JOURNAL OF CLINICAL MICROBIOLOGY, Mar. 1975, p. 330-334

Vol. 1, No. 3
Printed in U.S.A.

NOTES

Evaluation of Histoplasmin for the Presence of H and M Antigens: Some Difficulties Encountered in the Production and Evaluation of a Product Suitable for the Immunodiffusion Test

HERMAN GROSS, GEORGIA BRADLEY, LEO PINE, SAM GRAY, JAMES H. GREEN, AND W. KNOX HARRELL

Center for Disease Control, Atlanta, Georgia 30333

Received for publication 22 November 1974

The development of a single histoplasmin suitable for use in immunodiffusion testing of a variety of human histoplasmosis sera depends not only upon the presence of both antigens, but on relative concentration of one to the other, and perhaps on the chemical characteristics of the H and M antigens of a given strain.

In the serological diagnosis of histoplasmosis, mycelial phase histoplasmin serves two purposes. It is used as an antigen in the complement fixation test, and it is used to determine the presence of H or M precipitins in the immunodiffusion (ID) test. Generally, the formation of an H precipitin line with the patient's serum suggests an early and acute state of the infection; the formation of an M precipitin line is indicative of prior infection, a chronic progressive disease, a late stage of recovery by the patient, or an indication of a recent skin test with histoplasmin (5).

Histoplasmin suitable for use in the ID test is prepared and evaluated, respectively, by the Bacterial and Fungal Products Branch and the Fungus Immunology Branch, Center for Disease Control. Although progress has been made in the production and preparation of histoplasmin so that yields of H and M antigens are consistent (1-3), experiences of the above laboratories and those of the Products Development Branch have emphasized certain difficulties in the preparation and evaluation of the product that is to be used as a diagnostic reagent. Similarly, pertinent inquiries were received from several laboratories regarding culture procedure, strains, availability, and suitability of identifying sera. The purpose of this note is to emphasize certain procedures for producing histoplasmin and in particular to delineate the difficulties encountered in evaluating histoplasmin intended for use as a diagnostic reagent in the ID test.

In the ID test, proper identification of the H and M precipitin lines depends upon the use of histoplasmin of a correct and balanced H and M composition with a satisfactory human histoplasmosis serum reactive to both antigens. Under certain conditions the ID test may not show precipitin lines, may fail to separate the H from the M line regardless of the relative respective antigen concentration, may reverse the relative position of the H and M lines generally observed (9), or, due to the serum used or to the strain from which the antigen was obtained, may give broad diffuse ill-defined precipitin lines. In our experience, a reliable procedure for formation of distinct H and M precipitin lines is that of agar gel immunoelectrophoresis described below (Fig. 1).

Immunoelectrophoresis was conducted by using barbital buffer, pH 8.5 (0.0375 M) (5.53 g of barbituric acid, 30.9 g of sodium barbital 0.4 g of merthiolate and made to 2 liters for a $\times 2$ concentrated solution). Six standard glass slides (1 by 3 inches [2.54 $\times$ 7.62 cm]) were held in a Gelman slide holder, three slides in line on each side. These were coated with a 1% solution of agarose in the 0.0375 M barbital buffer (9.5 ml of melted agarose solution per three slides). The entire slide container was placed in the electrophoresis apparatus and the terminal slides at each electrode were connected to the buffer with a no. 3 Whatman filter paper wick. Electrophoresis was done at 250 V with 11 mA current for 1 h. Bromophenol blue dye marker was used to monitor the sample progress across the gel. Histoplasmin A50 is a product of strain 6623 concentrated by alcohol precipitation and puri-

