min. The eluate was passed through an L.K.B. Uvichord Optical Unit, and the absorption at 254 mμ was automatically recorded; 2.5-ml fractions were collected per tube.

Complement-fixation (CF) tests. The Laboratory Branch CF test procedure (18) developed at the Communicable Disease Center was used to evaluate all the antigens studied. In general, each 5-mg (dry weight) sample of antigen was titrated. When this was not possible, the titer was calculated to 5 mg (dry weight) based on the titration of the known weight of the antigen used. In three different experiments, in which varying concentrations of antigen were titrated and the titer for a 5-mg sample was calculated, agreement was observed with the results obtained by direct CF titration of a 5-mg sample. In a few cases, when variable titers were observed, the variations were insignificant.

RESULTS

Comparison of antigenicity and specificity of yeast-form H. capsulatum strains as antigens in the CF test. Fourteen strains of *H. capsulatum* were grown on CCS agar slants, harvested, and killed with 1:10,000 Merthiolate by incubation for 48 hr at room temperature. Antigen titrations against a single anti-*H. capsulatum* serum were performed with whole cells adjusted on a 5 mg/ml (dry weight) basis. All strains except 6617 and 6621, which were antigenically inactive, had titers ranging from 1:16 to 1:32. Antigen titrations with two sera each from human cases of histoplasmosis and North American blastomycosis showed significant cross-reactions with the heterologous sera. The titers against anti-*H. capsulatum* sera lots 16 and 1 varied in two experiments between 1:16 to 1:32 and 1:512 and 1:1,024, respectively, whereas those against the anti-*B. dermatitidis* sera lots 2623 and 5 varied between 0 to 1:16 and 1:8 to 1:64, respectively. The

results did not reveal any one strain to be more specific in its reactivity with the homologous sera than another.

Effect of media and morphology on chemical composition and antigen titer of several strains of H. capsulatum. The following experiments were performed to determine the effect of media on the chemical composition and antigenicity of different strains of *H. capsulatum*, with the purpose of selecting conditions to give the best yield of antigen. Three groups of strains were studied because of their morphological and antigenic differences. Two strains, A811 and 28, were used because of their reactivity with a large number of sera from cases of histoplasmosis (22), and because the yeast cells of these strains grew primarily as monilial chains rather than as pure yeasts. Two strains, F851 and L261, grew in a pure yeast form, and were reactive in the CF test with human sera. Two other strains, 6617 and 6621, also grew in pure yeast form, but they were not antigenic in the CF test. These strains were grown in liquid shake culture on three different media, and their relative antigenicity and chemical composition were determined. The results are given in Table 1.

Two strains, 6617 and 6621, were consistently nonantigenic in the CF test regardless of the medium on which they were grown; the other strains showed little change in antigenicity with change in the medium, and no significant antigenic differences were observed between them. Nor was there any significant change in the composition of the cells with a change in their growth medium. Although each strain maintained its characteristic carbohydrate-protein ratio from one medium to the other, there was considerable variation in chemical composition between the strains. However, there was no clear correlation among antigenic activity, morphology, and chemical composition. However, strains 6617 and 6621, which were unreactive in the CF test (Table 1), showed a significantly higher percentage of carbohydrate (15 to 31%) per milligram (dry weight) than the other strains. Strains A811 and 28 had relatively low concentrations of proteins (7 to 10%; Table 1), but strain 6617, in its yeast form, had essentially the same percentage of protein (5 to 7%). Likewise, the two antigenic yeast-form strains L261 and F851 were high in protein (31 to 37%), but the nonantigenic strain 6621 was equally high (21 to 29%).

These results emphasized that chemical composition and antigenic reactivity as determined by the CF test were primarily strain characteristics, and were not readily modified by a change of medium. Finally, as suggested by the initial results and excepting strains 6617 and 6621, all the strains of *H. capsulatum* appear to behave similarly as antigens.

Effect of chemical treatment on whole-cell antigens. The results obtained during various experiments suggested that less cross-reactivity occurred with the whole antigen treated with Merthiolate than with fractions obtained from the whole cells. It was thought that a significant degree of specificity might be obtained by retaining the whole-cell structure and extracting with chemical solvents. Strain F851 was grown on the CCS agar medium, and different samples of the cell suspension were treated with formalin, formamide, phenol, acetone, glacial acetic acid,

TABLE 1. *Comparison of chemical composition and antigenic activity of six strains of Histoplasma capsulatum grown on three different solid media*

