INFECTION AND IMMUNITY, Aug. 1971, p. 116-125
Vol. 4, No. 2

Printed in U.S.A.

Characterization of Antigens from the Yeast Phase of *Histoplasma capsulatum*

JOHN P. GARCIA AND DEXTER H. HOWARD

Microbiology Laboratory, Department of Public Health, City and County of San Francisco, San Francisco, California 94102, and Department of Medical Microbiology and Immunology, UCLA School of Medicine, Los Angeles, California 90024

Received for publication 10 August 1970

Preliminary studies established methods for obtaining maximum yields of viable cells. Liquid shaken cultures of *Histoplasma capsulatum* provided a maximum of 4×10^8 to 5×10^8 cells/ml regardless of the inoculum size. Under optimum conditions, cells were viable (90 to 95%) for 168 to 240 hr. Generation times ranged from 7.52 to 8.36 hr. Immunodiffusion, immunoelectrophoresis, and ultracentrifugation studies on phenol and ethylenediamine extracts of intact cells and cell walls revealed the presence of two components in the ethylenediamine extracts and three in the phenol preparations. The ethylenediamine extracts from intact cells and cell walls seemed to be identical although one of the components was more abundant in cell walls. Mice injected intraperitoneally with intact cells or cell walls were protected against intravenous challenge with *H. capsulatum*. Among the extracts, the water-soluble ethylenediamine extract from cell walls was most immunogenic. The other extracts gave only a light protection or none at all. Intact cells and cell walls were slightly toxic to mice. Two of the extracts were toxic when incorporated into Freund's complete adjuvant.

Antigens from the dimorphic fungus *Histoplasma capsulatum* have been studied extensively (12, 14, 23, 24, 26, 37, 38). However, relatively few efforts have been made to work with chemically or immunologically characterized fractions from cell walls of the fungus.

Current evidence suggests that the immunizing capacity of fungi resides in their cell wall components (20, 37). To establish relationships between antigenic fractions and in vivo behavior, it is obviously important to examine the parasitic phase of dimorphic zoopathogenic fungi. Accordingly, this study was initiated to isolate and characterize some components from the walls of the yeast cells of *H. capsulatum*.

MATERIALS AND METHODS

Organism. *H. capsulatum*, no. 505, which has been used in other studies from our laboratory (16), was employed throughout this work.

Media. Stock cultures of the yeast phase of the fungus were maintained on slants of potato-egg medium (22), and shaken cultures were grown in a liquid medium (36).

Growth characteristics of the yeast phase of H. capsulatum. Primary cultures were prepared by inoculating 1-liter round-bottom boiling flasks of liquid medium with the fungus grown on potato-egg medium. Such cultures were incubated at 37 C for 72 hr on a rotary shaker (Gyrotory Shaker, New Brunswick Scientific Co., New Brunswick, N.J.), and the growth from them was used to inoculate subsequent cultures. The total quantity of fluid per flask after inoculation was 346 ml.

Total cell counts were made on duplicate samples with a hemocytometer. Yeast cells were counted as individual cells, including those budding from and still attached to the mother cell. Viability was determined from wet mounts prepared with a 1:1 mixture of a cell suspension and a 1% (w/v) aqueous solution of eosin Y.

Cell preparations. Flasks of liquid medium were inoculated with a 72-hr broth culture of *H. capsulatum* and were incubated for 5 days at 37 C on a rotary shaker. Merthiolate was added to the cultures (final concentration, 0.01%) which were kept at 37 C for 5 days. This procedure effectively killed the cells.

The killed cultures were pooled, centrifuged at 1,300 $\times$ *g* for 20 min, and washed three times in sterile distilled water. A portion was lyophilized and used as "intact cells." The remainder was frozen and used to prepare the ruptured cells and cell walls as described below.

Frozen cells were suspended in sterile distilled water (10^9 to 10^{10}/ml) and ruptured in a tissue disintegrater (Mickle Laboratory Engineering Co., Gomshell, Surrey, England). Glass vials of 10-ml capacity were filled with 7 ml of the cell suspension plus 3 ml

of 0.2-mm glass beads. After 25 to 30 min in the disintegrater at 4 C, no intact cells remained. The suspension was decanted through a fine-mesh screen and was lyophilized. Preparations referred to as ruptured cells were these fractured frozen cells.

Cell walls were separated from the ruptured cells as follows. Lyophilized ruptured cells were suspended in sterile distilled water and centrifuged at 1,300 × *g* at 4 C for 25 min. The sediment was washed with sterile distilled water five to seven times. After the third and subsequent washings, the suspension was forced three times through hypodermic needles of

