CULTURAL STUDIES ON THE YEASTLIKE PHASE OF *HISTOPLASMA CAPSULATUM* DARLING

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DeMonbreun (1934) and Hansmann and Schenken (1934) were the first to report the growth in artificial culture of the etiological agent of histoplasmosis, *Histoplasma capsulatum* Darling. DeMonbreun was notably successful in culturing both growth phases of the fungus: the mycelial phase, on glucose agar at room temperature; and the yeastlike phase, on 10 per cent rabbit blood agar in sealed tubes at 37 C. This yeastlike phase, which appears in the parasitic condition in the human or animal host, is obtained in culture by inoculation of the culture medium directly from infected animals, since, with the exceptions of the strains studied by Ciferri and Redaelli (1934) and by Conant (1941), conversion of the mycelial to the yeastlike phase has not been demonstrated as occurring outside the animal body. Once the yeastlike phase is obtained, however, it may be maintained in most strains by repeated transfer every 5 to 14 days on 10 to 20 per cent blood agar at 37 C.

As described by DeMonbreun (1934), Conant (1941), and others, the colony of the yeastlike form on blood agar slants is white, moist, and of pasty consistency. The individual organism is a spherical or oval cell, 1.5 to 3 by 2 to 3.5 microns in diameter, with a thin, enveloping capsule. Reproduction occurs by the formation of a single bud at the more pointed end of the cell.

A few studies have been conducted on the cultural characteristics of the mycelial form, but very little has been done on the yeastlike phase. Moore (1935) compared the mycelial growth of two strains on about 15 different types of agar or broth. Howell (1940, 1941) studied the growth rates of the mycelium under varying temperatures and hydrogen ion concentrations. DeMonbreun, on the other hand, conducted some cultural studies on the yeastlike phase. Having originally isolated this latter phase from a mycelium and yeast cell mixture at the bottom of flasks of infusion broth (pH 7.2), he was successful in growing the oval cells on several types of solid media at 37 C, namely, on whole blood agar, fresh serum agar, Loeffler's blood serum, and Petragnani's medium (without malachite green). The maintenance of a proper degree of moisture and of a temperature of 37 C was considered of utmost importance. The nature of the culture medium also played an important role in determining the character of the growth, for when the yeastlike phase was inoculated onto glucose agar, Sabouraud's agar, potato, gelatin, or bread, notwithstanding the degree of moisture or temperature, the resulting growth was mycelial. Although attempts were made to culture the yeastlike phase in liquid media at 37 C, such as in infusion broth, serum, or infusion broth with 1 per cent serum, consistent growth without the development of mycelia was not obtained.

The primary stimulus to the following experiments was a need for a method whereby large numbers of the yeastlike cells of *H. capsulatum* could be easily obtained. A fluid medium in which the yeastlike phase would multiply rapidly and extensively and not revert to the mycelial form would present distinct advantages over blood agar slants. Accordingly, investigations were initiated to determine more fully the cultural requirements and characteristics of the yeastlike phase, and thereby to develop a liquid medium for its maximum growth.

MATERIALS AND METHODS

The 19 strains of *H. capsulatum* used were all obtained from the collection maintained in this laboratory. Saline suspensions of 3-to-4-month-old Sabouraud's agar cultures of mycelium and conidia were injected intraperitoneally into mice. After 2 to 5 weeks the mice were killed and the yeastlike phase of all but two of the strains was recovered on 20 per cent rabbit blood agar slants from the liver, spleen, or kidney. This phase was maintained at 37 C on 20 per cent rabbit blood agar slants in tubes which had been sealed to prevent drying of the agar. The cultures were transferred once a week, although every 2 to 3 weeks proved satisfactory for the maintenance of the yeastlike phase. In the following investigations, the fungus was grown at 37 C in 10 ml of the semisolid medium (to be described) in a 25-by-150-mm tube, which in most cases was sealed with paraffin to prevent evaporation.

EXPERIMENTS AND RESULTS

Since the yeastlike phase was known to exist, and possibly grow, in the blood of man and animals, and since blood and blood serum had been shown by DeMonbreun to maintain the yeastlike phase, a strain of *H. capsulatum* (6521) was inoculated into several liquid media in which sheep serum or plasma was the basic substance. Although cultures in each of these media were incubated at 37 C for 3 weeks under different oxygen tensions, no marked growth of the yeastlike phase was apparent.