Strain	3AA[a]				CCS[b]				BHI[c]			
	Total growth	CHO[d]	Protein[e]	Antigen titer[f]	Total growth	CHO	Protein	Antigen titer	Total growth	CHO	Protein	Antigen titer
	mg	%	%		*mg*	%	%		*mg*	%	%	
A811	122	11	7	32	145	9	7	16–32	122	10	7	32
28	120	12	10	64	152	11	7	16–32	165	9	7	32
L261	132	13	37	32	135	13	31	32	168	11	32	16–32
F851	178	6	34	32–64	168	9	32	32–64	165	12	38	32
6621	110	29	31	0	118	15	24	0	72	21	24	0
6617	158	31	7	0	152	24	6	0	82	25	5	0

[a] Synthetic medium containing cysteine, glutamic acid, aspartic acid, and tryptophan. Cells were grown on agar slants incubated 7 days at 37 C.
[b] Casein hydrolysate-cysteine-starch agar.
[c] Brain Heart Infusion agar.
[d] Carbohydrate as determined by the anthrone reaction.
[e] Protein as determined by the Folin-Ciocalteau reagent.
[f] Complement fixation titer, determined with 5 mg (dry weight) of cells.

TABLE 2. *Effect of chemical extraction on the specific reactivity of whole cells of Histoplasma capsulatum strain F851**

Extracted with	Antigen									
	Anti-*H. capsulatum*			Anti-*Blastomyces dermatitidis*			Anti-*Coccidioides immitis*		Normal	
	Serum 1		Serum 16 (expt 2)	Serum 5 (expt 1)	Serum 4135 (expt 2)	Serum 2623 (expt 2)	Serum 8		Serum 1 (expt 2)	Serum 704 (expt 2)
	Expt 1	Expt 2					Expt 1	Expt 2		
Acetic acid	512†	512	256	16	16	16	0	0	0	0
Formalin	512	1,024	512	32	32	±	8	±	0	±
Pyridine	512	1,024	256	32	8	0	0	0	0	0
Acetone	512	1,024	512	32	16	8	0	±	0	0
Urea	512	1,024	512	32	16	0	0	0	0	0
Toluene	256	—	—	32	—	—	0	—	—	—
Phenol	512	—	—	32	—	—	0	—	—	—
Formamide	512	—	—	64	—	—	8	—	—	—

* All suspensions made initially to 5 mg (dry weight) per ml.
† Results expressed as CF titers (reciprocal of dilution).

toulene, and urea as described in Materials and Methods. Box titrations were run to determine the optimal antigen concentrations. The antigens were then used against sera from proven human cases of North American blastomycosis and coccidioidomycosis. The results are given in Table 2. Although it appeared in the first experiment that acetic acid extraction gave a more specific antigen, this was not observed on the repeat experiment. Cross-reactivity varied with the sera tested. The titers of the different chemically treated antigens showed as much as fourfold differences when tested with a single serum. However, consideration of the variation between the two experiments showed that there was no one chemical treatment which significantly minimized or eliminated cross-reactivity.

Physical fractionation of cell components, methods of killing, and their effect on ease of breakage of the yeast-form cells. Cells frozen and thawed five times, formalized cells, and cells killed with pyridine were sonically treated, and the rates of breakage were compared (Fig. 1). Cells frozen and thawed were 90% disrupted within 3 min and 100% in 5 min, whereas cells treated with formalin or pyridine required 16 min or more for total destruction. Other experiments showed that there was no difference in the rates of breakage among untreated viable cells, cells frozen and thawed, and cells killed by incubation in 1:10,000 Merthiolate for 24 hr at room temperature. These results indicated that the formalin and pyridine treatments had altered the fragility of the cells, and had most probably affected the release of soluble antigens.

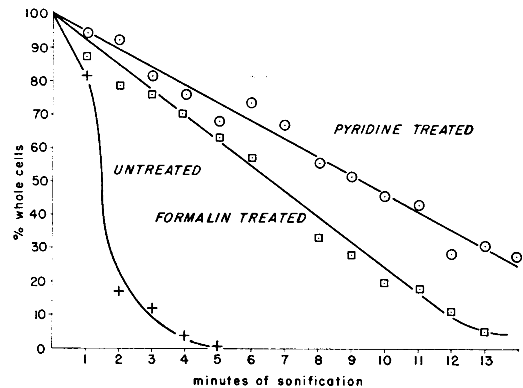

FIG. 1. *Relative rates of breakage of Histoplasma capsulatum yeast-form cells before and after chemical treatments. Cells of strain A811 were grown on the CCS agar medium, harvested, and divided into three equal suspensions. One was treated by freezing and thawing, a second with 0.5% formalin, and the third with pyridine according to the Labzoffsky et al. (10) procedure. The cells were washed three times with distilled water, after which they were resuspended in 0.001 M phosphate buffer (pH 8.0) and sonically treated as described in Materials and Methods. Wet mounts were made at 1-min intervals, and observed by use of the phase microscope. The percentage of broken cells was determined by direct counting of 10 different fields. Treatment was terminated after 16 min of sonic treatment.*

The latter was supported with the findings that a much greater amount of 254-mμ absorbing material was released from the frozen and thawed cells, and that differences in the protein peaks

occurred among the three soluble preparations when these extracts were chromatographed on Sephadex G-50 columns. Within the time limits needed to disrupt all the cells, only the extracts of the sonically treated frozen and thawed cells showed complement-fixing activity. This activity was lost after passage through the Sephadex column. It appeared best to work with cells that had not been treated chemically.