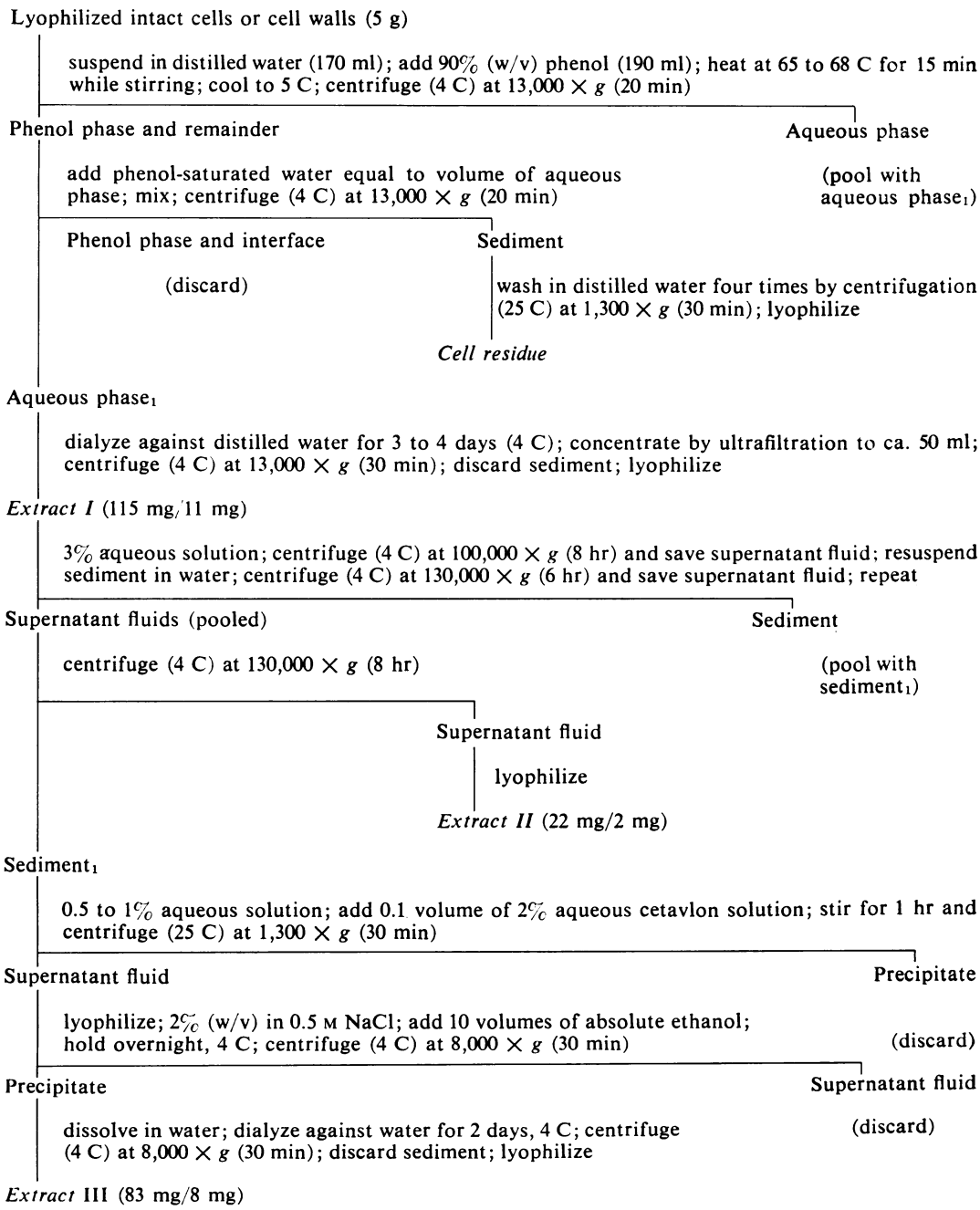

FIG. 1. *Procedure for phenol extraction. The values in parentheses following extracts I to III indicate recovered from intact cells/recovered from cell walls (no data for cell residue).*

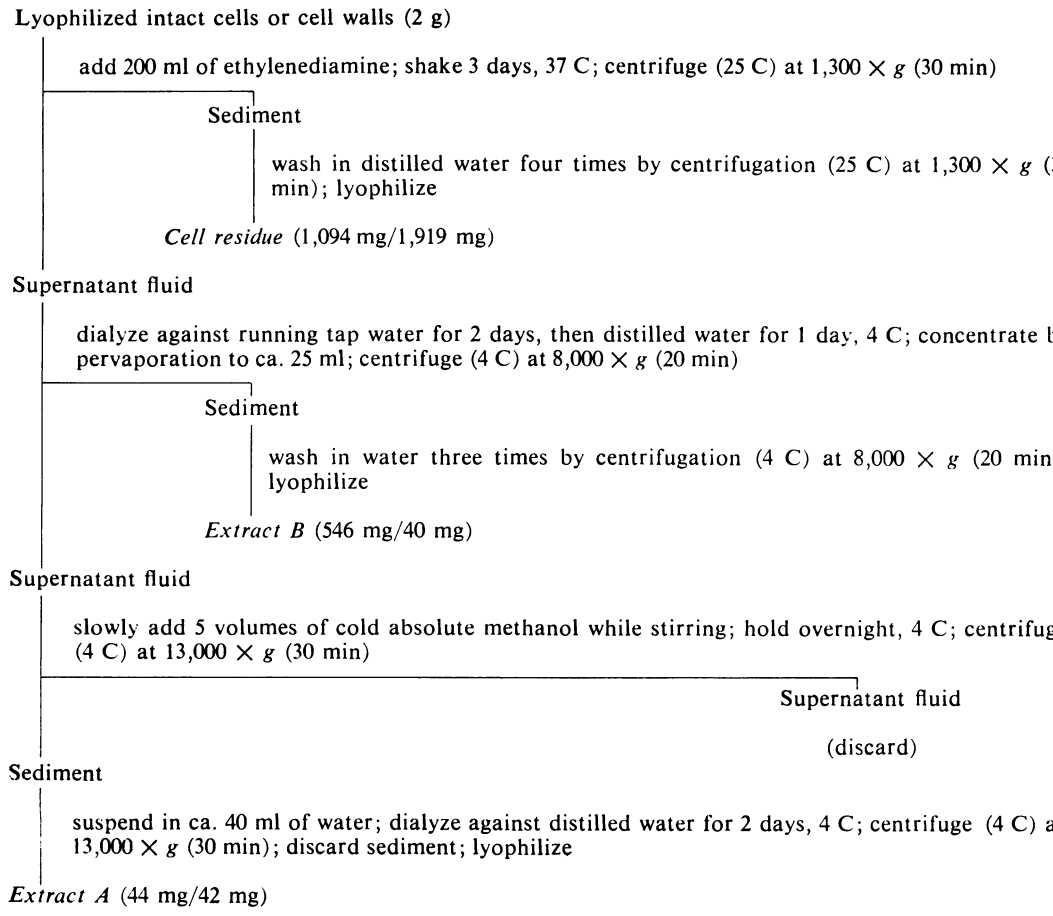

FIG. 2. *Procedure for ethylenediamine extraction. The values in parentheses following cell residue, extract B and extract A indicate recovered from intact cells/recovered from cell walls.*

decreasing diameters. When the supernatant wash fluid was clear, the sediment was lyophilized. Preparations referred to as cell walls were these sediments.

