Common Antigens Among Systemic Disease Fungi Analyzed by Two-Dimensional Immunoelectrophoresis

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The interpretation of immunological results in systemic mycoses has been complicated by cross-reactions among specimens from patients with blastomycosis, coccidioidomycosis, and histoplasmosis. The fungal preparations used in these tests evidently contained one or more antigens in common. Two-dimensional immunoelectrophoresis techniques were used to determine the number of antigens contained in several soluble extracts from *Blastomyces dermatitidis* and *Histoplasma capsulatum* that were common with those demonstrable in a coccidioidin-anticoccidioidin reference system. A total of 12 and 10 common antigens were found in preparations from *B. dermatitidis* and *H. capsulatum*, respectively. In addition, the crude preparations from each species of fungus exhibited some qualitative and quantitative differences in composition of antigens. Use of two-dimensional immunoelectrophoresis for standardization of fungus extracts, for monitoring separation of species-specific antigens, and for testing humoral antibody response should add further refinement to correlations with clinical disease.

The diagnosis of systemic mycoses has been aided by immunological tests when the etiological agent could not be demonstrated by culture or by staining of tissues without resorting to invasive procedures. Unfortunately, interpretation of immunological reactions frequently has been complicated by positive results with antigen preparations from more than one fungus (1, 5). One cause for this complication could be multiple infections. The mobility of modern populations has led to multiple exposures, and we have seen patients with healed histoplasmosis who developed primary coccidioidomycosis after emigrating to a new endemic area (10; unpublished data). A second explanation could be the presence of common antigens among these fungi. There has been evidence that infection by one of these fungi induced an immunological response detectable with antigen preparations from other fungi (3, 18). The analytical power of two-dimensional immunoelectrophoresis (IEP) offered an opportunity to differentiate between common and specific antigens among these fungi. This information would be useful not only for diagnostic objectives, but also for standardizing antigen preparations and for monitoring attempts to separate specific from common antigens.

The present study was concerned with identifying some of these common antigens. Soluble substances derived from *Blastomyces dermatitidis* and *Histoplasma capsulatum* were compared with a reference antigen-antibody system for *Coccidioides immitis* (9). The results demonstrated that the two heterologous fungi possessed identifiable antigens found also in the reference coccidioidin-anticoccidioidin system.

MATERIALS AND METHODS

Antigen preparations. Coccidioidin was prepared as described previously (9). Briefly, 12 isolates of *C. immitis* were grown separately in 2% glucose-1% yeast extract dialysate broth on a rotary shaker at 30 to 33°C. Fungus mats were separated by Büchner filtration, lysis of fungus cells was achieved with toluene-saturated distilled water at 37°C, and the filtered lysates were lyophilized. The original culture filtrates, to which aqueous thimerosal (1:10,000, vol/vol, final concentration) had been added, were used to dissolve the dried lysates. All 12 preparations were pooled and concentrated by ultrafiltration behind a UM05 membrane (Amicon Corp., Diaflo) with a nominal cutoff at 500 daltons and 95% retention at 1,000 daltons. The following antigens were obtained through the courtesy of Rebecca A. Cox: histoplasmins 75-0105 and H42, blastomycin KCB26, and alkali-soluble, water-soluble extracts from cell walls (6, 20) of *B. dermatitidis*, *C. immitis*, and *H. capsulatum*.

Antiserum. A single lot of antiserum was used as the reference antibody preparation. This had been raised in a burro by repeated subcutaneous injections

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with coccidioidin over an 8-month period as described previously (9). Immunoglobulins were precipitated with ammonium sulfate, dialyzed until free of sulfate, and made up to original volume with electrophoresis buffer (4).

Two-dimensional IEP. Published procedures were followed for the two-dimensional IEP methods used (13, 21). Optimal results were obtained with the following concentrated barbital buffer (grams per liter): sodium barbital, 15.47; barbital acid, 2.71; sodium azide, 1.0; calcium lactate, 0.4; Trizma base (Sigma), 44.3. This was stored at 4°C. Appropriate volumes for daily use were diluted 1:5 with deionized distilled water, yielding a buffer with ionic strength of 0.02 and a pH of 8.6. The diluted buffer was used in electrophoresis and for preparing gels and immunological reagents.

