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Characterization and Evaluation of a Soluble Antigen Complex Prepared from the Yeast Phase of *Histoplasma capsulatum*

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Soluble yeast-phase (YPS) antigen preparations (Reeves et al., 1972) were obtained from three strains of *Histoplasma capsulatum*. These were analyzed by agar-gel diffusion and complement fixation tests with human sera from known cases of histoplasmosis. Two of the preparations contained the H and M antigens normally found in histoplasmin, whereas the third preparation contained the H but not the M antigen. In addition, a group of unknown antigens, designated as the Y (yeast) antigens, were demonstrated in all three YPS preparations. On the basis of Sephadex gel filtration data, the molecular weight of the YPS M antigen was estimated to range from 117,000 to greater than 200,000; that of the YPS H antigen was estimated to range from about 60,000 to greater than 200,000; and that of the Y antigens was estimated to range from less than 10,000 to 100,000. Complement fixation tests with human homologous and heterologous sera showed that the Y antigens were specific for *H. capsulatum;* Y antigens were not detected in the single lot of histoplasmin used in this study. When 60 human histoplasmosis sera and serum samples from nine animals experimentally infected with *H. capsulatum* were analyzed, it was found that antibodies to the Y antigens occurred with about the same frequency as antibodies to the H antigen but with less frequency than that of antibodies to the M antigen. When used in rabbits as immunogens for the preparation of specific antisera to *H. capsulatum*, the components of the YPS preparations caused the formation of numerous cross-reacting antibodies. The data from this study show that the value of the YPS preparations for the serological diagnosis of histoplasmosis rests on the specificity of the H, M, and Y antigens and on the fact that the primary production of antibodies is restricted to these antigens in the course of natural infections. The YPS preparations were found to be stable for a period of at least 11 months under a variety of storage conditions and temperatures. Data obtained with various killing agents and metabolic inhibitors suggest an improved method for preparing the YPS antigens by using a suitable strain and killing the cells with iodoacetate.

Because the yeast phase is the parasitic form of the dimorphic fungus *Histoplasma capsulatum* that elicits a diagnositc antibody response (26), many investigators have attempted to find a yeast-phase antigen with maximal sensitivity and specificity for use in the complement fixation (CF) and agar-gel diffusion tests for the diagnosis of active histoplasmosis (3, 4, 9, 11, 14, 20, 22, 25, 27, 29, 34, 36, 38; G. H. Sweet, Fed. Proc. **26:**3037, 1967; N.W. Brough, Ph.D. thesis, Univ. of North Carolina, Chapel Hill, 1972). In a preliminary note, we described a soluble yeast-phase antigen preparation which proved to be sensitive and less cross-reactive in the CF test than standard whole-cell yeast antigens. In comparison with whole-cell yeast antigens, the soluble antigen preparation produced cleaner CF reactions with clearer end points (24).

In the present study, similar yeast-phase soluble (YPS) preparations from three strains of *H. capsulatum* have been investigated in order to separate and characterize their component antigens, to evaluate the specificity of these antigens, and to determine their utility as reagents for the serological diagnosis of histoplasmosis. In the second half of this report, the effects of chemical agents, temperatures, and incubation period were investigated in order to define the conditions most favorable for the production of the YPS antigens.

The YPS preparations contained antigens which were identified serologically as histoplasmin H and M antigens plus several unidentified

yeast (Y) antigens; the presence or relative concentrations of each of these antigens was characteristic of the strain used. The mechanism responsible for the release of the H and M antigens appeared to be autolytic in nature, whereas that of the Y antigens appeared to be a combination of autolysis and simple diffusion. It was found that high-titered YPS preparations could be easily obtained by suspending the yeast-phase cells in phosphate-buffered saline (PBS), pH 7.0; moreover, the presence of 0.02% iodoacetate helped prevent possible bacterial contamination and gave increased antigen yields.

MATERIALS AND METHODS

Strains, medium, and growth. Three strains of *H. capsulatum* were used in this study for the production of the experimental antigens, 6623, 6624, and A811. Strains 6623 and 6624, which were originally obtained from C. W. Emmons, National Institutes of Health, in 1952, and strain A811, a variant of strain 6624, have been previously described (19, 23). Strain 6623 was isolated from an opossum and was characterized by its profuse production of macroconidia when grown on a minimal vitamin medium. Strain A811 has a monial chain yeast form and essentially asporogenous mycelium; it was used by the Biological Reagents Section (BRS), Center for Disease Control, for the production of standard yeast-phase antigens for the CF test for some 8 to 10 years. Strain 6624 was originally isolated from a human case of histoplasmosis. It has a normal yeast-like morphology and a moderate production of macroconidia by the mycelial phase. It is the wild-type strain from which strain A811 was derived. These three strains appeared to offer a wide range of phenotypic characteristics from which to assess their capability for antigen production. A standard lot of histoplasmin (lot 7), used as a control in this study, was prepared by the BRS with *H. capsulatum* strain Ven-6, a strain which forms few spores and for which no yeast phase has been successfully isolated.

Strains 6623, 6624, and A811 were maintained in the yeast phase on the casein-hydrolysate-cysteine-starch (CCS) medium of Pine and Drouhet (19, 21), which was solidified with 1.5% agar. Stock cultures were grown on CCS agar slants aerobically at 37 C and were stored at 4 C. Transfers were made at 8- to 12-month intervals.

YPS antigen preparation. Freshly grown yeast cells were harvested from CCS agar slants into 0.01 M PBS, pH 7.0, and then used to inoculate 32-oz (about 960-ml) bottles containing 80 ml of CCS agar layered on the flat side. The bottles were carefully rotated to coat the entire agar surface with the inoculum and were incubated aerobically at 37 C for 6 to 8 days. One CCS agar slant provided enough yeast cells to inoculate three bottles.

