CONTINUOUS IN VITRO CULTIVATION OF SPHERULES OF COCCIDIOIDES IMMITIS
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ABSTRACT

BRESLAU, ABRAHAM M. (Veterans Administration Center and University of California Los Angeles Center for the Health Sciences, Los Angeles, Calif.), and Mike Y. Kubota. Continuous in vitro cultivation of spherules of Coccidioides immitis. J. Bacteriol. 87:468–472. 1964.—Spherules of Coccidioides immitis were grown in vitro in continuous culture for more than 4 years. Cultures were grown in modified Converse medium in a modified Lubarsky and Plunkett culture tube incubated at 40 C under increased CO₂ tension. Spherules that had transformed from hyphae were freed from mycelial fragments, and were allowed to settle to the bottom of the tube. The mycelium which normally degenerated at 40 C was removed repeatedly with a syringe and needle until the culture was almost pure spherules. For continuous culture, 10 to 12 ml of old medium were removed, and were replaced by fresh medium previously bubbled with a mixture of 20% carbon dioxide and 80% air.

Coccidioides immitis is a dimorphic fungus existing in soil primarily in the saprophytic mycelial form. In the parasitic form, the spherule is a thick-walled, nonbudding spherical structure.

Immunological studies by Levine, Cobb, and Smith (1960, 1961) with purified spherules as a vaccine source indicated a need for growing mycelium-free spherules. Roessler et al. (1946), Levine et al. (1960, 1961), Converse (1955, 1956, 1957), Converse and Besemer (1959), and Lones and Peacock (1960a, b) reported success in growing the spherules in vitro with the flask-shaking method described by Roessler et al. (1946). Spherules and endospores were harvested at the peak of growth by filtering the culture through a fine stainless-steel screen, surgical gauge, and nylon hosiery. However, a mycelium-free spherule culture was not maintained over long periods of time.

1 Deceased, November 1962.

This report describes a method whereby the spherule phase of C. immitis was continuously maintained in culture for more than 4 years.

MATERIALS AND METHODS

Cultures were grown in a modified culture tube described by Lubarsky and Plunkett (1955). The culture tube (32 × 205 mm) had an extension arm 8 mm in diameter, 20 to 25 mm in length, attached as a straight tube 35 to 40° tangential to the bottom curvature of the tube and plugged with a vaccine cap. The tube was placed on a wooden rack and incubated at 40 C. A gas mixture of 20% carbon dioxide and 80% air, obtained from General Dynamics Corp., Los Angeles, Calif., was bubbled through the medium to maintain increased CO₂ tension.

Stock cultures of Silveira, Mauser, and Bary strains were carried on Sabouraud or Brain Heart Infusion agar slants in 2-oz prescription bottles, in the manner described by Huppert (1957). A 2- or 3-month-old agar-slab culture was used as inoculum. The mycelial mat was broken up in saline suspension with progressively smaller sized (15, 18, 20 gauge) hypodermic needles and syringe. When the suspension was fine enough to pass through a 20-gauge needle with ease, a heavy (3 to 5 ml) inoculum was injected into the culture tube containing 60 to 65 ml of medium. After initial inoculation, biweekly subcultures were made to determine which strain gave the best spheralation.

For continuous culture studies, 10 to 12 ml of medium were removed, and fresh medium previously bubbled with the carbon dioxide-air mixture was replaced through the vaccine cap. Samples of the culture were mounted on slides, stained with lacto-phenol cotton blue, and examined microscopically; they were graded 0 to 10 as a numerical means to determine the progress to spheral formation. All work outside the incubator was done in a Berkeley-type bacteriological gloved box heated to 40 C.
TABLE 1. Effect of enrichment additives on transformation and spherulation of Coccidioides immitis

