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Skin-Reactive Antigens of Histoplasmin.* (31911)

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A culture filtrate from a mycelial-phase growth of *Histoplasma capsulatum,* which is defined as histoplasmin, is capable of inducing delayed skin reactions in infected animals(1) and man(2). However, histoplasmin is a gross mixture of constituents of the medium and products of cell growth, and estimates of the number of antigenic components have varied from one(3) to six(4). Studies of polysaccharide antigens derived from histoplasmin and from culture filtrates from yeast-phase growths have suggested that several chemically and antigenically different polysaccharides can be found in *H. capsulatum* and that antigenic strain differences can exist(5). Further evidence for occurrence of serotypes has been provided more recently(6).

The present work was undertaken with the aims of (1) demonstrating that the skin reactivity is a delayed hypersensitivity reaction, and (2) by using histoplasmins and fractions thereof obtained from various strains of *H. capsulatum,* to investigate variation of skin reactivity with composition of the fraction and with the strain used in its production.

Materials and methods. Preparation of histoplasmins. H. capsulatum strains 6651 (obtained through the courtesy of Dr. S. Marcus, University of Utah) and 103 (isolated from a patient with histoplasmosis at the Mayo Clinic) were maintained in the mycelial state on inhibitory mold agar (IMA) at room temperature. Strain 6651 was grown in Salvin's medium(3) and strain 103, on a tryptose phosphate medium(7) in the dark, at room temperature for 9 months. Each culture was then filtered through cheesecloth and paper to remove gross particles, through a

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5.0-μ Millipore® filter,† and, finally, through a 0.45-μ Millipore filter. The clear, yellowish brown solutions were then dialyzed in the cold against repeated changes of cold, deionized water until chloride-free and then lyophilized. Yields of dry products were 2.78 g (from 4 liters) from strain 6651 (preparation 1622) and 6.29 g (from 2 liters) from strain 103 (preparation 1423). The products were stored in the dry state at —10°C or were reconstituted in deionized water and maintained in the frozen state. No loss in reactivity, when examined by immunodiffusion or skin-testing methods, was noted for at least 6 months.

Ammonium sulfate precipitation. Aqueous solutions of the histoplasmin preparations were saturated by dialysis in the cold against a saturated solution of ammonium sulfate adjusted to pH 7.0. The precipitate was separated by centrifugation, washed 4 times with saturated ammonium sulfate, and then dissolved in water, dialyzed against daily changes of deionized water until sulfate-free, and lyophilized. The supernate and pooled washings were combined, dialyzed free of sulfate, and lyophilized.

Pevikon block electrophoresis(8). For a typical run, 400 g of Pevikon‡ was thoroughly washed with water and then suspended in 400 ml of veronal buffer (pH 8.60, ionic strength 0.1). The slurry was poured into a container 11 by 30 by 2 cm and excess buffer was removed by drainage from sponge wicks at either end until the proper consistency was obtained. A 1- by 8-cm slot was cut, 10 cm from the cathode, across the width of the Pevikon block and the excised material was mixed with about 3 ml of histoplasmin (containing 10 to 30 mg of solids) which had been previously dialyzed against the starting buffer. This slurry was then repacked, the entire block was covered with Saran wrap, and electrophoresis was conducted at 160 v and 19 ma for 16 hours in the cold. At the end of this time the block was sectioned at 1-cm intervals and the sections were extracted with 0.85% NaCl. The extracts were filtered through a coarse, sintered-glass funnel and each filtrate was made up to a constant volume. Extracts were analyzed by the anthrone(9) and the Folin-Lowry(10) techniques for carbohydrate and protein. Selected fractions were pooled, dialyzed, and lyophilized. Preparations for skin testing were prepared from these fractions in concentrations of 0.1, 1.0, 10.0, 50.1, and 100.0 μg/0.1 ml in 0.85% NaCl.

TABLE I. Delayed Skin Reactivity in Sensitized Guinea Pigs to Disc-Electrophoresis Fractions from Histoplasmin 1423.

