Antibodies in Histoplasmosis

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ABSTRACT

Production of precipitating and complement-fixing antibody in rabbits and other animals was induced by immunization with live yeast-phase cells of *Histoplasma capsulatum*. Results of studies of polysaccharide antigens from three strains of *H. capsulatum*, by quantitative complement-fixation with human and rabbit antisera, strongly suggest the presence of type specificity. The variations of titer during 11 weeks in one patient with histoplasmosis and the variations of titer among a group of patients with histoplasmosis were studied by use of quantitative complement-fixation tests.

Although histoplasmosis was defined as a disease entity in 1908 (4) and knowledge as to its distribution and pathogenesis has accumulated rapidly (25), details of the chemical and antigenic compositions of *Histoplasma capsulatum* are uncertain. At least six antigens are present in the culture filtrate from a mycelial-phase culture (histoplasmin) when patients' sera are used in testing (6), although recent work (24) indicates that some of these antigens may be absent in certain strains of *H. capsulatum*. The problem is complicated by reports that the properties of antigenic substances isolated from yeast-phase cultures differ from the properties of those from mycelial-phase cultures (2, 3, 5, 9, 11, 18–21, 25). There may be further complications because it has been suggested that strain differences (antigenic) exist in *H. capsulatum* (13); additional evidence for this is presented herein.

To date, no single, discrete antigen has been isolated from *H. capsulatum*, although a number of reports describe the isolation of crude polysaccharides from yeast- and mycelial-phase cultures (2, 3, 9, 18, 19, 23); histoplasmin has been fractionated on diethylaminoethyl (DEAE) cellulose, and preparations with *m* and *h* antigen activity have been separated (5).

To carry out immunochemical studies on polysaccharides separated from yeast-phase culture filtrates of *H. capsulatum*, precipitating antibody of high titer was needed. Methods for producing high-titer precipitating antibody were studied, and reactions of the antibody with various crude antigens derived from *H. capsulatum* were determined by quantitative precipitin and complement-fixation tests. Some data are also presented on reactions with human sera from patients with histoplasmosis.

MATERIALS AND METHODS

**Experimental animals.** Female New Zealand white rabbits, having an initial weight of 2 to 3 kg, were used throughout. Animals were fed Wayne Rabbit Ration and water *ad libitum*.

**Immunization.** Two strains of *H. capsulatum* were used: 6651, obtained through the courtesy of S. Marcus, University of Utah, and 103, isolated at the Mayo Clinic from a patient with histoplasmosis and obtained through the courtesy of J. A. Ulrich. Organisms were maintained in the yeast phase at 37 C on slants of hormone-blood-agar containing penicillin and streptomycin (41 units per ml of medium). Cells were grown in Salvin's liquid medium at 37 C (13) for 20 days, and were harvested by centrifugation at 4 to 6 C. The cell suspension was washed three times with 2 volumes of cold water and then was resuspended in cold 0.85% NaCl. Sterile solutions and glassware were used in all procedures. The final suspension was diluted with 0.85% NaCl to a final concentration of 2.2 mg/ml (dry weight of organisms; equivalent to 3.3 X 10⁶ organisms per milliliter) and stored at 4 to 6 C. The suspension was checked by staining the cells (with polyvinyl alcohol-cotton blue and Gram stain) and by culture to determine the presence of *H. capsulatum* and the absence of bacterial contaminants. The percentage of viable organisms was not determined. In a number of animals, subcutaneous nodules developed at the site of injection and could be shown, by culture, to contain *H. capsulatum*. However, no weight loss was evident.

**Formalin-killed cells.** Formalin-killed cells were prepared by adjusting a culture of *H. capsulatum* to a final concentration of 0.5% Formalin and keeping it at 4 C for 24 hr before harvesting and washing the cells. Suspensions of Formalin-killed cells were prepared at 2.5 and 12.3 mg/ml (dry weight), and 1 ml was injected intravenously at 2- to 3-day intervals. Formalin-killed cells
were also incorporated in complete Freund’s adjuvant at a final concentration of 6.1 mg/ml (dry weight) and were administered in a divided dose, intradermally and subcutaneously, at weekly intervals for a total of three 1-ml injections. Adult male mice (Webster strain) were also used; they were injected intraperitoneally three times at weekly intervals with complete adjuvant containing Formalin-killed *H. capsulatum*, 6.1 mg/ml (dry weight). In some cases, sarcoma 180 cells were given intraperitoneally to induce ascites formation (1). Immunization schedules are given in Table 1.