<table>
<thead>
<tr>
<th>Medium</th>
<th>Additive</th>
<th>Final conc</th>
<th>Visual observations</th>
<th>Morphology</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>Converse basal medium</td>
<td>1:2.5</td>
<td>3 5 6 6 5</td>
<td>Spheres 15 to 30 μ in diameter</td>
</tr>
<tr>
<td>CA</td>
<td>Amino acid</td>
<td>1:1,000</td>
<td>2 4 6 6 5</td>
<td>Same as above</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1:500</td>
<td>4 4 6 6 5</td>
<td>Same as above</td>
</tr>
<tr>
<td>CVA</td>
<td>Vitamins</td>
<td>1:10,000</td>
<td>3 5 7 6 7</td>
<td>Same as above; few spheres with endospores</td>
</tr>
<tr>
<td>CVBA</td>
<td>Biotin</td>
<td>1:1,000</td>
<td>3 5 6 6 6</td>
<td>Some abortive spherules</td>
</tr>
<tr>
<td>CVBAP</td>
<td>Cytosine</td>
<td>1:100,000</td>
<td>4 5 7 7 8</td>
<td>Spheres 20 to 35 μ in diameter; good sporangia and endospores</td>
</tr>
<tr>
<td>CVBAT</td>
<td>Tamol N</td>
<td>1:1,000</td>
<td>4 5 5 4 4</td>
<td>Same as above</td>
</tr>
</tbody>
</table>

* Bary strain.
† Transformation and spherulation graded 0 (no spherules) to 10 (100% spherules).

The experimental medium used was Converse basal spherule medium modified in the following manner: Converse medium (less NaCl), diluted 1:2.5; amino acid (casein hydrolysate, vitamin-free; Nutritional Biochemicals Corp., Cleveland, Ohio), final concentration 1:500; vitamin (Beectax, biotin-free; Winthrop Laboratories, Rensselaer, N.Y.), final concentration 1:10,000; biotin, final concentration 2.5 μg/ml phosphate buffer. The optimal additive concentrations were determined by biweekly subculturing of the Bary strain with higher or lower (or both) concentrations of each additive.

**Results**

The results of the optimal medium study with the Bary strain are shown in Table 1, which also lists Tamol N and cytosine as additives to the medium. Their use was discontinued when their addition resulted in plasmolysis of the spherules.

When the Bary strain was inoculated from a slant into medium in a flask and shaken at 34 and 37 C to duplicate experiments of earlier investigators, only profuse mycelial growth resulted. Even at 40 C, the inoculum germinated into mycelium. Although some degeneration was noted with continuous shaking, no spherulation occurred. When the carbon dioxide-air mixture was bubbled through the medium, many spherules were seen, but the culture was still overcrowded with mycelial growth.

TABLE 2. Effect of strain differences in spherulation of Coccidioides immitis

<table>
<thead>
<tr>
<th>Strain</th>
<th>Visual observations*</th>
<th>Morphology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mauser</td>
<td>0 0 0 0 0</td>
<td>Thin, mycelial growth; no transformation of hypha.</td>
</tr>
<tr>
<td>Silveira</td>
<td>1 1 3 2 2</td>
<td>Few transformed hyphae; few spherules 10 to 20 μ in diameter.</td>
</tr>
<tr>
<td>Bary</td>
<td>2 5 4 6 7</td>
<td>Many transformed hyphae; many spherules 20 to 35 μ in diameter.</td>
</tr>
</tbody>
</table>

* Transformation and spherulation graded 0 (no spherules) to 10 (100% spherules).

Even under increased CO₂ tension, the Mauser strain failed to produce spherules, whereas only an insignificant amount (10 to 30%) of spherulation occurred with Silveira strain. Continued cultivation of this strain did not clear the culture of its mycelial forms. The Bary strain was not only more productive, but spherules had a lesser tendency to germinate and revert to mycelium. In a continuous culture, the Bary strain hyphae had almost totally transformed into spherules within 3 weeks, and continued to multiply by means of asexual endospores. When a mycelial broth inoculum was used, transformation of hyphae into spherules was observed within 72 to 96 hr. When the spherules were inoculated into fresh medium,
they quickly germinated and reverted into mycelium. After 72 to 96 hr, these mycelial forms again transformed into spherules. The comparative spherulation studies of the various strains are listed in Table 2. The Barry strain was selected for continuous culture studies.

