Growth of Spherules of *Coccidioides immitis* in a Chemically Defined Liquid Medium. (22144)

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The dimorphic fungus, *Coccidioides immitis,* undergoes a completely different type of growth *in vivo* from that *in vitro*. The parasitic form, or spherule, appears in animal tissue as a non-budding, spherical structure 20-80 μ in diameter with a thick, refractile, double wall, and is filled with numerous small endospores 2-5 μ in diameter. Reproduction takes place by rupture of the spherule wall, freeing the endospores, which increase in size and develop into typical large endospore-filled spherules. The saprophytic form appears in laboratory media as a mycelium with branching, septate hyphae which break up into numerous thick-walled, rectangular, ellipsoidal, or spherical arthrospores 2-4 μ in diameter. The arthrospores germinate and repeat the cycle. Observation of the parasitic phase in solid or semi-solid complex medium has been reported by several authors. MacNeal and Taylor(1) observed multiplication of spherules in infected pus when added to ascitic fluid or gelatinized horse serum containing sterile kidney slices. Lack(2) noted development of spherules from chlamydospores inoculated into Hall tubes containing glucose broth and partially coagulated egg albumin. Baker and Mrak(3) observed the development of spherules in a variety of solid media after 45 to 60 days, when active growth had ceased due to drying of the medium. Conant and Vogel(4) observed spherules on Sabouraud's dextrose slants following exposure of the cultures to Tween 80 (polyoxyethylene (20) sorbitanmonooleate). Burke (5) reported the appearance of spherules usually after 4 to 10 weeks, but occasionally after 11 days in a partially defined solid medium containing coconut milk.

Experiments in this laboratory resulted in the production of spherules in a liquid synthetic medium, following the observation of the parasitic phase in cultures grown in the presence of lithium hydroxide and tamol "N" (an arryl sulfonic acid). The following presentation is devoted to a description of the medium, the development of the spherules, and the morphology of the cultures.

Methods and materials. 1. Inoculum used was a culture of *C. immitis,* strain M11, grown for 28 days at 34°C on a reciprocating shaking machine operating through a 4½ inch stroke at 96 strokes/minute. Growth medium for the inoculum was a glucose, acetate, inorganic salts medium, Basal #2, Roessler *et al.*(6) supplemented with 4 ppm Zn^{++} as $ZnSO_4$. The inoculum (0.1 ml) was approximately 10 x 10^6 viable arthrospores. 2. The experimental medium was a 5-fold dilution of the inoculum medium, containing .022 M glucose, .016 M ammonium acetate, .003 M each dipotassium and monopotassium phosphate, .0016 M magnesium sulfate, .0000124 M zinc sulfate and distilled water,

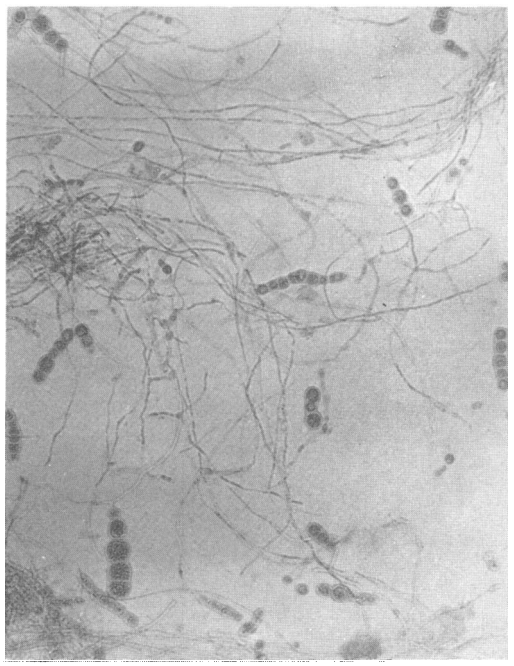

FIG. 1. Representative field of *C. immitis* culture showing growth of spherules, 200 ×.