The YPS antigens were prepared as has been previously described (24). Yeast cells were harvested from the CCS agar bottles into enough 0.001 M borate-buffered saline, pH 8.6, containing 0.02% merthiolate (thimerosal; Eli Lilly & Co.) to give an approximate 30% (vol/vol) suspension. The cells were allowed to stand in this solution at room temperature for three days, at which time no colonies were obtained by streaking samples on CCS agar slants. The cell suspensions were filtered through several layers of sterile gauze to remove any pieces of agar which had been collected. The filtered cells were washed once, resuspended in merthiolate saline to 10% (vol/vol) concentrations, and incubated at 4 C for 2 months. The supernatants from each cell suspension were collected by centrifugation at 600 $\times$ *g* and were stored at 4 C. The cells were resuspended to volume in merthiolate saline. This process was repeated three times with each cell suspension with a 2-week incubation period at 4 C set between harvests. After the last harvest, the supernatants from each cell suspension were pooled, concentrated with Carbowax (polyethylene glycol, 20 M; Union Carbide Corp.), dialyzed against 50 volumes of PBS containing 0.02% sodium azide, and clarified by centrifugation at 1,500 $\times$ *g*.

Serology. Human convalescent sera from culturally or histologically proven cases of histoplasmosis, coccidioidomycosis, blastomycosis, and cryptococcosis were obtained from the BRS and from the Serum Bank in the Scientific Resources Branch, Center for Disease Control. Histoplasmin (lot 7), a CF antigen derived from *Coccidioides immitis* (lot 13), and a yeast-phase CF antigen of *Blastomyces dermatitidis* (lot 24) were obtained from the inventory of the BRS. Sera from three dogs which had been inoculated with viable yeast-phase cells of *H. capsulatum* were kindly supplied by E. Chick, University of Kentucky Medical School, Louisville. Blastomycosis rabbit sera produced during a previous study (22) were used as heterologous controls in the CF test. Sera from four goats which had been inoculated with viable yeast-phase cells of *H. capsulatum* were kindly supplied by W. K. Harrell in the BRS. Sera from two cynomolgus monkeys which had been inoculated intraperitoneally with viable yeast-phase cells of *H. capsulatum* during a previous study (12) were obtained from the inventory of the BRS.

Agar-gel diffusion tests were performed by the method of Ouchterlony (18) by using an agar medium developed by P. Nichols and previously described (7). H and M antigens purified from histoplasmin by diethylaminoethyl chromatography (1) and histoplasmin lot 7 were used as controls. The combined use of the purified M and H antigens with human antisera having only anti-H and anti-M antibody (serum no. 5735), only anti-M antibody (serum no. 61), or anti-M antibody plus antibody to unknown yeast-phase antigen (serum lot 41) served as the basis for identification of H and M precipitin lines.

The Laboratory Branch complement fixation test was performed in microtiter as described (39).

Chemical determinations. Protein was determined by the method of Lowry et al. (15), with bovine albumin as a standard. Total carbohydrate was determined by the phenol-sulfuric acid method of Dubois et al. (6), with rhamnose as a standard. All chemicals used in this study were of reagent quality.

Sephadex gel filtration. Sephadex G-200 gel was

poured and packed with PBS containing 0.02% sodium azide into a column (1.2 by 50 cm); the void volume was determined by chromatographing 5 ml of a 0.2% solution of Dextran Blue 2000. Samples containing approximately 20 mg of protein were eluted with the same buffer with ascending flow at a rate of 9 ml/h and were collected in 5.3-ml samples. The level of protein in the effluent was observed by continuous monitoring of the optical density at 257 nm. The serological activity of the fractions was determined by pooling the fractions into 10.6-ml samples, which were then concentrated fivefold with Carbowax and tested by CF with a standard human histoplasmosis serum. All chromatographic procedures were conducted in a cold room at 4 C. The ranges of approximate molecular weights of the material in the fractions were determined by the calculations of Squire (35).

Rabbit immunization. Adult white rabbits, New Zealand strain, were prebled and were given subcutaneous inoculations dorsally with a vaccine composed of complete Freund adjuvant (BBL, Cockeysville, Md.) emulsified with an equal volume of aqueous antigen which contained 1 mg of protein. The antigens used were the crude YPS preparations of each strain and histoplasmin lot 7. The volume of injected vaccine never exceeded 1 ml. The rabbits were bled at weekly intervals from an ear vein; sera were stored at −20 C without a preservative. The development of antibodies was monitored by CF and agar-gel diffusion tests; the crude YPS antigens and histoplasmin lot 7 were used as controls. Eight weeks after the first injection, the rabbits were given an intramuscular booster of aqueous antigen containing 1 mg of protein. Two weeks after the booster injection, the animals were exsanguinated.

RESULTS

Characteristics of YPS antigens from each strain. YPS-A811 preparation had a substantially lower protein-to-carbohydrate ratio and a lower content of serologically active material than the preparations of strains 6623 and 6624 (Table 1). All of the YPS preparations contained the H antigen of histoplasmin, but only YPS-6623 (Fig. 1) and YPS-6624 (Fig. 2) contained the M antigen. The H band in YPS-6623 (Fig. 1) is only partly discernible because it lies under the M band. Under the conditions of this test, the H band is diffuse and appears to contain more than one band (Fig. 1-3), but it has appeared as a discrete fine band with other sera or under other test conditions. All of the YPS preparations contained a group of unidentified antigens which formed precipitin bands with serum lot 41, but not with serum no. 5735. Both of these sera were from known human cases of histoplasmosis, but serum no. 5735 contained H and M precipitins that were readily discernible in agar-gel diffusion tests, whereas lot 41 contained only M precipitins. Serum lot 41 did, however, contain antibodies to the H antigen which could be detected in the CF test with purified H antigen. When tested at their optimal antigen dilutions, all three YPS preparations failed to react in the CF test with two human blastomycosis and two human coccidioidomycosis sera. None of the unidentified YPS antigens could be detected in histoplasmin lot 7 in agar-gel tests with serum lot 41.

