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H and M Antigens of *Histoplasma capsulatum*: Preparation of Antisera and Location of These Antigens in Yeast-Phase Cells

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Antiserum has been prepared in rabbits against the H and M antigens of *H. capsulatum* with immunoelectrophoretic precipitin arcs used as vaccines. The antiserum is specific for *H. capsulatum* in the immunodiffusion test and can be used as reference serum for identifying antibodies to these antigens in sera from suspected cases of histoplasmosis. We found that (i) H and M antigens are not located on the surface of yeast-phase cells and (ii) complement fixation releases the antigens reactive in the complement fixation test from yeast-phase cells.

The immunodiffusion (ID) test has been shown to be a sensitive, specific, and simple test for the presumptive laboratory diagnosis of histoplasmosis (1, 8, 9, 11, 12, 18, 23). Bauman and Smith (1) reported that the ID test detected precipitating antibodies in the sera from 63 of 70 (90%) proven cases of histoplasmosis. With the same sera, the complement fixation (CF) test, with histoplasmin and yeast-phase (YP) antigens, was positive with 72.8 and 94.3%, respectively. The ID test determines the presence of antibodies in the patient's serum to the H and M antigens of *Histoplasma capsulatum*. H precipitins in the serum suggest an early and acute stage of the infection; M precipitins indicate a prior infection, a chronic progressive disease, a late stage of recovery, or a recent skin test with histoplasmin (9).

The identification of H and M antibodies in sera by the ID test requires the use of a properly prepared antigen (2, 4-6) and a control antiserum containing antibodies to the H and M antigens (6). An adequate supply of human sera that gives good H and M precipitin lines is difficult to obtain. Most of these sera either lack one of the antibodies or produce diffuse lines in the ID test, making it difficult to determine whether lines of identity are formed with the test serum (6). Control antisera, prepared in animals and containing H and M precipitins in the proper proportion to one another, would eliminate the uncertainties inherent in working with human sera. Kaplan et al. (10) infected cynomolgus monkeys with viable, YP *H. capsulatum*. These animals developed high CF and latex agglutination titers and M precipitins. Two of the 10 animals inoculated also developed H precipitins. Sharbaugh et al. (19) infected Hereford calves with viable, mycelial fragments of *H. capsulatum*. These animals also developed high CF and latex agglutination titers and H and M precipitins. Although satisfactory control antisera were obtained from the monkeys and calves, many laboratories do not have the facilities for handling these animals, particularly when the animals have been infected with an organism such as *H. capsulatum*. In the past, we attempted to prepare control antiserum for the CF and ID tests for histoplasmosis by inoculating calves and goats with viable YP *H. capsulatum* cells (W. Kaplan, L. Kaufman, and K. Harrell, unpublished data). Essentially, the cows developed no CF antibodies or H and M precipitins, but all of the goats developed CF antibodies and M precipitins. One of the four goats also developed H precipitins. In the ID test, the goat antiserum containing H and M precipitins produced somewhat weak, diffuse precipitin lines. Although useful, they were not entirely satisfactory for determining lines of identity with test sera.

The H and M antigens can be separated from one another by immunoelectrophoresis (IE) (6). We isolated the H and the M antigen-antibody complex with this technique and used the material to immunize rabbits. The resulting antisera gave specific, sharp precipitin lines in the ID test when reacted against a crude histoplasmin preparation. When these two antisera were pooled, an antiserum was obtained that gave sharp, well-separated H and M precipitin lines. This antiserum can be used as reference

material for the identification of H and M antibodies in sera from patients with suspected histoplasmosis.

MATERIALS AND METHODS

Control antigens and antisera. The crude histoplasmin used for the ID and IE tests was prepared as previously described (7) with the District and Ven 6 strains of *H. capsulatum*. This consisted of growing the two strains on Smith's asparagine medium (21) for 6 months at room temperature as static cultures, separating the mycelial mat by filtration, and concentrating the supernatant approximately 10-fold. Histoplasmin for the CF test was prepared in the same manner, except that it was not concentrated. Partially purified H and M antigens of *H. capsulatum* were prepared as described by Bradley et al. (2). For absorption studies, YP cells of *H. capsulatum* strain A811 and *Blastomyces dermatitidis* strain B373 were grown on brain heart infusion agar at 37°C for 7 days. The cells were harvested with saline containing 1% thimerosal and washed two times with the suspending fluid. *H. capsulatum* strain A811 was also used for the YP CF antigen (7).