Extraction of cell preparations. Intact cells and cell walls were treated with phenol (40; M. S. Redfearn, Ph.D. Thesis, Univ. of Wisconsin, 1960) and cetavlon (39) to extract polysaccharides and with ethylenediamine (7, 21) to extract glycoproteins. Protocols for these procedures are presented in Fig. 1 and 2.

Antisera. Antisera against intact cells, ruptured cells, and cell walls (three rabbits per preparation) were produced in female New Zealand rabbits weighing about 3 kg. Materials were suspended in Difco hemagglutination buffer (*p*H 7.2 to 7.3) at a concentration of 20 mg/ml. Animals were injected intravenously with 0.25 ml of the buffer-diluted material every other day for a week; the intravenous series was followed by two sets of intramuscular and subcutaneous injections (in Freund's complete adjuvant) spaced 2 weeks apart. The rabbits began to develop local necrotic lesions so the protocol was changed in that Freund's incomplete adjuvant was used for the last series of injections that were administered once a month until the animals were bled. Four to six months after initial immunization, blood was collected from the femoral vein and the sera were stored at −20 C. Gamma globulin was fractionated from whole serum by precipitation with ammonium sulfate at 40% saturation (4).

Immunological and immunochemical procedures. Precipitin reactions were performed by double diffusion in agar-gel utilizing 1% Oxoid Ionagar no. 2 (Consolidated Laboratories, Inc., Chicago Heights, Ill.) in Veronal buffer (*p*H 8.6 and $\mu = 0.1$), containing 0.01% Merthiolate. The antigens, usually 0.1 to 10 mg/ml, were dissolved in distilled water or the buffer. Tests were done on 1 by 3 or 2 by 3 inch (ca. 2.54 by 7.62 or 5.08 by 7.62 cm) glass slides containing 3 or 7 ml of the gel, respectively. Slides were washed in buffer, dried, and stained with amido black (11). To check the serological relationship between two antigens and one antiserum, each antiserum was adsorbed directly in the agar wells (1, 8).

Several of the soluble antigens were resolved by immunoelectrophoresis (11). The gel was 1% Ionagar no. 2 in the following buffers: acetate, *p*H 4.6 (3);

phosphate (*p*H 6.0), tris(hydroxymethyl)aminomethane (Tris), *p*H 7.4, Veronal (*p*H 8.6), and glycine, *p*H 9.8 (2); borate, *p*H 8.6 (5). All buffers contained 0.01% Merthiolate and had an ionic strength of 0.05. The antigens, prepared as for the precipitin reactions, were electrophoresed for 90 min at 4 ma per inch.

Extracts to be analyzed in the Beckman model E analytical ultracentrifuge were dissolved (10 mg/ml) in 0.015 M Tris buffer (*p*H 7.75) and placed in a synthetic boundary cell, 0.4 ml of material in the cell and 0.2 ml of buffer in the cup. Samples were sedimented at 259,700 × *g* at 20 C for 64 to 192 min, and pictures were taken at 8- or 16-min intervals.

Sephadex (Pharmacia, Uppsala, Sweden) G-50, G-75, G-100, and G-200 were used to fractionate some extracts. Fractions were collected on a fraction collector (Gilson Medical Electronics, Middleton, Wis.) and the optical density of the effluent was read at 280 nm on a Uvicord II (LKB Instruments, Inc., Rockville, Md.).

Chemical analysis. Nitrogen content was determined by micro-Kjeldahl (17). When valid, protein and amino acid nitrogen were calculated by subtracting glucosamine nitrogen from total nitrogen. Glucosamine content was estimated (10) by hydrolyzing samples with 6 N HCl for 12 hr at 100 C, and the amount of glucosamine nitrogen was calculated from the per cent (6.3%) nitrogen it contained.

Toxicity and immunogenicity of cell preparations and extracts. Six- to eight-week-old female Swiss albino mice were injected intraperitoneally with 0.5 ml of an appropriate concentration of nonviable cellular material or extract in hemagglutination buffer containing 0.01% Merthiolate. When Freund's adjuvant was included, the ratio of adjuvant to antigen was 1:1 (v/v); the total volume injected was the same (0.5 ml). Fourteen days later, survivors were challenged with 2 LD_{50} of *H. capsulatum* suspended in 0.2 ml of hemagglutination buffer and injected into the ophthalmic plexus (29). The LD_{50} dose was 1.4×10^7 viable cells, based on 21 days of survival calculated by the method of Reed and Muench (32).

RESULTS

Growth characteristics of the yeast phase of H. capsulatum. Growth curves up to the "stationary phase" for different inoculum sizes are illustrated in Fig. 3. The slope of the curves and the final cell concentrations were nearly identical. The only obvious variation in growth patterns was the time required to enter the stationary period. In all instances this was reached by 120 hr; the shortest time required was about 60 hr. A lag phase was observed only with the two smallest inocula.

Plots of per cent viability versus time for each culture are shown in Fig. 4. For the first 168 to 240 hr (depending on the size of the inoculum), approximately 95% of the cells were viable. The larger the inoculum, the sooner the cells in the culture began to die. The generation times varied between 7.52 and 8.36 hr and were independent of inoculum size.