Separation and characterization of antigen groups by gel filtration. A preliminary attempt was made to separate and purify the YPS antigens with ammonium sulfate precipitation; however, more than 60 to 70% of the serologically active material, as determined by CF, was

TABLE 1. *Characteristics of the YPS crude preparations and histoplasmin lot 7 and their pooled and concentrated Sephadex G-200 fractions*

Strain	Pool	Range of V_e/V_o for each pool	CF titer[a]	Protein (μg/ml)	Carbohydrate (μg/ml)	Protein/ Carbohydrate	Agar gel bands
YPS-6623	Crude		256	1,264	108	11.7	H, M, unknowns
YPS-6623	1	0.803-1.447	256	744	60	12.4	H and M
YPS-6623	2	1.461-1.724	8	1,757	80	22.0	H only
YPS-6623	3	1.737-2.658	256	2,486	110	22.6	Unknowns
YPS-6624	Crude		128	1,209	128	9.4	H, M, unknowns
YPS-6624	1	0.921-1.553	16	304	74	4.1	H and M
YPS-6624	2	1.566-1.829	2	593	106	5.6	H only
YPS-6624	3	1.842-3.158	128	1,179	42	28.1	Unknowns
YPS-A811	Crude		32	975	311	3.1	H, unknowns
YPS-A811	1	0.921-1.553	0	213	69	3.1	H only
YPS-A811	2	1.566-1.829	0	304	113	2.7	H only
YPS-A811	3	1.842-2.816	64	1,012	242	4.2	Unknowns
Histoplasmin	Crude		256	6,200	9,600	0.65	H and M
Histoplasmin	1	0.842-1.763	32	913	933	0.98	H and M
Histoplasmin	2	1.776-2.026	0	609	1,163	0.52	None
Histoplasmin	3	2.039-3.132	0	768	497	1.6	None

[a] End-point dilution factors obtained with human histoplasmosis serum lot 41.

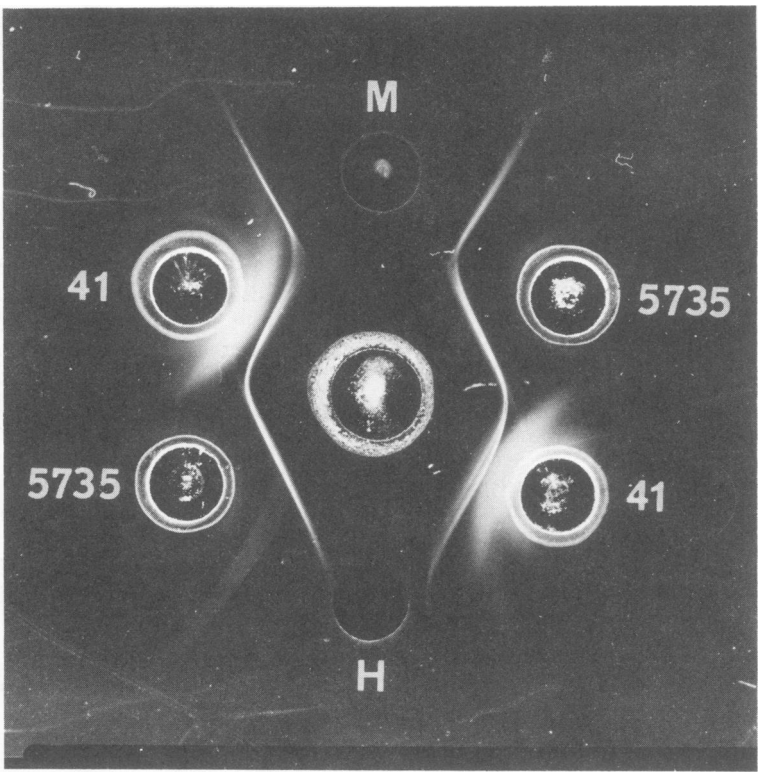

FIG. 1. *Agar-gel diffusion pattern of YPS-6623 (center well) and diethylaminoethyl-purified histoplasmin H and M antigens with human histoplasmosis sera no. 5735 (M and H precipitins) and lot 41 (M and anti unknown precipitins).*

lost by all three preparations during this procedure. Of the active material recovered, most was found in the precipitates formed at the 60% level of saturation.

The Sephadex G-200 elution patterns of the three YPS preparations and of histoplasmin lot 7 are shown in Fig. 4. Marked strain differences of the YPS antigens were quite apparent; these differences were observed in repeated experiments. The YPS-6623 antigens eluted from the column in a series of broad peaks of CF activity, ranging from the void volume to a V_e/V_o (elution volume/void volume) ratio of 2.333. YPS-6624 eluted with a peak near the void volume and with a larger peak centered near a V_e/V_o value of 2.45. YPS-A811 showed no serological activity near the void volume but did show a broad peak of CF activity centered near a V_e/V_o value of 2.3. In contrast, histoplasmin lot 7, fractionated by the same procedure, eluted with a broad peak of CF activity near the void volume only.