**Antiserum.** Human antisera were collected from patients with histoplasmosis (proved by culture). All rabbits were bled 7 days after the last injection and at weekly intervals thereafter for a total of six postimmunization bleedings. Blood samples obtained before immunization were all negative when tested by microimmunodiffusion and quantitative and qualitative complement-fixation tests. All antisera were maintained, without preservative, in the frozen state until used.

**Test antigens.** Histoplasmins were prepared from cultures of *H. capsulatum* 6651 (preparation 1622) and 103 (preparation 1423) grown in Salvin’s medium in the dark at room temperature for 9 months. Cultures were filtered through a membrane filter (pore size, 0.45 μ; Millipore Filter Corp., Bedford, Mass.); the filtrates were dialyzed exhaustively against distilled water in the cold and then lyophilized. Preparations were kept at −10 °C until used; they were reconstituted with either 0.85% NaCl or appropriate buffers. Polysaccharides were prepared from *H. capsulatum* strains 6651, 103, and G-17M as described previously (13). Properties of the antigens used are given in Table 2. Nitrogen was determined by the Markham method (12), carbohydrate content by an anthrone method (22), and nucleic acid by calculation from the absorption peak at 260 μm (10).

**Other methods.** Quantitative precipitin tests were performed as described by Kabat (7). Quantitative complement-fixation tests with polysaccharide and histoplasmin solutions were performed with 100 C'H₉₀ units of fresh guinea pig complement per tube. All sera were heated at 56 °C for 30 min. Complement-fixation tests (14) with whole yeast-phase cells were performed with 3 C'H₉₀ units. All sera and antigen solutions were diluted with standard barbital buffer (pH 7.3) containing Ca²⁺ and Mg²⁺ (7). Microimmunodiffusion was carried out as previously described (13).

**RESULTS**

**Precipitating and complement-fixing antibody.** None of the mice given complete adjuvant containing *H. capsulatum* produced any detectable amounts of precipitating antibody in their ascitic fluid. Rabbits given Formalin-killed cells or complete adjuvant containing *H. capsulatum* did not produce precipitating antibody detectable by microimmunodiffusion, but complement-fixing antibody was found. In groups of rabbits in which various courses of immunization were used, antibody production was directly related to dose (Fig. 1), and disappearance of precipitating antibody from the serum was rapid (Fig. 2). Similar determinations were done for complement-fixing antibody (Fig. 3); the results were similar to those found for precipitating antibody.

**Complement-fixation tests with cellular and soluble antigens.** Antisera were studied for their complement-fixing antibody content by two complement-fixation methods, because it has been suggested (16, 17) that, in both man and rabbits, complement-fixing antibody remains at high levels for long periods, although precipitating antibody disappears rapidly. Data shown in Fig. 4 in general indicate a marked diversity in results between the two tests, probably as a reflection of the use of a soluble polysaccharide preparation in one and of intact yeast-phase cells in the other.

**Comparison of crude antigens.** Two crude histoplasmin antigens (1423 and 1622) obtained from different strains of *H. capsulatum* and two crude polysaccharide antigens (H-25 and H-29) also obtained from different strains (Table 2) were compared by quantitative complement-fixation tests (Fig. 5). Marked differences in number of C'H₉₀ units fixed are evident; the two different polysaccharide preparations appear to cross-react, whereas the two histoplasmins obtained from different strains appear to fix almost the same amount of complement but at two different antigen levels.

Sera from patients with histoplasmosis were also used in quantitative complement-fixation tests with several crude polysaccharide antigens (Fig. 6). These antigens were prepared by a previously outlined method (13); nitrogen, carbohydrate (anthrone), and ribonucleic acid (RNA) contents, given in Table 2, varied markedly

### Table 1. Schedule of immunization of rabbits with *Histoplasma capsulatum*

<table>
<thead>
<tr>
<th>Rabbits</th>
<th>Schedule* (no. of injections)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15, 16, and 17b</td>
<td>1</td>
</tr>
<tr>
<td>18, 19, and 20b</td>
<td>3</td>
</tr>
<tr>
<td>27, 28, 29, and 30</td>
<td>1</td>
</tr>
<tr>
<td>31, 32, 33, and 34</td>
<td>3</td>
</tr>
<tr>
<td>35, 36, 37, and 38</td>
<td>5</td>
</tr>
</tbody>
</table>

*All immunizations were with *H. capsulatum* 6651 given intravenously as a 2.2-mg/ml suspension (3.3 × 10⁵ cells per milliliter of live cells in 0.85% NaCl.

b Rabbits in these two groups were previously immunized with four weekly subcutaneous injections of 0.5 ml of formalin-killed *H. capsulatum*, 6.1 mg/ml in complete Freund’s adjuvant.
Fig. 1. Quantitative precipitin curves of antisera from rabbits after immunization with one to five inoculations of living Histoplasma capsulatum 6651. Antigen used throughout was a crude polysaccharide preparation (H-15) Subscripts refer to when (week postimmunization) blood sample was taken. See Table 1 for immunization schedules.