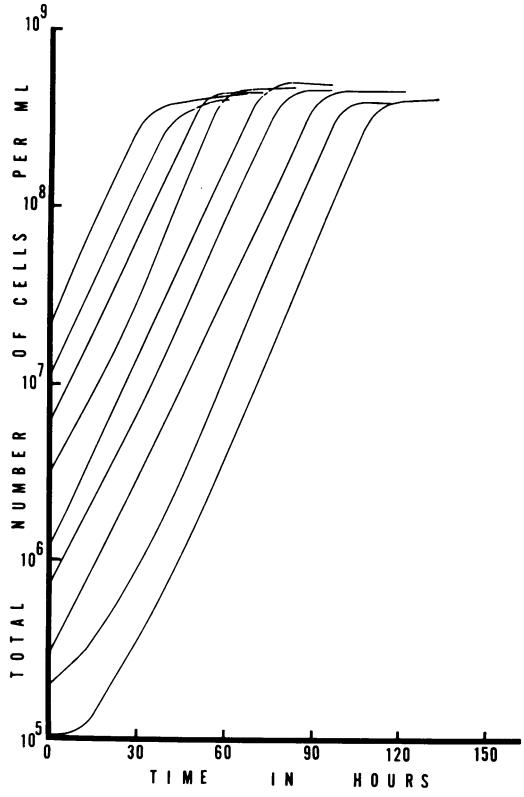

FIG. 3. *Growth curves for the yeast phase of Histoplasma capsulatum grown at 37 C in shaken flasks containing liquid medium. Inoculum sizes were: 0.25-, 0.49, 0.74, 1.97, 2.95, 7.87, 15.9, 28.5, and 54.1 × 10^8 cells, respectively.*

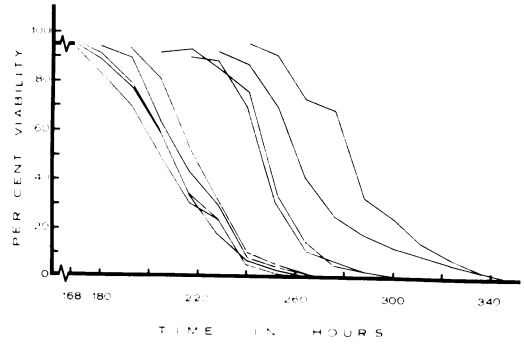

FIG. 4. *Viability of the yeast phase of Histoplasma capsulatum grown at 37 C in shaken flasks containing liquid medium. Each curve represents a separate culture; these represent the same cultures as those in Fig. 3.*

Properties of cells and extracts. Intact cells or

cell walls treated as shown in Fig. 1 and 2 had the following solubility and chemical characteristics. The ethylenediamine extracts A, from either intact cells or cell walls, were water-soluble, whereas the 'B' extracts were soluble only in ethylenediamine (insoluble in concentrated and dilute acid or base and a variety of organic solvents, e.g., ethylene glycol, diethylene glycol, formamide, and 8 M urea). Phenol extracts I, II, and III were water-soluble.

Results of nitrogen and glucosamine analyses are recorded in Table 1. Total nitrogen and glucosamine determinations were done in triplicate.

Water-insoluble preparations, i.e., the ethylenediamine B and cell residue, and the particulate cell preparations (intact cells and cell walls) were analyzed chemically but were not otherwise studied.

Immunochemical and biophysical properties of cell preparations and extracts. Gamma globulin preparations from whole immune sera gave cleaner reactions in immunodiffusion tests and were thus used in the observations. The number of precipitin bands detected by immunodiffusion against some of the soluble extracts is shown in Table 2.

TABLE 1. *Chemical determinations on cell preparations and extracts*

Preparation/extract	Weight per mg (dry weight) of sample (μg)			
	Total nitrogen	Glucosamine		Protein and amino acid nitrogen[b]
		Nitrogen[a]	Total	
Intact cells (IC)	58.0	7.4	117.6	ND[c]
IC/phenol cell residue	63.0	11.6	184.5	ND
IC/phenol II	11.2	0.8	12.7	ND
IC/phenol III	5.7	0.5	8.0	5.1
IC/ethylenediamine A	83.0	0.5	7.9	ND
IC/ethylenediamine B	54.3	0.2	3.3	54.0
IC/ethylenediamine cell residue	74.3	7.7	121.7	ND
Cell walls (CW)	35.7	17.7	280.2	13.8
CW/ethylenediamine A	51.3	0.4	6.0	45.3
CW/ethylenediamine B	82.7	0.4	6.3	82.2
CW/ethylenediamine cell residue	46.3	7.2	114.0	37.4

[a] Glucosamine nitrogen calculated from total weight (nitrogen = 6.3% of *N*-acetyl-glucosamine).

[b] Protein and amino acid nitrogen = total nitrogen − glucosamine nitrogen.

[c] ND, not done since nucleic acid nitrogen was not accounted for.

TABLE 2. *Number of precipitin bands observed in immunodiffusion test*

Extract (source)	No. of bands with antiserum (γ-globulin fraction) against		
	Intact cells	Cell walls	Ruptured cells
Ethylenediamine A (intact cells)	2	1	2
Ethylenediamine A (cell walls)	2	1	2
Phenol I (intact cells)	1	1	2–3

The ethylenediamine A extracts, from either intact cells or cell walls, demonstrated reactions of complete identity between each other, regardless of the form of the cells used to induce antibody formation. Also, one of the precipitin bands obtained with the phenol I extract (sometimes the only band present) always showed complete identity with one of the bands from the ethylenediamine extract A of intact cells. However, the same band from the phenol I preparation showed only partial identity with the ethylenediamine A extract from cell walls. The second band from the phenol I preparation, observed with antibody against ruptured cells, was nonidentical with the ethylenediamine A extract from either cell walls or intact cells. The results of adsorption studies confirmed these observations.

Since gamma globulin against ruptured cells gave the greatest number of intense bands, it was selected for additional studies with the extracts.

Two bands for both the ethylenediamine extracts and two or three bands for the phenol preparation were also discovered by immunoelectrophoresis. Variations in *p*H and buffer did not reveal additional bands or improve the resolution of those originally observed.

The patterns obtained by running the extracts in the analytical ultracentrifuge are illustrated in Fig. 5 and 6. Ethylenediamine extracts A, from intact cells and from cell walls, gave identical curves consisting of a single homogeneous peak (Fig. 5). The phenol I extract from intact cells resulted in two peaks, the largest of which resolved into two subfractions after 16 min at 59,789 rev/min (Fig. 6).

