Serological Comparison of Spherules and Arthrospores of *Coccidioides immitis*[1]

MARSHALL E. LANDAY,[2] ROBERT W. WHEAT, NORMAN F. CONANT, AND EDWIN P. LOWE

Department of Microbiology and Immunology, Duke University Medical Center, Durham, North Carolina 27706, and Department of the Army, Fort Detrick, Frederick, Maryland 21701

Spherule and arthrospore cellular preparations were sonic-treated and separated into their respective supernatant and sediment components. Complement-fixation tests with antispherule and antiarthrospore pooled rabbit sera revealed that the soluble antigens exhibited more serological activity than the sediment preparations. After autoclaving, an arthrospore cellular antigen exhibited increased activity with either antisera, whereas autoclaved spherules exhibited increased activity only with antispherule serum. Complement-fixation tests with coccicioidin and spherule culture supernatant preparations revealed quantitative or qualitative differences in antigenic determinants between these two morphological phases of *Coccidioides immitis*.

Serological studies of *Coccidioides immitis* have been made by many investigators, including Smith et al. (18), Ajello et al. (1), Campbell (3), and Huppert and Bailey (7). Most of these investigators employed mycelial antigens and sera from infected human beings or animals. Because the spherule is the growth form found in infected hosts, the pertinence of investigating serological differences between spherules and arthrospores is obvious. Furthermore Levine, Cobb, and Smith (13) demonstrated differences in protective capacities between these morphological phases. Landay et al. (11) showed that spherule and arthrospore cellular antigens exhibited serological differences and similarities in the complement-fixation assay. It was also observed that sonic treatment of spherules and of arthrospores increased the complement-fixing activity of these cells.

This study was initiated to investigate the antigenicity of soluble and particulate fractions of these sonic-treated antigens. Furthermore, the effect of heat on spherule and arthrospore cellular preparations was studied, and a comparison of coccidioidin and spherule culture supernatant antigens was also made.

MATERIALS AND METHODS

Complement-fixation antigens: sonic-treated antigens. Spherules of *C. immitis* were grown in the syn-

thetic medium of Converse (4), killed with Formalin, tested for sterility, washed three times in Seibert's buffered saline containing several drops of Tween 80 and 1:10,000 merthiolate (w/v), and then suspended in this diluent at a concentration of 12 mg (dry weight) per ml. A cellular antigen was prepared in an identical manner from arthrospores of *C. immitis* grown by the method of Sinski et al. (17).

Yeast cells of *Histoplasma capsulatum*, *Blastomyces dermatitidis*, and *Candida albicans* were grown for 4, 5, and 2 days, respectively, on Pine's histocitrate medium (16) at 37 C. The resulting growths were killed with Formalin, tested for sterility, washed three times in Seibert's modified buffer, and suspended in the diluent at a concentration of 12 mg/ml.

The cellular antigens were subjected to treatment in a Branson S-75 sonifier for varying periods of time until microscopic evidence of effective breakage was observed. The times required for effective disruption of each antigen were: *C. immitis* spherules and arthrospores, 30 min each; *H. capsulatum*, 15 min; *B. dermatitidis* and *C. albicans*, 10 min each. Each whole sonic-treated preparation comprised one complement-fixing antigen. A soluble and insoluble portion of each sonic-treated antigen were separated in a Servall refrigerated centrifuge at 10,500 × *g* for 15 min. The supernatant fluid constituted the second complement-fixing antigen. A third antigen was prepared from the sediment by washing three times in Seibert's buffer and resuspending it in this diluent at the original volume-to-volume ratio.

Autoclaved antigens. The cellular suspensions (12 mg/ml) of spherules and of arthrospores were autoclaved at 15 psi at 121 C. Each whole autoclaved suspension comprised one complement-fixing antigen. Samples of these suspensions were then separated into their supernatant and sediment components by centri-

fugation as outlined above for the sonic-treated antigens.