The *H. capsulatum* antiserum used for detecting H and M antigens in the IE test was prepared by inoculating goats with live YP cells of *H. capsulatum* (unpublished data). This antiserum gave weak but detectable precipitin arcs. The human sera were from patients with proven cases of histoplasmosis and contained antibodies to the H and M antigens or only to the M antigen.

Serological test procedures. IE was conducted as previously described (6), except that it was continued until the dye marker had migrated 4 to 4.5 cm. The ID test was performed in 1% agarose dissolved in the barbital buffer used for IE. For the latter test, two 1-in^2 (about 2.5-cm^2) templates, each containing six funnel-shaped wells around a central well, were placed on a microscope slide (1 by 3 inches), and the molten agar was allowed to flow between the template and the slide. The dimensions of the wells and agar thickness were the same as described by Busey and Hinton (3). Dilutions of the antigen or sera were made with the barbital buffer and added to the wells with a microliter pipette. Both the IE and ID test slides were incubated in a moist chamber at 37°C for 18 to 24 h.

The CF titers were determined by the Center for Disease Control Laboratory Branch CF test (15).

Preparation of the antisera. Vaccines for immunizing rabbits were prepared as follows. The H and M antigens were separated from one another by IE (see Fig. 1b), and each precipitin arc was cut out of the agar and placed in 0.02 M phosphate-buffered saline, pH 7.4 (PBS). The area in which the precipitin arcs overlapped was excluded. The arcs from several IE runs were pooled, and when enough had been collected to immunize the desired number of rabbits, the arcs were washed five times with 500-ml volumes of PBS over a period of several days. As much of the PBS as possible was removed by straining through cheesecloth, and the antigen-antibody-agar complex was macerated by forcing it through a 21-gauge needle. An equal volume of complete Freund adjuvant was added, and the mixture was repeatedly forced through a 21-gauge needle until a stable emulsion was formed. Since we did not have a method of quantitating the amount of H or M antigen in the antigen-antibody complex, the number of suspended precipitin arcs included in each vaccine preparation was used as a rough measure of antigen concentration.

For primary immunization, rabbits were inoculated intradermally in four to six sites in the back and also in the front footpads. Three-fourths of the vaccine dose was given intradermally; the remainder was divided, and equal amounts were injected into the footpads. Two groups of rabbits were given a second intradermal inoculation, with one-fourth the first dose 7 days after the initial inoculation. The concentration of the vaccine dose varied with different groups of rabbits (from 36 to 84 precipitin arcs per rabbit). There was no observable difference in the reactivity of antiserum obtained from the different groups of rabbits. After the primary immunization, the rabbits were rested 6 weeks, and a booster inoculation was given intravenously with 0.2-ml amounts of either crude histoplasmin or purified H or M antigen. Test bleedings were taken 7 to 10 days after the booster inoculation.

RESULTS

Preparation of anti-M sera. Five successive lots of anti-M sera were prepared (Table 1). The vaccine for the first lot was precipitin arcs obtained by electrophoresing crude histoplasmin and reacting this with the H and M antiserum prepared in goats. When positive human sera were used for reference in the ID test, this antiserum, labeled M-lot 1, formed a weak precipitin line with the H antigen, a strong line with the M antigen, and a second strong line that was distinct from both the H and M lines (Table 1). When the M-lot 1 antiserum was used to develop precipitin arcs after electrophoresis of crude histoplasmin, two precipitin arcs were observed in the area of the M antigen (Fig. 1b, c). Since these two antigens had similar electrophoretic mobilities but were distinct in the ID test, the second antigen was labeled non-M (NM). Antiserum prepared with the M-lot 1 and crude histoplasmin precipitin arcs (M-lot 2) also reacted with the M and NM antigens in the ID test, but did not react with the H antigen. Three additional lots of M antisera were prepared in an attempt to obtain an antiserum specific for the M antigen. Each of the latter three lots was prepared with precipitin arcs containing an M antigen separated from the H antigen by column chromatography and referred to as purified M antigen. IE showed that this preparation contained both the M and NM antigens (Fig. 1c). The precipitin activity

