Immunological Procedure for the Rapid and Specific Identification of *Coccidioides immitis* Cultures

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Received for publication 30 August 1976

An immunological procedure for the rapid and specific identification of *Coccidioides immitis* isolates has been developed. The specificity of the procedure is based on the fact that *C. immitis* produces antigens that are not produced by morphologically similar fungi. The procedure involved the transfer of heavy mold-form inocula to flasks that contained small volumes of brain heart infusion broth. Shake cultures were grown at 25°C on a gyratory shaker at 150 rpm. The concentrated supernatant antigens so obtained from broth cultures were tested in parallel with coccidioidin by a microimmunodiffusion technique against *C. immitis* antiserum. The ability of the immunological procedure to identify *C. immitis* cultures was evaluated by testing the supernatant antigens of 166 unknown isolates, many of which, by gross or microscopic examination or both, resembled *C. immitis* or belonged to the family *Gymnoascaceae*. Each culture was also identified by conventional laboratory procedures. Comparative evaluation showed that the immunological test for *C. immitis* was 100% sensitive and specific. The diagnostic precipitin bands that were produced by the interaction of the *C. immitis* supernatants and antisera were shown to consist of (i) a heat-stable antigen comparable to that active in the tube precipitin test and (ii) two heat-labile antigens, one of which was associated with complement fixation reactivity. With pure cultures, this immunological procedure permitted the identification of *C. immitis* isolates within 5 days.

Coccidioidomycosis is an important, serious respiratory infection of man and lower animals that may disseminate to involve all the viscera, the central nervous system, and the skin. The etiological agent, *Coccidioides immitis*, is found in certain semi-arid regions of North, Central, and South America (5). Within the *C. immitis*-endemic areas in the United States, an estimated 100,000 new infections occur annually (1). Because of the high rate of *C. immitis* infection for people living and traveling in the endemic areas, laboratories in all areas should be capable of identifying the etiological agent.

Definitive identification of *C. immitis* cultures involves the demonstration of arthrospores in the mycelial form of the fungus and conversion of the mycelial form to the diagnostic tissue form. Conversion is necessary because certain saprophytes morphologically resemble *C. immitis* (4) in their mycelial form, and some *C. immitis* isolates fail to form arthrospores or are difficult to convert to their tissue form (7). Definitive identification of *C. immitis* by conventional means can take 3 weeks or longer. To

obviate the use of expensive laboratory animals and to speed up identification, workers have attempted to devise in vitro procedures that would readily permit the conversion of the mycelial growth of *C. immitis* to the diagnostic endospore-forming spherules (2, 9, 11).

The present study was undertaken to determine whether mycelial-form cultures of *C. immitis* consistently and rapidly produced specific exoantigens that could be readily and accurately detected by using immunodiffusion (ID) procedures (10). Detection of such antigens would serve to identify an isolate as *C. immitis*.

MATERIALS AND METHODS

Control antiserum. Frozen human serum from a patient with coccidioidomycosis (lot 18311) was obtained from the Serum Bank Section, Center for Disease Control, Atlanta, Ga. It was used throughout the study.

Control antigen. Coccidioidin lot 1, prepared in Trypticase-yeast extract-dextrose broth, was obtained from the Biological Reagents Section, Center for Disease Control. This antigen was used throughout the study.

Unknown cultures. One hundred and sixty-six fungus cultures were obtained from a variety of sources. The species represented in these cultures included: *Arachniotus reticulatus*, *Arachniotus* spp., *Auxarthron* spp., *A. umbrinum*, *Blastomyces dermatitidis*, *Briosia cubispora*, *C. immitis*, *Geotrichum candidum*, *Histoplasma capsulatum* var. *capsulatum*, *H. capsulatum* var. *duboisii*, *H. farciminosum*, *Malbranchea pulchella* var. *sulfurea*, *Myxotrichum* sp., *Oidiodendron* sp., *Paracoccidioides brasiliensis*, *Pseudoarachniotus* sp., *Sporendonema purpurescens*, *Trichosporon capitatum*, *T. cutaneum*, and *T. penicillatum*.

