

IN VITRO PRODUCTION OF THE SPHERULE PHASE OF COCCIDIOIDES IMMITIS¹

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Received for publication January 17, 1955

The *in vitro* growth of the spherule (sporangium) phase of *Coccidioides immitis* has posed a problem of prime interest in the study of the disease coccidioidomycosis. The fungus is dimorphic in that it has a cultural, or saprophytic, phase in which growth consists of mycelium and arthrospores, and a tissue or parasitic phase consisting of thick-walled nonbudding spherules (sporangia) varying in size from 20 to 80 μ in diameter. Endospores measuring 2 to 5 μ in diameter are developed in the mature spherules by cleavage planes, released into the tissues by rupture of the spherule wall, and serve as a means of reproduction and dispersal. The freed endospores enlarge and develop into the characteristic endospore-containing spherules.

The physiological requirements for the production of the tissue phase are not known, although studies have been made concerning the developmental stages of spherules in the tissues of mice, along with their relation to host reaction (Tarbet, Wright, and Newcomer, 1952). *In vitro* production of the spherule phase has been achieved with some degree of success by several investigators by means of special media (Baker and Mrak, 1941; Burke, 1951; Burke, Salvin, and Gerhoff, 1952; Brueck and Buddingh, 1951; Conant and Vogel, 1954; Lack, 1938; MacNeal and Taylor, 1914; Moore, 1941; Newcomer, Wright, and Tamblyn, 1952; Vogel and Conant, 1952 *a, b*). These special media provided no freedom from the presence of the mycelial phase and resulted in the production of the vegetative phase accompanied by relatively few spherules.

¹ This paper was presented at the joint meeting of the Northern California and Hawaiian and Southern California Branches of The Society of American Bacteriologists at the Annual meeting of the A.A.A.S. at Berkeley, California, December 26, 1954.

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As a result of a study of the literature, indicating that the production of spherules is the result of conditions exerted by the host upon the invading organisms, it was decided that the controlled use of liquid tissue culture media could reproduce some of these conditions in the test tube. With this in mind, the following experimentation was undertaken to produce the tissue phase *in vitro*.

MATERIALS AND METHODS

The tissue culture medium was composed of the following: Sterile Tyrode's solution, 4 parts; sterile rooster serum (from a 7-9 pound young cockerel), 2 parts; chick embryo extract, 2 parts. The extract was made from 9 day old embryos pulverized in a Waring blender with the addition of 0.2 ml Tyrode's solution per embryo. This was then filtered through a Seitz filter.

Special culture tubes were prepared to allow an oxygen and carbon dioxide mixture to bubble through the media. These tubes, hereafter referred to as spherule tubes, were prepared as follows: A "U" tube extension, 8 mm in diameter, was attached to the bottom of a 22 by 160 mm pyrex test tube. This was plugged with a rubber serum cap to allow the aseptic introduction or extraction of liquids. The mouth of the spherule tube was closed by means of a two-holed rubber stopper containing a gas inlet tube and a gas outlet tube. The inlet tube extended the length of the spherule tube, and the outlet tube extended one inch into the spherule tube. Both gas tubes extended one inch above the stopper and were plugged with absorbent cotton. Two 20 liter glass bottles, one containing a mixture of CO₂ and O₂ and the other water, were arranged so that the gas mixture, displaced by measured amounts of water from the water bottle, was forced through rubber tubing into the inlet of the spherule tube and allowed to bubble through the media (figure 1). By means of Y and T connector tubes, a series of six spherule tubes