TABLE 1. *Antibody response of rabbits to H. capsulatum immunoelectrophoresis precipitin arc vaccines*

Antisera produced	Vaccines used for primary inoculation[a]		Antibody response detected by immunodiffusion[b]			
	Antigen	Antiserum	H	M	NM	Other
M-lot 1	Crude histoplasmin	Goat lot 1	w	+	+	Anti-goat
M-lot 2	Crude histoplasmin	M-lot 1	−	+	+	w, unknown
M-lot 3	Purified M	M-lot 2	−	+	+	w, unknown
M-lot 4	Purified M	Human M	−	+	+	Anti-human
M-lot 5	Purified M	M-lot 4	−	+	+	w, unknown
H-lot 1	Crude histoplasmin	Goat lot 1	+	−	w	Anti-goat
H-lot 2	Crude histoplasmin	H-lot 1	+	−	w	−
H-lot 3	Purified H	H-lot 2	+	−	−	−
NM-lot 1	Purified M	H-lot 2	−	w	+	w, unknown

[a] Immunoelectrophoresis precipitin arcs mixed with equal volume of complete Freund adjuvant and inoculated intradermally and into front footpads.

[b] H, Purified H antigen; M, purified M antigen containing non-M (NM) antigen; w, weak precipitin line; +, strong precipitin line; −, no line.

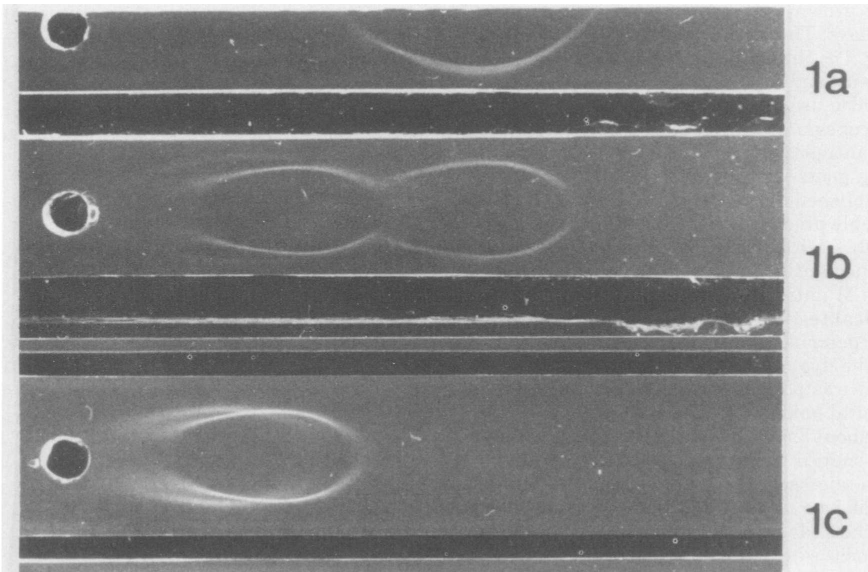

FIG. 1. *Immunoelectrophoresis of (a) purified H antigen; (b) crude histoplasmin; (c) purified M plus NM antigens. Pool 1 H and M antiserum added to the troughs.*

of these three lots was comparable to that of the M-lot 2 antiserum (Table 1). The precipitin arcs used to prepare M-lot 4 antiserum were a complex of the purified M antigen and a human serum that reacted only with the M antigen. Since the M-lot 4 antiserum reacted with both the M and NM antigens, the human serum used also contained antibodies to the NM antigen, but at a level not observable by either ID or IE. The M-lot 5 antiserum gave lines of identity with an M precipitin line of a positive control human serum that contained only M precipitins (Fig. 3).