Test procedure. The methods for preparing inocula and for producing, harvesting, concentrating, and analyzing supernatants of brain heart infusion broth cultures for *C. immitis* and other antigens were essentially identical to those of Standard and Kaufman (10). A heavy mass of mycelial growth, at least 2 to 4 cm², was transferred in duplicate into 30 ml of brain heart infusion broth. After 3 and 6 days of incubation at 25°C, the shake cultures were killed with merthiolate. Each supernatant was concentrated (in approximately 3 h) 10× and 25× with an Amicon Minicon macrosolute B-15 concentrator (Amicon Corp., Lexington, Mass.). Micro-ID templates with 17 seven-well patterns were used. *C. immitis* antiserum was placed in the central well of each ID pattern and preincubated for 1 h at 25°C. Reference coccidioidin was then placed in the upper and lower wells of each pattern, and the unknown antigens (concentrated supernatants) were placed in duplicate in the lateral wells. ID plates charged with reactants were incubated in a moist chamber at 25°C for 24 h. A fungus isolate was considered to be *C. immitis* when its concentrated supernatant, upon reacting with *C. immitis* antiserum, produced a precipitin line or lines identical to any of those formed by reference coccidioidin and *C. immitis* antiserum. If the unknown antigen produced lines that were diffuse or overlapping, then they were diluted 1:2 to better relate them to the control precipitin lines.

RESULTS

Preliminary studies with 11 confirmed cultures of *C. immitis* revealed that 3- and 6-day-old cultures produced between one and three exoantigens in brain heart infusion broth. These antigens were similar to those found in the lot 1 coccidioidin used in the coccidioidomycosis ID test. Histoplasmin containing H and M antigens and blastomycin containing A antigen (8) produced no bands after reaction with the *C. immitis* antiserum (Fig. 1).

To determine the accuracy of the immunological procedure for identifying *C. immitis* cultures, we studied 166 coded cultures, the identities of which were unknown to us, in parallel by the immunological procedure and by conventional morphological procedures (Table 1). Sixty-nine of the isolates produced one or more exoantigens that reacted with *C. immitis* antiserum. However, only 66 of the exoantigen-

FIG. 1. Immunodiffusion of *C. immitis* broth culture supernatants. Contents of peripheral wells: 1 and 4, coccidioidin lot 1; 2, histoplasmin; 3, blastomycin; 5 and 6, coccidioidin from two different *C. immitis* cultures. The center well contained human coccidioidomycosis case serum.

positive supernatants produced one to three lines of identity with the precipitates formed by the reference reagents. These 66 isolates were immunologically identified as *C. immitis*, and their identity was confirmed by conventional methodology. Four of these 66 isolates failed to produce arthrospores on initial examination. Supernatants from three other unknown isolates produced a band that was unrelated to any of the three coccidioidomycosis reference bands. Immunologically, these fungi were not identified as *C. immitis*. Morphologically, they were identified as species of *Auxarthron*.

Supernatants from 97 of the unknown fungi studied failed to react with the *C. immitis* antiserum, and these fungi were not immunologically identified as *C. immitis* (Fig. 2, Table 1). These cultures were identified by conventional procedures as saprophytes or pathogens other than *C. immitis*.

Since the *C. immitis* cultures produced three precipitin bands that were related to the reference system, studies were undertaken to determine the identity of these reference bands. Serum containing only antibody to the heat-labile F (IDCF) antigen (6) was tested in parallel with *C. immitis* antiserum against heated (30 min at 60°C) and unheated coccidioidin and concentrated *C. immitis* supernatants (Fig. 3). The heated coccidioidin contained only antigen comparable to that found active in the tube precipitin test; this will be referred to as the IDTP antigen. The study indicated that the band forming closest to the serum well resulted from an antigen-antibody reaction that corresponds to that occurring in the tube precipitin test. The

TABLE 1. Accuracy evaluation of the immunological procedure for identifying *Coccidioides immitis* cultures with 166 unknown fungus isolates

Type of precipitin reactions	No. of unknown fungus supernatants producing reactions	Identification by immunological test	Identification by conventional tests	No. identified
1 to 3 bands of identity	66	<i>C. immitis</i>	<i>C. immitis</i>	66
1 band of non-identity	3	Not <i>C. immitis</i>	<i>Auxarthron</i> spp.	3
No bands	97	Not <i>C. immitis</i>	<i>Arachniotus</i> spp.	4
			<i>Auxarthron</i> spp.	2
			<i>Blastomyces dermatitidis</i>	9
			<i>Briosia cubispora</i>	1
			<i>Geotrichum candidum</i>	3
			<i>Histoplasma capsulatum</i> var. <i>capsulatum</i>	31
			<i>H. capsulatum</i> var. <i>duboisii</i>	15
			<i>H. farciminosum</i>	8
			<i>Malbranchea pulchella</i> var. <i>sulfurea</i>	2
			<i>Myxotrichum</i> sp.	1
			<i>Oidiodendron</i> sp.	1
			<i>Paracoccidioides brasiliensis</i>	10
			<i>Pseudoarachniotus</i> sp.	1
			<i>Sporendonema purpurescens</i>	1
			<i>Trichosporon capitatum</i>	2
			<i>T. cutaneum</i>	5
			<i>T. penicillatum</i>	1