To characterize the antigens responsible for the serological activity of these peaks, the fractions from each gel filtration were combined into three pools (Table 1); the pools were concentrated 10- to 15-fold with Carbowax, tested by CF and agar-gel diffusion, and analyzed for protein and carbohydrate. The results show that with YPS-6623 and YPS-6624, the fractions nearest the void volume (pool 1) contained H and M antigens, the pool made up of the fractions after the void volume (pool 2) contained H antigen, and the fractions showing the last eluted peaks of CF activity (pool 3) contained only the unidentified antigens. YPS-A811 differed in that the first two pools contained no detectible CF activity. After a further fivefold concentration of these pools, H antigen was identified in both of them by agar-gel diffusion; however, CF titers could not be determined because the pools had developed anticomplementary activity as a result of the concentration. The histoplasmin peak (pool 1) contained only the H and M antigens. The remaining histoplasmin pools, made up to correspond roughly to the YPS preparation pools, contained no detectible serological activity. The effect of strain differences was again apparent from various aspects of the data presented in Table 1.

Serological characterization and specific-

ity of soluble low-molecular-weight yeast antigens. To simplify the identification of the YPS antigens, the unidentified antigens were designated collectively as the Y antigens. The response of the two human histoplasmosis sera used to examine the YPS preparations in agar-gel diffusion (Fig. 1-3) indicated that not all human histoplasmosis sera can be expected to have antibodies to the Y antigens. To estimate the frequency of antibodies to the Y antigens and their possible importance in the serodiagnosis of histoplasmosis, 60 human histoplasmosis sera were tested by agar-gel diffusion with diethylaminoethyl-purified H and M antigens and with the third pool of the Sephadex G-200 fractions of YPS-6623 (Table 1). Of the 60 sera, 23 (38.3%) contained Y precipitins, 19 (31.7%) contained H precipitins, and 46 (76.7%) contained M precipitins. Of the 23 sera which contained the Y precipitins, 2 (8.7%) did not contain either the H or M precipitins, 1 (4.3%) contained H precipitins but not M precipitins, 15 (65.2%) contained M precipitins but not H precipitins, and 5 (21.7%) contained both H and M precipitins. Thus, antibodies to the Y antigens occurred in convalescent human histoplasmosis sera as frequently as did antibodies to the H antigen but less frequently than antibodies to the M antigen. Because of a lack of data on the individual human sera used in this study, it was not possible to determine whether the presence of Y precipitins is specifically associated with a particular clinical stage of histoplasmosis as has been suggested for the H and M precipitins (2, 10); however, the fact that the Y precipitins were found most frequently with M precipitins and less frequently with H and M precipitins together would seem to suggest that Y precipitins develop either primarily in chronic cases of histoplasmosis or in the latter stages of acute infections.

Sera from three dogs, four goats, and two monkeys that had been inoculated with viable yeast cells of *H. capsulatum* were examined for the presence of H, M, and Y precipitins (see Materials and Methods for the sources of these sera). Sera from one of the dogs contained M and Y precipitins but no H precipitins. Sera from the other two dogs were negative for all precipitins. Sera from all of the goats contained M precipitins, and sera from one of the goats contained H precipitins, but Y precipitins were

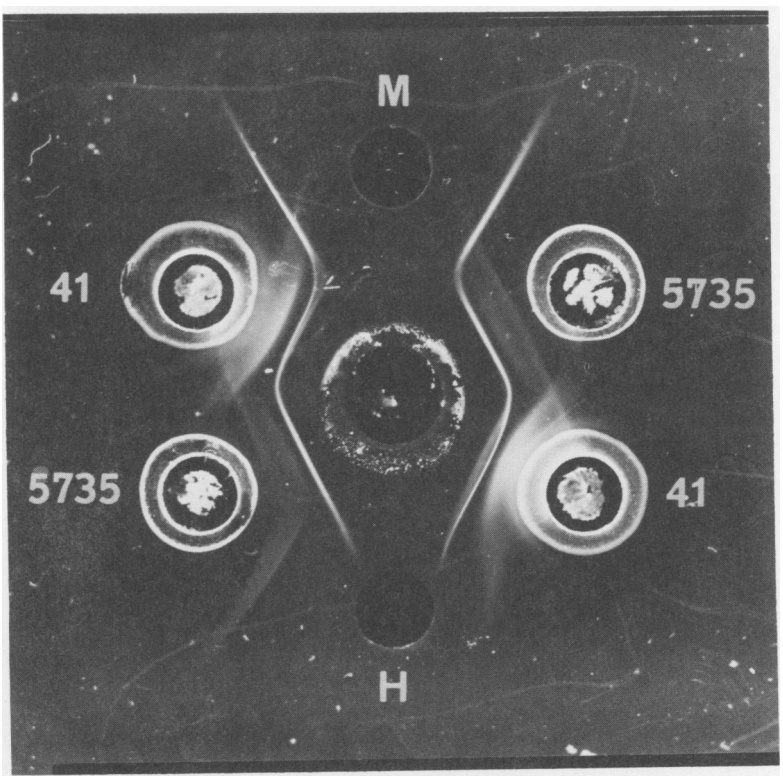

FIG. 2. *Agar-gel diffusion pattern of YPS-6624 (center well) and diethylaminoethyl-purified histoplasmin H and M antigens with human histoplasmosis sera no. 5735 and lot 41.*