The procedures for preparing agarose on chemically clean glass plates (8 by 10 cm) and the conditions for performing two-dimensional IEP have been described (9).

RESULTS

The several antigen preparations were examined for antigens in common with those demonstrated with the coccidioidin-anticoccidioidin immunoglobulin reference system. We had reported previously that three dilutions of anticoccidioidin immunoglobulin in the second dimension IEP were required to reveal the maximum number of antigen-antibody precipitates with this reference system, as reproduced in Fig. 1 (9). In the present study, tandem-crossed IEP proved to be the most sensitive method for detecting common antigens by distortion of the symmetrical antigen-antibody precipitates formed by the reference system (Fig. 2). Intermediate gel two-dimensional IEP did not reveal any additional common antigens. The common antigens that have been identified up to the present time are presented in Table 1, which includes the results with spherulin reported previously (9). Twenty-six antigens had been identified in coccidioidin and, of these, a combined total of 10 were found in the three preparations from *H. capsulatum* and 12 in the two extracts from *B. dermatitidis*. Six antigens, numbers 18, 19, 20, 23, 25, and 26, were found most frequently among all the preparations studied.

DISCUSSION

The interpretation of immunological reactions as aids for diagnosing systemic mycotic infection has been complicated when a single specimen yielded positive results with antigen preparations from several different fungi. This has occurred with both the complement fixation and intradermal tests used extensively for more than 3 decades (1, 3, 5, 11, 19). Nevertheless, empirical correlations with diagnosis and clinical course

have been derived from the accumulated experience with these tests and antigen preparations. The development of Ouchterlony-type immunodiffusion tests for these mycoses represented a major advance, because reference systems were established for identifying a limited number of species-specific antigen-antibody reactions (7, 8, 12). In general, results with immunodiffusion not only supported those with complement fixation, but also enabled differentiation between homologous and heterologous reactions.

It was also apparent from this past experience that humoral antibody production was being induced by more than one antigen, and that several reactions carried differing clinical interpretations and significance. For example, in their studies of coccidioidomycosis, Smith et al. (19) reported that the tube precipitin reactivity of coccidioidin resisted autoclaving, but complement-fixing activity in the same coccidioidin was completely destroyed. The tube precipitin test was positive in early disease and provided a specific indicator for a diagnosis of coccidioidomycosis. In addition, this test commonly reverted to negative within a few months but might recur during exacerbation of preexisting disease. The complement fixation test, in contrast, usually converted to positive after the tube precipitin test and tended to remain positive for longer periods of time. Diagnostic indications depended on conversion from negative to positive or on significant increases in titer during the course of the disease, and persistently high complement fixation titers generally paralleled extent and severity of the infection. Subsequently, other investigators demonstrated that immunoglobulin M was the principal class of antibody reactive in the tube precipitin test and immunoglobulin G in the complement fixation test (16, 17). Thus, coccidioidin contained at least two antigens, which differed in heat sensitivities and in reactivity with different classes of antibody. Furthermore, positive results with these antigens provided different types of clinical information. Similarly, Heiner (7) found at least six antigen-antibody reactions by immunodiffusion in histoplasmosis. One of the antigens was nonspecific, since it was found in uninoculated, concentrated Sabouraud liquid medium. A second antigen yielded positive results with sera from patients with blastomycosis, coccidioidomycosis, and histoplasmosis. Four additional antigens reacted only with specimens from patients with histoplasmosis. Antibody against three of these antigens (labeled h, x, and y) was found only in sera from patients with proven histoplasmosis. Antibody against the fourth (labeled m) was detected not only in cases of histoplas-

FIG. 1. Two-dimensional IEP with coccidioidin (C) against anticoccidioidin-precipitated immunoglobulin made up to original serum volume and diluted for tests to 1:5 (A), 1:2 (B), or 1:10 (C). Well with coccidioidin is at cathodal end of plate. (Reproduced from reference 9.)

mosis, but also among healthy individuals with delayed hypersensitivity to histoplasmin. Clinical interpretations related to serological results have been reported also for other mycoses (reviewed in references 1, 5, and 11). Therefore, identification of specific antigen-antibody reac-

tions has been helpful for aiding diagnosis and for deriving clinical correlations.