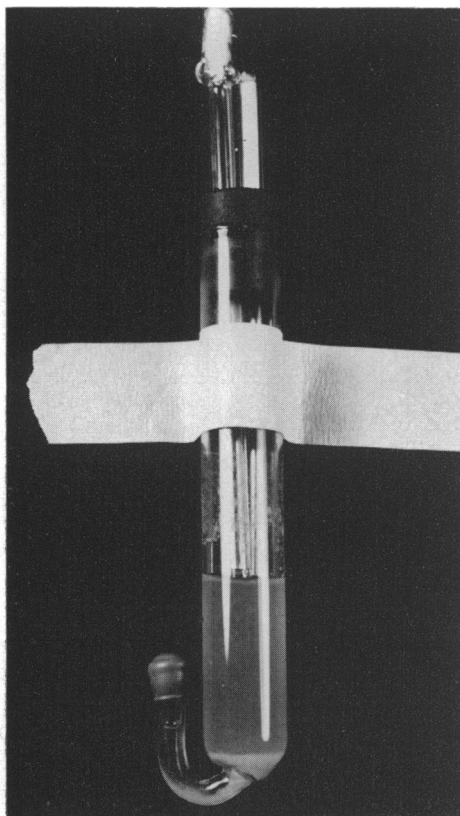


Figure 1. Spherule tube. Note spherule material at base.

could be used at any one time, with the gas flow being controlled by means of adjustments on Hosecock clamps. A wooden rack was used to hold the spherule tubes in an upright position in a 37 C incubator.

RESULTS

Experiment 1. To each of two spherule tubes, cleansed and sterilized preparatory to use, 8 ml of tissue culture medium were added, and the tubes then placed in a constant temperature chamber at 37 C and attached to the gas mixture inlet tubes. At the end of one hour, during which time the temperature of the media reached 37 C, 0.5 L of the gas mixture consisting of 76.7 per cent CO₂ and 23.3 per cent O₂ was allowed to bubble through the media. An inoculum consisting of arthrospores and mycelial fragments suspended in 0.75 ml of Tyrode's solution was introduced into each of the spherule tubes (figure 2). *C. immitis* strain Baker I, isolated from soil, was the source of the inoculum. At 6, 18, 24, 48,



Figure 2. *Coccidioides immitis* arthrospores and mycelia used in spherule experiment. 2,500 X.

60, 72, 84, and 96 hour intervals from the start of the experiment, 0.75 L of the gas mixture was allowed to bubble through the media in each of the spherule tubes during a one hour interval. Samples consisting of 0.25 ml were withdrawn from each tube prior to the addition of gas at the 18, 24, and 48 hour intervals. Seventy-two hours after the start of the experiment, a sample consisting of 2.25 ml was withdrawn from each of the spherule tubes and replaced by 3.0 ml of fresh tissue culture medium. The next 0.25 ml samples were withdrawn after 96 hours. The experiment was concluded at the end of 108 hours, and the contents of each tube were fixed by the addition of enough formalin to produce a 2 per cent formalin concentration.

Results: Samples taken at intervals from 18 through 72 hours show the arthrospores gradually rounding up and enlarging to form immature spherules without cleavage planes (figures 3 and 4). The samples taken at 72 hours showed some of the immature spherules beginning to germinate. At this point, fresh tissue culture media were added to each of the spherule tubes which arrested further germination and mycelial growth. The sample taken at 96 hours showed conclusive spherule formation with cleavage planes and endospores. Some of the spherules were in the process of bursting and releasing the endospores into the surrounding medium. Samples taken at the end of the experiment showed many stages of spherule production (figures 5, 6, and 7). At no time was there a

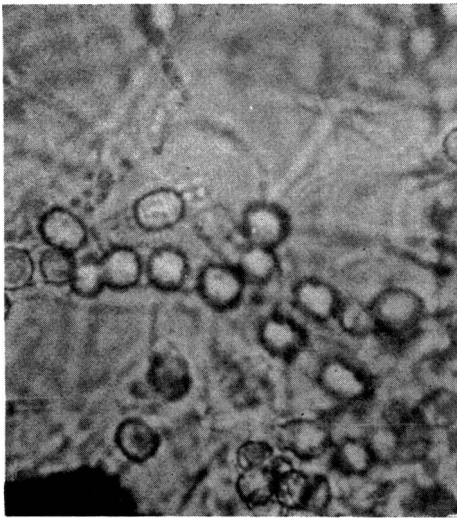


Figure 3. *Coccidioides immitis* arthrospores in process of forming spherules (18 hr). 3,000 X.