Spherule culture supernatant antigen. Spherules were grown in the Converse medium and killed by the addition of 0.5% Formalin. The supernatant fluid growth medium was clarified by centrifugation at 2,000 × *g* for 10 min in a model SBV International Centrifuge. This supernatant fluid was dialyzed against 0.85% NaCl for 24 hr at 4 C. Rubber bands were placed on the dialysis bag so that the fluid volume would not increase. After dialysis, 1:10,000 Merthiolate (w/v) was added, and a dry weight determination was made (7.9 mg/ml).

Coccidioidin antigen (18). This material was obtained from C. E. Smith, of the University of California at Berkeley, and a dry weight determination was made (7.32 mg/ml).

Immunizing antigens. Spherules grown in the Converse medium were freed of a large part of their mycelium by the chloroform extraction method of Levine (12). The spherules were then washed three times in Seibert's modified buffered saline, and were suspended in this diluent at a concentration of 12 mg/ml.

After preimmunization sera were drawn from the marginal ear veins of four white rabbits, the animals were divided into two pairs. One pair was immunized over a 5-week period with the purified spherule cellular suspension, the other with the arthrospore cellular suspension. Each animal received a total dose of 144 mg of antigen by intravenous injection. Approximately 4 days after the last injection, the animals were bled by cardiac puncture. Sera obtained from the two animals in each pair were pooled.

Serological assay. The complement-fixation assay used in this study has been previously described (11).

RESULTS

Complement-fixation tests with preimmune sera. When a 1:10 dilution of preimmune serum was tested with a 1:8 dilution of spherule cells or with a 1:8 dilution of whole sonic-treated spherules, negative results were observed. When a 1:8 dilution of preimmune serum was tested with a 1:8 dilution of arthrospore cells or a 1:8 dilution of whole sonic-treated arthrospores, negative results were again observed.

Complement-fixation tests with immune sera. Spherule pooled antiserum versus sonic-treated antigens. The antispherule serum exhibited titers of 1,280 when tested with a 1:8 dilution of the whole sonic-treated spherule, the sonic-treated spherule supernatant, and the sonic-treated spherule sediment antigens. These preparations demonstrated antigen titers of 1:512, 1:512, and 1:64, respectively, in assays with this antiserum (Table 1).

Antibody titers of 1,280, 1,280, and 640, respectively, were observed when antispherule serum was reacted with a 1:8 dilution of the sonic-treated arthrospore, the supernatant, and the sediment antigens. These preparations

exhibited antigen titers of 1:512, 1:512, and 1:64, respectively, in these assays (Table 1).

Spherule antiserum exhibited antibody titers of 1,280, 640, and 1,280, respectively, when tested with a 1:8 dilution of sonic-treated *H. capsulatum*, the supernatant, and the sediment preparations. These antigens displayed titers of 1:128, 1:64, and 1:64, respectively, in the tests (Table 1).

An antibody titer of 10 was observed when spherule antiserum was reacted with a 1:8 dilution of sonic-treated *B. dermatitidis* antigen. This antiserum did not fix complement when tested with the supernatant or sediment preparations (Table 1).

Antispherule serum exhibited titers of 640, 320, and 320, respectively, when tested with a 1:8 dilution of sonic-treated *C. albicans*, the supernatant, and the sediment antigens. These preparations displayed antigen titers of 1:64, 1:64, and 1:16, respectively, in these assays (Table 1).

Spherule pooled antiserum versus autoclaved antigens. Antibody titers of 1,280, 1,280, and 640, respectively, were observed when this antiserum was reacted with a 1:8 dilution of the whole autoclaved spherule, the autoclaved spherule supernatant, and the autoclaved spherule sediment preparations. These antigens exhibited titers of, 1:256, and 1:8, respectively, in the assays with antispherule serum (Table 2).

Spherule antiserum demonstrated titers of 640, 640, and 160, respectively, when tested with a 1:8 dilution of autoclaved arthrospore, the supernatant, and the sediment preparations. The antigens exhibited titers of 1:512, 1:512, and 1:16, respectively, in these tests (Table 2).