When the experimental medium in the culture tube was warmed to 40 C and bubbled with the carbon dioxide-air mixture before inoculation from a slant, spherulation occurred with only a minimum of mycelial growth. As the inoculum in the continuous culture transformed into spherules, they were freed from the mycelial fragments with the action of a syringe and needle. When bubbling was stopped, the heavier spherules, sporangia, and endospores dropped to the bottom of the tube, followed by the fluffier mycelium. When an adequate spherule precipitate was seen on the bottom of the tube, the upper mycelial layer was carefully removed. Selective removal in this manner finally resulted in an almost pure spherule culture (Fig. 1). The few remaining mycelial fragments usually degenerated and disappeared. When a spherule harvest was necessary, the precipitate was swept off the bottom with a hypodermic needle. Only a part of the precipitated spherules was harvested at any given time, to allow continued multiplication of the spherules.

Spherules are very sensitive to temperature change and dessication. A drop of a few degrees for any length of time, or drying of the spherules on the tube wall above the medium from too vigorous bubbling, resulted in germination and reversion to mycelium. If the germ tube had not germinated beyond a four- or five-cell stage, it quickly transformed into spherules (Fig. 2) upon washing down into the medium.

Selective pH studies at pH 5.0, 5.5, 6.0, and 6.5 were first carried out in the experimental medium with 0.1 M sodium phosphates. Spherules were maintained at all pH levels; pH 6.0 was selected as optimum from visual microscopic examinations. Spherules originally transformed from hyphae usually measured 15 to 35 μm. When the spherules were maintained in the continuous culture, a gradual decrease of spherule size was noted. Cleavage (Fig. 3) occurred in some spherules measuring only 5 μm.

Potassium phosphate buffer (0.1 M, pH 6.0) was substituted for sodium phosphate to reduce the sodium content of the medium. This substitution increased the size of spherules from 4 to 8 μm, with occasional spherules measuring 12 to 15 μm (Fig. 4).

**Discussion**

Our studies indicated that temperature is a critical factor for the survival of mycelium. Below 40 C, much of the hyphae remained viable and grew rapidly. At 40 C, mycelial growth became static and degeneration was noted. Yet, spherules survived. Slide-mount examination from a 2- or 3-month-old agar slant indicated that our inoculum was predominantly arthrospores and dead mycelium. Whereas the arthrospores germinated at lower temperatures, they transformed directly into spherules at 40 C. The few viable hyphae quickly degenerated. This confirms Converse’s (1956) observation that arthrospore inoculum transformed into spherules, without the intermediate mycelial stage, at that temperature.

However, temperature alone was not the only requirement for spherulation. The presence of metabolic or bubbled CO₂ (Lones and Peacock, 1960a; Bartnicki-Garcia and Nickerson, 1962a, b) was needed for transformation. This was confirmed when the arthrospores were inoculated into flasks and incubated at 40 C. Without CO₂, the inoculum germinated and the mycelium grew profusely, whereas the inoculum had transformed into spherules under increased CO₂ tension.

The CO₂ requirement for spherulation was further confirmed when spherules, subcultured into fresh medium without bubbled CO₂, germinated. Germination was eliminated when only a part of the old medium was removed and replenished with CO₂-bubbled fresh medium.

Converse and Besemer (1959) reported that addition of 0.005 M glutathione increased spherule sizes to 50 to 80 μm. Various concentrations of glutathione were added to our medium in an attempt to increase our spherule size, but without success. The obvious differences noted may be due to transformation of hyphae into spherules according to Converse’s observation and to continued asexual multiplication in our culture.

**Acknowledgments**

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