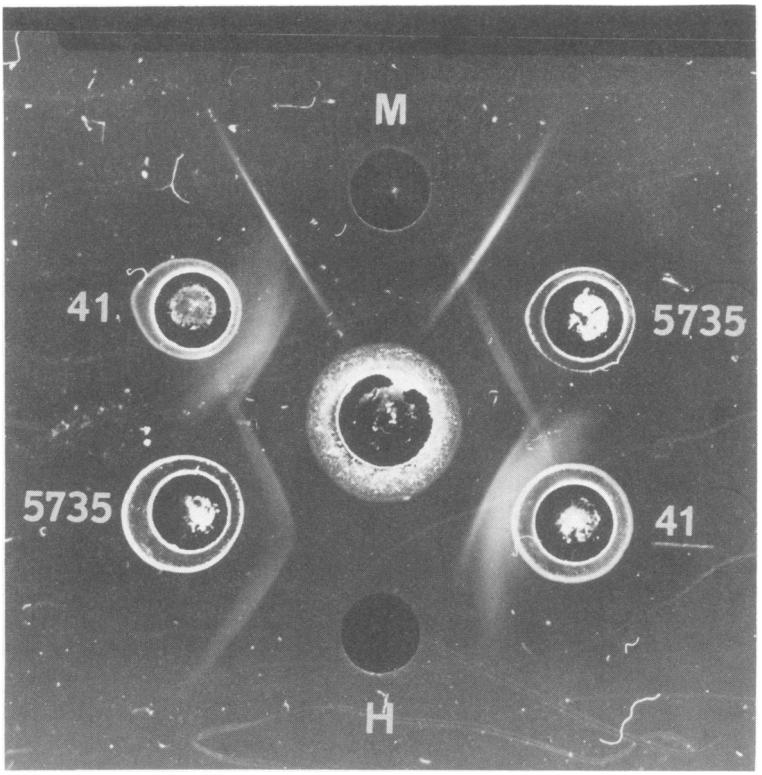

FIG. 3. *Agar-gel diffusion pattern of YPS-A811 (center well) and diethylaminoethyl-purified histoplasmin H and M antigens with human histoplasmosis sera no. 5735 and lot 41.*

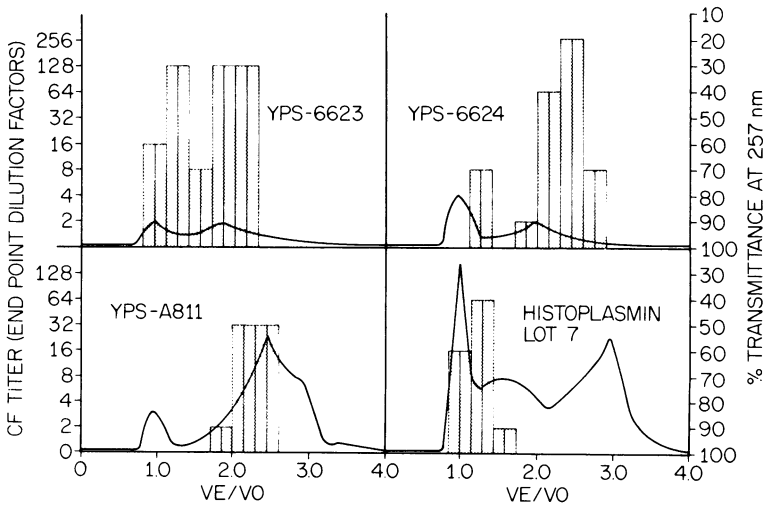

FIG. 4. *Sephadex G-200 chromatography of the YPS preparations and histoplasmin lot 7. Percentage of transmittance at 257 nm is indicated by the solid line, and CF activity with human histoplasmosis serum lot 41 is indicated by the vertical bars. Ve/Vo means elution volume/void volume.*

not found. Sera from both of the monkeys contained M and Y precipitins but no H precipitins. Thus, of the nine animals tested, one (11.1%) had developed H precipitins, three (33.3%) had developed Y precipitins, and seven (77.8%) had developed M precipitins. Although the number of samples was small and may have lacked statistical significance, the pattern of

immune response found in the experimental animals compared well with that found in the convalescent human histoplasmosis sera used in this study.

No attempt was made to distinguish between the reactions of the individual antigens in the Y group because the Y band in all cases was wide and diffuse and showed at least two or more discrete bands with all the sera containing Y precipitins.

On the basis of the preceding work, it appeared that the Y antigens might be an important tool for the serodiagnosis of histoplasmosis; however, the question of whether or not these antigens are specific for *H. capsulatum* still remained. Using agar-gel diffusion tests, a number of human blastomycosis and coccidioidomycosis sera were examined for Y precipitins with YPS-6623 Sephadex G-200 pool 3 with completely negative results. Because the agar-gel technique lacks the sensitivity of the CF reaction, the experiment was repeated by testing human blastomycosis, coccidioidomycosis, and cryptococcosis sera for complement-fixing antibodies to histoplasmin lot 7 and YPS-6623 and their Sephadex G-200 pools. The results presented in Table 2 show that the Y antigens did not react with these heterologous sera, whereas the pools containing the H and M antigens reacted to a small number of the sera. Thus, antibodies to the Y antigens appear to be specifically diagnositc for infections with *H. capsulatum*.

With YPS-6623 Sephadex G-200 pool 3 used as a control, the Y antigens in the YPS preparations were examined for bands of identity by agar-gel diffusion with human histoplasmosis serum lot 41. At least one band of identity was formed (Fig. 5), and several Y antigens are evident in each preparation. A strong M band can be seen with YPS-6623, and a more diffuse and fainter M band can be seen with YPS-6624. H bands were not formed with this serum.

TABLE 2. *Complement fixation results[a] for YPS-6623 and histoplasmin lot 7 and their Sephadex G-200 pools[b] with human heterologous sera*

Antigen	Blasto-mycosis case sera (no. positive/ no. tested)	Coccidioido-mycosis case sera (no. positive/ no. tested)	Crypto-coccosis case sera (no. positive/ no. tested)
YPS-6623	1/21	4/26	0/4
Pool 1	1/21	2/26	0/4
Pool 2	0/10	2/26	0/4
Pool 3	0/24	0/26	0/4
Histoplasmin	0/21	3/26	0/4
Pool 1	1/21	3/26	0/4

[a] Antigens were tested at their optimal antigen dilutions; results were considered positive if an antigen reacted with a serum diluted 1:8 or greater.