Fraction No.*	Reactivity (mm^2)†
Spacer gel	64
1	4
2	25
3	100
4	4
5	4
6	25
7	25
.85% NaCl	0
8.0 mg of H-1423 in solution	144

* Each fraction obtained from a 5-mm wide section of the gel, starting at the cathode.

† Area of induration produced 24 hr after injection.

Antisera. Antisera were prepared against each strain of *H. capsulatum* in New Zealand white rabbits by 5 weekly intravenous injections of 2.58×10^8 live yeast-phase cells in saline suspension. The animals were bled 7 to 10 days after the last injection and the antisera were fractionated by ammonium sulfate precipitation to obtain a gamma-globulin-rich material.

Disc electrophoresis. The method of Ornstein and Davis(11,12) was used. Immunodiffusion was done by a microimmunodiffusion method(13). In addition, disc electrophoresis was also performed on 1.5 mg of histoplasmin 1423, and the column was extruded and then cut into 5-mm wide sections which were gently homogenized by expulsion from a tuberculin syringe. These suspensions were diluted to a final volume of 1.0 ml with saline and were then used in skin testing in sensitized guinea pigs (0.1 ml). Results are outlined in the Table.

Results. Original histoplasmins.§ Analysis

† Millipore Filter Corp., Bedford, Mass.

‡ Pevikon C-870, Stockholms Superfosfat Fabriks, A. B., Stockholm, Sweden.

§ Defined as histoplasmin which had been dialyzed, lyophilized, and reconstituted to a known concentration by weight.

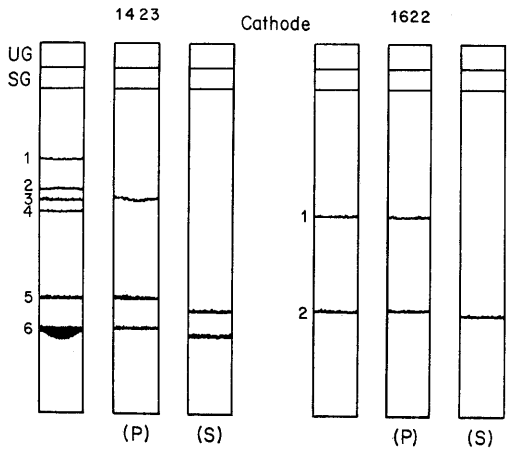

Fig. 1. Disc-electrophoretic separation of histoplasmins and $(NH_4)_2SO_4$ fractions (stained with amido-black). UG = upper gel; SG = spacer gel.

of original histoplasmin 1622 and 1423 shows almost no differences in the protein and carbohydrate content (9.1 to 10.7% N and 9.6 to 10.7% carbohydrate as glucose). However, disc-electrophoretic patterns of the two histoplasmins revealed marked differences in protein composition (Fig. 1). Histoplasmin 1423 contained at least 6 bands, while histoplasmin 1622 showed 2 bands.

Results of skin tests indicated that histoplasmin 1622 was more active in animals injected with the homologous as well as the heterologous strain and that the material was still active at a level of 0.1 μg (Fig. 2).

Ammonium sulfate fractions. It was found that ammonium sulfate precipitates most of the protein, leaving a carbohydrate-rich supernate (Hermans, P. E., and Markowitz, H. Unpublished data). Differences in composition could also be shown by disc electrophoresis (Fig. 1). Original histoplasmins and ammonium sulfate fractions were compared by skin testing, and the dose-response curves are given in Fig. 2. The ammonium sulfate supernate and precipitate from preparation 1622 were not as active as the original material in any concentration used (Fig. 2 *B*). The protein-rich precipitate was more active than the supernate at a level of 100 μg but the supernate was more active at a lower concentration. The precipitate of preparation 1423 was very active in skin testing (Fig. 2 *C*) although no precipitin activity could be found by immunodiffusion.