Purification of extracts. Purification of the "crude" phenol extract I (Fig. 1) resulted in two fractions, phenol II and III. The phenol III portion produced a single precipitating antigen when tested by immunodiffusion, whereas the phenol II preparation formed two to three bands. One of the bands was serologically identical with the band found in extract III. The other two, one

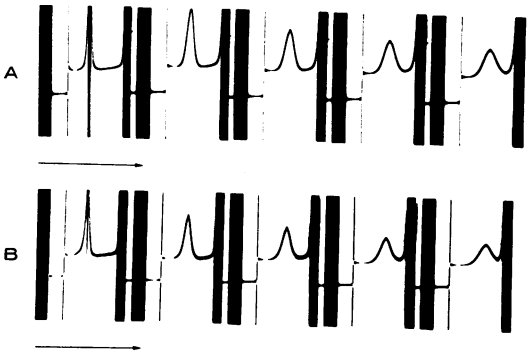

FIG. 5. *Ultracentrifugal patterns of ethylenediamine A extracts from H. capsulatum. The extracts (10 mg/ml) were dissolved in 0.015 M Tris buffer (pH 7.75) and sedimented at 59,780 rev/min (259,700 × g) at 20 C in a synthetic boundary cell. Photographs were taken at 16-min intervals, starting at 0 time. (A) Extract from intact cells; (B) extract from cell walls.*

FIG. 6. *Ultracentrifugal pattern of phenol extract I from H. capsulatum (intact cells). The extract (10 mg/ml) was dissolved in 0.015 M Tris buffer (pH 7.75) and sedimented at 59,780 rev/min (259,700 × g) at 20 C in a synthetic boundary cell. Photographs were taken at 16-min intervals starting at 0 time.*

very faint and not always detected, were non-identical with the band from extract III.

Curves for gel filtration of the ethylenediamine extracts A appear in Fig. 7 and 8. Sephadex G-75 with a flow rate of 10 ml/hr gave the best separation. The ethylenediamine extract A from cell walls gave a prominent peak followed by a small shoulder, and a small peak appeared near the end of the run (Fig. 7). All fractions were tested by immunodiffusion. Fractions from the central portion of the major peak produced rather diffuse bands of precipitation, whereas fractions from the beginning and end of the curve gave sharp distant single bands. These samples from extreme ends of the curve were compared and showed spur formation. The final peak gave no precipitin bands even when this material was concentrated 10-fold.

The chromatographic run with the ethylenediamine extract A from intact cells is reproduced in Fig. 8. A profile similar to that obtained with the extract from cell walls was observed, except that the shoulder was resolved into a definite

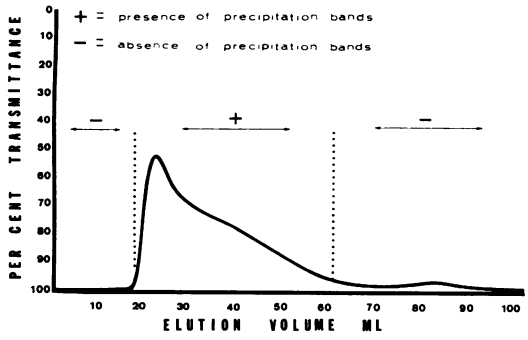

FIG. 7. *Elution pattern of ethylenediamine extract A from H. capsulatum (cell walls) on Sephadex G-75. Samples (15 mg in 3 ml) were eluted with distilled water and collected at 10 ml/hr in 1-ml fractions. Per cent transmittance at 280 nm was plotted and precipitating activity was tested with gamma globulin against ruptured cells.*

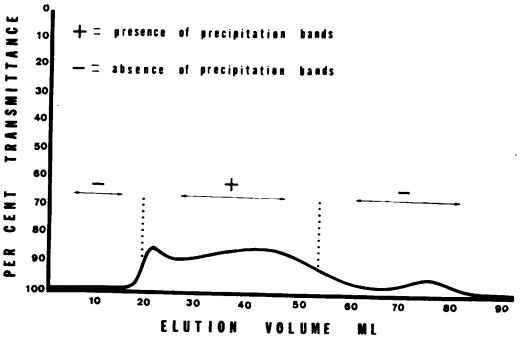

FIG. 8. *Elution pattern of ethylenediamine extract A from H. capsulatum (intact cells) on Sephadex G-75 under same conditions as Fig. 7.*

TABLE 3. *Immunogenicity and toxicity of Histoplasma capsulatum in mice*

Immunogen (5 mg)	Deaths during 14 days after immunization		Deaths after challenge with viable cells[a]	
	No.	Per cent	No.	Per cent
Intact killed cells	1/34[b]	2.9	4/17[b]	23.5
Controls[c]			0/16	0
Cell walls	3/38	7.9	6/18	33.3
Controls			1/17	5.8
Nonimmunized	0/30	0	12/15	80.0
Controls			0/15	0

[a] Inoculated with 2 LD_{50} of viable *H. capsulatum* 14 days after immunization. Mice were observed for 21 days.

[b] Ratio of deaths to the total number of mice.

[c] Half of the immunized mice were used as controls and received buffer in place of challenge dose.