To clarify the antigenic relationship between the M and the NM antigens, we prepared an antiserum from precipitin arcs composed of purified M antigen and an anti-H serum (H-lot 2, Table 1) that reacted with the H and NM antigens, but not with the M antigen. This antiserum also gave strong precipitin lines with the NM antigen and, in some ID slides, a weak precipitin line with the M antigen. Figure 2 shows the lines of nonidentity between the M and NM antisera. We have not observed any reaction of this antiserum with the ID antigens of *B. dermatitidis* or *Coccidioides immitis*, and therefore assume that it is specific for *H. capsulatum*. The diagnostic significance of these antibodies is not known.

Preparation of anti-H sera. Three successive lots of anti-H sera were prepared (Table 1). H-lot 1 antiserum was prepared from precipitin arcs composed of crude histoplasmin and the goat anti-*H. capsulatum* serum, and H-lot 2

was prepared from precipitin arcs of crude histoplasmin and the H-lot 1 antiserum. Each of these antisera reacted with the H and the NM antigens, suggesting that some of the NM antigen was mixed or trailed very closely behind the H antigen when crude histoplasmin was electrophoresed. The third lot of H antiserum (H-lot 3) was prepared from precipitin arcs composed of a purified H antigen and the H-lot 2 antiserum. This H antigen preparation did not contain either the M or NM antigen as shown by IE (Fig. 1a). The H-lot 3 antiserum reacted only with the H antigen in the ID test and gave a line of identity with a H precipitin line of a positive control human serum (Fig. 3).

The final product containing antibodies to the H and M antigens was prepared by mixing equal volumes of the M-lot 5 and the H-lot 3 antisera (labeled pool 1). To obtain sharp, well-separated H and M precipitin lines in the micro-ID test, we had to dilute this mixture 1:16 with normal rabbit serum. Figure 3 illustrates the use of this antiserum in identifying H and M antibodies in human case serum. The control serum (pool 1) is in wells 1 and 4, and different human sera are in wells 2, 3, 5, and 6. The human sera in wells 2, 3, and 5 contain H and M antibodies, and the serum in well 6 contains only M antibodies. The human serum in well 3 also contains antibodies to the NM antigen. This is one of the few human sera we have studied that show this latter precipitin line.

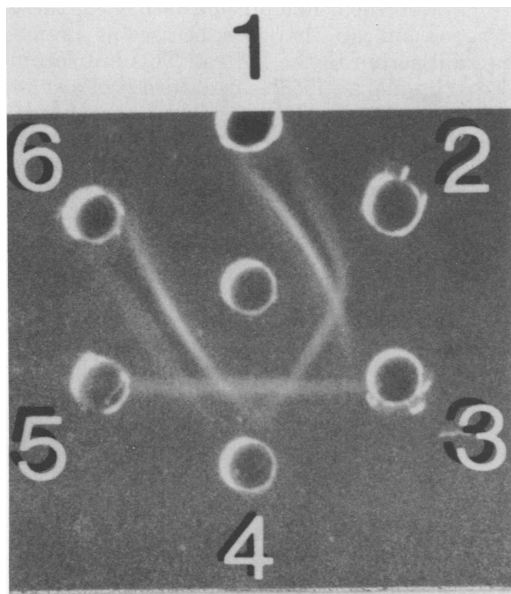

FIG. 2. *Immunodiffusion of H, M, and NM antisera. Center well, crude histoplasmin; wells 2 and 5, M antiserum; well 3, NM antiserum; well 4, H antiserum; wells 1 and 6, empty.*