[b] See Table 1.

Extensive chemical and serological analyses of a second Sephadex G-200 gel filtration of YPS-6623 showed that antigens which cross-reacted with rabbit anti-blastomycosis and human anti-coccidioidomycosis sera were present in those fractions containing material with molecular weights greater than 200,000 (V_e/V_o = 1.0 to 1.24). Similarly, high-molecular-weight anticomplementary antigens were present in these same fractions and in one of the fractions containing material with a molecular weight less than 10,000. Agar-gel diffusion analyses showed that at least three separate Y antigens were present in those fractions containing material with molecular weights of 100,000 and less (V_e/V_o = 1.65 to 3.0).

Immunogenicity of the YPS preparations. Crude YPS preparations of each strain were used to immunize three rabbits. The serological results (CF and agar-gel double diffusion) showed that each antigen was immunogenic, giving respective H, M, and Y antibodies. But strong cross-reactions of these sera were observed with heterologous antigens of *B. dermatitidis* and *C. immitis*. Use of histoplasmin as an immunogen also produced cross-reactive antibodies.

Stability of the YPS preparations. Suitability of the YPS preparations as standard diagnostic antigens depends not only on their sensitivity and specificity, but on their stability as well. During an 11-month period, samples of the YPS preparations stored at −70, −20, 4, 25, and 37 C and lyophilized and stored at 4 C were tested for loss of CF titer. No loss of activity was observed with those antigens stored at −70 or −20 C. Those stored at 4 C lost about one tube dilution of titer, and those stored at 25 and 37 C lost from one to four tube dilutions of titer. These results show that a good shelf life can be expected for crude YPS preparations if they are stored at a temperature of 4 C or lower.

Testing of reagents and conditions which favor increased production of the YPS antigens. The effect of various chemical agents and their relationship to the release of specific antigens of the YPS complex was investigated by the following series of experiments. Yeast-phase cells of *H. capsulatum* strains 6623 and 6624 were harvested into two saline solutions, one containing 0.02% merthiolate and one containing 0.2% formalin. The suspensions were left at 25 C for three days, after which the kill-

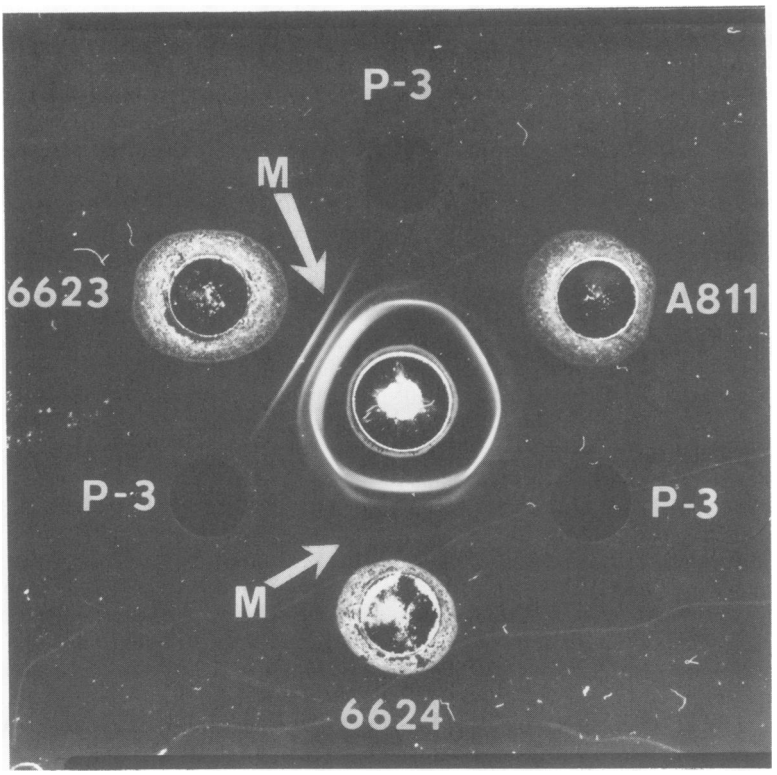

FIG. 5. *Agar-gel diffusion pattern of human histoplasmosis serum lot 41 (center well) with YPS-6623, YPS-6624, YPS-A811, and the Sephadex G-200 pool 3 of YPS-6623 (P-3). The M precipitin lines of YPS-6623 and YPS-6624 are indicated by the arrows.*

ing of the cells was confirmed by plating the suspensions on CCS agar. The suspensions were filtered through two layers of gauze to remove bits of agar and were washed once and resuspended in their respective solutions of merthiolate and formalin. The final cell suspensions contained 10% solids by volume. Each suspension was divided into three equal parts for storage at 4, 25, and 37 C. Samples were taken at weekly intervals, clarified by centrifugation, and tested for CF activity with human histoplasmosis sera lot 41. The results showed that the merthiolate-killed cells yielded more YPS antigen than the formalin-killed cells with both strains. Incubation at either 4 or 25 C proved to be more beneficial for antigen release than incubation at 37 C. Maximal antigen solubilization seems to have been achieved within 1 week of incubation with strain 6624, but required 6 to 7 weeks with strain 6623; however, maximal antigen production with 6623 was fourfold greater than that with 6624.

The effect of the various parameters on the specificity of the antigens was examined by testing representative samples for CF activity with two human blastomycosis and two human coccidioidomycosis sera. The YPS samples from the merthiolate-killed cells of both strains did not react with the heterologous sera, whereas almost all of the formalin-treated samples of both strains did react, particularly those that had been incubated at 37 C. The more frequent association of cross-reactivity with formalin-treated yeast cell antigens than with merthiolate-treated yeast cell antigens has been previously reported (22).