TABLE 4. *Immunogenicity and toxicity of extracts of Histoplasma capsulatum in mice*

Immunogen (source)	Extract	Amt (mg)	Deaths during 14 days after immunization		Deaths after challenge with viable cells[a] (observed 21 days)	
			No.	Per cent	No.	Per cent
Intact cells	Phenol cell residue	5.0	0/21[b]	0	18/21	85.7
	Phenol I	0.2	0/21	0	21/21	100
	Ethylenediamine A	0.2	0/21	0	19/21	90.5
	Ethylenediamine B	0.2	0/21	0	18/21	85.7
	Ethylenediamine B[c]	0.2	2/21	9.5	18/19	94.7
Cell walls	Ethylenediamine A	0.05	0/21	0	20/20	100
	Ethylenediamine A	0.2	0/19	0	15/19	78.9
	Ethylenediamine A	0.5	0/21	0	18/18	100
	Ethylenediamine A[c]	0.2	7/21	33.3	13/14	92.8
	Ethylenediamine B	0.2	0/19	0	19/19	100
	Ethylenediamine cell residue	5.0	0/21	0	19/21	90.4
Controls	Adjuvant[c]		0/21	0	19/19	100
Controls	Buffer		0/21	0	19/19	100

[a] Mice inoculated with 2 LD_{50} of viable *H. capsulatum* 14 days after immunization.
[b] Ratio of deaths to the total number of mice.
[c] Freund's complete adjuvant used in a ratio of 1:1 (v/v).

peak. An immunodiffusion test revealed the presence of two bands whenever precipitation occurred.

Phenol extracts II and III from intact cells were tested by immunodiffusion with the fractions isolated above on the Sephadex column. All precipitin bands showed reactions of nonidentity except for one band from the phenol II preparation that coalesced with all of the fractions.

Immunogenicity and toxicity of cell preparations and extracts. Mice injected with either intact nonviable cells or cell walls were protected when challenged with 2 LD_{50} of viable yeasts (Table 3). Approximately 77% of the mice receiving intact cells survived, and 67% of those immunized with cell walls survived at least 21 days. All of the control animals not receiving a challenge dose survived for this period, with the exception of one mouse in the control group receiving cell walls.

Toxicity of the intact cells and cell walls was measured as deaths occurring during the period of immunization, i.e., during the 14 days after injection of the nonviable inocula and prior to the challenge with viable cells. One of 34 and 3 of 38 mice died that were given intact cells or cell walls, respectively.

The immunogenicity of the extracts gave various results, ranging from partial protection (21% survival) to no protection (see Table 4). The residues remaining after extraction conferred very little protection when compared to whole cells or cell walls (Tables 3 and 4). Incorporating the extracts into Freund's complete adjuvant improved their effect in some instances (Table 4). However, the use of adjuvant also augmented the toxicity of the extracts (Table 4).

DISCUSSION

Growth of yeast cells of H. capsulatum. Preliminary studies established methods for obtaining large volumes of viable cells of *H. capsulatum*. Cells in approximately the same stage of growth were desirable, since different stages probably contain different antigens, quantitatively if not qualitatively. Various workers (25, 27, 33) have devised liquid media for the cultivation of *H. capsulatum* in the yeast phase. In this study good growth was obtained in the medium of Salvin (32, 33, 36). The data from the growth curves indicate that a maximum yield of 4×10^8 to 5×10^8 cells/ml was obtained regardless of the sizes of inocula used. This is consistent with Salvin's (33) observations.

The use of dye exclusion techniques (6, 13) allowed rapid determination of the viability of *H. capsulatum*. From the time of inoculation until at least 96 hr after a culture had entered the stationary phase, approximately the same percentage of cells were viable regardless of the inoculum size. No lag period was observed when the inoculum was 3×10^5 cells/ml or greater. Thus, by using cells grown for 5 days (120 hr), a maximum yield of cells that were in the stationary phase and 94 to 95% viable was assured.

Pine and Peacock (28) reported generation times for *H. capsulatum* of 9 to 11 hr in a minimal vitamin medium supplemented with glucose and citrate. Reca and Campbell (30) studied the growth of the fungus in Trypticase soy broth. They did not report a generation time, but by calculation from their data the generation time was about 11.7 hr. In the present work the generation time of the fungus ranged from 7.52 to 8.36 hr and did not vary by more than 0.84 hr for different sizes of inocula. No correction was made for the death rate during the exponential growth phrase. During this period of growth, cells were at least 93% viable.

Preparation and characterization of extracts from H. capsulatum. In recent years, interest has centered on obtaining pure antigenic preparations from *H. capsulatum* (12, 14, 23, 24, 26, 38).

Sensitive immunological procedures, however, indicate that these preparations still contain several antigenically reactive components. In the present study, relatively large quantities of cellular components were required to obtain appreciable yields of extracts, particularly the soluble extracts. The insoluble portions, by their very nature, could not be used in many of the analyses. The ethylenediamine extract B from intact cells or cell walls was insoluble in urea. This is contrary to Korn and Northcote's (21) findings with a similar extract from the cell walls of bakers' yeast.

Antiserum prepared against the ruptured cells was the most reactive in immunodiffusion tests (based on the number and intensity of precipitin bands observed), perhaps because greater amounts as well as different antigens are available in a ruptured cell preparation. Only one band was observed when antibodies against the intact cells were tested with the phenol I extract, but this band was more intense than the bands seen with the other extracts. That cell walls contained less antigenic material was further evident by the appearance of only one precipitin band when antiserum against this preparation was run against different extracts. Presumably, the more soluble components were released and washed away during preparation of the cell walls. That components remained was shown by the ability of antiserum prepared against ruptured or intact cells to produce two bands when tested against the cell wall extract.

The serological identity between the ethylenediamine A extracts, demonstrated by the coalescence of precipitin bands, shows that they have a common antigenic factor. The same is true for reactions between the phenol I extract and one of the antigens in the ethylenediamine extract A of intact cells. The spur formation observed in reactions between the phenol I and ethylenediamine A from cell walls indicates nonidentical, but serologically related, determinants. This reaction was observed when the test serum was antibody globulin against intact cells or cell walls. When antibody against ruptured cells was employed in the same test pattern, another band appeared for the phenol I extract. This gave a reaction of nonidentity, indicating that these antigens have no determinants in common.

Precipitin bands were more clearly differentiated by immunoelectrophoresis. When migration did occur, all bands moved the same distance in the agar-gel. This would indicate a similar charge on antigenic components under these particular test conditions. The lack of movement of polysaccharides from the phenol I extract, except in a borate buffer, indicates they are neutrally charged. The components detected in the ethylenediamine A extracts are slightly charged, presumably because of the protein moiety of the antigen.