The delayed hypersensitivity response measured by intradermal tests with fungal extracts may be also a composite reaction to more than one antigen. Levine et al. (15) demonstrated that

FIG. 2. Tandem IEP with coccidioidin (C) and histoplasmin (H75-0105) against anticoccidioidin-precipitated immunoglobulin made up to original serum volume and diluted for tests to 1:2 (A) or 1:10 (B). Wells with antigens are at cathodal end of plate.

TABLE 1. Common antigens found in soluble preparations from some fungi causing systemic disease with respect to a coccidioidin-anticoccidioidin reference system

<i>C. immitis</i> ^a			<i>H. capsulatum</i>			<i>B. dermatitidis</i>	
C	S	C-ASWS	75-0105	H42	H-ASWS	KCB26	B-ASWS
1	—	—	1	—	—	1	—
2	—	—	—	—	—	—	—
2a	—	—	—	—	—	—	—
3	—	—	—	—	—	—	—
4	—	—	—	—	—	—	—
5	—	—	—	—	—	—	—
6	—	—	—	—	—	—	—
7	—	—	—	7	—	7	—
8	8	—	—	8	—	8	—
9	9	—	—	9	—	9	—
10	—	—	—	—	—	—	—
11	—	—	—	—	—	—	—
12	—	—	—	—	—	—	—
12a	12a	—	—	—	—	—	—
13	—	—	—	—	—	—	—
14	—	—	—	—	—	—	—
15	—	15	—	—	—	—	—
16	16	16	—	—	—	—	—
17	—	—	—	—	—	17	—
18	18	18	18	18	18	18	18
19	19	19	19	19	19	19	19
20	20	20	20	20	20	20	20
21	—	—	—	—	—	21	—
22	—	—	—	—	—	—	—
23	23	23	23	23	23	23	—
24	—	—	—	—	—	—	—
25	25	25	25	25	25	25	25
26	26	26	26	26	26	26	26
—	A	—	—	—	—	—	—
—	B	—	—	—	—	—	—

^a C, Coccidioidin; S, spherulin; ASWS, alkali-soluble-water-soluble extracts from cell walls.

spherulin was more sensitive than coccidioidin for eliciting delayed hypersensitivity in humans and found that heating spherulin reduced this sensitivity to that of coccidioidin as measured in an experimental murine model of coccidioidomycosis (14). This indicated that spherulin contained both heat-sensitive and heat-resistant antigens that were capable of eliciting responses to intradermal injection.

It is apparent, therefore, that information of clinical value can be obtained from immunological reactions to several different antigens. This knowledge has been acquired in the past with crude preparations containing multiple antigens and with immunological tests of limited analytical capability. In the present study with a more powerful analytical method, we have answered several questions. First, each of these crude preparations contained many antigens that could be identified with a hyperimmune serum. Second, the several preparations from each species differed both qualitatively and quantitatively in their antigenic components. For example, histoplasmin 75-0105 contained six antigens

in common with histoplasmin H42 and one not found in histoplasmin H42, and lacked three detected in histoplasmin H42 (Table 1). Spherulin contained considerably more of antigen 12a than did coccidioidin (9). Third, the almost universal presence of six antigens suggested that one or more of these have been responsible for cross-reactions in immunological tests, and the elimination of these should alleviate this problem. Thus, the presence of multiple antigens in common, combined with qualitative and quantitative differences in composition of antigens among the soluble extracts from these three fungus species, has led to problems for deriving clinical interpretations and to potential discrepancies in results obtained with different batches of antigenic extracts from a single species.

The analytical power of two-dimensional IEP can be directed toward standardizing antigen preparations and monitoring the separation of species-specific antigens. The availability and distribution of standardized reference immunological reagents should produce more comparable results from different laboratories. Success

in the production of species-specific antigens and the use of these for serological and cell-mediated immune responses should add further refinement to correlating the host's immunological responses with the diagnosis, course, and prognosis of clinical disease.

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