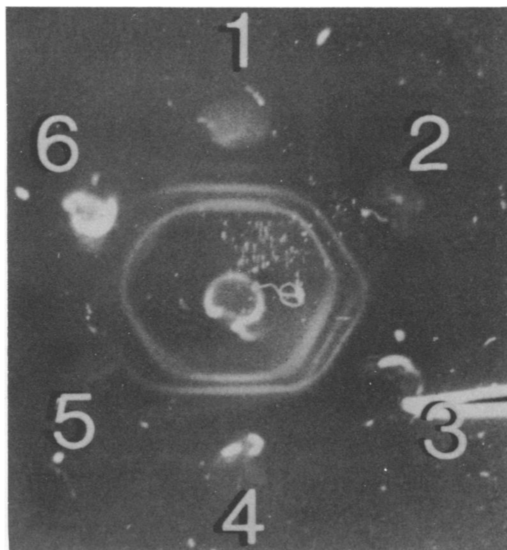

FIG. 3. *Use of rabbit H and M antiserum to identify antibodies in sera from patients with proven cases of histoplasmosis. Center well, crude histoplasmin; wells 1 and 4, rabbit H and M antiserum: wells 2 and 5, human sera containing H and M antibodies; well 3, human serum containing H, M, and NM antibodies; well 6, human serum containing only M antibodies.*

The pool 1 antiserum did not react with *B. dermatitidis* or *C. immitis* antigens in the ID test. However, in some IE preparations, a weak, diffuse precipitin line was observed with *H. capsulatum* and *B. dermatitidis* ID antigens. This line was distinct from the M, NM, and H precipitin lines. In retrospect, we determined that antibodies to this nonspecific antigen were present in the M antiserum. Absorption of the pool 1 antiserum with washed YP cells of either *B. dermatitidis* or *H. capsulatum* removed these antibodies.

Additional serological studies of pool 1 H and M antiserum. The CF titers of the H, M, pool 1 of H and M, and NM antisera were unusually high (Table 2). The H-lot 3 antiserum was reactive with the crude histoplasmin and the purified H antigen, but was negative with the purified M antigen at the starting dilution of 1:64. The M-lot 5 antisera was reactive with all three test antigens, but was approximately 10-fold less active with the purified H antigen than with the other two. The NM-lot 1 antiserum gave results comparable to results obtained with the M-lot 5 antiserum.

The results obtained when the pool 1 H and M antiserum was absorbed with YP cells of *H. capsulatum* and *B. dermatitidis* were interesting. Equal packed-cell volumes of the two orga-

TABLE 2. *Representative CF titers of H. capsulatum H and M antisera*

Antiserum	CF antigen used			
	Crude histoplasmin	Purified M	Purified H	Yeast phase
H-lot 3	4,096[a]	<64	4,096	ND[b]
M-lot 5	2,048	4,096	256	ND
NM-lot 1	4,096	4,096	<64	ND
H + M[c]	4,096	2,048	4,096	4,096
H + M absorbed[d]	4,096	2,048	4,096	4,096

[a] Reciprocal of titer; starting dilution, 1:64.
[b] ND, Not done.
[c] Consists of equal volumes of H-lot 3 and M-lot 5 antisera.
[d] Absorbed with equal volumes of serum and washed packed yeast-phase cells of *H. capsulatum* and *B. dermatitidis*.

nisms were mixed together and washed with saline. The pool 1 antiserum was absorbed at a ratio of 1 part serum to 1 part packed cells. The CF titer of the absorbed antiserum was the same as the titer of the unabsorbed antiserum with crude histoplasmin, purified H and M antigen, and YP CF antigen (Table 2). In one experiment, the ratio of absorbing cells to serum was increased to 10:1 without a significant change in the CF titer. As mentioned earlier, the pool 1 antiserum was diluted 1:16 with normal rabbit serum for use in the ID test. This optimal dilution for such use did not change after absorption. Thus, we concluded that the H and M antigens were not on the surface of the YP cells of *H. capsulatum*. This was further substantiated when the globulin fractions of the H, M, and NM antisera were labeled with fluorescein isothiocyanate. These conjugates did not stain the YP cells of either *H. capsulatum* or *B. dermatitidis*.