Many other variations of the medium were tried, but in all there appeared very little, if any, growth of the yeastlike phase. However, when the viscosity of the medium was increased by the addition of 0.175 per cent Difco agar, the yeastlike phase grew most abundantly and was nearly free of mycelium. The term "nearly free" is used since examination of several slides containing the yeastlike phase would reveal only an occasional abortive hypha. The medium on which this profuse growth appeared had the following composition (Difco products): proteose peptone, 10 g; neopeptone, 3.25 g; tryptone, 3.25 g; glucose, 2.0 g; sodium chloride, 5.0 g; disodium phosphate, 2.5 g; agar, 1.75 g; and distilled water to make 1,000 ml. This medium was the basic one used in most of the ensuing experiments and will be referred to hereafter as "YP medium."

Hydrogen ion concentration. The YP medium was dispensed in 10 ml amounts in 25-by-150-mm culture tubes, and duplicate tubes were adjusted to pH readings of 3.9, 4.3, 4.85, 5.4, 5.85, 6.3, 6.9, 7.05, 7.3, 7.5, 7.7, 8.1, 8.6, and 9.6. The tubes

were then inoculated with the yeastlike phase of strain 6521 and incubated at 37 C for 3 weeks.

The results showed maximum growth of the yeastlike phase at pH's between 6.3 and 8.1. In these tubes, as well as those in which less growth occurred, almost no mycelium was found. The experiment was repeated with two other strains, and similar results were obtained.

Temperature. In order to determine the optimum temperature for growth of the yeastlike phase, 7 strains of *H. capsulatum* were grown at 4 temperatures, 25, 31, 37, and 43 C, in YP medium. The inoculum in all cases was the yeastlike phase.

At 25 C and at 31 C the resultant growth was mycelial in character, whereas at 37 C the yeastlike phase grew extensively with no mycelium evident. At 43 C, with the exception of one strain which showed extensive growth, the yeastlike phase grew much less abundantly than at 37 C, although again no hyphae appeared. Accordingly, of the temperatures studied the best growth of the yeastlike phase occurred at 37 C.

FIG. 1. GROWTH OF THE YEASTLIKE PHASE OF STRAIN 6521 OF *H. CAPSULATUM* AT pH 7.3 IN YP MEDIUM IN AGAR PERCENTAGES OF 0.0, 0.005, 0.01, 0.05, 0.1, 0.2, 0.3, 0.4, AND 0.5, (FROM LEFT TO RIGHT)

Viscosity. In tubes containing 10 ml of the basic YP medium, the percentage of agar was varied from 0.0 to 0.5 as follows: 0.0, 0.005, 0.01, 0.05, 0.1, 0.2, 0.3, 0.4, and 0.5 per cent. Each of these percentages was inoculated in duplicate with the yeastlike phase of strain 6521 and incubated at 37 C.

After 3 weeks' incubation examination of the cultures revealed marked development of the yeastlike phase in the tubes containing an agar percentage of 0.1 and higher. Virtually no growth was discernible in the tubes with agar percentages of 0.05 and less. The most cells developed in the medium containing 0.2 per cent agar, with slightly less in the one having 0.1 per cent agar. The yeastlike colonies in the YP media with agar percentages of 0.3 to 0.5 were extensive and also unmixed with mycelial elements, but, owing probably to the solidity of the medium, they were restricted to the very surface of the medium (figure 1). Additional studies at agar concentrations between 0.1 and 0.3 per cent indicated that 0.175 per cent agar provided a viscosity for the most extensive development of the yeastlike phase, to the exclusion of the mycelial phase.

These experiments to determine the optimum viscosity of the medium for the growth of the yeastlike phase of *H. capsulatum* were repeated with 5 other strains, and results similar to those obtained with strain 6521 were obtained. When

growth was extensive, it appeared in the upper portion of the medium (figure 1). When growth was markedly restricted, as in the tubes with no agar, no yeast cells were apparent in the upper part of the medium, although very small quantities developed at the bottoms of the tubes.

The reason for the luxuriant growth of the yeastlike phase in a medium with a low percentage of agar was obscure. Two possible explanations were tested: (1) the agar contained an essential growth substance, or (2) the agar produced a narrow zone of a particular oxygen or carbon dioxide tension essential for the development of the yeastlike phase. Experiments were conducted to test these two hypotheses.

Difco agar, which was the type used in the foregoing experiments, was washed for 48 hours in slowly running tap water and then incorporated into tubes of YP medium in a percentage of 0.175. These tubes and similar ones without agar were inoculated in duplicate with 12 strains of *H. capsulatum* and incubated at 37 C for 3 weeks. Again, in all cases, growth of the yeastlike phase was luxuriant in the tubes of 0.175 per cent agar and virtually nonexistent in those containing no agar.