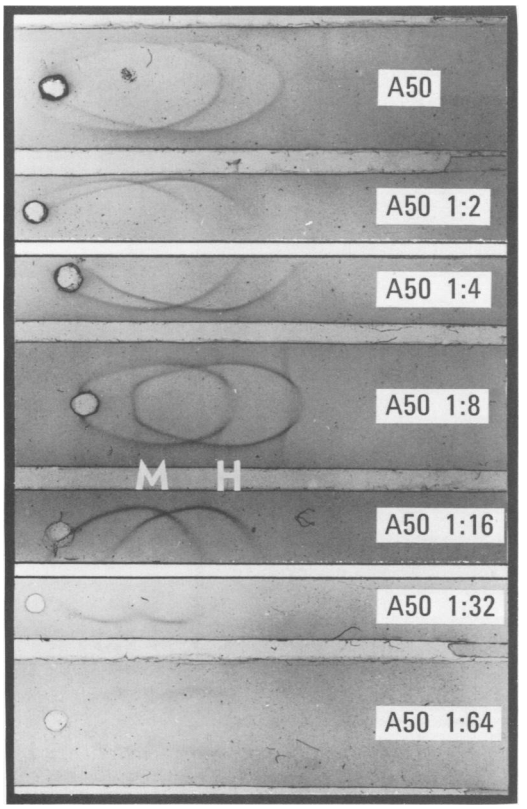

FIG. 1. *Immunoelectrophoresis assay of histoplasmin A50 H and M antigens against human histoplasmosis serum 10717.*

fied by passage through Sephadex G 100 (1). Slides were stained with 0.5% amido black.

Satisfactory preparation of histoplasmin for the production of H and M antigens depends primarily upon choosing the correct strain and the correct form of the inoculum. C. E. Smith's asparagine medium (8) is generally satisfactory. Using mycelial fragments obtained by scraping the surface of agar slants gives great variation among flasks and generally an overall poor yield. Therefore, we prefer to use a strain known to produce H and M antigens (strain 6623 [ATCC 26320], strain 6624 [ATCC 12700], or strain District) and a strain from which a yeast phase can be obtained as inoculum (2, 3). Shake cultures are preferred since production of the antigens is more rapid (1 to 2 months at 25 C), but when a shaker is not available or does not have the capacity for the volumes needed, stagnant cultures similarly inoculated with yeast phase give equally reliable and consistent results but require longer periods (5 to 6 months). When a yeast-phase inoculum was used from strain 6623 or 6624, solid and confluent mats were formed within 2 weeks in all flasks incubated statically. With strains which do not produce a yeast phase (District), several primary shake cultures are made with a mycelial suspension scraped from an agar slant; then at 2 or 3 weeks, a single flask is chosen which demonstrates a heavy homogenous suspension of mycelial elements. Secondary shake flasks are inoculated with this source of inoculum. After the cultures are harvested (4), the supernatant is generally concentrated 10-fold, and the optimal concentration for the ID test is determined against several sera.

Evaluation of the histoplasmin formed rests primarily on the use of an adequate serum to demonstrate the presence of H and M antigens; secondarily, it depends upon the characteristics of the histoplasmin formed, i.e., upon the relative concentration of H antigen formed to that of M and perhaps upon the chemical nature of the two antigens. Thus, for several years we have used human serum 5735 to evaluate the factors influencing the histoplasmin production of many strains (2, 3) for the determination of the chemical composition of partially purified H and M antigens (1) and for the characterization of yeast-phase complement-fixing antigens known to contain H and M antigens (6) (Fig. 2A). Initially, strong and well-defined precipitin lines were observed for H and M antigens even though relative titers of H to M might be equal or might differ 32-fold (Fig. 2 of reference 2). It became obvious that the character of the precipitin lines could change radically because of antigen concentration or changes in composition due to purification (compare Fig. 4 and 5 of reference 1).

