A STUDY OF THE SPORULATION OF *HISTOPLASMA CAPSULATUM*

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Darling (1906, 1907, 1908, 1909) first described the disease histoplasmosis and its etiologic agent, *Histoplasma capsulatum* in a group of papers written from the Ancon Hospital, Canal Zone. Since the encapsulated round or oval bodies found in the endothelial cells in lesions of the lungs, liver, spleen, and intestines of patients closely resembled the etiologic agent of kala azar, Darling suspected that the organisms were protozoa.

DaRocha-Lima (1912) made a comparative study of material obtained from Darling and from a case of kala azar and concluded that *H. capsulatum* was related more closely to the fungi than to the protozoa. It remained for DeMonbreum (1934) to cultivate for the first time the fungus, *H. capsulatum*, from a terminal histoplasmosis case and prove experimentally that it was the etiologic agent of the disease. In culture he demonstrated large spores, 20 μ in diameter, which were formed below the surface of the medium on two or three cell pedicels. As the spores grew, they became spherical and the walls increased in thickness; they contained fat globules of uniform size which resembled asci. He postulated from his observations that as the medium dried the spores became crenated and tuberculated.

Although many workers have studied the spores produced by *H. capsulatum* in the interest of taxonomy, few have studied the life history and sporulation of the organism. Cozad and Furcalow (1953) posed a question as to whether all large spores were potentially tuberculated, or whether there might occur three types of spores, the small, large, and tuberculate. Conant (1941) studied the life cycle of the organism *en masse*, and his drawings and descriptions were used to great advantage in this study of sporulation in both solid and liquid media.

MATERIAL AND METHODS

Histoplasma capsulatum (Communicable Disease Center, Chamblee, Georgia, *H. capsulatum*, stock culture number A-534) was isolated by the senior author from a Sabouraud's agar slant on the ninth day of incubation from the blood clot of a whole blood specimen submitted from a 4 month old infant (J.J.) for a histoplasmosis complement fixation test. The complement fixation test was reported as negative. Study of the culture obtained revealed "nimbospore" forms, heretofore unreported. This will be described in detail under discussion.

A bit of the original culture was placed on a blood agar slant, and the stopper was sealed with paraffin. Unlike Conant (1941) and others whose strains took 7 to 21 days to convert from the mycelial to the yeast phase, this inoculation reverted to a yeast phase culture in 4 to 6 days and then was used to inoculate solid media to produce the mycelial phase for inoculation as follows:

The depressions of several microculture slides were filled with Sabouraud's glucose agar by means of a sterile dropping pipette. After the medium cooled, a bit of the mycelial phase of the above culture, or transplants of it, was seeded on each microculture, covered with a sterile coverslip, and incubated at room temperature. The slides were supported by bent glass tubing over a small volume of sterile water in a petri dish to insure proper humidity. Both mature tuberculate spores and nimbospores were produced from these mycelial subcultures in 4 to 6 days.

Depressions of microculture slides filled with Sabouraud's glucose broth were inoculated with portions of the yeast phase of the strain and observed for germination. These cultures were sealed against evaporation by the introduction of several small droplets of mineral oil between the coverslips and slides. The yeast phase germinated and bore mature spores in 6 to 9 days following the cycle as depicted by Conant's (1941) illustrations.