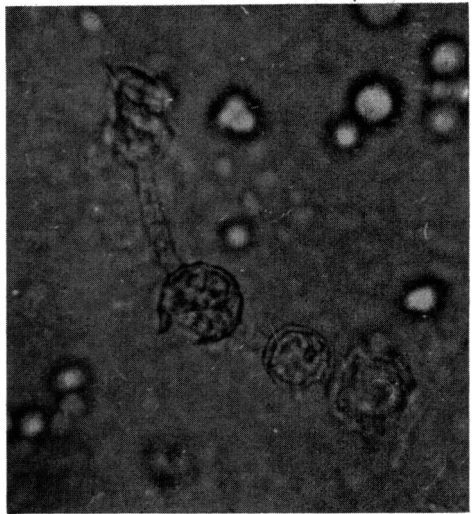


Figure 5. Spherules with maturing endospores. Note that the spherules are still in chains (48 hr). 1,500 X.

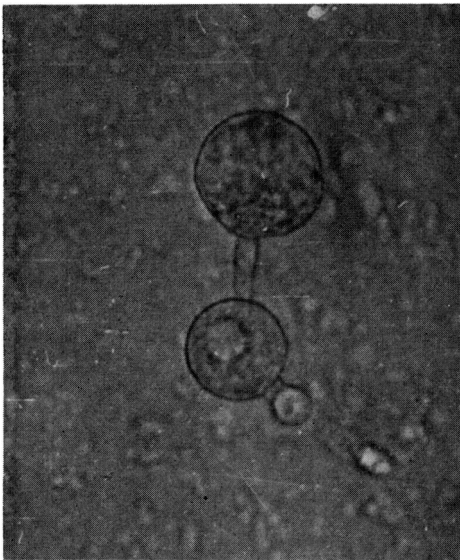


Figure 4. Immature spherules (24 hr). Note lower has hollow center indicating start of cleavage. Cell connector is still intact. 2,000 X.

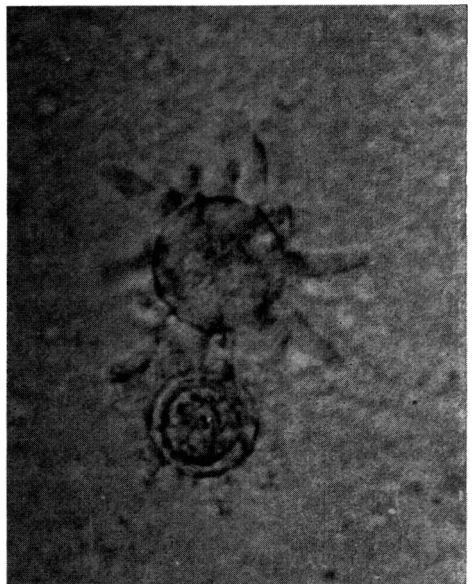


Figure 6. Spherules producing germ tubes (72 hr). 2,000 X.

mycelial mat produced in either of the spherule tubes, and the only mycelium seen was that which was produced at the period when the spherules started to germinate. The greatest amount of spherule growth appeared to be concentrated at the lowest portion of the spherule tube and in the U tube extension (figure 1). Sterility tests revealed no contamination.

Experiment 2. Since the preceding experiment

showed that it is possible for *C. immitis* to produce spherules *in vitro* in the aforementioned culture medium, the following experiment was devised to see if this could be repeated with a different CO₂ and O₂ content and also to see if sera other than that of roosters could be utilized.