Because of the safety hazards involved when working with viable cells, all cells were frozen and thawed five times prior to sonic treatment, since this procedure with sonic treatment was shown by use of cultural and animal inoculations to kill all the cells.

Relationship of time of sonic treatment to antigens released, their cytological source, and their antigenic characteristics. The following experiment was run to ascertain the effect of sonic treatment in the release of antigenic material from the cell walls, and to determine the specificity of these antigens. Viable cells of strain A811 were used. Due to the possibility of infection, the zero-time CF determination was made with cells treated with 0.5% formalin in 0.85% NaCl. The protocol for the experiment is given with Fig. 2, and the results of the experiment are given in Fig. 2 and 3. With the assumption that the formalized cells had the same CF titer as viable cells, sonic treatment of the cells for 5 min resulted in a titer increase from 1:64 to 1:512 (Fig. 2). Upon sonic treatment of the washed cell wall residue, the cell walls themselves increased in antigenicity from 1:128 to 1:256 at 8 min. The soluble supernatant fluid obtained by sonic treatment of the cell walls showed no increase in titer until 12 min, at which time the titer rose from 1:128 to 1:384. This experiment was repeated twice with essentially the same results. The results showed that increased sonic treatment unmasked additional antigenic sites in the cell wall, and that a soluble fraction of high antigenic activity was released somewhat later. However, this soluble fraction was highly labile. With 5 days of storage at 5 C, the soluble fraction dropped from an original titer of 1:384 to 1:64 (Fig. 3). At 7 days, a further decrease to 1:32 was observed, and this was maintained for the next 14 days.

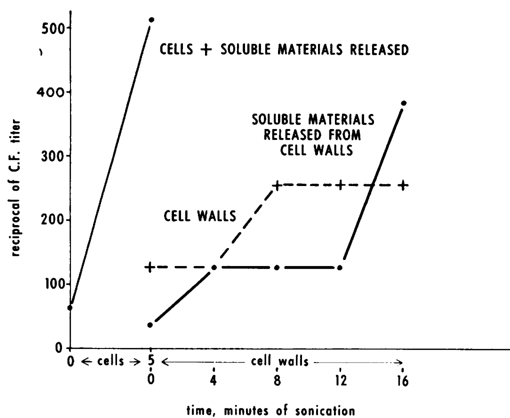

FIG. 2. *Effect of sonic treatment with time on the release of soluble and insoluble antigens from viable cells of Histoplasma capsulatum yeast-form cells, strain A811. A 3-ml amount of packed yeast-form cells of strain A811 grown on agar plates of the CCS medium was suspended in 17 ml of 0.01 M phosphate buffer (pH 8.0). These cells were sonically treated for 5 min at 1-min intervals with alternate periods of cooling. A 2-ml amount was withdrawn at 5 min and diluted to 6 ml with buffer, and the residual suspension was centrifuged at 37,000 × g for 20 min at 5 C. The pellet was resuspended in buffer solution to 15 ml. The cell wall suspension was then sonically treated as before for an additional 16 min; 1-ml portions were withdrawn and diluted to 3 ml. They were titrated with the CF test, starting with a 1:4 dilution of the final suspension.*

The cell walls and the active cell wall supernatant fluid were then tested for antigenicity and specificity. The results showed that significant decreases in titer occurred in cell wall and supernatant materials stored 5 to 25 days at 5 C. Both fractions, soluble and particulate, showed higher cross-reactivity with the anti-*B. dermatitidis* sera than did the whole-cell control antigen.

Reactions of "Labzoffsky" antigens with human histoplasmosis sera. Labzoffsky et al. (10) observed that three fractions of the yeast-form antigen reacted specifically with sera of rabbits