Pevikon block electrophoresis. The separa-

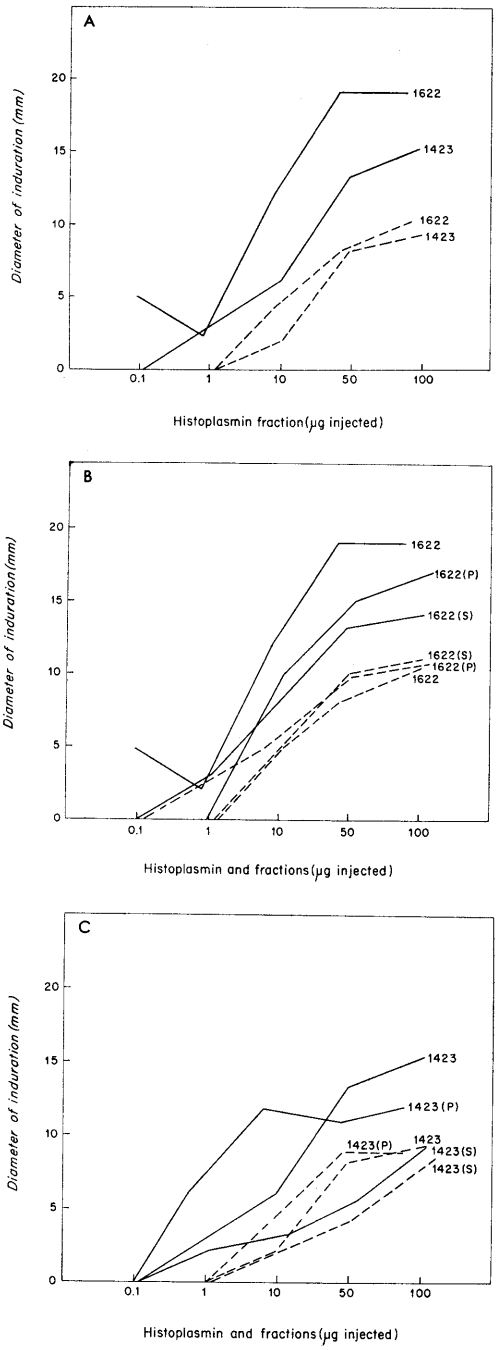

Fig. 2. Delayed skin reactions in sensitized guinea pigs to histoplasmin (*A*) and $(NH_4)_2SO_4$ fractions (*B* and *C*). *Solid lines* = animals sensitized to strain 6651; *broken lines* = animals sensitized to strain 103; (P) = $(NH_4)_2SO_4$ precipitate; and (S) = $(NH_4)_2SO_4$ supernate.

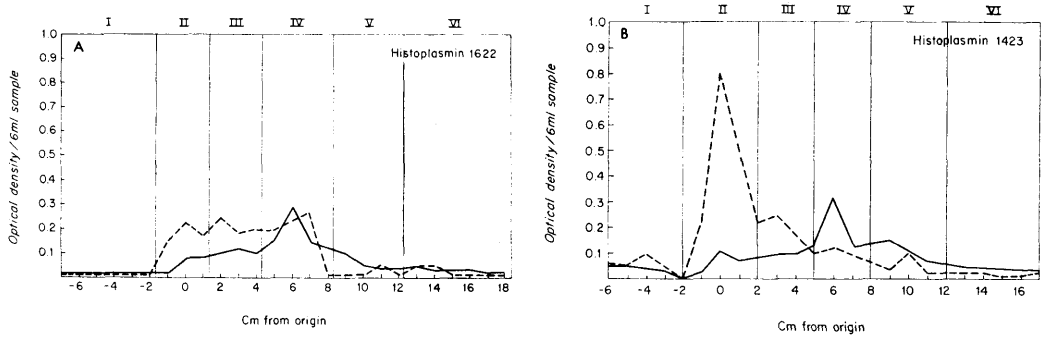

Fig. 3. Results of Pevikon block electrophoresis of histoplasmin; 1.0 ml (27.6 mg) of histoplasmin 1622 or 1.0 ml (30.0 mg) of histoplasmin 1423, dialyzed against starting buffer, were used as starting materials. *Broken lines* = carbohydrate by an anthrone method; *solid lines* = protein by the Folin-Lowry technique.

tions obtained by electrophoresis are given in Fig. 3. Some resolution of antigenic material was possible but by immunodiffusion it could be shown that each fraction still contained several reactive components (Fig. 4 for an example).