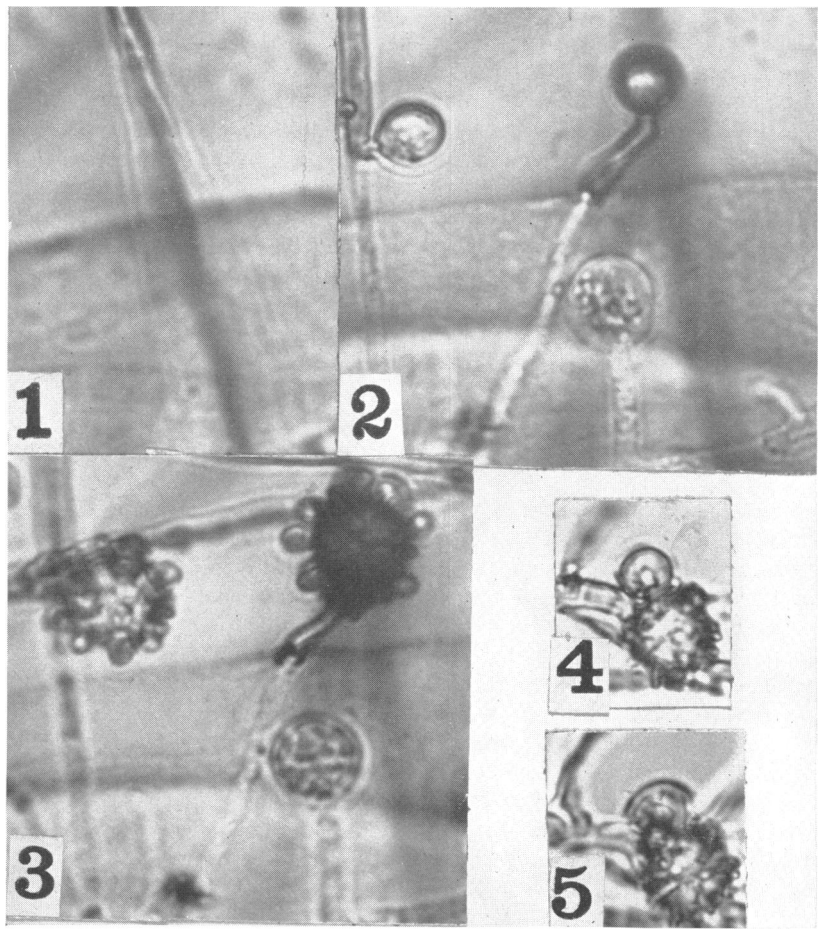
Both solid and liquid media produced the usual types of spores, varying from small,

smooth spores to the diagnostic tuberculate spores and the new type heretofore undescribed.

DISCUSSION

Upon examining the cultures microscopically, one can see that throughout the growth area spores are constantly developing. They start as a swelling at some point on the tubular wall of one of the septate hyphae or at the tip (figures 1, 2, 3). It is impossible in the beginning to tell whether these swellings are the origin of branching hyphae or of the sessile or pediceled spores. If the swelling should develop into a spore, it will be seen first as a rounded swelling on the wall of the hypha or on the end of its pedicel gradually growing larger. As it grows in size the cell walls become thicker until they become mature spores. This sporulation process follows the same development in liquid as well as solid media. These mature spores may be of two kinds, tuberculate spores and the previously unreported nimbospores. Because of the halo or cloud-like appearance of the thick wall around the latter spores, they have been referred to by the senior author as nimbospores. A photomicrograph (figure 6) was taken of a group of nimbospores stained with lacto-phenol-cotton blue. It shows the thick wall apparently laid down in layers.

To eliminate, so far as limited facilities would permit, the possibility that the sheath might be of a polysaccharide nature, tests for callose



Figures 1, 2, 3. Illustrate the development of pediceled tuberculate spore. This represents a period of 48 hours (× 1,350).

Figures 4 and 5. Immature aerial nimbospore becoming mature (× 1,350).