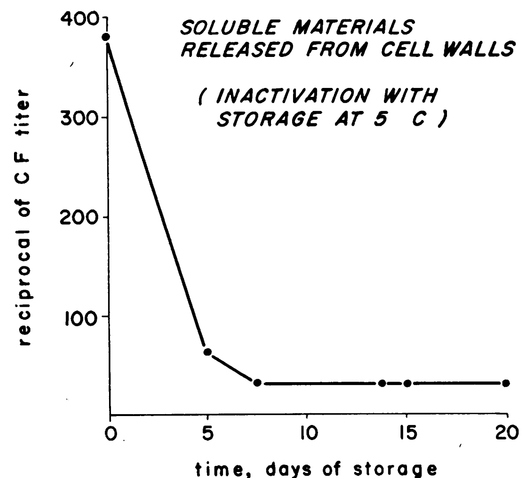

FIG. 3. *Loss of CF activity from soluble materials released from cell walls by yeast-form cells of Histoplasma capsulatum strain A811.*

hyperimmunized with killed cells of *H. capsulatum*. Yeast fractions were prepared according to their methods, and were tested against sera from human cases by use of the CF test. After each fractionation, the antigens were evaluated for antigencity and specificity. On the basis of dry weight, pyridine extraction generally increased the antigenic activity of the whole cells; this was not consistent. However, sonic treatment of cells extracted with pyridine consistently increased the antigenicity of the cell suspension, with the resultant particulate and soluble materials showing high individual antigenic titers.

Of the antigens tested, the cell wall residues after extraction with acetone and phenol exhibited the highest antigenic activity in the CF test, exhibiting complement-fixing titers of 1:256 to 1:1,024 at 5 mg (dry weight) per ml original concentration. One such preparation, a white upper cell wall layer obtained after centrifugation, had a titer of 1:256 with a human histoplasmosis serum, 1:8 and 1:16 with two human blastomycosis sera, and 1:16 and 1:32 with two human coccidioidomycosis sera. Similarly, all other fractions of *H. capsulatum*, although of lower antigenic activity, showed a higher cross-reactivity with heterologous human sera than did the whole-cell yeast-form control antigen. The latter had no complement-fixing activity with the blastomycosis sera, and a titer of 1:8 with both coccidioidomycosis sera.

Physical and chemical fractionation of two strains of H. capsulatum and their chemical and antigenic analyses. The results suggested that two major components, the cell wall and the material closely associated with it, were responsible for the antigenic reactions most desired. Therefore, a comparison was made of the reactivity of these fractions from two different strains, and the chemical characteristics of these fractions were determined. Two strains, A811 and F851, were chosen because they showed major morphological and physiological differences as described in Table 1 and the text relevant to it. The cells of these two strains were grown on the CCS medium and sonically treated for 5 min, and the resultant suspensions were fractionated as indicated in Table 3. From the data presented, it is evident that the per cent composition represented by the fractions obtained from the two strains differed significantly.

Two fractions, C and D, derived from strain F851 were essentially absent in strain A811. Fraction C appeared to be high in protein and low in carbohydrate; observation of fraction C under phase optics suggested that this fraction

TABLE 3. *Component variation within two yeast-form strains of Histoplasma capsulatum as reflected by physical fractionation**

Strain	Fraction	Description	Dry wt of fraction	Analysis†	
				Carbohydrate	Protein
			%		
A811	A	Frozen and thawed cells, sonically treated	100.0	6.0	9.4
	B	5,000 × *g* pellet	99.9	16.0	30.4
	C	37,000 × *g* pellet	0.1	0.0	0.0
	D	37,000 × *g* supernatant fluid	0.0	0.0	0.0
F851	A	Frozen and thawed cells, sonically treated	100.0	17.0	11.1
	B	5,000 × *g* pellet	95.7	23.0	22.7
	C	37,000 × *g* pellet	3.6	15.3	44.7
	D	37,000 × *g* supernatant fluid	0.1	75.3	37.5

* Cells of strains A811 and F851 were grown for 7 days at 37 C in prescription bottles containing 75 ml of CCS medium. Cells were washed off the agar surface, suspended in 50 ml of distilled water, and washed three times (10 ml of water in nylon centrifuge tube and frozen and thawed five times). The cells were again centrifuged and resuspended to give 30 ml, cooled in Dry Ice-alcohol to 1 to 4 C, and sonically treated for five 1-min intervals with alternate periods of cooling. A fraction obtained after sonic treatment was labeled fraction A. Cells were centrifuged at 5,000 × *g* for 20 min, and supernatant fluid was removed. The cell debris was washed three times with 25 ml of distilled water, and the washings were added to the original supernatant fluid. The washed cell residue was labeled B. The combined washings were centrifuged at 37,500 × *g* for 5 hr at 5 C. The pellet was labeled fraction C. The supernatant fluid was lyophilized and brought to a volume of 5 ml with distilled water, and was labeled fraction D.

† Carbohydrate and protein were determined by the anthrone and Folin methods, respectively, and are expressed as per cent dry weight.

was composed of cell membranes or collapsed cell protoplasts.

Fraction D of this strain, on the other hand, showed a much higher carbohydrate content than fraction C. These two fractions of strain F851 were highly active antigenically (Table 4). Fraction D also showed a relative high specificity when tested against lot 5 anti-*B. dermatitidis* serum, and did not differ significantly from that of antigen A811 (A811-A, Table 5).