Other methods of killing the cells were evaluated for the release of the specific YPS antigens initially observed with merthiolate (Table 3). Strain 6623 yeast cells were grown and harvested into the test solutions (see Table 3 and footnote) as described for the previous experiment. Samples of the solutions used to remove the cells from the agar (standing solutions) and the wash solutions were also tested for CF activity. Plating on CCS agar showed that after the suspensions had been incubated at 25 C for 3 days, cells suspended in PBS, borate saline, sodium arsenite, or sodium lauryl sulfate were still viable. After 7 days only the PBS-suspended cells were still viable, and after 10 days no viable cells could be detected in this suspension. The results with human histoplasmosis serum lot 29 (antibodies to the H and M

antigens) showed that, in general, only small amounts of the H and M antigens were released after 7 to 10 weeks of incubation (Table 3); reasonable titers were obtained with cells suspended in the PBS, borate saline, iodoacetate, sodium arsenite, and sodium lauryl sulfate solutions. In contrast, none of the mercury-containing compounds, including merthiolate, gave good yields of the antigens. The results with histoplasmosis monkey serum lot 1 (antibodies to the M and Y antigens) showed that rather large quantities of the Y antigens were released into all of the suspending solutions shortly after the cells were harvested (Table 3). The washing procedure removed more antigenic material from the cells so that at the start of the incubation period, the cell suspensions contained only about 5 to 20% of the antigenic material present after harvest. Approximately 3 to 9 weeks of incubation at 4 C were needed for the suspensions to show a level of antigenic material equivalent to that found in the suspensions of freshly harvested cells. The release of Y antigens was slowest in the formalin- and mercury-containing compound solutions (as with the H and M antigens; Table 3), but the total antigen released in each of the suspensions was substantial. It should be noted that the release of Y antigens into the harvest solutions did not seem to be related to whether or not the cells had been killed. Agar-gel diffusion analyses confirmed that all of the suspensions contained soluble H, M, and Y antigens. The specificity of the different antigen preparations was determined by testing for CF activity with three human blastomycosis and two human coccidioidomycosis sera. Of all the preparations, only the formalin-treated preparation reacted with the heterologous sera. The monkey serum used to test for the release of the Y antigens (Table 3) was tested and found to contain CF antibodies to both *B. dermatitidis* and *C. immitis*, but because the test preparations did not react with the heterologous sera (except for the formalin preparation), the titers obtained with the monkey serum are considered to be the result of the

TABLE 3. *Complement fixation titers[a] of YPS antigens released in the presence of various reagents[b] when tested with two different histoplasmosis sera*

Sample	Phosphate-buffered saline, pH 7.0[c]		0.2% Formalin in PBS, pH 7.0		0.02% Iodoacetate in PBS, pH 7.0[d]		0.02% Merthiolate in PBS, pH 7.0[e]	
	Human lot 29	Monkey lot 1	Human lot 29	Monkey lot 1	Human lot 29	Monkey lot 1	Human lot 29	Monkey lot 1
Standing solution	0[f]	128	0	128	2	128	2	128
Wash solution	0	32	0	64	0	64	0	64
Start	0	16	0	16	0	16	0	16
Week 1	0	32	0	16	0	32	0	16
Week 2	2	64	0	16	0	64	0	32
Week 3	2	128	0	32	0	128	0	64
Week 4	2	128	0	32	2	128	0	64
Week 5	4	128	0	32	2	128	0	64
Week 7	8	256	0	32	4	256	0	128
Week 9	8	256	2	64	4	256	0	128
Week 10	16	256	2	64	8	256	0	128
Week 11	32	256	2	64	8	256	0	128
Week 16	32	512	2	64	16	256	2	128
Week 17	32	512	2	64	32	256	2	128
Week 22	32	512	4	128	64	512	2	128

[a] End-point dilution factors; human histoplasmosis serum lot 29 contained antibodies to the H and M antigens; monkey histoplasmosis serum lot 1 contained antibodies to the M and Y antigens. Antigens were prepared from strain 6623.

[b] All cell suspensions contained 10% solids by volume. Incubation temperature was 4 C. Samples taken for serological analysis were first cleared by centrifugation at 1,000 × *g* for 20 min. All inhibitory compounds were made up in PBS (pH 7.0) and, unless otherwise stated, final pH of the suspensions was 7.0. Antigen samples were not concentrated prior to testing.

[c] Yeast cells suspended in borate-buffered saline, pH 8.6, gave results similar to those obtained with PBS-suspended cells.

[d] Yeast cells suspended in 0.02% sodium arsenite or 0.02% sodium lauryl sulfate gave results similar to those obtained with iodoacetate-treated cells.

[e] Yeast cells suspended in 0.02% *p*-chloromercuribenzoate, 0.02% mercuricthiocyanate, or 0.02% phenylmercuric nitrate gave results similar (within one to two dilutions) to those obtained with merthiolate-treated cells.

[f] Titer less than 2.

presence of the M and Y antigens. A comparison of the CF titers of each antigen preparation obtained with human serum lot 29 and monkey lot 1 indicates that the titers obtained with the monkey serum were due mostly to the presence of the Y antigens.

These results show that suspension of *H. capsulatum* yeast-phase cells in PBS, pH 7.0, with or without iodoacetate, sodium arsenite, or sodium lauryl sulfate, gave the highest yields of the YPS antigens. Our laboratory experience with contamination in the absence of a bacterial inhibitor and the yields of H, M, and Y antigens observed when iodoacetate was present suggest that extraction with 0.02% iodoacetate in PBS is at present the best method for preparation of the YPS antigens.