Results of skin tests with the isolated Pevikon-block fractions are shown in Fig. 5. There was no marked increase in reactivity of any fraction over the original histoplasmin, although materials with higher nitrogen and tyrosine contents were more active. All of the fractions that had precipitin activity also were active in skin testing.

Evidence of delayed hypersensitivity reactions. Skin testing at various time intervals after sensitization showed that a maximal response occurred at 4 weeks and that, thereafter, a relatively constant reaction was obtained. Each lesion reached a peak in terms of induration and erythema at 24 hours. Skin biopsy revealed a perivascular, mononuclear infiltrate (Fig. 6) typical of the delayed reaction(14). In passive transfer studies (peritoneal exudate cells transferred from sensitized guinea pigs to nonsensitized ones [15]), skin testing with histoplasmin gave a positive reaction. After completion of skin testing, all animals were bled and the antisera were analyzed by immunodiffusion for precipitin activity. No reactions were observed.

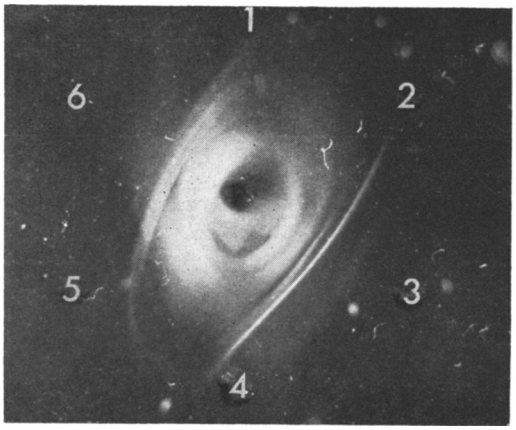

Fig. 4. Immunodiffusion of histoplasmin (1622P) fractions, obtained by Pevikon block electrophoresis, *versus* rabbit anti-*H. capsulatum* 6651 serum, 1 = fraction I; 2 = II; 3 = III; 4 = IV; 5= I; 6 = III.

Discussion. Although histoplasmin has been extensively used in skin testing procedures, details of the efficacy of various antigenic components are not available. The present work represents separation of antigenic components by a salting-out technique and electrophoresis of the resulting fractions. Assay of the various fractions by a dose-response technique(16) yields a more quantitative result which can be used to measure activity of an antigen in the delayed hypersensitivity reaction.

Histoplasmin could be effectively fractionated into materials containing a high concentration of carbohydrates and a protein-rich fraction, both of which possessed antigenic activity. The ammonium sulfate precipitate gave a slightly better reaction in the homologously sensitized animals; the carbohydrate-rich fraction was active in much lower amounts (Fig. 2). A paradox seems to be present in that a histoplasmin preparation from strain 103 was somewhat lower in reac-

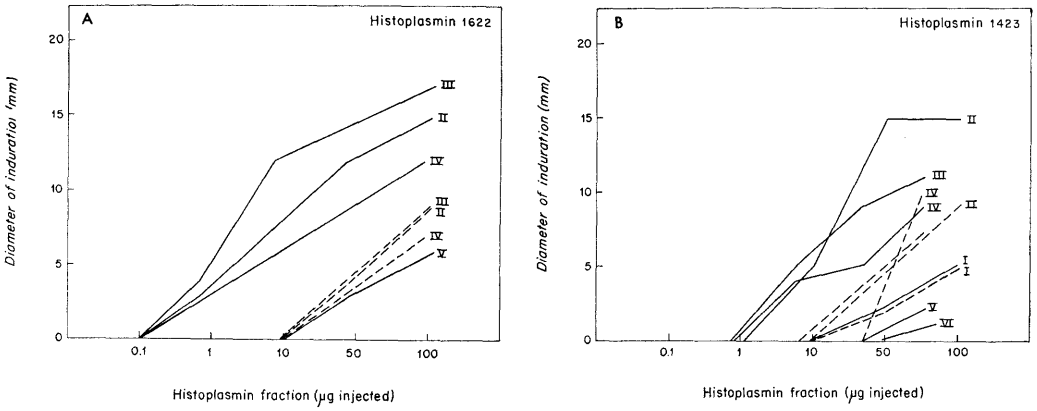

Fig. 5. Delayed skin reactions in sensitized guinea pigs to Pevikon-block electrophoretic fractions of histoplasmin. *Solid lines* = animals sensitized to strain 6651; *broken lines* = animals sensitized to strain 103.