The fractions containing the crude cell walls (fractions B) were divided into separate portions, treated with pepsin or trypsin, washed, and subjected to chemical extractions to obtain purified chitin residues and mannan. The antigenicity of all these preparations against three human anti-*H. capsulatum* sera is shown in Table 4. Their relative specificities with sera from human cases of histoplasmosis and North American blastomycosis are indicated in Table 5. Of the preparations tested, the control antigen A811 (Table 5) gave a specificity ratio (anti-*H. capsulatum* serum titer/anti-*B. dermatitidis* titer) of 32, the highest specificity for anti-*H. capsulatum* serum obtained. Fraction D had a high antigenicity and specificity as described above. Pretreatment of fraction B with enzymes consistently decreased the specificity ratio. Trypsin appeared more active in this respect. It was not until the pure chitinous wall was obtained, by use of the procedure of Northcote and Horne (12) with acetic acid and sodium hydroxide extraction, that a higher ratio (anti-*H. capsulatum* serum/anti-*B. dermatitidis* serum) of 16 was observed. The mannans were isolated in very small amounts and were not active antigenically.

Treatment of fractions B of both strains did not give any remarkable differences in specificity ratios. Chemical analyses of the purified cell walls (Table 6; A811 and F851 chitin residues pretreated with trypsin and pepsin) showed that the compositions of the cell wall chitin of the two strains were essentially identical. Of the components determined, the relatively high concentration of methyl pentoses and apparently amino acids is of interest, since these compounds have not been reported previously in the purified chitinous cell wall of the *H. capsulatum* yeast-form cell. Antigenically, the titers of the chitinous residues did not vary significantly, although the strains differed in morphological aspects. Any differences in specificity between the two strains were therefore most probably the result of masking components or absence of nonspecific components on the surface of the cell walls of either strain. The fact that the effect of proteinases was to increase the antigenic reactivity but decrease specificity could be due to the destruction

TABLE 4. *Antigenic reactivity of the various fractions of the yeast cells of Histoplasma capsulatum*

Strain	Fraction*	CF titers with human anti-*H. capsulatum* sera		
		Lot 1	Lot 16	Lot 1598
A811	Original cells, formalin-killed	512	32	—
	B, 5,000 × *g* pellet	2,048	64	—
	C, 37,000 × *g* pellet	0	0	—
	D, 37,000 × *g* supernatant fluid	0	0	—
	B, trypsinized	512	32	64
	B, pepsinized	1,024	64	128
	"Chitin" from trypsinized B	4,096	128	512
	"Chitin" from pepsinized B	—	64	—
	Mannan	—	0	—
F851	Original cells, formalin-killed	512	32	—
	B, 5,000 × *g* pellet	2,048	32	—
	C, 37,000 × *g* pellet	8,192	128	—
	D, 37,000 × *g* supernatant fluid	2,048	512	—
	B, Trypsinized	4,096	128	512
	B, Pepsinized	4,096	128	512
	"Chitin" from trypsinized B	8,192	128	128
	"Chitin" from pepsinized B	8,192	128	256
	Mannan	0	0	—

* See Table 4 for description of fractions B, C, and D. Fractions B were treated with proteinases as described in Materials and Methods. Chitin and mannans were isolated by the method of Northcote and Horne (12). Titers are given on the basis of 5 mg (dry weight) per ml.

TABLE 5. *Titers against human anti-Histoplasma capsulatum and anti-Blastomyces dermatitidis sera of fractions of yeast-form H. capsulatum cells after sonic treatment*

Strain	Fraction*	Human antisera (lot no.)		Titer of anti-*H. capsulatum*/anti-*B. dermatitidis*	Specificity ratio
		H. capsulatum	*B. dermatitidis*		
A811	Original cells, formalin-killed	1	5	512/0	
		1	5	256/8	32
	B, 5,000 × *g* pellet	1	5	256/16	16
	C, 37,000 × *g* pellet	1	5	0	0
	D, 37,000 × *g* supernatant fluid	1	5	0	0
	B, trypsinized	1598	5	64/32	2
	B, pepsinized	1	5	1024/128	8
	Chitin from trypsinized B	1	5	256/16	16
	Chitin from pepsinized B	1	5		
	Mannan	1	5	0	0
F851	Original cells, formalin-killed	1	5	512/32	16
	B, 5,000 × *g* pellet	1	5	256/32	8
	C, 37,000 × *g* pellet	1	5	AC	
	D, 37,000 × *g* supernatant fluid	1	4	128/8	16
		1	5	512/64	8
	B, trypsinized	1598	5	128/64	2
	B, pepsinized	1	5	512/32	16
	Chitin from trypsinized B	1598	5	32/16	2
	Chitin from pepsinized B	1	5	512/32	16
	Mannan	1	5	0	0
A811	Control	1	5	512/16	32
		1598	5	128/16	8

* See Table 4 for description of fractions B, C, and D. Titers given have not been normalized to 5 mg (dry weight) per ml, but represent the values obtained with the samples as used.