These observations suggest that, in the CF test, complement fixation changes the surface structure of the YP cells in a manner that releases CF antigens from inside the cells into the surrounding medium. To test this hypothesis, we set up a CF test so that, after the antigen-antibody-complement mixture was incubated (at 4°C) overnight, the yeast cells were removed by centrifugation and the CF titer was determined by using the supernatant material as antigen. To insure that any H and M antigens that had leaked out of the cells were removed (17), we washed the YP CF antigen two times with saline just before setting up the test. The CF titer resulting from the supernatant material being used as an antigen was 1:256 to 1:512, compared with a CF titer of 1:4,096 obtained in the routine test. Two controls were run in parallel with the latter test. The antiserum was omitted from the mixture incubated overnight at 4°C in one, and in the other the complement was omitted. After the YP antigen was removed, the antiserum or complement was added and the two tests were incubated for an additional 6 h at 4°C to permit CF. After the hemolytic system was added, neither of these tests demonstrated any CF activity. Therefore, we concluded that fixation of complement in this system alters the surface of the YP cells, thus releasing the CF antigens. The antigens released by CF were not concentrated enough to react in the ID test, and therefore we could not identify them.

All of the antisera studied were anticomplementary in the CF test at a dilution of 1:16 to 1:32, which is not unusual for rabbit serum. When the anticomplementary activity was taken into consideration, none of the antisera listed in Table 2 reacted with CF antigens of *B. dermatitidis* or *C. immitis*.

DISCUSSION

One of the major limitations to the use of the ID test as an aid in the laboratory diagnosis of histoplasmosis has been the shortage of reference antisera for the identification of antibodies to the H and M antigens of *H. capsulatum* in the patients' sera. The use of specific immune precipitates from IE slides for immunization of rabbits has produced an antiserum that gives strong, well-separated precipitin lines for these antigens and can, therefore, be used as a reference antiserum in the ID test. This antiserum also contained antibodies to a third *H. capsulatum* antigen that we have called a non-M antigen, since it migrates with the M antigen in IE but was found to be distinct from this antigen in the ID test (Fig. 2). Because this antiserum did not react with ID antigens of *B. dermatitidis* or *C. immitis*, we concluded that the non-M antigen, like the H and M antigens, is specific for *H. capsulatum*. Only a few human sera from patients with proven cases of histoplasmosis have reacted with the non-M antigen, and therefore the clinical significance, if any, of these antibodies is not known.

Reeves et al. (16, 17) have shown that H and M antigens are present in the YP cells of some but not all strains of *H. capsulatum*. These antigens, plus several others which they called Y, were present in the suspending medium in which the thimerosal-killed cells were suspended. Our data show that the H and M antigens are not located on the surface of the YP cells. Absorption of H and M antiserum with washed YP cells of *H. capsulatum* did not reduce the CF or ID titer of this antiserum. Fur-

thermore, a fluorescent-antibody conjugate prepared from the H, M, and NM antisera did not stain the homologous YP cells. These conjugates still reacted with the H, M, and NM antigens in the ID test, showing that the conjugation procedures had not altered the reactivity of these antibodies. H and M antibodies could not be demonstrated by the ID test in antiserum prepared with intact, killed YP cells of *H. capsulatum* (14), another indication that these are not surface antigens.

These studies have also shown that CF releases CF antigens from YP cells. When washed YP cells that had been incubated at 4°C overnight in the presence of the H and M antiserum and complement were removed by centrifugation, the supernatant reacted to a titer of 1:256 to 1:512 after the hemolytic system was added. Controls in which either the antiserum or the complement was omitted from the overnight incubation and then added after the YP cells were removed had no CF activity. The CF titer obtained with the supernatant antigen differed significantly from that obtained with the YP antigen routinely used in the CF test (1:256 to 512 versus 1:2,048 or higher). This difference probably reflects the presence of the soluble H, M, and Y antigens (17) in the routine antigen and those released by CF. The YP cells used to demonstrate the release of CF antigens by CF were washed just before the test was set up and therefore did not contain the former antigens.

A number of investigators have used antigen-antibody precipitin complexes as vaccines for preparing specific antisera (20, 22, 23). The use of such complexes in this study had the following benefits: (i) the H and M (NM) antigens were separated from one another enough that antiserum specific to each could be prepared; (ii) the antigen-antibody complex mixed with adjuvant was very antigenic in rabbits, whereas in previous studies with only the H or the M antigen plus adjuvant, no antibodies to these antigens were elicited (2); and (iii) the pool of the H and M antisera was specific for *H. capsulatum* in the ID test and could be used as a reference antiserum to identify precipitins to the H and M antigens in sera from patients suspected of having histoplasmosis.

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