Conversion and Maintenance of *Histoplasma capsulatum* in Tissue Culture. (22838)

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The conversion of the diphasic fungus, *Histoplasma capsulatum,* to its tissue phase, has always been difficult, in our experience. However, many artificial media, such as a modification of Francis' cystine glucose blood agar (1), brain-heart infusion glucose blood agar and Salvin's synthetic medium(2), have been successfully used by many investigators. In recent years Kurung's egg(3), and Littman's liver-spleen media(4) were developed more or less specifically for the conversion and maintenance of *H. capsulatum* in its yeast phase. In addition to the artificial media Randall and his co-workers have advocated the use of tissue culture methods for the study of this fungus. They were successful in the *in vitro* cultivation of the tissue phase in media containing horse tissue, chicken fibroblast-like cells and the Earl's strain "L" mouse cells (5-7).

In preparation for studies on efficacy of antifungal agents it became evident that a more suitable, rapid and consistent method of obtaining the tissue phase of *H. capsulatum* had to be found. Therefore, the HeLa tissue culture method was explored in an effort to determine the efficiency of another animal material in which to study the fungus. Studies were made of the conversion and maintenance of the fungus with and without HeLa cells and its maintenance with and without rotation of the culture drum.

Methods and materials. The 13 isolates of *H. capsulatum* used in these studies were obtained from recent human cases. They were isolated from clinical specimens shipped to this laboratory. HeLa cells were cultivated in 250 ml bottles employing a medium composed of 20% human serum, 8% of a 1% yeast extract, 1% of a 20% glucose solution, 68% Hank's balanced salt solution (B.S.S.), 3% of a solution of 2.8% sodium bicarbonate and 0.001% phenol red. One hundred units of penicillin, 100 μg of streptomycin, and 0.2 μg of butyl parahydroxybenzoate were added to each ml of medium. The cells were trypsinized and stock solution of HeLa cells was made in HeLa maintenance medium composed of 63.5% HeLa mixture 199, 3% of a 5% sodium bicarbonate solution, 1% of a 20% solution of glucose, 25% tryptose phosphate broth and 7.5% calf serum. (Final pH of this medium was 7.4.*) No antibiotics were added. The cultures were initiated in 15 by 125 mm test tubes having bakelite caps and neoprene liners. Approximately 50,000 cells were suspended in each 1 ml of maintenance medium. Tubes were incubated at 37°C for 72 hours at a slant of 5°. After microscopic examinations were made of the tubes, usable ones were washed twice with phosphate buffered saline and replenished with 1 ml of maintenance medium. In attempting conversion of fungus to tissue phase a small amount of mycelium of each isolate was transferred to 1 ml of HeLa maintenance medium containing HeLa cells. These tubes were incubated, in a slanting position, at 37°C in the roller drum for 7 days. Microscopic examination was made at weekly intervals of growth, to determine degree of conversion. Then 0.1 ml of the culture was transferred, using sterile pipette, to a fresh tube of HeLa maintenance medium containing HeLa cells. This procedure was followed at the end of each week until isolates were completely converted to the yeast phase. The above procedure was followed in another experiment with the exception that the maintenance medium did not contain HeLa cells. It was of interest to learn whether HeLa cells were necessary to maintain *H. capsulatum* in its yeast phase. Accordingly, a series of tubes containing 1 ml of HeLa maintenance medium with HeLa cells and another series without HeLa cells were inoculated with a standard inoculum of yeast cells. The tubes were then placed in the roller drum and periodic read-

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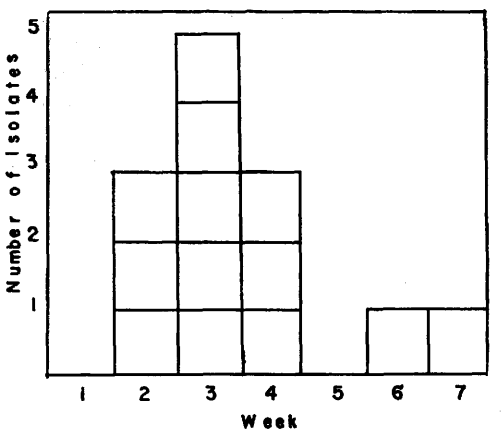

FIG. 1. Conversion of *Histoplasma capsulatum* to the yeast phase in the presence of HeLa cells. Illustrated are the No. of weeks required to convert 13 isolates in tissue culture medium.

ings made to determine amount and rate of growth. In addition to above experiments a study was made to determine the conditions necessary for propagation and maintenance of large numbers of yeast cells. Twenty ml of HeLa maintenance medium were dispensed into a series of sterile 100 ml centrifuge bottles with neoprene stoppers. Five isolates of the fungus previously converted to their yeast phase were selected. Five ml of a standard yeast inoculum were added to duplicate centrifuge bottles of each isolate. One set was placed in the roller drum; the other set remained stationary at 37°C. Daily readings were made to determine rate of growth and hydrogen-ion change of medium. Direct macroscopical observations of bottles were made to determine if rotation was necessary for maximum growth and maintenance of fungus in its yeast phase.