Spherule pooled antiserum versus spherule culture supernatant antigen. When a 1:16 dilution of this antigen was reacted with antispherule serum, an antibody titer of 1,280 was observed. The antigen (7.9 mg/ml) displayed a titer of 1:256 in the assay (Fig. 1a).

Spherule pooled antiserum versus coccidioidin. An antibody titer of 640 was observed when spherule antiserum was reacted with a 1:6 dilution of coccidioidin. This antigen displayed a titer of 1:768 in the test (Fig. 1a).

Arthrospore pooled antiserum versus sonic-treated antigens. Antibody titers of 80 were observed when this antiserum was reacted with a 1:8 dilution of the whole sonic-treated spherule, the sonic-treated spherule supernatant, and the sonic-treated spherule sediment antigens. These preparations demonstrated antigen titers of 1:256, 1:256, and 1:32, respectively, in assays with antiarthrospore serum (Table 1).

Arthrospore antiserum exhibited titers of 640 when tested with a 1:8 dilution of sonic-treated

TABLE 1. *Spherule and arthrospore pooled antiserum versus sonic-treated antigens*

	Antigen concn (mg/ml)	1:8ᵃ	1:16	1:32	1:64	1:128	1:256	1:512	1:1,024	1:2,048	1:4,096
Spherule pooled antiserum											
Sonic-treated spherule	12	1,280	1,280	1,280	1,280	1,280	1,280	320	Neg		
Supernatant		1,280	1,280	1,280	1,280	1,280	1,280	320	Neg		
Sediment		1,280	1,280	640	160	Neg					
Sonic-treated arthro-spore	12	1,280	1,280	640	640	640	640	80	Neg		
Supernatant		1,280	1,280	640	640	640	320	80	Neg		
Sediment		640	640	320	80	Neg					
Sonic-treated *Histo-plasma capsulatum*	12	1,280	1,280	1,280	640	40	Neg				
Supernatant		640	640	320	40	Neg					
Sediment		1,280	1,280	640	40	Neg	Neg				
Sonic-treated *Blasto-mycen dermatitidis*	12	10	Neg								
Supernatant		Neg	Neg								
Sediment		Neg	Neg								
Sonic-treated *C. al-bicans*	12	640	320	320	40	Neg					
Supernatant		320	160	80	40	Neg					
Sediment		320	160	Neg							
Arthrospore pooled antiserum											
Sonic-treated spherule	12	80	80	80	40	20	10	Neg			
Supernatant		80	80	40	20	20	10	Neg			
Sediment		80	40	20	Neg						
Sonic-treated arthro-spore	12	640	640	640	640	640	80	10	Neg		
Supernatant		640	640	640	640	320	40	10	Neg		
Sediment		640	640	320	80	20	Neg				
Sonic-treated *H. cap-sulatum*	12	640	640	320	160	40	10	Neg			
Supernatant		640	320	160	80	20	10	Neg			
Sediment		640	160	160	10	Neg					
Sonic-treated *B. der-matitidis*	12	640	320	160	20	Neg					
Supernatant		640	320	80	Neg						
Sediment		40	Neg								
Sonic-treated *C. al-bicans*	12	40	40	20	10	10	Neg				
Supernatant		20	20	10	10	Neg					
Sediment		20	10	Neg							

ᵃ First antigen dilution exhibiting no anticomplementary activity.

arthrospore, the supernatant, and the sediment preparations. These antigens exhibited titers of 1:512, 1:512, and 1:128, respectively, in the assays (Table 1).

Antibody titers of 640 were observed when antiarthrospore serum was tested with a 1:8 dilution of any of the sonic-treated *H. capsulatum* antigens. These preparations displayed antigen titers of 1:256, 1:256, and 1:64, respectively, in the tests (Table 1).