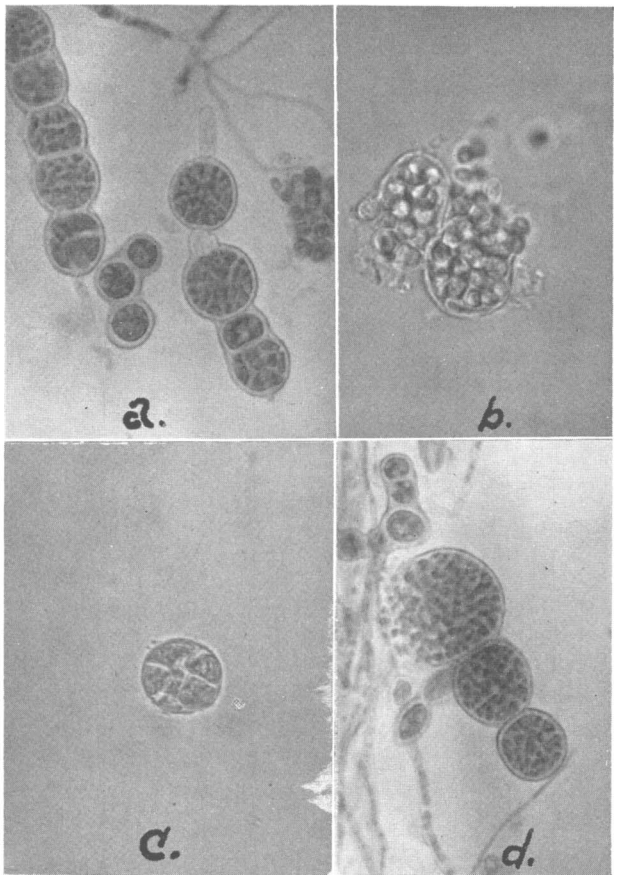

FIG. 2. (a) Spherules in various stages of development, 450 ×. (b) Two mature spherules with ruptured walls. Endospores are being extruded into the surrounding medium, 650 ×. (c) Young spherule showing developing cross-walls, 450 ×. (d) Chain of mature spherules. Note apparent rupture of wall of largest spherule and start of endospore release, 450 ×.

with final pH 6.6. This was dispensed into 250 ml Erlenmeyer flasks, 50 ml per flask, and autoclaved 15 minutes at 121.5°C. The flask closures were constructed from modified thistle tubes filled with gauze covered cotton and inserted through rubber stoppers. 3. Experimental cultures were incubated with shaking at 34°C and examined microscopically at 24-hour intervals. 4. *Evaluation.* Visual estimation of growth was made with unaided eye. Viable plate counts were made with a 1% peptone, 2% glucose, 0.1% autolysed yeast, 2% agar medium, with incubation at 34°C for 48 hours. Microscopic examinations were made on lactophenol-blue wet mounts. For photomicrographic purposes the edges of the cover slips on the wet mounts were sealed with Duco cement.

Results. (1) *Development of the parasitic form.* Spherules appeared after 72 hours incubation, following initial mycelial growth, with maximum production occurring between 96 and 120 hours. They occurred at terminal and intercalary locations of the hyphae similar to typical chlamydospore development on solid medium, and developed from chains of arthrospores, the typical spore-form of the *in vitro* type of growth in liquid medium. Upon continued incubation there appeared to be simultaneous germination of arthrospores into hyphae which produced both new arthrospores and spherules, and rupturing of spherule walls with the release of endospores which developed into new, mature, endospore-filled spherules. The parasitic form of growth appeared to comprise 50 per cent or more of the

total growth of the cultures.

(2) *Spherule morphology.* The spherules were morphologically similar to those occurring in animal tissue. They occurred as spherical or ovoid structures, 3 to 80 μ in diameter with double refractile walls, and contained varying numbers of endospores, depending on the size and stage of development of the spherules. The diameter of the endospores varied from 1 to 5 μ with the majority appearing to be one-tenth to one-fifth the volume of mature arthrospores which are normally 3 to 5 microns in diameter. The accompanying figures show representative fields of view and detailed morphology of a typical culture of *C. immitis* grown under the conditions described.

(3) *Other strains.* Although the majority of this work was accomplished with *C. immitis,* strain M11, a rodent isolate from Arizona contributed by Dr. C. E. Smith of Berkeley, Calif., 38 other strains isolated from humans and animals have been tested under identical conditions. Sixty per cent of these developed spherules in the experimental medium, but to a lesser extent than strain M11.

4. *Growth measurements.* The total growth of spherule cultures (15 x 10^6 per ml) in 5 fold diluted medium, as measured by viable plate counts, was approximately one-tenth that of arthrospore cultures (130 x 10^6 per ml) grown in the inoculum medium.

Discussion. Conditions for the growth and maintenance of the parasitic form of *C. immitis* in a chemically defined liquid medium are not yet fully understood although physical, chemical, and nutritional studies should throw further light on this subject. There seems to be a critical stage during the early development of the cultures at which time the environment exerts its greatest effect in stimulating production of spherules. Harvesting the spherules must be done not later than the fourth or fifth day of incubation as a gradual decrease in numbers takes place thereafter, accompanied by a slow reversion of the cultures to the saprophytic phase. A possible explanation might be a selective change of environment involving the exhaustion of nutritional factors or the accumulation of metabolic products inhibiting growth of the parasitic form.

The use of a chemically defined liquid medium for production of spherules would materially benefit physical, chemical, and metabolic studies of the parasitic phase of the organism. It would also permit large scale production of antigenic material, free from foreign protein matter.

Summary. A method for growth of the parasitic phase (spherules) of *Coccidioides immitis* in a chemically defined liquid medium is described. A description of the development and morphology of the structures is included.

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