Results. Conversion to yeast phase with HeLa cells: In Fig. 1, the results of the conversion studies are presented. From these results it can be seen that there are differences in rate of conversion in HeLa maintenance medium from the original mycelial phase. At the end of 2 weeks incubation in roller drum, 3 isolates had completely converted to the tissue phase. Examination of several microscopical preparations from these tubes failed to reveal any fragments of mycelium. The greatest number of isolates (five) converted by the end of 3 weeks. Three additional isolates completely converted by the end of four weeks. The twelfth isolate converted by 6 weeks and the thirteenth reached complete conversion by the end of seventh week.

Conversion studies in absence of HeLa cells: Results of attempts at conversion to the yeast phase without HeLa cells are shown in Fig. 2. Six of the 13 isolates successfully converted completely to the yeast phase in the medium without HeLa cells. The first complete transition, 3 isolates, occurred by the end of fourth week. Four additional isolates converted, 2 in the fifth and 2 in sixth week of incubation. Six isolates failed to convert and very few, if any, yeast cells were seen in the microscopical preparation at the end of seventh week, when experiment was terminated.

Maintenance and propagation of yeast phase after conversion in presence and absence of HeLa cells: When the fungus has been converted completely to the yeast phase, HeLa cells are not absolutely necessary for propagation and maintenance of the culture. They do, however, enhance the rate of growth. In 4 of the 5 isolates studied, maximum growth occurred on the fourth day of incubation in medium containing HeLa cells. The

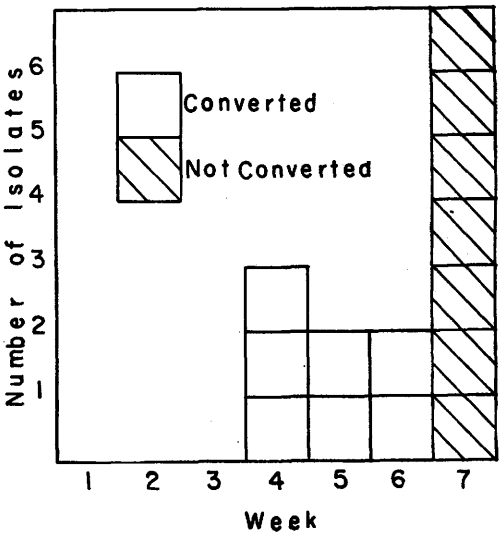

FIG. 2. Conversion of *Histoplasma capsulatum* to the yeast phase without HeLa cells. Illustrated are the weeks of transition for the 7 isolates which converted completely. Six isolates failed to convert by the end of 7 wk.

fifth isolate reached its maximum growth on sixth day.

In the medium without HeLa cells the same 5 isolates required 8 or 9 days to obtain comparable growth as reached in medium containing HeLa cells.

Determination of increase in numbers of cells by volumetric measurements showed an average increase of 30-fold. There was little difference between tubes containing HeLa cells and those without HeLa cells in the medium. In the small test tubes containing 1 ml of medium without HeLa cells there was an indication of reversion, in that a small amount of mycelium was found in 2 of the 5 isolates. During later studies in which large centrifuge bottles containing 20 ml of medium were used, no mycelium was detected in any cultures of the 5 isolates through 10 series of propagations.

Maintenance after conversion with and without rotation: The ultimate use of propagated yeast was to be in antifungal evaluation studies, which required large quantities of yeast cells. The amounts of yeast in small tubes were inadequate for experimentation. The successful conversion and maintenance of the yeast phase in medium without HeLa cells led to studies using 20 ml of medium in 100 ml centrifuge bottles with neoprene stoppers. This was the maximum amount of medium plus the quantity of inoculum that could be used in the slanting position of the roller drum.

The same 5 isolates showed a marked difference in rate of growth in the 100 ml centrifuge bottles when subjected to rotation or held stationary. Four of the 5 isolates reached maximum growth on fourth day of rotation, the fifth isolate on the fifth day. None of the bottles maintained in stationary position in the same incubator reached a comparable growth at end of 10 days.