tivity in the homologously sensitized animal than was that from a related strain (6651). The former strain of *H. capsulatum* also appears to be unique in elaborating an antigenic fraction (1423P) which had no detectable precipitin activity but was highly active in skin tests.

Histoplasmin 1423 differed antigenically from histoplasmin 1622 and appeared to lack at least two of the antigens present in the latter (Hermans, P. E., and Markowitz, H. Unpublished data). Results obtained with fractions from disc electrophoresis of histoplasmin 1423 (Table) also suggest the presence of at least two fractions, with different mobilities, which are capable of giving a delayed reaction.

Fig. 6. Skin reaction of guinea pig (sensitized 6 weeks previously with histoplasmin 1423) 24 hr after challenge. Biopsy 2 hr after challenge showed no evidence of infiltration. (Hematoxylin and eosin; x63.)

Pevikon block electrophoresis proved effective in achieving further separation of antigens; at pH 8.6, carbohydrate-rich material showed only slight migration while protein-rich fractions moved toward the anode. The carbohydrate-rich material was most active in skin tests, and tests with ammonium sulfate fractions also supported this conclusion. The lower activity of isolated fractions on a weight basis, compared to the starting preparation, may indicate denaturation during separation or a synergistic effect of several antigens; however, no explanation can be offered here. Although fractions with higher carbohydrate content seem to be more active on a weight basis, again, a possible explanation could be the presence of a protein constituent with much higher activity.

The reaction evoked in the skin tests performed in this work met most of the criteria for delayed hypersensitivity in that (1) the reaction was not evident in 4 hours, was evident at 18 hours, reached a maximum at 24 to 48 hours, and rapidly subsided thereafter;

(2) erythema and induration were uniformly present; (3) delayed reactivity could be transferred by cells; and (4) a typical histologic picture could be demonstrated by skin biopsies.

Summary. Histoplasmins from two strains of *Histoplasmia capsulatum* have been fractionated by salting-out and electrophoretic procedures. Fractions obtained in this manner have been assayed for ability to induce a delayed type of hypersensitivity in sensitized guinea pigs. Carbohydrate-rich fractions appear to be more effective in this regard, and one histoplasmin fraction has been found which exhibits no precipitin activity but is highly effective in the skin test. The skin test reaction with histoplasmin is also demonstrated to be an example of a delayed hypersensitivity reaction.

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Influence of Mother's Milk on Incidence of Spontaneous Aortic Lesions in Weanling Rabbits.* (31912)

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A recent survey of aortas from approximately 1100 untreated rabbits disclosed a relatively high incidence of spontaneous, nonlipid medial lesions(1). These lesions have a variable gross, but fairly uniform microscopic appearance(2). They are always confined to the inner one-half to one-third of the media. Fragmentation and disruption of the elastic lamina with accumulation of acid mucopolysaccharide is seen, frequently accompanied by calcification of the fragmented portions of the elastic fibers. The morphologic characteristics of these spontaneous medial lesions and of the diet-induced atheroma are dissimilar at all stages of their development (3). The early atheromatous plaque is characterized by accumulation of lipid-filled foam cells in the intima and of lipid droplets in the subjacent media. The development of atheroma, however, is influenced by the presence of the spontaneous lesions. There is an increased tendency for lipid deposition at the raised borders of nodular medial lesions projecting from the luminal surface and in areas of acid mucopolysaccharide accumulation within the medial lesions. Atheroma formation is absent at sites of medial calcification. On the basis of our survey(1) and studies which cover rabbits from birth to maturity (2,4), it seems that these medial lesions are laid down by the sixth week, and in most cases are irreversible, but do not necessarily

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