FIG. 2. Specificity of the ID technique for *C. immitis*. Contents of peripheral wells: 1 and 4, coccidioidin lot 1; 2, broth supernatant antigen from *Arachniotus* sp.; 3, broth supernatant antigen from *Myxotrichum* sp.; 5, broth supernatant from *Geotrichum candidum*; 6, broth supernatant from *Trichosporon* sp. The center well contained human coccidioidomycosis case serum.

band forming closest to the antigen well was comparable to the IDCF line. Another band was formed between the IDCF and IDTP lines and is referred to as IDHL (ID heat labile). The IDCF and IDHL bands frequently overlaid each other (Fig. 2 and 3), but, with appropriate concentration to attain equivalence, they may be separated (Fig. 1). The IDHL antigen is not abundant in our control coccidioidin and is therefore difficult to demonstrate. However, it is influenced and becomes more evident when placed in a well adjacent to one containing IDHL and other antigens. The antigens in the IDCF and IDHL bands are considered heat labile, since these bands failed to appear with heated coccidioidin and supernatants (Fig. 3). The IDTP band was the one most commonly noted in the supernatants. However, detection of any of the three bands was considered diagnostic for *C. immitis*.

DISCUSSION

Definitive identification of *C. immitis* cultures requires conversion of their mold form to the tissue form, since one may encounter atypical cultures of *C. immitis* as well as saprophytes that bear arthrospores closely resembling those of the pathogen.

FIG. 3. Identity of the *C. immitis* precipitin bands. Contents of peripheral wells: 1, antiserum with *F* antibody only; 2, tube precipitinogen coccidioidin; 3 and 5, control coccidioidin, lot 1; 4, heated *C. immitis* broth culture supernatant; 6, unheated *C. immitis* broth culture supernatant. The center well contained *C. immitis* antiserum with all three precipitins.

This study revealed that *C. immitis* cultures produce at least three exoantigens that are specific. All of the 66 fungi that produced either the IDTP, IDCF, or the IDHL diagnostic bands were identified by conventional methods as *C. immitis*. The immunological method had 100% specificity and accuracy. None of the 27 saprophytic fungi that resembled *C. immitis* produced any of these exoantigens (Table 1). Three of the 27 saprophytic fungi, namely the *Auxarthron* spp., produced an antigen that was shared with *C. immitis* but was unrelated to the diagnostic IDTP, IDCF, and IDHL antigens. The IDTP antigen was usually the first of the diagnostic antigens to be detected. The IDHL antigen usually appeared next, and the IDCF antigen last. Some *C. immitis* cultures produced one to three additional antigens that formed bands unrelated to the reference bands. It is apparent that the reference reagents used for the immunological identification of *C. immitis* must contain the IDTP antigen and preferably the combination of diagnostic IDTP, IDCF, and IDHL antigens. A line of identity only with any of these three antigens is diagnostic. We cannot emphasize this too strongly.

Our study indicated that the 10 $\times$ and 25 $\times$ concentrates yielded equally potent precipitinogens. All of the *C. immitis* cultures used in this study were identified by analysis of their 3-day-old culture supernatants. In many instances, the antigens from 6-day-old culture supernatants were too concentrated and drove the de-

veloping precipitates into the center serum well.

The immunological test appears entirely specific. Tests with concentrated supernatants from 100 saprophytic and pathogenic fungi other than *C. immitis* failed to demonstrate any antigens that corresponded to the IDTP, IDCF, and IDHL antigens of *C. immitis*.

Unlike the in vitro techniques used to demonstrate endospore formation (2, 9, 11), the immunological test does not require sporulating cultures. A few of the *C. immitis* isolates studied, although nonsporulating, produced the specific antigens that made their identification possible.

The immunological method described reduces the time necessary for identifying *C. immitis* cultures to 5 days as compared with the 3 weeks usually required by routine morphological and in vivo conversion procedures. Although the in vitro conversion procedure of Sun et al. (11) also yields identification within 5 days, it requires sporulating cultures. The immunological method not only is sensitive, specific, and rapid, but it also minimizes laboratory manipulation of a potentially hazardous, infectious fungus.

ACKNOWLEDGMENTS

We thank Milton Huppert (V. A. Hospital, Long Beach, Calif.) and Wesley Press (Arizona State Health Department, Phoenix, Ariz.) for graciously supplying us with some of the *C. immitis* isolates and reagents.

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