In order to test the possibility that some inorganic element was exerting an effect on the growth of the yeastlike phase, as much as ten times the concentration of agar used in the YP medium, namely up to 1.75 g per 100 ml of medium, was ashed and added to the medium. The tubes were then inoculated with 7 strains and incubated at 37 C for 3 weeks. In no case did the yeastlike phase appear.

To eliminate completely the possibility of there being some necessary organic or inorganic growth substance in the agar, semisolid "silica gel" was prepared according to the method of Anderson and MacSween (1942), with the exception that a ratio of one part silicate solution to 25 parts of nutrient medium was employed instead of the 1:9 ratio recommended. The purpose of the latter modification was to produce a medium of approximately the same viscosity as one containing 0.175 per cent agar. The nutrient base was YP medium in all cases. The silica gel semisolid medium, after inoculation with 14 strains and incubation at 37 C for 3 weeks, brought about luxuriant growth of the yeastlike phase in all cases but one, with virtually no mycelial fragments discernible.

Oxygen requirements. Since the yeastlike phase grew near the surface of the YP medium, the possibility presented itself that the organism concerned was strongly aerobic and that the semisolid YP medium served to keep the cells on or near the surface of the medium. Accordingly, experiments were initiated to determine the oxygen requirements of the cells.

Tubes containing 10 ml of the YP medium were inoculated with the yeastlike phase and incubated at 37 C under each of the following conditions: (1) in complete anaerobiosis (produced by a suction pressure of 30 inches of mercury), (2) under one-half inch of sterile liquid petrolatum, (3) under 10 per cent carbon dioxide in air, (4) under 20 per cent carbon dioxide in air, (5) under 40 per cent carbon dioxide in air, (6) under 80 per cent carbon dioxide in air, and (7) under 100 per cent oxygen.

The 5 strains subjected to conditions of complete anaerobiosis grew about as extensively as the controls exposed to ordinary room air at 37 C. Under sterile liquid petrolatum the same 5 strains multiplied again only in the yeastlike phase, although to a slightly lesser degree than the controls. Under 10, 20, 40, and 80 per cent carbon dioxide, luxurious development of the yeastlike phase was observed in all cases, with virtually no abortive hyphae present. When in an atmosphere of 100 per cent oxygen, growth of the yeastlike phase was somewhat less than the controls, with one strain having a noticeable amount of mycelial development.

In addition, a flask with YP medium minus agar was inoculated with the yeastlike phase and shaken constantly and vigorously for 4 weeks at 37 C. No extensive growth of the yeastlike phase was then apparent. However, if the inoculum of yeastlike cells was carefully floated on cork shavings, oil drops, or paraffin chips on the surface of YP medium minus agar, marked growth of the yeastlike phase resulted on the surface of the medium.

Age of culture. The yeastlike phase of *H. capsulatum* was maintained in culture both on 20 per cent rabbit blood agar slants and in YP medium. The cultures on the blood agar were transferred every 7 days, whereas those in the YP medium were transferred only once a month. Nevertheless, several times isolates on the blood agar slants spontaneously reverted to the mycelial phase and had to be restored by inoculations from the YP medium. This spontaneous transformation to mycelium at no time occurred in the YP medium. Thus, it would seem that the semisolid medium is more suited for the maintenance of the yeastlike phase in culture.

Growth of other species on YP medium. Since the yeastlike phase of *H. capsulatum* grew so well on YP medium, it seemed logical to inoculate other species of pathogenic fungi therein to obtain their respective parasitic phases. The species studied in this regard were *Blastomyces dermatitidis*, *Coccidioides immitis*, and *Sporotrichum schenckii*.

When the yeastlike phase of *B. dermatitidis* from rabbit blood agar slants was inoculated into the YP medium and incubated at 37 C, the 5 strains tested developed primarily as mycelium. In the studies on 6 strains of *C. immitis*, with inocula consisting of either a saline suspension of conidia or a suspension of spherules from an infected mouse testis, there was no obvious development of the yeastlike phase in the YP medium at 37 C. Of the 6 strains of *S. schenckii* incubated in the YP medium, two developed entirely in the yeastlike form, but the others appeared both in the mycelial and budding phases.

SUMMARY

Seventeen strains of *Histoplasma capsulatum* were grown in a fluid medium in the yeast-like phase.

The best growth occurred at hydrogen ion concentrations between 6.3 and 8.1, at a temperature near 37 C, and in a medium containing a mixture of organic nitrogen compounds.

No growth appeared in the "YP medium" unless a small percentage of agar, silica gel, oil, or some similarly functioning substance was added.

The oxygen and carbon dioxide tensions were of little importance.

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