The Fine Structure of the Yeast-like Cells of Histoplasma in Culture

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Summary: The fine structure of the yeast-like cells of Histoplasma capsulatum Darling and H. duboisii Vanbreuseghem has been studied and compared by electron microscopy. The micro-morphology of single and budding cells, as well as some details of the budding process, are described. No evidence of a capsule or slime layer on the outside of the cell wall of either species was demonstrated. In both species the cell walls were found to be laminated and in general slightly thicker in H. duboisii than in H. capsulatum. The possibility of the laminations being associated with the presence of chitin in the framework of the cell walls is discussed. The cells of the two species, strikingly similar in structure, were found to be highly differentiated, resembling animal cells in some of their micromorphological features, namely in the presence of endomembrane systems, in mitochondrial numbers and forms, and in nuclear fine structure.

Since the discovery by Darling (1906) of Histoplasma capsulatum, this fungus has been the subject of considerable cytological and histochemical study (Rocha-Lima, 1913; De Monbreun, 1934; Ciferri & Redaelli, 1934; Conant, 1941; Dowding, 1948; Kligman & Baldridge, 1951; Milne, 1957). These contributions have elucidated some morphological features of the fungus, such as the presence of a cell wall and the nucleus of the yeast-like cells, as well as the structure of the hyphae and the different types of spores developed in the saprophytic growth cycle. However, due to the small size (1–5 μ) of the yeast-like cells of this species, in the parasitic form as well as in culture, it is not possible with either the ordinary light microscope or the phase-contrast microscope to obtain a precise picture of the cellular components and it has been even more difficult to determine whether or not the cell is surrounded by a thin capsule or slime layer.

Recently, a different type of Histoplasma has been described in cases of tropical-African origin (Catanei & Kervran, 1945; Duncan, 1947; Dubois, Janssens & Brutsaert, 1952; Dubois & Vanbreuseghem, 1952; Vanbreuseghem, 1958), distinguishable from the classical type by its large intracellular parasitic form, which is by linear measurement three to six times the size of H. capsulatum. It was named Histoplasma duboisii by Vanbreuseghem (in Dubois, Janssens & Brutsaert, 1952) but its status as a species is still controversial. Comparative studies of both forms of Histoplasma have appeared in the literature (Drouhet & Schwarz, 1956a, b; Mariat & Segretain, 1956; Vanbreuseghem, 1956; Drouhet, 1957; Schwarz & Drouhet, 1957; Schwarz, 1958a).
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and more recently Schwarz and Emmons (Schwarz, 1958b) have intimated acceptance of the new species.

Notwithstanding the differences in size and in tissue reaction of the parasitic forms (Edwards, Edwards & Hazen, 1959), the two types of Histoplasma seem to be indistinguishable in culture. The mycelial phase shows the same morphological characteristics and the yeast phase consists principally of the classical small, thin-walled, ovoid, yeast-like cells (1–5μ in length), among which a variable number of larger, thick-walled, budding cells (over 5μ in length) are found. A good description of the latter was given by Duncan (1958), who also noted that H. duboisii seemed to have a more marked tendency than H. capsulatum to develop the large cells in response to unfavourable conditions.

This paper presents the results of a comparative cytological investigation, using the electron microscope and thin sectioning techniques, of the two types (or species) of Histoplasma in the yeast phase.

METHODS

Four strains of Histoplasma in the yeast phase were employed, two of H. capsulatum Darling (nos. 4890 and 5854), and two of H. duboisii Vanbreuseghem (nos. 58136 and 58134). Strain no. 4890 was obtained from the late Dr Rhoda Benham of Columbia University, New York, New York, and no. 5854 was isolated in 1957 in this laboratory from sputum from a patient with pulmonary histoplasmosis. Strains nos. 58136 and 58134 were received under nos. RV 7408 and RV 4910, respectively, through the courtesy of Dr R. Vanbreuseghem of the Institut de Médecin Tropicale, Antwerp, Belgium. They were in the mycelial phase when received and were converted to the yeast phase by passage through mice.

The yeast-phase cultures were obtained from solid and liquid media. Francis's glucose-cystine blood agar slants (Campbell, 1947), and a defined liquid medium (Salvin, 1950) dispensed in 125 ml. Erlenmeyer flasks in 50 ml. amounts were used. The cultures on agar slants were sealed with paraffin and incubated at 37° for 48–72 hr.; the broth cultures were shaken manually twice each day for about 3 min., during the incubation period of 48–72 hr. at 37°.