When serum 5735 was expended, evaluation of antigens with other sera soon demonstrated that the formation of precipitin lines were affected not only by antigen concentration, but also by the nature of the serum, by which product (serum or antigen) was used in the center well of the rosette, by the composition of the products of the neighboring wells, and finally by the strains producing the antigen. Thus, histoplasmin A50, a concentrated and partially purified product of strain 6623, showed equal concentrations of H and M antigens (titer of each 1:64) when assayed by immunoelectrophoresis with serum 10717 (Fig. 1). The same serum (Fig. 2B) and serum 61 (M only) (Fig. 2C) tested against various dilutions of histoplasmin A50 in the standard rosette failed to differentiate H and M antigens, although serum 10717 readily identified H and M antigens of

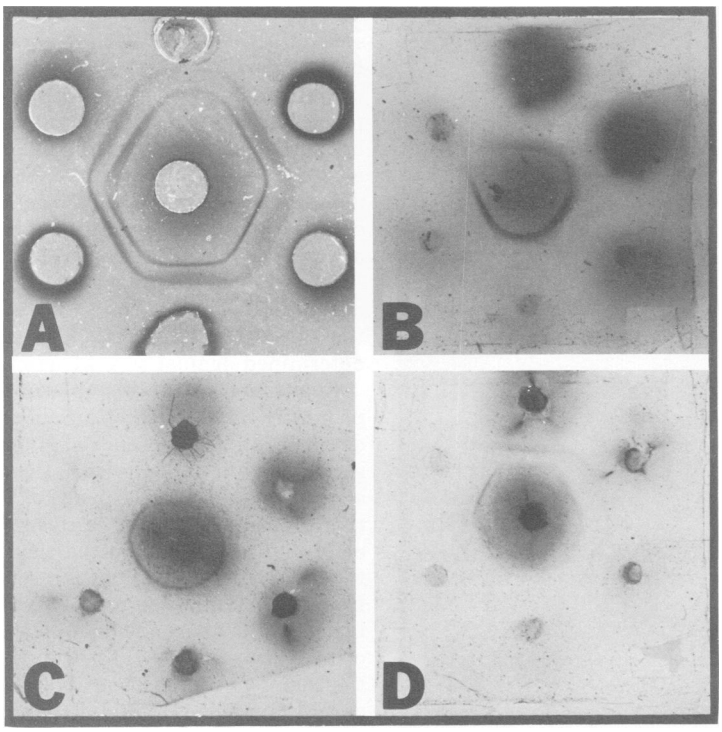

FIG. 2. *Comparative reaction of histoplasmin A50 with different human sera. Standard agar slides (1 × 3 inch) were prepared (1) and overlayed with plastic template according to the procedure of Wadsworth (1957). (A) Center well, serum 5735. Starting at top well proceeding clockwise: A50 (1:8); A50 (1:8); standard antigen lot 7; A50 (1:8); A50 (1:8); and standard antigen lot 7. (B) Center well, serum 10717. Top well and proceeding clockwise: A50 (undiluted); A50 (1:2); A50 (1:4); A50 (1:8); A50 (1:16); and A50 (1:32). (C) Center well, serum 61. Top well and proceeding clockwise: A50 (undiluted); A50 (1:2); A50 (1:4); A50 (1:8); A50 (1:16); and A50 (1:32). (D) Center well, serum 10717. Top well and proceeding clockwise: 6624 (undiluted); 6624 (1:2); 6624 (1:4); 6624 (1:8); 6624 (1:16) and 6624 (1:32).*

strain 6624 (Fig. 2D). The use of various sizes of wells and well spacings for the ID test did not adequately separate the H and M precipitin lines although it was possible to clearly demonstrate the H and M antigen content in certain arrangements and dilutions of well components (Fig. 3). When A50 was tested against a variety of sera, it consistently gave a single line, although a mixture of A50 with histoplasmin 6624, which had a high H concentration relative to M (Fig. 2D), showed the clear presence of H and M in four of the sera when an adequate dilution of the mixture was tested (Fig. 4). Similarly, the addition of a purified M fraction to a concentrate of District histoplasmin permitted satisfactory separation of H and M lines. When serum 10717 was tested against histoplasmin A50, crossing over of H and M lines was not observed, and the H line retained its normal position next to the serum wells regardless of dilution (Fig. 2). It would, therefore, be difficult to explain the overall results only on the basis of the relative ratio of H antigen to M antigen of the A50 histoplasmin since it would be anticipated that precipitin antibody to each antigen would vary among sera. Obviously the character of the serum, containing antibody to each antigen, plays a major role in the formation of precipitin lines and their relative positions. The results show that certain combinations of H and M antigen are not separated under a variety of conditions of the ID test with specific sera known to contain H and M precipitins.