Fig. 2. Production of precipitating antibody as a function of time and dose of Histoplasma capsulatum 6651. Data shown as means of titer for all antisera.

among the strains. However, different preparations from the same strain were similar analytically. Variations in number of C'H_{50} units fixed depend on the antiserum used. Thus, while serum from patient B reacted almost equally with all of the preparations, serum from patient A fixed almost three times as much complement when the polysaccharide preparation was from G-17M (H-27) as when it was one of the other two antigens. When serum from patient C was used, preparation H-15 was far more effective than either of the other antigens. Since the same serum and identical conditions were used in assay of all three polysaccharide antigens, it seems necessary to assume that the antigenic preparations differ. Levels of complement-fixing antibody also varied markedly among the three sera, because serum B diluted 1→200 fixed a maximum of 35 C'H_{50} units, serum C diluted 1→156 fixed a maximum of 35 C'H_{50} units, and serum A diluted 1→200 fixed a maximum of only 15 C'H_{50} units.

Variation of complement-fixing antibody with time course of disease and among different sera. Because histoplasmosis may be a chronic disease and its onset is difficult to determine, the complement-fixing antibody levels noted in Fig. 6 may represent one or the other extreme of antibody production. Accordingly, the serum of one patient, admitted to the hospital with recent onset
of symptoms, was tested at weekly intervals [the first sample was drawn at admission, and the remaining 10 samples, at weekly intervals after institution of therapy with amphotericin B (E. R. Squibb & Sons, New York, N.Y.)]. As shown in Fig. 7 (upper), a very rapid decrease in titer was noted within 1 week. The titer decreased slowly thereafter until the 9th week, at which time only 2 C'H50 units were being fixed. Also, the amounts of antigen required to give maximal fixation varied from week to week.

Quantitative complement-fixation curves were also obtained with a variety of sera from other patients with histoplasmosis. Although all patients were seen when the disease was clinically evident (symptoms and isolation of the organism), the time after infection could not be ascertained. Thus, the variation in titer shown in Fig. 7 (lower) could not be ascribed to any known factor(s).

![Figure 3](image)

**Fig. 3.** Quantitative complement-fixation curves of antisera from rabbits after immunization with living Histoplasma capsulatum 6651. Antigen was H-15. Subscripts refer to week (postimmunization) blood sample was taken. (upper) Rabbit 17; dilution of antiserum, 1 → 200. (lower) Rabbit 19; values in parentheses refer to dilution of the antiserum used in the tests.

![Figure 4](image)

**Fig. 4.** Comparison of yeast-phase cells (○) and soluble antigens (×) in complement-fixation tests with hyperimmune rabbit antiserum to Histoplasma capsulatum 6651. Studies were carried out with a 1 → 1,000 dilution of serum from rabbit 19 (upper) and a 1 → 2,000 dilution of serum from rabbit 20 (lower). Antigens were solution of polysaccharide preparation (H-15) and suspension of yeast-phase H. capsulatum 6651, 4.0 × 10^6 cells per tube.

<table>
<thead>
<tr>
<th>Prepn no.</th>
<th>Phase and strain no.</th>
<th>N</th>
<th>Carbohydrate (as glucose)</th>
<th>[α]D^20°</th>
<th>RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1423b</td>
<td>Mycelial, 103</td>
<td>10.7</td>
<td>10.7</td>
<td>+17</td>
<td>—</td>
</tr>
<tr>
<td>1622b</td>
<td>Mycelial, 6651</td>
<td>9.1</td>
<td>9.6</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>H-27c</td>
<td>Yeast, G-17M</td>
<td>3.66</td>
<td>15.4</td>
<td>+8</td>
<td>13.0</td>
</tr>
<tr>
<td>H-15c</td>
<td>Yeast, 6651</td>
<td>2.45</td>
<td>9.7</td>
<td>+49</td>
<td>9.0</td>
</tr>
<tr>
<td>H-28c</td>
<td>Yeast, 103</td>
<td>4.98</td>
<td>40.5</td>
<td>+57</td>
<td>12.2</td>
</tr>
<tr>
<td>H-29c</td>
<td>Yeast, 103</td>
<td>6.07</td>
<td>31.0</td>
<td>—</td>
<td>13.2</td>
</tr>
</tbody>
</table>

* a: Expressed in degrees. Aqueous solutions, c = 5 to 10 mg/ml.
  b: Histoplasmins.
  c: Crude polysaccharides.
retention of antigenic materials for long periods. Thus, one might expect an extremely complex pattern of antibody response.