DISCUSSION

The results of this study have shown that a soluble antigen complex recovered from yeast-phase cells of *H. capsulatum* is a stable and specific reagent that can be easily prepared and used for the diagnosis of histoplasmosis in both CF and agar-gel diffusion tests. The increased specificity and other desirable characteristics described for this soluble complex (24) are shown here to be due to cell products which react antigenically as the H and M antigens found in mycelial histoplasmin and to a group of unidentified antigens (Y antigens) which appear to be specific for the yeast phase of *H. capsulatum*. Of these antigens, the H and M antigens of the complex would appear to be of prime importance; however, antibodies to the Y antigens were also found in both human convalescent sera and sera from experimentally infected animals. These antibodies were found about as frequently as antibodies to the H antigen but less frequently than antibodies to the M antigen. Although the data presented here were insufficient to show whether or not a diagnosis of an active infection could be made on the basis of the presence of Y antibodies, the specificity of the Y antigens and the frequency of occurrence of their antibodies suggest that these antigens strongly enhance the diagnostic importance of the YPS complex.

To our knowledge, the presence of the H and M antigens in yeast-phase cells of *H. capsulatum* has been reported by only one other investigator. N. W. Brough (Ph.D thesis, University of North Carolina, Chapel Hill, 1972) extracted formalin-killed, disrupted *H. capsulatum* yeast cells with saline and found that the homogenates contained the C, H, and M antigens described by Heiner (10) as well as three unidentified antigens which formed precipitin lines in agar gel with human histoplasmosis sera. The C antigen (which is common to *H. capsulatum*, *B. dermatitidis*, and *C. immitis*) could not be detected in the YPS preparations by agar-gel diffusion tests during our study. It is possible that the H and M antigens observed in the YPS preparations are of a different chemical composition than those isolated by Bradley et al. (G. Bradley, L. Pine, and M. W. Reeves, unpublished data) from histoplasmin, since the latter were found to have protein-carbohydrate ratios of 2:1 rather than 8:1 or greater as observed for the YPS antigens. The diffuse or multiple bands observed with the YPS H antigens (Fig. 1-3) would seem to indicate that either several types of molecular species of H antigen exist in these preparations or that other unidentified antigens (not Y antigens) are present which are closely associated with the H antigen.

Tompkins (38) reported a soluble, specific antigen extracted from *H. capsulatum* yeast-phase cells with either dioxane or phenol. The antigen had a molecular weight of about 5,000, absorbed well at 260 nm, and appeared to be a combination of nuclear bases and peptides. In agar-gel diffusion tests, this antigen reacted with only 3 of 140 human sera found to be positive in the CF test with whole yeast-cell antigens; however, these three sera had been taken from the only cases from which *H. capsulatum* had been isolated. In the results presented in this study, the Y antigens were found in Sephadex G-200 fractions containing material with a molecular weight of about 5,000 which absorbed well at 260 nm. It is possible that one of the Y antigens may be identical to Tompkins' antigen.

Many antigen preparations derived from the yeast-phase cells of *H. capsulatum* have been reported as a result of precipitation of antigens from yeast culture media (13, 16, 17, 28, 34, 37), disruption of yeast cells by grinding or sonication (3, 5, 20, 27, 31, 36; G. H. Sweet, Fed. Proc. **26:**3037, 1967), and extraction by extensive and complex procedures (14). None of these preparations have been shown to contain the H and M antigens, and only a few of these antigens have been shown to be specific for *H. capsulatum*. None have been used for the routine diagnosis of histoplasmosis. Although it seems that an infinite variety of antigens can be extracted from *H. capsulatum* yeast-phase cells, we consider such antigens to be of little practical value unless it can be shown that antibody is formed to them during active infections and that they are thus capable of acting as diagnostic reagents. For this reason we have evaluated the

YPS preparations only with sera from humans and animals known to have been infected with *H. capsulatum* and other related fungi.

Our results lead us to conclude that the YPS preparations described here and earlier (24) have an excellent potential as a diagnostic reagent for the detection of histoplasmosis. The antigens are highly stable, in the crude form are not anticomplementary, and have a specificity in the CF test comparable to that of histoplasmin and better than that found with whole yeast-cell suspensions. The complex is soluble and thus permits an easy and uncomplicated reading of the CF test. The presence of the Y antigens extends the range of sensitivity and specificity of the CF and agar-gel diffusion tests and thus increases the potential numbers of positive sera which may be identified. Preparation of the YPS antigens is simple, and the procedure required is no more complex than that presently used for the preparation of histoplasmin. The use of YPS preparations would eliminate the use of two separate antigens which are presently used in the CF and agar-gel diffusion tests (whole yeast-cell suspensions and mycelial culture filtrates) since these preparations can be used for both tests.

Conditions most favorable for the preparation of the YPS antigens have been determined to be suspension of the yeast-phase cells in PBS, pH 7.0, and incubation at 4 C for at least 3 months. The addition of 0.02% iodoacetate is advisable to prevent bacterial contamination and to eliminate the hazard of infection with *H. capsulatum*. Treatment with sodium arsenite and sodium lauryl sulfate yielded adequate amounts of the YPS antigens, but these reagents were not as effective as iodoacetate in killing the yeast cells. As compared with the other reagents tested, merthiolate had an adverse effect on the release of the YPS antigens. The effect of formalin was also detrimental and resulted in the solubilization of high levels of cross-reactive antigens (22). Cross-reactive and anticomplementary substances were also present in the merthiolate-prepared extracts, but these substances were not very serologically active in the crude YPS preparations.

The quantity and quality of the YPS antigens produced were found to be strain dependent (Table 1), a fact which has been previously noted in the preparation of whole yeast-cell and mycelial culture filtrate antigens (7, 8, 30, 32, 33); therefore, careful selection of strain is necessary to ensure adequate production of the YPS complex.

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