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Pure Spherules of *Coccidioides immitis* in Continuous Culture

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Investigation of host-parasite relationships involving the parasitic form of *Coccidioides immitis* has been difficult because, previously, spherules and endospores have not been grown continuously in tissue culture medium without detectable formation of hyphae. Arthroconidia were harvested from mycelial cultures and inoculated into tissue culture flasks which contained RPMI 1640 medium supplemented with 10% calf serum and N-Tamol (Rohm & Haas Co., Philadelphia, Pa.). Flasks were purged with 5% CO_2, sealed, and placed on a reciprocating shaker at 35°C. Hyphae which arose during incubation were removed by filtration. Arthroconidia readily converted to the spherule-endospore form within 12 days. Six days after complete conversion, spherules and endospores were transferred to RPMI 1640 without N-Tamol. The spherule-endospore cycle was maintained in tissue culture medium for 84 days without the formation of detectable hyphae.

Coccidioides immitis is a dimorphic fungus which grows as a filamentous saprophyte in the soil of the semiarid regions of North and South America. Infectious arthroconidia become airborne and are inhaled into the lungs where they enlarge to become thick-walled spherules containing endospores. Endospores are released and mature into new spherules.

The mold form of *C. immitis* can be grown on a variety of nutrient agars; however, the parasitic form is more difficult to culture in vitro because endospores readily germinate to form new hyphae. Initially, many complex media (3, 10, 14) were used to convert and propagate spherules in vitro. Although some hyphae persisted, low yields of spherules and endospores could be cultured by these in vitro techniques. These techniques were modified, resulting in better spherule and endospore yields (4–8, 15). The most successful medium for the continuous growth of spherules is modified Converse medium which is a mixture of salts, glucose, and the detergent N-Tamol (Rohm & Haas Co., Philadelphia, Pa.). Spherule-endospore cultures have been grown over long periods in modified or unmodified Converse medium (2, 15); unfortunately, the medium is not suitable for mammalian cells, and cultures routinely contain some hyphae. Teramura (M.S. thesis, University of Health Sciences/The Chicago Medical School, Chicago, Ill., 1981) demonstrated the conversion of arthroconidia to spherules in the presence of spleen cells in RPMI 1640 medium; however, these endospores germinate and produce hyphae.

In this communication, we report our success in maintaining the spherules and endospores in tissue culture medium without significant hyphal growth. Our ability to maintain spherules for several months in medium that will readily support the growth of mammalian cells should provide an important model system for studying the host-parasite relationship.

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MATERIALS AND METHODS

Fungal isolates. Two strains of *C. immitis* were tested. The El Paso strain was originally isolated from a patient at William Beaumont Army Medical Center, El Paso, Tex. CMS-1 was isolated from a patient in Palos Heights, Ill., and was provided by James Bolan of Palos Community Hospital, Palos Heights, Ill. Both isolates were grown on Sabouraud dextrose agar in 50-cm^2 screw-cap culture bottles at 25°C.

Isolation of arthroconidia. To encourage arthroconidia formation, cultures were left for 4 to 8 weeks, and the agar was allowed to dehydrate. Arthroconidia were harvested by the spin bar technique of Huppert et al. (9). Briefly, 15 ml of sterile phosphate-buffered saline (PBS) was added to each bottle to wet the mycelial mat. Arthroconidia were released into suspension by agitating the PBS with a sterile magnetic stir bar. After 30 min, the suspensions of arthroconidia and hyphal elements were filtered through four layers of sterile gauze to remove the hyphal elements. The filtrates were washed three times with PBS by centrifuging for 5 min at 800 × *g*. Cell numbers were determined by fixing portions of the cell suspensions in 10% glutaraldehyde and counting individual cells with a hemacytometer. Cell counts were performed immediately before and directly after gauze-filtration steps. Arthroconidia were suspended at a final concentration of 2×10^7 cells per ml in PBS and were sealed in sterile 30-ml vaccine bottles containing glass beads. Arthroconidia were routinely discarded after 3 months of storage at 4°C.

Medium. Arthroconidia were converted to spherules by using RPMI 1640 medium supplemented with 2.835 μg of $Na_2PO_4 \cdot 7\ H_2O$ per liter, 10% heat-inactivated calf serum (Hyclone, Logan, Utah), 0.8 mg of N-Tamol per ml, 40 U of penicillin G per ml, and 40 μg of streptomycin (GIBCO Laboratories, Grand Island, N.Y.) per ml.