Arthrospore antiserum exhibited titers of 640, 640, and 40, respectively, when reacted with a 1:8 dilution of sonic-treated *B. dermatitidis*, the supernatant, and the sediment preparations. These antigens exhibited titers of 1:64, 1:32, and 1:8, respectively, in these assays (Table 1).

Antibody titers of 40, 20, and 20, respectively, were observed when this antiserum was tested with a 1:8 dilution of sonic-treated *C. albicans*, the supernatant, and the sediment antigens. These preparations exhibited antigen titers of

TABLE 2. *Spherule and arthrospore pooled antiserum versus autoclaved antigens*

	Antigen concn (mg/ml)	Complement-fixing antigen dilution									
		1:8[a]	1:16	1:32	1:64	1:128	1:256	1:512	1:1,024	1:2,048	1:4,096
Spherule pooled antiserum											
Autoclaved spherule	12	1,280	1,280	1,280	1,280	640	320	Neg			
Supernatant		1,280	640	640	640	320	160	Neg			
Sediment		640	Neg								
Autoclaved arthrospore	12	640	640	640	640	640	320	80	Neg		
Supernatant		640	640	640	640	640	320	160	Neg		
Sediment		160	20	Neg							
Arthrospore pooled antiserum											
Autoclaved spherule	12	40	20	20	10	Neg					
Supernatant		40	20	10	10	Neg					
Sediment		40	10	Neg							
Autoclaved arthrospore	12	320	320	320	320	160	80	20	Neg		
Supernatant		320	320	320	320	160	80	20	Neg		
Sediment		160	10	Neg							

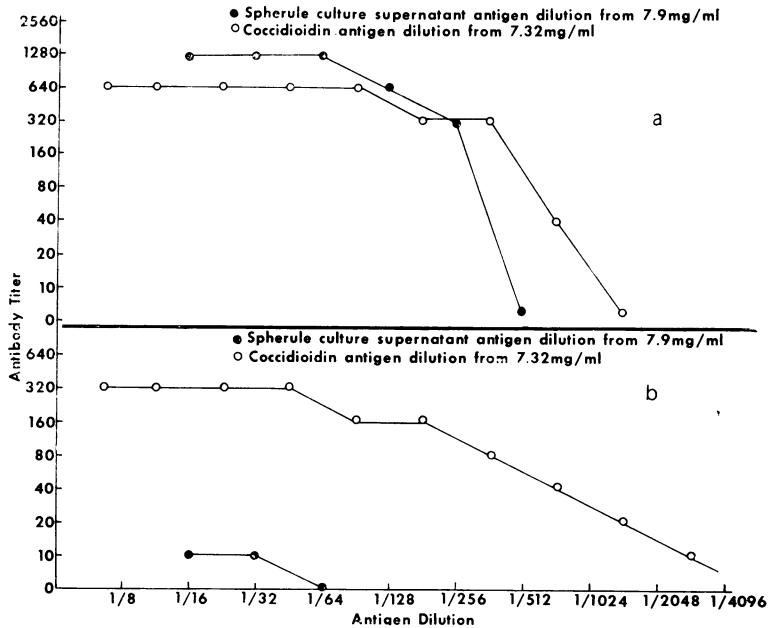[a] First antigen dilution exhibiting no anticomplementary activity.

FIG. 1. (*a*) *Spherule pooled antiserum versus antigens from the fluid growth media.* (*b*) *Arthrospore pooled antiserum versus antigens from the fluid growth media.* (*All antigen dilutions exhibiting no anticomplementary activity were tested.*)

1:128, 1:64, and 1:16, respectively, when assayed with antiarthrospore serum (Table 1).

Arthrospore pooled antiserum versus autoclaved antigens. This antiserum demonstrated titers of 40 when reacted with a 1:8 dilution of the whole autoclaved spherule, the autoclaved spherule supernatant, and the autoclaved spherule sediment preparations. These antigens exhibited titers of 1:64, 1:64, and 1:16, respectively, in the assays (Table 2).