The patterns obtained with the analytical ultracentrifuge confirmed the presence of three components in the phenol I extract. The third component did not appear in some of the immunodiffusion and immunoelectrophoretic tests. However, only a single homogeneous peak was obtained when either of the ethylenediamine A extracts was analyzed. This was in contrast to the two precipitin bands detected by diffusion and electrophoresis in agar-gel. Thus one must be cautious in interpreting results from ultracentrifugation alone. It is possible that the concentration of one of the components was too small or that both were so similar in molecular size as to preclude their resolution.

One component, isolated from the "crude" phenol extract I, appeared in extract III. Some of this antigen was evidently carried over into extract II since this extract produced the three precipitin bands observed for the crude extract. Furthermore, one of these bands coalesced with the bands from extract III.

The two components in the ethylenediamine extract A of cell walls were finally separated by gel filtration on Sephadex G-75. The resolution, however, was not good. The two antigens were isolated by taking samples from the beginning and the end of the major curve. It is quite likely that the final peak observed contained breakdown products. When the extract was lyophilized two additional times, this peak increased in size with a concomitant decrease in the area of the major peak. Moreover, the compounds from the second peak lacked any precipitating ability. Thus one should avoid lyophilizing such materials. Pine et al. (26) found a loss in the complement-fixing activity of materials simply by storing them at 5 C for several days.

The phenol extract III from intact cells was completely different from the ethylenediamine A extracts isolated on the Sephadex column. This absence of any common determinant group might have been predicted on the basis of the chemical differences between the two. The phenol II preparation did share one of its antigens with the rest of the extracts. However, the remaining antigens were also nonidentical when tested with other extracts.

The similarity between the ethylenediamine A extracts from intact cells and cell walls was observed in immunodiffusion, immunoelectrophoresis, and ultracentrifugation analyses. The data from the Sephadex fractionations indicated that the first component to appear from cell wall extracts is in much greater concentration than its counterpart from the intact cell extracts. The second components occur in approximately equal concentration. Antigens from these extracts might well be the same. The greater quantity of the first component from cell walls might be interpreted as being a less soluble component that is not easily released into the interior of the cell. It is probable that the antigens found in these extracts originate in the cell wall. Intact cell extracts obviously contain material from the cell walls, but it is unlikely that the purified cell walls contain appreciable amounts of contaminating material from the interior of the cell. Of course, it is possible that these compounds are produced in both cytoplasm and cell walls.

It is difficult to say how much, if any, alteration of antigens occurred during the extraction procedures. Markowitz (24) suggests that isolation procedures may result in chemical changes. The final peak observed in the present study during the Sephadex fractionation and its increase after lyophilization of the extract suggest that changes may indeed occur.

Toxicity and immunogenicity of extracts from H. capsulatum. Protection against experimental histoplasmosis by immunization with Formalin-killed yeast cells has been demonstrated by several workers (15, 18, 34, 35, 37). Salvin's group dried cells with acetone, whereas the other workers suspended washed cells directly in saline prior to inoculation. Cells for the study reported herein were Merthiolate-killed and lyophilized prior to suspension. Despite these variations in methodology, the survival in the present study of 76.5% of mice immunized with whole killed cells in comparison with only 20% survival of non-immunized controls is comparable to results obtained by others. One of 34 mice in our study that received killed whole cells died. Salvin and Ribi (37) also found that a similar amount (5 mg) of whole cells produced deaths.

Cell walls were also tested in our study for their immunizing capacity. The 66.7% survival obtained in challenged mice is close to the value of 68% for similar preparations found by Salvin and Ribi (37). Our results support the contention that protective antigens reside in the cell walls (37). A similar conclusion has been reached in studies with the spherules of *Coccidioides immitis* (20).

The extract showing the best protection was the ethylenediamine A preparation from cell walls administered at a level of 0.2 mg. Greater (0.5 mg) or lesser (0.05 mg) amounts failed to confer immunity. Others (38) have observed the same effect. Presumably, the lower level is not sufficient to induce protective antibodies and the higher dose possibly results in immune paralysis. The comparable extract from intact cells produced virtually no immunity since only 2 of 21 (9.5%) mice so immunized survived challenge. If the ethylenediamine A extracts from intact cells and cell walls were identical, then the first component appearing in Sephadex fractionation of these extracts may have been responsible for the protection. The first component was found in much greater quantity in extracts of the cell wall, and this was the cell preparation that showed greatest protection.

The effect of Freund's complete adjuvant incorporated with two of the antigens was studied. Rather than enhancing resistance, it increased the toxicity of the preparations. Similar results have been reported by Kong and Levine (19) when adjuvant was added to killed cells of *C. immitis*.

ACKNOWLEDGMENTS

This study was supported by Public Health Service training grant no. T1-A1-132-07 from the National Institute of Allergy and Infectious Disease.

We are most grateful to James F. Godfrey for technical assistance with the ultracentrifugal studies.

LITERATURE CITED

1. Björklund, B. 1952. Specific inhibition of precipitation as an aid in antigen analysis with gel diffusion method. Proc. Soc. Exp. Biol. Med. 79:319–324.
2. Bloemendal, H. 1963. Zone electrophoresis in blocks and columns. Elsevier Publishing Co., New York.
3. Boyd, W. C. 1945. A nomogram for acetate buffers. J. Amer. Chem. Soc. 67:1035–1037.
4. Campbell, D. H., J. S. Garvey, N. E. Cremer, and D. H. Sussdorf. 1964. Methods in immunology. W. A. Benjamin, Inc., New York.
5. Crowle, A. J. 1961. Immunodiffusion. Academic Press Inc., New York.
6. Devereux, E. D., and F. W. Tanner. 1927. Observations on the growth of yeasts in pure nutrient solutions. J. Bacteriol. 14: 317–333.
7. Domer, J. E., J. G. Hamilton, and J. C. Harkin. 1967. Comparative study of the cell walls of the yeastlike and mycelial phases of *Histoplasma capsulatum*. J. Bacteriol. 94:466–474.