Cultivation of spherules and endospores. Arthroconidia (2×10^7) were inoculated into 25-cm^2 air-tight tissue culture flasks (Corning Glass Works, Elmira, N.Y.) containing 10 ml of medium. The flasks were incubated with caps vented for 24 h at 37°C in a humidified 5% CO_2 atmosphere. The caps were then tightened, and the flasks were incubated at 35°C for an additional 24 h on a reciprocating shaker. The speed of the shaker was adjusted so that there was optimal agitation of the cells with minimal tide-line formation. At 48-h inter-

vals, hyphal elements were removed by filtering the medium through four layers of sterile PBS-wetted gauze. The filtrates which contained spherules and endospores were centrifuged at 255 × *g* for 5 min. Spherules and endospores were resuspended at a concentration of 2×10^5 cells per ml in fresh growth medium. Flasks were purged with 5% CO_2, capped tightly, and returned to the shaker for incubation for 48 h, after which the procedure described above was repeated.

RESULTS

After inoculation of arthroconidia, the flasks were incubated at 37°C in a 5% CO_2 atmosphere for 24 h, at which time we observed rounding and swelling of the arthroconidia. Flasks were then sealed and incubated on a reciprocating shaker for 24 h at 35°C. Examination of the cultures at this time indicated that about 9% of the arthroconidia had converted to spherules. Gauze filtration effectively removed the hyphal fragments. Continued incubation with filtration every 2 days for four to six passages was effective in eliminating hyphal elements and allowing the continuous reproduction of spherules and endospores (Fig. 1).

Although cell counts performed before filtration failed to detect hyphae in the established cultures, we continued to filter cultures every 2 days to maintain morphologically pure cultures. However, filtration may not be necessary after pure cultures have been established. Cells were maintained as spherules and endospores for 84 days, at which time the cultures were terminated (Fig. 2). During continuous passage, cells grew asynchronously, increasing in number from 4 to 14 times in 48 h. Spherules normally ranged in size from 25 to 35 μm; however, spherules as large as 62 μm were observed on occasion (Fig. 3). Spherule size was not dependent upon the presence or absence of N-Tamol.

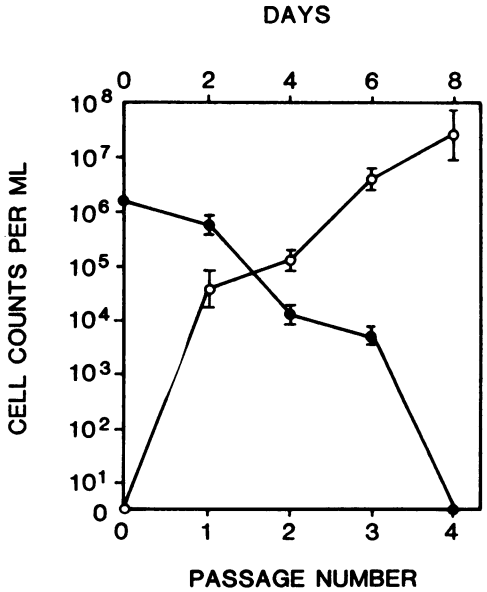

FIG. 1. Elimination of hyphal elements (●) and reproduction of spherules and endospores (○) in RPMI 1640 with 10% calf serum and N-Tamol. These data are representative of three experiments. Values are means ± standard deviations of triplicate cultures with the CMS-1 strain of *C. immitis*. Comparable results were obtained with the El Paso strain.

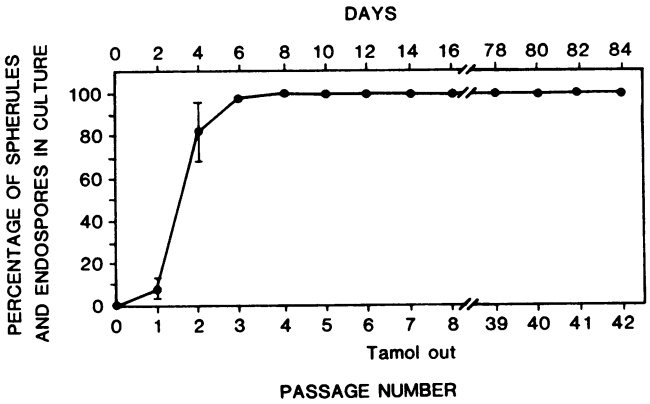

FIG. 2. Spherules of *C. immitis* can be maintained in long-term culture. Absence of hyphal elements in cultures was established by microscopic evaluation over an 84-day period. N-Tamol was removed from the culture medium at passage 7. These data are representative of three experiments. Values are means of triplicate cultures ± standard deviations, with the El Paso strain. Comparable results were obtained with the CMS-1 strain.

Early studies were performed with RPMI 1640 without N-Tamol. Conversion in the absence of N-Tamol was much slower, and pure cultures were never established. When N-Tamol was included initially, rapid conversion was apparent, and N-Tamol could be removed after the culture was established. Cell densities of 2×10^6 cells per ml or greater must be consistently maintained in the absence of N-Tamol to ensure maintenance of a pure spherule culture.