Arthrospore antiserum exhibited titers of 320, 320, and 160, respectively, when tested with a

1:8 dilution of autoclaved arthrospore, the supernatant, and the sediment preparations. These antigens demonstrated titers of 1:512, 1:512, and 1:16, respectively, in the tests (Table 2).

Arthrospore pooled antiserum versus spherule culture supernatant antigen. When a 1:16 dilution of this antigen was reacted with antiarthrospore serum, an antibody titer of 10 was observed. The antigen (7.9 mg/ml) displayed a titer of 1:32 in the assay (Fig. 1b).

Arthrospore pooled antiserum versus coccidioidin. An antibody titer of 320 was observed when arthrospore antiserum was reacted with a 1:6 dilution of coccidioidin. This antigen displayed a titer of 1:3,072 in the test (Fig. 1b).

DISCUSSION

Previous investigators, including Landay et al. (11), Hill and Campbell (6), and Martin (14) have revealed that disruption of fungal cells by sonic-treatment or grinding has led to increased serological activity. We observed that the sonic-treated spherule supernatant antigen retained all the activity of the whole sonic-treated spherule preparation, whereas the sonic-treated spherule sediment did not (Table 1). A similar observation was made with sonic-treated arthrospore antigens. Kong, Levine, and Smith (9) stated that supernatant material from disrupted spherules did not protect mice against coccidioidomycosis. Smith et al. (18) observed that in human coccidioidomycosis a high complement-fixation titer indicated a poor prognosis. We therefore considered it possible that nonprotective protoplasmic antigens provide the stimulus for the production of nonprotective antibodies which are detected in advanced cases of coccidioidomycosis.

Landay et al. (11) reported that in the complement-fixation test spherule cells reacted minimally with arthrospore serum, whereas this antigen exhibited maximal reactivity with antispherule serum. The arthrospore cellular preparation was a heterogenous complement-fixing antigen since it reacted strongly with either antisera.

In this study, the whole autoclaved arthrospore preparation exhibited increased antigen titers with either sera, whereas autoclaved spherule cells demonstrated increased activity with antispherule serum, and only a minimal increase with arthrospore antiserum. It therefore appeared that autoclaving arthrospores caused a release of additional heat-stable determinants which reacted with sera from both phases of *C. immitis.* Autoclaving spherule cells, however, made available

a high proportion of antispherule determinants and relatively few cross-reacting ones.

Of particular interest was the fact that the autoclaved supernatant antigens exhibited more serological activity than their respective autoclaved sediment antigens (Table 2). Kong and Levine (8) stated that *C. immitis* protective antigens were found in the cell wall and that heat treatment reduced, but did not eliminate, this immunogenicity. It was therefore deemed possible that the activity of the autoclaved sediments represented heat-stable protective determinants, whereas non-protective heat-stable antigens were found in the autoclaved supernatant portions.

Fiese (5) reported that autoclaved coccidioidin failed to fix complement with sera from patients with proven coccidioidomycosis. The results discussed above show that autoclaving frequently increased the complement-fixing activity of our antigens with rabbit sera. Martin (14), however, stated that sera from different animal species varied in their capacities to fix complement with certain protein and carbohydrate materials.

Two other antigens, spherule culture supernatant fluid and coccidioidin, were also tested by the complement-fixation technique. Spherule antigen again reacted minimally with antiarthrospore serum (Fig. 1a and b), whereas coccidioidin reacted strongly with sera from either phase of *C. immitis.* Since coccidioidin was derived from mycelium, as were arthrospores, these results might again indicate quantitative or qualitative differences in antigenic determinants between the spherule and mycelial phases of *C. immitis.*

Since the spherule is the morphological form of the fungus found in diseased tissues, one would expect the antigenic content of these bodies to provide the stimulus for antibody production. We therefore suggest that an efficacious complement-fixing antigen to detect cases of coccidioidomycosis may be prepared from the spherule phase of this organism.

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