TABLE 6. *Chemical analyses of fractions from Histoplasma capsulatum yeast-form strains A811 and F851*

Strain and fraction	Description	Carbohydrate (anthrone)	Reducing sugar	Hexose (cysteine-H_2SO_4)	Hexosamine	*N*-acetyl hexosamine	Methyl pentose	Amino acids	Protein (Folin)
F851-A	Washed whole cells*	6.2†	16.2	20.6	5.7	0.6	4.3	29.2	12.9
A811-B	Crude cell walls	6.3	20.6	16.0	5.7	0.4	5.4	53.4	24.0
F851-B	Crude cell walls	8.2	23.8	19.9	6.6	0.5	5.6	46.0	15.1
F851-C	37,000 × *g* pellet	7.6	19.9	24.6	5.6	0.3	5.9	95.0	30.4
F851-D	37,000 × *g* supernatant fluid	9.6	26.4	26.0	2.8	0.0	7.6	26.3	—
A811-B	Trypsinized cell walls	7.7	27.0	28.1	6.2	0.0	8.2	47.7	12.3
A811-B	Pepsinized cell walls	12.0	32.1	40.7	8.7	0.3	7.9	27.4	10.9
F851-B	Trypsinized cell walls	13.0	44.8	86.4	2.9	3.0	1.1	32.5	—
A811-B	Chitin of trypsinized cells walls; extracted with sodium hydroxide and acetic acid	12.7	34.9	28.9	1.3	0.3	8.7	9.3	0.0
A811-B	Chitin of pepsinized cell walls; extracted as above	13.3	35.5	47.6	1.2	0.0	8.7	7.5	0.0
F851-B	Chitin of trypsinized cell walls; extracted as above	10.0	31.1	44.3	1.2	0.0	12.9	11.0	0.0
F851-B	Chitin of pepsinized cell walls; extracted as above	14.1	37.5	45.7	1.3	0.3	9.1	11.0	0.0

* Cells were first frozen and thawed three times as described in the text.
† Results expressed as per cent dry weight.

of proteins which served to mask antigenic sites, or which contributed to the cross-reactivity of the cell fraction. These cell wall proteins therefore appeared to differ from the protein of fraction C of strain F851.

DISCUSSION

Campbell and Binkley (4) and Campbell (3) have discussed the problem of nonspecific reactivity of the antigens used in the CF test for histoplasmosis. Both histoplasmin and whole yeast-form cells of *H. capsulatum* are valuable and mutually complementary in the presumptive diagnosis of infections due to *H. capsulatum*. Both products, however, are capable of reacting with sera from cases of North American blastomycosis and coccidioidomycosis, although generally to a lower degree. An optimal antigen is one which is highly sensitive and specific in reaction to antibodies against *H. capsulatum*. Toward this goal certain basic evaluations of whole and broken cells have been made. Campbell and Saslaw (5) made extracts from ground whole yeast-form cells, and found them to be stable but of low antigenic activity coupled with a high cross-reactivity with antiserum for *B. dermatitidis*. Schubert et al. (23) evaluated the antigens of a single strain of *H. capsulatum* using histoplasmin, whole yeast-form cells, and the supernatant fluid of a sonically treated yeast suspension. They observed that the yeast extract reacted essentially in the same way as the whole yeast-form cell when tested with sera from a proven case of histoplasmosis, although the extract gave somewhat higher titers. Lindberg (Ph.D. Thesis, The Univ. Michigan, Ann Arbor, 1950) reported that whole, formalin-killed, yeast-form cells had all the antigenicity of ground cells, extracts of ground yeast-form cells, ground mycelium, mixed mycelium and yeast, and the cell-free culture filtrate. Salvin and Ribi (21), using CF procedures, compared the antigenicity of cell walls and protoplasm from cells killed with ether or Merthiolate to the activity of heat-killed whole yeast-form cells. They reported that, on a weight basis, the cell walls were much more reactive than the protoplasm. Evidence was also presented that the cell wall possessed soluble antigens of lesser activity than the wall itself. Our results substantiated the very high reactivity of purified cell walls, but pointed out that soluble components may be equally active though less stable.

Labzoffsky et al. (10) fractionated the cell components more extensively than previous workers, and obtained several active purified soluble fractions. Although we obtained highly antigenic preparations by using Labzoffsky's methods, tests with human sera indicated that they all lacked either stability or specificity reported by Labzoffsky et al. (10) with rabbit sera. All the soluble fractions which we obtained were extremely labile, and lost activity rapidly when stored at 5 C. In addition, the results repeatedly showed that disrupted cells had increased reactivity with antibody to *B. dermatitidis*.