DISCUSSION

Growth of hyphae-free spherules and endospores under moderate conditions which could support the growth of mammalian cells has not been previously reported. Our goal was to culture *C. immitis* in a medium that could be used to

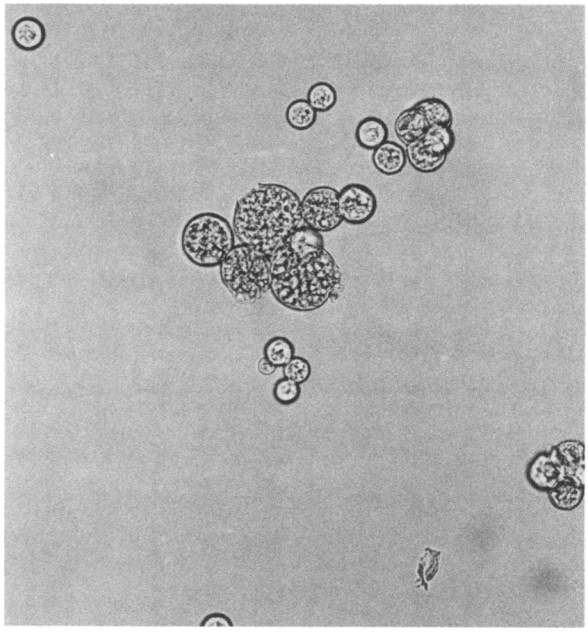

FIG. 3. Spherules of *C. immitis* El Paso in 10% glutaraldehyde. Magnification, ×350.

grow and maintain lymphocytes. We showed that spherules can be grown in RPMI 1640 supplemented with 10% calf serum at 37°C with an initial 5% CO_2 atmosphere and can be maintained in long-term cultivation. To facilitate conversion and establish hyphae-free isolates, cultures were filtered at 48-h intervals, and 0.8 mg of N-Tamol was added per ml; however, once the cultures were established, N-Tamol was no longer necessary. The spherules produced in this culture were within the expected in vivo size range (6). The culture conditions used for maintaining cultures of spherules will also support the growth of lymphocytes, thus permitting the investigation of cellular interactions between the fungus and the immune system.

Numerous studies on the in vitro cultivation of spherules have been reported. In general, other investigators have observed that spherules can be grown for limited periods in tissue culture medium with mammalian cells (1; K. Teramura, M.S. thesis); however, in the absence of feeder cells, endospores germinate to produce hyphae which rapidly overgrow the culture. Successful long-term cultivation of spherules has been obtained in media that are not conducive for growth of mammalian cells. The best-known medium is that formulated by Converse (7). This medium has been used to demonstrate conversion of the mycelial form to the spherule form of *C. immitis* in vitro. Converse (7) demonstrated that N-Tamol greatly enhanced conversion and endosporulation. Levine et al. (11, 12) indicated that total conversion could not be achieved with a modified Converse medium. Although modified Converse medium is preferential for growing large numbers of spherules, it is unsuitable for the cocultivation of mammalian cells. The medium used in our studies was tissue culture medium supplemented with serum and N-Tamol. N-Tamol is apparently required during the conversion of arthroconidia to spherules and possibly for several transfers thereafter. After that time, N-Tamol did not have to be included in the medium for the successful maintenance of the spherule-endospore cycle. Cells from an established culture are free from N-Tamol and can be used without concern for the effects of residual detergent on observed interactions.

Other investigators have obtained spherule production by increasing the incubation temperature or the CO_2 concentration. Lubarsky and Plunkett (13) demonstrated that increased CO_2 aided production of spherules in a complex chicken embryo extract system, and the method of Breslau and Kubota (2) required 20% CO_2. Converse (6) and Breslau and Kubota (2) obtained conversion with the degeneration of hyphae when the temperature was raised to 40°C. A temperature drop from 40 to 37°C resulted in germination of endospores and reversion to the hyphal form. Conversion in our system required an initial purging with 5% CO_2, which is compatible with conditions required in tissue culture systems. Sequential filtration of hyphal elements during early conversion selected for fungal cells which will remain in the parasitic cycle when maintained at 2×10^6 cells per ml or greater at 35 to 37°C.

Pure cultures of *C. immitis* spherules and endospores can be maintained over a long period in RPMI 1640 medium in 5% CO_2 at 37°C. We are currently investigating the effects of natural killer cells on spherules generated with our culture conditions, and we will be evaluating the stability of *C. immitis* spherules in the presence of lymphoid cells. This system should also be of value for studying interactions of the fungus and other cells of the immune system.

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