The gas mixture was varied to increase the CO₂ content approximately 14 per cent and to reduce the O₂ content by approximately 55

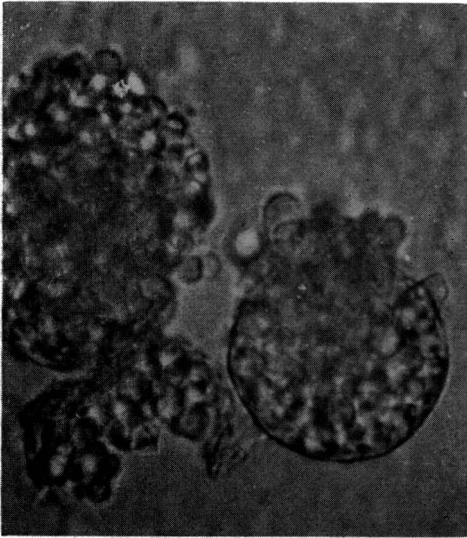


Figure 7. Endospore release by mature spherules (108 hr). 2,000 X.

per cent in comparison to that used in the preceding experiment.

Six spherule tubes, A to F inclusive, were used. Eight ml of the same type of tissue culture medium as used in the preceding experiment were added to tube A and to tube B. To tubes C and D, the following was added: horse serum (Difco), 2 ml; chick embryo extract, 2 ml; Tyrode's solution, 4 ml. To tubes E and F, the following was added: human cord serum (Difco), 2 ml; chick embryo extract, 2 ml; Tyrode's solution, 4 ml. Conditions and procedures for this experiment were the same except that 0.4 L of the gas mixture was bubbled through each tube each time instead of 0.75 L. The length of time of this experiment and the time and amount of sampling were the same as in the preceding experiment. At the end of 48 hours, each spherule tube was replenished with 3 ml of its original type of media. *C. immitis* strain Baker I, isolated from soil, was used as a source of arthrospores.

Results: Tubes A and B yielded a great profusion of spherules in all stages of growth, far more than that produced in the preceding experiment, and without any trace of germination or mycelia. Tubes C, D, E, and F showed spherule formation but not nearly as profuse as in the first experiment. No contamination was found to be present.

Experiment 3. Since the preceding experiments involved only one strain of *C. immitis*, this ex-

periment, repeating that part of experiment 2 in which rooster serum was used, was set up to test the following six strains: Baker II, isolated from soil, Taft IV, isolated from soil; TP 18, isolated from a pocket mouse (*Perognathus* sp.); KR 9, isolated from a kangaroo rat (*Dipodomys* sp.); TP 3, isolated from a pocket mouse (*Perognathus* sp.); GH I, isolated from a patient at General Hospital, Los Angeles County.

Results: The results of this experiment were similar to those obtained in tubes A and B in the preceding experiment.

DISCUSSION

The use of a tissue culture medium, under controlled conditions, appears to be a means for the *in vitro* production of spherules of *C. immitis*. It is necessary to replenish the medium after 48 hours in order to prevent the production of the vegetative phase, i.e., germination and the production of mycelia. The fact that replenishment is necessary seems to imply that there is either an exhaustion of nutrients or certain other factors contained in the medium which may be responsible for the organism's *in vivo* activities *in vitro*.

The use of the medium containing rooster serum was found to be more successful in comparison to horse serum or human cord serum. This may be due to the amount and kind of growth factors present in each of the sera which play an unknown part in activating *in vitro* spherule production. Also, the carbon dioxide-oxygen ratio has an important effect. The significant factor is unknown, but the reduction of oxygen seems to simulate tissue conditions to a greater extent. The effect of increased carbon dioxide on the organism has, as yet, not been investigated.

This method of abundant spherule production may be an aid in testing agents against the tissue phase of *C. immitis*, and also may be used in obtaining spherules for antigen production.

SUMMARY

A new and satisfactory method for the production of *Coccidoides immitis* spherules has been described. It was found that in order to prevent reversion to the vegetative phase of the life cycle, the medium had to be replenished after 48 hours.

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