Unfortunately, human sera of the characteristics of serum 5735 are not readily obtained; attempts to use animal sera have been limited since sera from a monkey, cow, rabbit, or goat have invariably shown antibodies to antigens other than H or M.

Thus, the development of a single histoplasmin suitable for use in ID testing of a variety of human histoplasmosis sera depends not only

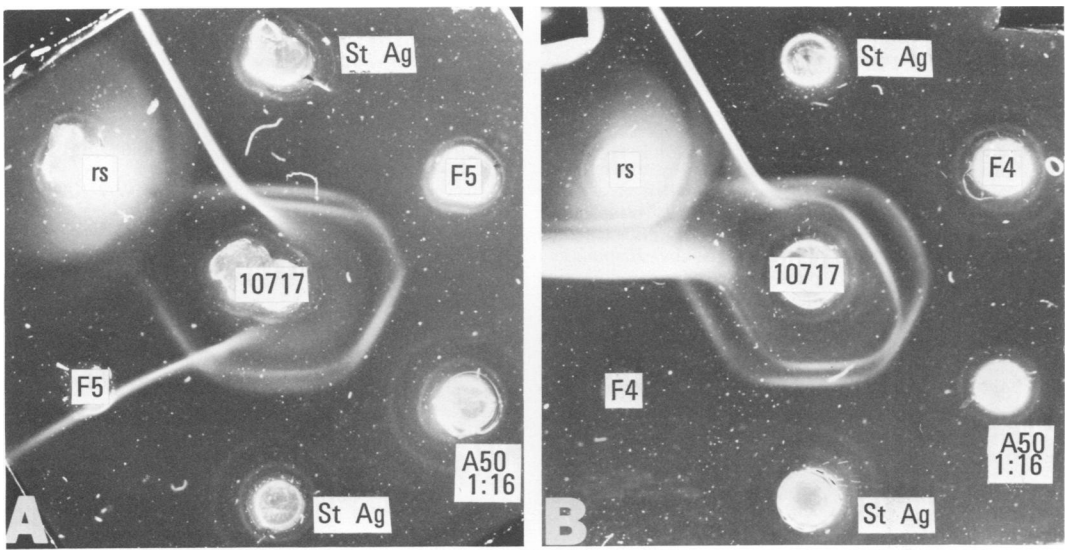

FIG. 3. *The effect of composition of neighboring wells on microimmunodiffusion identification of H and M antigens of histoplasmin A50 (1:16 dilution). St Ag, standard antigen histoplasmin, Lot 7; rs, anti-H rabbit serum; F5 is an H antigen enriched fraction eluted from diethylaminoethyl cellulose, pH 5; F4 is that fraction eluted at pH 4 (1). Center wells contain serum 10717.*