The fixation and embedding procedures were as follows:

(A) From the agar slant cultures, the growth was transferred directly by means of a nichrom wire loop to a 2 % (w/v) OsO₄ solution in veronal + acetate buffer at pH 7·4; or the growth was washed off in a physiological salt solution, centrifuged and then suspended in a small volume of the veronal + acetate buffer (pH 7·4) to which an equal volume of 3 % (w/v) OsO₄ was added, so as to have a final concentration of 1·5 % OsO₄.

(B) The broth cultures were centrifuged in large tubes for 15–20 min. at about 1900 rev./min., and the sediment suspended in 3–5 ml. of veronal + acetate buffer solution (pH 7·4) and mixed with an equal volume of a cold 3 % (w/v) OsO₄ solution in the same buffer. The final mixture (1·5 % OsO₄) was kept at 4–5° during 20–30 min. Thereafter, the fixation proceeded at room temperature.
After 1 hr. this mixture was centrifuged as indicated above, and the sediment resuspended in 3 ml. of a buffered 1·5 % OsO₄ solution (pH 7·4).

In all instances, the last step of fixation was accomplished in a small tube suitable for the Servall centrifuge, using about 5 ml. of the fixing fluid, the fixation time varying from 1 to 3 hr. Fixation of 1 hr. and 40 min. in 2 % OsO₄, and 2 hr. in 1·5 % OsO₄ gave the best results. After fixation, the commonly used schedule of washing in buffer and dehydrating in graded aqueous ethanol was followed. Before each change of solution, the material was centrifuged at low speed to a compact pellet and between changes the cells were agitated gently by hand.

Embedding was done in a mixture of 90 % butyl methacrylate and 10 % methyl methacrylate, with 1·5 % of Lucidol as the initiator, at 48° for 36–40 hr.

Sections were cut with a diamond knife in the Porter–Blum microtome. They were supported on formvar lightly stabilized by carbon, and observed in RCA and Siemens electron microscopes at initial magnifications of ×2600 to ×20,000.

RESULTS

The cells on either solid or liquid media were similar in structure, hence they will be considered together.

Single yeast-like cells

*Histoplasma capsulatum.* The yeast-like cells of *H. capsulatum* are generally oval to spherical in outline. Some irregular forms have been observed, but it is believed that such irregularities result from fixation and embedding procedures. The measurements of 116 cells averaged 1·73 μ by 2·47 μ in their short and long axes respectively, with variations from 0·53–4·60 μ in the long axis. The cell is delimited by a laminated cell wall of an average thickness of 90 μ which appears to be smooth in outline. The outer limit of the cell wall is a thin layer of high electron-density (Pl. 1, figs. 1, 2) averaging 16 μ in width, accompanied by inner layers of medium density. The innermost layer is intimately apposed or adherent to the plasma membrane, being evident only when the cytoplasm is pulled away from the cell wall as in plasmolized or degenerated cells. It then appears similar in density and thickness to the outer layer described above.

The plasma membrane (or cytoplasmic membrane—O’Hern & Henry, 1956; Agar & Douglas, 1957) is of high electron density and averages 8 μ in thickness (Pl. 1, fig. 1; Pl. 2, figs. 4, 6). In few cells is the plasma membrane regular. More commonly it is extremely rugose or serrate and exhibits considerable vesiculation, indicative of pinocytotic activity.

The nucleus occupies a large portion of the cell and is usually ovoid, at times irregular; it averages 1·08 μ by 1·63 μ in short and long axes. The nucleus possesses one or more nucleoli which have a generally homogeneous matrix of granules of high density (Pl. 1, figs. 1, 2) and which may be fragmented or vacuolated. The chromatic material appears mostly scattered throughout the matrix. The nucleus is bounded by a thin membrane of 10 μ thickness which
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in turn is surrounded by a fairly regular perinuclear cisterna of an average width of 28 mµ. The outer envelope of the nucleus averages 10 mµ in thickness and at times is seen to be continuous with membranes of the endoplasmic reticulum (Pl. 2, fig. 8). Pores are found at irregular intervals along the nuclear border (Pl. 3, fig. 8), oftentimes in front of mitochondria which are adjacent to the nucleus. The outer nuclear membrane may be continuous with the outer membrane of a neighbouring mitochondrion.