The overall results of the investigations reported here re-emphasize observations reported previously by several investigators. First, it is commonly observed that various sera obtained from human cases of histoplasmosis and blastomycosis can show a wide diversity of reactivity in the CF tests with one given antigen. Consequently, the relative sensitivity and specificity of an antigen should be verified with several low, medium, and high titrated homologous and heterologous antisera. Unfortunately, the amounts and numbers of such sera from human cases of blastomycosis were limited in this study. Second, there has been a common suggestion that the choice of the strain of *H. capsulatum* is perhaps the most critical step in obtaining a higher order of specificity. This has been suggested by Campbell and Saslaw (5) and by Schubert and Ajello (22), who compared the variation in strains of *H. capsulatum* as complement-fixing antigens. Recently, Markowitz (11) emphasized the potential differences in "strain" production of yeast-form polysaccharide eliciting precipitin and delayed hypersensitivity reactions in sensitized animals. Our comparative results, with the CF titration, showed that all the strains tested with the exception of two were remarkably alike. Our methods, however, varied from those of Markowitz, and our antigens were not evaluated with as large a number of sera as used by Schubert and Ajello (22).

It is known that media influence the production of antigens in the growth of *C. immitis*. Rowe, Newcomer, and Landau (20) have shown that a marked increase in precipitinogens resulted when cultures were grown in Roessler's medium, and when glucose was increased from 0.2 to 4.2%. Recently, it was observed by Pine, Kaufman, and Boone (16) that growth medium for yeast-form *H. capsulatum* could influence quantitatively the production of antigen reacting with a fluorescent antibody (FA) specific for *H. capsulatum*. It is interesting to note that, under the conditions of growth used in this study, the various media did not influence the antigenic nature of the yeast-form cells of the strains tested. The apparent characteristic composition of the three yeast-form groups analyzed here (A811,

28; L261, F851; and 6617, 6621) emphasized strain differences which were also apparent by other means, such as growth rates, substrate requirements, ease of conversion, etc. (17), but which did not reflect antigenic differences when the CF test was used.

In all of our tests, the Merthiolate-killed whole-cell antigens of *H. capsulatum* strain A811 were more specific for histoplasmosis human sera than were broken cells or fractions derived from them. We found that the purified cell wall residues were highly antigenic, and were relatively specific after removal of protein and other materials by a combination of enzyme digestion and chemical extraction. The results point to the possibility that those reactive chemical sites more specific for *H. capsulatum* lie on the surface of, or in, the cell walls. In this regard, it is interesting to note that FA specific for the yeast-form of *H. capsulatum* (9) is a cell wall stain. It is also important to note the rather high and equal specificity of the purified cell walls of two different morphological strains, A811 and F851. These preparations were free from protein, and were essentially identical by chemical analyses and qualitative composition of sugars. This suggests that the basic cell wall polysaccharide complex is in itself antigenically and chemically the same in other strains, and that variation within strains is probably due to additional protein-carbohydrate complexes associated with the cell wall. The results show that, within the limits of the numbers of sera used to test them, the whole-cell antigens tested from various strains are the same with but a few exceptions, even though these strains may vary considerably in their chemical composition, morphological aspects, and physical properties. The soluble antigens released from the cells were high in antigenicity but quite unstable; the particulate fractions were lower in antigenicity but were of a greater stability. All fractions tested showed a cross-reactivity equal to or greater than that observed with the whole yeast-form antigen.

Until other isolated purified fractions are obtained which have the high specificity and sensitivity desired, the Merthiolate-treated whole-cell yeast-form antigen presently used represents an antigen with more desirable characteristics of stability and specificity than the fractions described here.

ACKNOWLEDGMENT

I wish to thank Leo Kaufman for his suggestions throughout this work, and for his very helpful criticisms and assistance in the preparation of the manuscript.