8. Dray, S., and G. O. Young. 1959. Two antigenically different α-globulins in domestic rabbits revealed by isoprecipitins. Science 129:1023–1025.
9. Flodin, P. 1963. Dextran gels and their applications in gel filtration, 3rd. ed. Meijels Bokindustri, Halmstad, Sweden.
10. Gatt, R., and E. R. Berman. 1966. A rapid procedure for the estimation of amino sugars on a micro scale. Anal. Biochem. 15:167–171.
11. Grabar, P., and P. Burtin (ed.). 1964. Immuno-electrophoretic analysis. Elsevier Publishing Co., New York.
12. Greene, C. H., L. S. DeLalla, and V. N. Tompkins. 1960. Separation of specific antigens of *Histoplasma capsulatum* by ion-exchange chromatography. Proc. Soc. Exp. Biol. Med. 105:140–141.
13. Hanks, J. H., and J. H. Wallace. 1958. Determination of cell viability. Proc. Soc. Exp. Biol. Med. 98:188–192.
14. Heiner, D. C. 1958. Diagnosis of histoplasmosis using precipitin reactions in agar gel. Pediatrics 22:616–627.
15. Hill, G. A., and S. Marcus. 1959. Resistance induced against *Histoplasma capsulatum*: quantitative aspects. J. Infec. Dis. 105:26–30.
16. Howard, D. H. 1964. Intracellular behavior of *Histoplasma capsulatum*. J. Bacteriol. 87:33–38.
17. Kabat, E. A., and M. M. Mayer. 1961. Experimental immunochemistry, 2nd. ed. Charles C Thomas, Publisher, Springfield, Ill.
18. Knight, R. A., G. Hill, and S. Marcus. 1959. Immunization of mice with polysaccharides of *Histoplasma capsulatum*. Proc. Soc. Exp. Biol. Med. 100:356–358.
19. Kong, Y. M., and H. B. Levine. 1967. Experimentally induced immunity in the mycoses. Bacteriol. Rev. 31:35–53.
20. Kong, Y. M., H. B. Levine, and C. E. Smith. 1963. Immunogenic properties of nondisrupted and disrupted spherules of *Coccidioides immitis* in mice. Sabouraudia 2:131–142.
21. Korn, E. D., and D. H. Northcote. 1960. Physical and chemical properties of polysaccharides and glycoproteins of the yeast-cell wall. Biochem. J. 75:12–17.
22. Kurung, J. M., and D. Yegian. 1954. Medium for maintenance and conversion of *Histoplasma capsulatum* to yeastlike phase. Amer. J. Clin. Pathol. 24:505–508.
23. Labzoffsky, N. A., J. B. Fischer, and J. J. Hamvas. 1957. Studies on the antigenic structure of *Histoplasma capsulatum*. Can. J. Microbiol. 3:975–985.
24. Markowitz, H. 1964. Polysaccharide antigens from *Histoplasma capsulatum*. Proc. Soc. Exp. Biol. Med. 115:697–700.
25. Pine, L. 1954. Studies on the growth of *Histoplasma capsulatum*. I. Growth of the yeast phase in liquid media. J. Bacteriol. 68:671–679.
26. Pine, L., C. J. Boone, and D. McLaughlin. 1966. Antigenic properties of the cell wall and other fractions of the yeast form of *Histoplasma capsulatum*. J. Bacteriol. 91:2158–2168.
27. Pine, L., and E. Drouhet. 1963. Sur l'obtention et la conservation de la phase levure d'*Histoplasma capsulatum* et d'*H. duboisi* en milieu chimiquement défini. Ann. Inst. Pasteur 105:798–804.
28. 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.
29. Pinkerton, W., and M. Webber. 1964. A method of injecting small laboratory animals by the ophthalmic plexus route. Proc. Soc. Exp. Biol. Med. 116:959–961.
30. Reca, M. E., and C. C. Campbell. 1967. Growth curves with yeast phase of *Histoplasma capsulatum*. Sabouraudia 5:267–273.
31. Reed, L. J., and H. Muench. 1938. A simple method of estimating fifty per cent end points. Amer. J. Hyg. 27:493–497.
32. Salvin, S. B. 1949. Cysteine and related compounds in the growth of the yeast-like phase of *Histoplasma capsulatum*. J. Infec. Dis. 84:275–283.
33. Salvin, S. B. 1950. Growth of the yeastlike phase of Histoplasma capsulatum in a fluid medium. J. Bacteriol. 59:312–313.
34. Salvin, S. B. 1953. Immunization of mice against *Histoplasma capsulatum*. J. Immunol. 70:267–270.
35. Salvin, S. B. 1955. Further studies on immunization of mice against *Histoplasma capsulatum*. Amer. J. Hyg. 61:72–81.
36. Salvin, S. B. 1956. Growth of *Histoplasma capsulatum* in liquid medium. Public Health Monograph no. 39, p. 61.
37. Salvin, S. B., and E. Ribi. 1955. Antigens from yeast phase *Histoplasma capsulatum*. II. Immunologic properties of protoplasm vs. cell walls. Proc. Soc. Exp. Biol. Med. 90: 287–294.
38. Salvin, S. B., and R. F. Smith. 1959. Antigens from the yeast phase of *Histoplasma capsulatum*. III. Isolation, properties, and activity of a protein-carbohydrate complex. J. Infec. Dis. 105:45–53.
39. Scott, J. E. 1960. Aliphatic ammonium salts in the assay of acidic polysaccharides from tissues. Methods Biochem. Anal. 8:145–197.
40. Westphal, O., O. Lüderitz, and F. Bister. 1952. Uber die extraktion von Bakterien mit Phenol/Wasser. Z. Naturforsch. 7b:148–155.