Discussion. In our experience the tissue culture method has proved very satisfactory for conversion and maintenance of *H. capsulatum* in the yeast phase. It is apparent from Figs. 1 and 2 that presence of HeLa cells in the medium has a marked influence upon rate and final outcome of the yeast phase transition. The ease with which 13 isolates of fungus converted to the tissue phase suggests that the method is rapid and reproducible. Large quantities of cells can be produced by this technic. It has the advantage that the yeast phase can be readily propagated in a liquid medium by weekly transfers. After acclimation to a liquid menstruum and using the roller drum method, these cells can be easily incorporated in antifungal studies.

In addition to *H. capsulatum* this method has proved satisfactory in preliminary studies, for conversion and maintenance of *Blastomyces dermatitidis, Sporotrichum schenckii* and *Coccidioides immitis*. The last named fungus was most difficult to convert to the tissue phase. Data available at this time suggest that the ratio of medium to inoculum is critical. Too much mycelium tends to prevent formation of large numbers of spherules. It is of significance that copious mycelium developed at 37°C when small pieces of infected liver or spleen tissues from mice were inoculated into HeLa maintenance medium. Very few spherules were observed in the first transfer from infected tissue. The ease of mycelium production at blood temperature in a defined liquid medium may be correlated with the frequent reports of mycelium in lungs infected with *C. immitis*.

Candida albicans and *Cryptococcus neoformans* were easily propagated in tissue medium without HeLa cells.

The results of these investigations stimulated further use of tissue culture methods in evaluating antifungal agents against systemic fungi. The technic lends itself very well to evaluation of the effects of the antifungal agent on HeLa cells and tissue phase of the fungus.

It is recognized that tissue culture method is expensive and requires that personnel be proficient in the technic. However, the widespread adoption of the method in virology and other studies has made this laboratory tool available in most research institutions.

Conclusions. 1. Tissue culture method can be successfully used to convert *Histoplasma capsulatum* to the tissue phase. 2. Six of 13 isolations did not convert to the tissue phase in absence of HeLa cells; with all isolates conversion was more complete and rapid in

presence of cells. 3. The yeast phase can be propagated and maintained in tissue culture mediums without HeLa cells in 100 ml centrifuge bottles. 4. Rotation of cultures is necessary to assure maximum growth of yeast cells in tissue culture medium. 5. The technic can be successfully used to convert other diphasic systemic fungi, *Blastomyces dermatitidis, Sporotrichum schenckii,* and *Coccidioides immitis* to their tissue phase. 6. *Candida albicans* and *Cryptococcus neoformans* can be propagated in tissue culture medium without HeLa cells.

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Effect of *Rauwolfia serpentina* on Thyroid and Pituitary Glands of Young Rats.* (22839)

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The active components of *Rauwolfia serpentina* are held to exert their effects by way of the central nervous system, and it is believed that their influence on the endocrine glands is of minor consequence (Gaunt *et al.* 1). However, in the present study the prolonged administration of a preparation of the whole root to a group of young rats resulted in a significant diminution of the weights of both the pituitary and thyroid glands.

Procedure. The rats employed were of the Sprague-Dawley strain, females, and 21 days of age at the beginning of the experiment. Twenty were treated and 16 littermates remained uninjected as controls. The *Rauwolfia serpentina* was given in aqueous solution by subcutaneous injection and at a daily dosage equivalent to 1 mg per kilo body weight. A fresh solution was prepared and adjusted once weekly to the weight of the animals. The injections were continued until the animals were from 65 to 70 days old when they were sacrificed with illuminating gas. At autopsy the body weight and the weights of the hypophysis, thyroid, adrenals, and ovaries were obtained.

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Results. The rats remained active and in good condition throughout the period of treatment, although the injected animals ate somewhat smaller quantities of food and eventually their body weight was generally less than that of the controls. The day of vaginal opening, indicating the onset of complete ovarian function, occurred from the 43rd to the 52nd day of age in the experimental group as compared to the 41st to the 46th day in the controls.

The organ and body weights of the individual rats found at autopsy are given in Table I, while Fig. 1 presents the data for the thyroid and pituitary weights arranged on a semi-logarithmic scale. The oblique lines (broken lines for the controls; continuous for the experimental) represent the average log-organ to body weight relationship as estimated by the method of least squares. The vertical distance between the two lines in each case offers comparison between the organ weights with allowance for body weight. For the pituitary gland this vertical difference is .0338 (in units of logarithms) which corresponds to an 8% smaller size (after adjustment for body weight) in the experimental than in the control group. This dif-