LITERATURE CITED

1. BELCHER, R., A. J. NUTTEN, AND C. M. SAMBROOK. 1954. Determination of glucosamine. Analyst **79**:201–208.
2. CAMPBELL, C. 1947. Reverting Histoplasma capsulatum to the yeast phase. J. Bacteriol. **54**: 263–264.
3. CAMPBELL, C. C. 1960. The accuracy of serologic methods in diagnosis. Ann. N.Y. Acad. Sci. **89**: 163–177.
4. CAMPBELL, C. C., AND G. E. BINKLEY. 1953. Serologic diagnosis with respect to histoplasmosis, coccidioidomycosis, and blastomycosis and the problem of cross reactions. J. Lab. Clin. Med. **42**:896–906.
5. CAMPBELL, C. C., AND S. SASLAW. 1948. The use of yeast phase antigens in a complement fixation test for histoplasmosis. II. Results with ground antigens. J. Lab. Clin. Med. **33**:1207–1211.
6. CUMMINS, C. S., AND H. HARRIS. 1956. The chemical composition of the cell wall in some gram-positive bacteria and its possible value as a taxonomic character. J. Gen. Microbiol. **14**: 583–600.
7. DISCHE, Z. 1955. New color reactions for determination of sugars in polysaccharides. Methods Biochem. Analy. **2**:313–358.
8. DISCHE, Z., AND L. B. SHETTLES. 1948. A specific color reaction of methyl pentoses and a spectrophotometric micromethod for their determination. J. Biol. Chem. **175**:595–603.
9. KAUFMAN, L., AND W. KAPLAN. 1961. Preparation of a fluorescent antibody specific for the yeast phase of *Histoplasma capsulatum*. J. Bacteriol. **82**:729–735.
10. LABZOFFSKY, N. A., J. B. FISCHER, AND J. J. HAMVAS. 1957. Studies on the antigenic structure of *Histoplasma capsulatum*. Can. J. Microbiol. **3**: 977–985.
11. MARKOWITZ, H. 1964. Polysaccharide antigens from *Histoplasma capsulatum*. Proc. Soc. Exptl. Biol. Med. **115**:697–700.
12. NORTHCOTE, D. H., AND R. W. HORNE. 1952. The chemical composition and structure of the yeast cell wall. Biochem. J. **51**:232–236.
13. PARK, J. T., AND M. J. JOHNSON. 1949. A submicrodetermination of glucose. J. Biol. Chem **181**:149–151.
14. PINE, L. 1955. Studies on the growth of *Histoplasma capsulatum*. II. Growth of the yeast phase on agar media. J. Bacteriol. **70**:375–381.
15. PINE, L., AND E. DROUHET. 1963. Sur l'obtention et la conservation de la phase levure d'*Histoplasma capsulatum* et d'*H. duboisii* en milieu chemiquement defini. Ann. Inst. Pasteur **105**: 798–804.
16. PINE, L., L. KAUFMAN, AND C. J. BOONE. 1964. Comparative fluorescent antibody staining of *Histoplasma capsulatum* and *Histoplasma duboisii* with a specific anti-yeast phase *H. capsulatum* conjugate. Mycopathol. Mycol. Appl. **24**:315–326.

17. PINE, L., AND C. L. PEACOCK. 1958. Studies on the growth of *Histoplasma capsulatum*. IV. Factors influencing conversion of the mycelial phase to the yeast phase. J. Bacteriol. **75**:167–174.
18. PUBLIC HEALTH SERVICE. 1965. Standardization diagnostic complement fixation method and adaptation to micro test. Public Health Monograph 74. U.S. Public Health Serv. Publ. 1228.
19. REISSIG, J. L., J. L. STROMINGER, AND L. T. LELOIR. 1955. A modified colorimetric method for the estimation of *N*-acetyl amino sugars. J. Biol. Chem. **217**: 959–966.
20. ROWE, J. R., V. D. NEWCOMER, AND J. W. LANDAU. 1963. Effects of cultural conditions on the development of antigens by *Coccidioides immitis*. I. Immunodiffusion studies. J. Invest. Dermatol. **41**:343–350.
21. SALVIN, S. B., AND E. RIBI. 1955. Antigens from yeast phase of *Histoplasma capsulatum*. II. Immunologic properties of protoplasm vs. cell walls. Proc. Soc. Exptl. Biol. Med. **90**:287–294.
22. SCHUBERT, J. H., AND L. AJELLO. 1957. Variation in complement fixation antigenicity of different yeast phase strains of *Histoplasma capsulatum*. J. Lab. Clin. Med. **50**:304–307.
23. SCHUBERT, J. H., L. AJELLO, J. S. COOPER, AND L. C. RUNYON. 1955. Evaluation of histoplasmin and yeast phase antigens derived from a single strain of Histoplasma capsulatum in the complement fixation test. J. Bacteriol. **69**:558–562.
24. SCOTT, T. A., AND E. H. MELVIN. 1953. Determination of dextran with anthrone. Anal. Chem. **25**:1656–1661.
25. SUTHERLAND, E. W., C. F. CORI, R. HAYNES, AND N. S. OLSEN. 1949. Purification of the hyperglycemic-glycogenolytic factor from insulin and from gastric mucosa. J. Biol. Chem. **180**: 825–837.
26. TROLL, W., AND R. K. CANNAN. 1953. A modified photometric ninhydrin method for the analysis of amino and imino acids. J. Biol. Chem. **200**: 803–811.