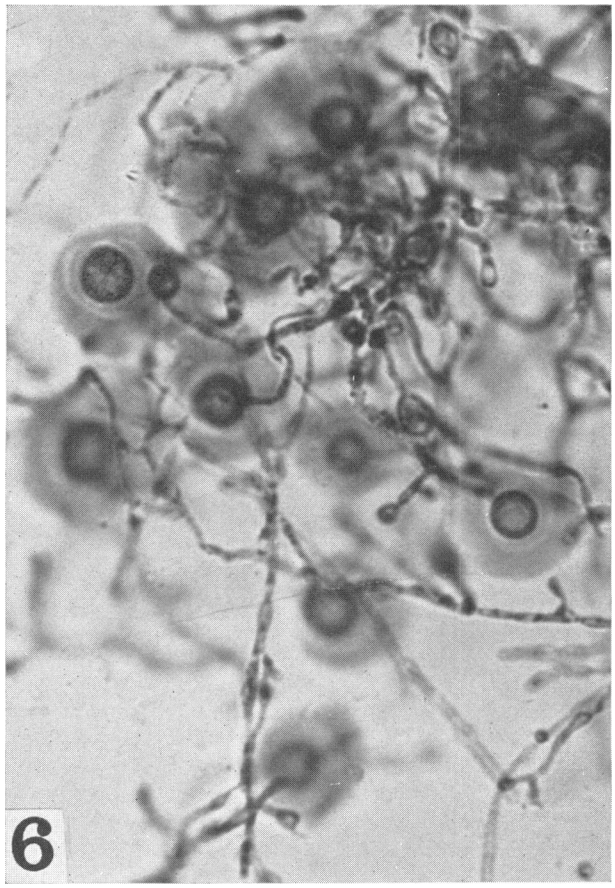


Figure 6. A temporary slide mount prepared with lacto-phenol-cotton blue showing a cluster of nimbospores (× 1,350).

and cellulose were made. These two common cell constituents appeared to be absent from the walls of those nimbospores observed.

In examining Sabouraud's glucose agar cultures of the J.J. strain of *H. capsulatum*, smooth, extremely thick-walled spores were observed scattered just below the surface of the medium. These nimbospores usually are found on mycelia growing in or closely applied to the medium (figure 6). More rarely they are found growing on the aerial mycelia (figures 4, 5). These spores may be observed easily after 10 days of growth on Sabouraud's glucose agar. The area within the cell wall itself averages 9.65 by 9.51 μ in diameter, surrounded by a thick wall of gelatinous material which increases the diameter of the whole to an average of 21.61 by 21.03 μ. When viewed through a microscope or camera a band of refracted light is visible around the outside of the thick wall of the nimbospore as well as around the nontuberculate spores (figures 3, 4, and 5).

In August of 1951, when this strain was isolated, numerous subcultures were made for future study. The nimbospores from cultures two years old were studied closely along with a critical examination for changes in other spores. There was a considerable decrease in the number of tuberculate spores, whereas a great increase in diameter of the nimbospores was noted. The average diameter of the nimbospores had become 31.16 by 30.69 μ. Transplants were still viable after two years of incubation at room temperature.

A nimbospore which developed in the thin portion of the slant in a culture tube was observed microscopically from time to time over a period of two years. The nimbospore grew in diameter

by increasing the thickness of the cell wall. Except for this increase in diameter the mature nimbospore remained as previously described. It never became tuberculate, but it did continue to grow in diameter.

A nimbospore in a microculture was observed over a period of time. This nimbospore never became tuberculated even after the medium was so dry that the edges curled.

The nimbospores were observed also in Sabouraud's glucose broth. They may be observed in both the floating and submerged colonies although they were much more difficult to observe in the fluid medium because of the transparency of the fluid as well as the thick cell wall.

Five additional strains of *H. capsulatum* were examined exhaustively for nimbospores. Of the five strains only three had produced nimbospores. These nimbospores varied in size and were far fewer in number as compared to those produced by the J.J. strain.

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SUMMARY

Spores of *Histoplasma capsulatum*, in general, are formed from swellings on the wall of one of the septate hyphae or at the tip. They increase in size and the cell walls thicken until they mature into tuberculate spores or nimbospores.

A heretofore unreported type of mature spore is found in a strain of *H. capsulatum*. It is a heavy, thick-walled, nontuberculate spore found on Sabouraud's glucose agar as well as Sabouraud's glucose broth, and is referred to as a nimbospore because of the halo or cloud-like appearance of the cell wall. Three of five other strains produced nimbospores in smaller sizes and fewer numbers.

In August of 1951, subcultures of the original isolation were made. Subcultures of these proved viable after two years of incubation.

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