Elucidation of the mechanism of the immune response is further complicated by the fact that no single, purified antigen is available for quantitation of the antibody response. However, it appears that, at least experimentally in rabbits, Formalin-killed cells of *H. capsulatum* are poor antigens. Live cells are more effective, but an intensive schedule of immunization is necessary

**FIG. 5.** Quantitative complement-fixation curves. All dilutions were made with borbitol buffer containing Ca++ and Mg++. Antigen (µg) represents the total amount of antigen added per 2.5 ml of solution mixed with 2.5 ml of antiserum and 5.0 ml of a dilution of guinea pig serum containing 100 C'H₅₀ units for each point assayed (7). All determinations were done in duplicate. (top) Histoplasmin 1622, strain 6651 (O); histoplasmin 1423, strain 103 (>). Rabbit anti-6651 serum (rabbit 19) was diluted 1 → 2,000. (middle) Polysaccharide preparation H-29, strain 103 (O); polysaccharide preparation H-25 (13), strain 6651 (>). Rabbit anti-6651 serum (rabbit 20) was diluted 1 → 2,000. (bottom) Same symbols for polysaccharide preparations H-25 and H-29 but with rabbit anti-103 serum (pool 6974) diluted 1 → 25.

**DISCUSSION**

Histoplasmosis is one of a few infectious diseases caused by a fairly slowly growing organism. *H. capsulatum* in its infective form is in the mycelial phase, and yet appears as a yeast-phase organism in infected tissues. In addition, the organism has the capacity of producing granulomatous lesions with effective walling-off and...
to produce a high titer of precipitating antibody. Immunization of rabbits with live cells in Freund's adjuvant appears to be just as poor, except that a good response is obtained for what is probably 19S complement-fixing antibody. Mice also seem to respond poorly in producing precipitating antibody; however, the goat appears to respond well to live yeast-phase cells in Freund's adjuvant (unpublished data). The response in rabbits to strain 103 is much poorer than that to strain 6651, and prolonged immunization over 8 to 12 months still produces only about 30 to 40 μg of antibody N per ml of serum. Other strains, not used in this study, may be capable of greater antigenic stimulation.

Other evidence has suggested that levels of complement-fixing and precipitating antibodies can be of prognostic significance in histoplasmosis (8). However, the observation of persistence of a particular type of antibody may also be artifact dependent on the antigenic preparation and the sensitivity of the serological test used. Thus, Fig. 4 illustrates a difference in complement-fixing antibody titers, depending on the antigen used. By using whole yeast-phase cells, or a soluble component which can be eluted from them (P. E. Hermans and H. Markowitz, unpublished data), antibody can be detected in sera which do not react at all when soluble polysaccharide preparations prepared from culture filtrates are used.

The most likely possibility is that different antigenic components are present in cell walls and in material found in culture filtrates. The importance of the sensitivity of the test used is still another factor; with a passive hemagglutination test (P. E. Hermans and H. Markowitz, to be published), antibody can still be detected almost 2 years after an apparent cure, at a time when the patients appear to be well clinically and other serological tests are negative.

Still another confusing factor is the probability that serotypes of *H. capsulatum* exist. This has been suggested before (13) and was demonstrated recently by the use of immunofluorescence reactions (8). In essence, further evidence for this point of view, as presented in Fig. 5 and 6, is that different polysaccharide preparations cross-reacted with antisera to each strain of *H. capsulatum* (Fig. 5). Reactions of polysaccharide preparations from three different strains of *H. capsulatum* (Fig. 6) are also evidence of differences in chemical constitution of the antigens. This apparently is not due to variations during growth, because numerous preparations, all from cells grown in the same medium (Salvin's medium), are chemically similar and behave in identical fashion immunologically. When grown in different media, such as cryptose-phosphate or Pine's media (9, 15), variations in content of antigens but not absence of a specific component can be noted on immunodiffusion. In addition, studies, to be presented later, on differences in carbohydrate composition, electrophoretic analyses, delayed hypersensitivity reactions, and immunofluorescence reactions also indicate the existence of serotypes of *H. capsulatum*.

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**LITERATURE CITED**