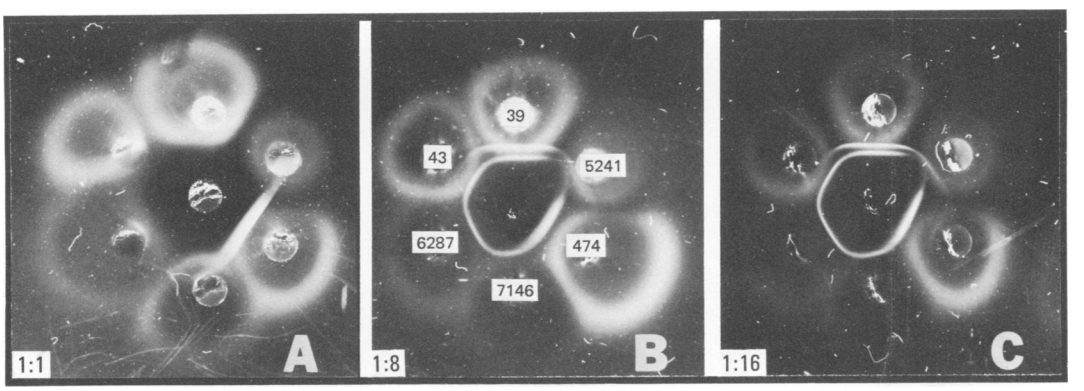

FIG. 4. *Effect of antigen concentration on evaluation of H and M antibodies in human sera. Six sera tested against combined histoplasmins A50 + 6624. (A) Undiluted; (B) 1:8; (C) 1:16. Note obvious antigen excess with undiluted antigen; presence of H precipitin line in sera at 2 and 4 o'clock at 1:8 were not observed until photographed.*

upon the presence of both antigens, but on relative concentration of one to the other, and perhaps on the chemical characteristics of the H and M antigens of a given strain. If a single histoplasmin proves unsatisfactory in the ID test, an adequate histoplasmin for the identification of H and M precipitins may be made by combining two or more histoplasmins derived from different strains; the correct ratios of one histoplasmin to the other must be determined by trial, and in all cases, the optimal concentration of the mixture must be determined for a large variety of sera for a given ID system. The use of a purified H and M antigen (1) in the ID test would eliminate certain difficulties involved in using a single histoplasmin containing both. However, our results indicate that an immunoelectrophoresis test similar to that described here would clearly delineate both H and M antibodies and their respective antigens. Our results suggest that the counterelectrophoresis system of Kleger and Kaufman (6) would appear to be the method most likely to unequivocally discern the presence of both H and M precipitins in patient sera, although we have not used this method.

LITERATURE CITED

1. Bradley, G., L. Pine, M. W. Reeves, and C. W. Moss. 1974. Purification, composition, and serological characterization of histoplasmin H and M antigens. Infect. Immunity **9**:870-880.
2. Ehrhard, H. B., and L. Pine. 1972. Factors influencing the production of H and M antigens by *Histoplasma capsulatum*: development and evaluation of a shake culture procedure. Appl. Microbiol. **23**:236-249.
3. Ehrhard, H. B., and L. Pine. 1972. Factors influencing the production of H and M antigen by *Histoplasma capsulatum*: effect of physical factors and composition of medium. Appl. Microbiol. **23**:250-261.
4. Harrell, W. (ed.) 1973. Preparation of *Histoplasma capsulatum* (Histoplasmin) CF antigen, p. 98-100. *In* Procedural manual for production of bacterial, fungal, and parasitic reagents. Center for Disease Control, Atlanta, Georgia.
5. Kaufman, L. 1973. Value of immunodiffusion tests in the diagnosis of systemic mycotic diseases. Ann. Clin. Lab. Sci. **3**:141-146.
6. Kleger, B., and L. Kaufman. 1973. Detection and identification of diagnostic *Histoplasma capsulatum* precipitates by counterelectrophoresis. Appl. Microbiol. **26**:231-238.
7. Reeves, M. W., L. Pine, and G. Bradley. 1974. Characterization and evaluation of a soluble antigen complex prepared from the yeast phase of *Histoplasma capsulatum*. Infect. Immunity **9**:1033-1044.
8. Smith, C. E., E. G. Whiting, E. E. Baker, H. G. Rosenberger, R. R. Beard, and M. T. Saito. 1948. The use of coccidiodin. Am. Rev. Tuberc. **57**:330-360.
9. Wiggins, G., and J. H. Schubert. 1965. Relationship of histoplasmin agar-gel bands and complement-fixation titers in histoplasmosis. J. Bacteriol. **89**:589-596.