The cytoplasm of the cell contains tubules and vesicles of the endoplasmic reticulum (Pl. 1, fig. 2; Pl. 5, fig. 16), scattered and clumped granules, lipid bodies and mitochondria. In some cells, there appear to be vesicles and lamellae, suggestive of the reticular apparatus of Golgi, apparently associated with the presence of lipid (Pl. 1, fig. 2; Pl. 8, fig. 7). The form of this organelle does not appear as distinct as it usually does in higher cells. The mitochondria are abundant, in general 4–5 per cell, usually oval in outline, averaging 470 mµ by 660 mµ. They contain relatively few cristae mitochondriales and generally have a matrix of fine granules or filaments of low density, thus giving the appearance of being virtually empty (Pl. 1, fig. 2). The lipid bodies (Pl. 1, fig. 2) are common, usually 2 per cell (Pl. 4, fig. 12). They are, in general, of regular outline, oftentimes limited by a membrane, but in some instances they appear as scattered irregular masses.

Histoplasma duboisii. The single yeast cells of H. duboisii are generally similar to those of H. capsulatum. They possess the same nuclear and cytoplasmic components and are of the same shape (Pl. 2, figs. 5, 6). H. duboisii cells average 1.92 µ by 2.75 µ in their short and long axes, respectively, with variations from 0.77 to 5.86 µ in the longer axis. The cell wall is laminated, averaging 100 mµ in thickness, having an outer and denser layer of an average of 10 mµ and an innermost layer of the same thickness. The plasma membrane usually exhibits considerable pinocytotic activity and averages 9 mµ in thickness.

The Histoplasma duboisii cells, observed under the present conditions, appear to be of two types or stages, differing in the density or packing of the cytoplasmic and nuclear components: (a) light cells which show a virtually empty cytoplasm (Pl. 2, fig. 6), i.e. the constituent granules are clumped, as previously observed in H. capsulatum; (b) dark cells (Pl. 2, fig. 5) which possess a closely packed, finely granular cytoplasmic matrix and a nuclear matrix, fuller and more finely granular in nature than the light cells. The other cellular components are similar to those of H. capsulatum, and need not be described further.

Budding yeast-like cells

Histoplasma capsulatum. Budding cells of H. capsulatum were observed in various stages in cultures of 48–72 hr. The earliest indication of a budding process seems to be a thickening of the cell wall around a small nipple-like evagination of the cytoplasm (Pl. 3, fig. 10). The plasma membrane in the evacuating area may show extreme vesiculation. The evagination continues until a small thumb-like process, containing only cytoplasmic matrix, is formed (Pl. 4, fig. 11). The head of this process then enlarges and becomes distinguishable from the neck but still contains only cytoplasmic matrix. In the
next apparent stage, membranes may be seen in the neck and in the head of the new formation (Pl. 4, fig. 12), with the nucleus of the mother cell appearing in the area nearest the neck of the bud (Pl. 4, fig. 13). In older buds mitochondria appear (Pl. 4, fig. 12). In an apparently later stage, nuclear material is seen in the parent cell, in the neck, and also in the proximal portion of the bud (Pl. 4, fig. 18). This is better illustrated in *H. duboisii* (Pl. 6, fig. 18). In a more advanced step a definitive nucleus appears in the bud itself (Pl. 4, fig. 14; Pl. 5, figs. 15, 16). (Since this study was completed, Hashimoto, Conti & Naylor, 1958, have well shown the nuclear migration and vegetative division in bud-formation in *Saccharomyces.*) The plasma membrane of the bud shows the same vesiculation as that of the mother cell (Pl. 5, fig. 16). After the appearance of the definitive nucleus in the bud, the neck becomes constricted and while still maintaining this narrow connexion, the bud enlarges in size, approximating the size of the parent cell, and acquires the mature complement of cytoplasmic components, i.e. numerous mitochondria, endoplasmic reticulum, lipid granules and Golgi apparatus (Pl. 4, fig. 14; Pl. 5, figs. 15, 16). The connexion between mother and daughter cells becomes quite tenuous, and eventually their respective cytoplasts are separated by their plasma membranes. This step is followed by an invasion, between the two cells, of less dense, laminar material of the still continuous cell wall. Secondly, a dense line, normal to the long axes of the cells, appears in the new cell wall between mother and daughter cells (Pl. 5, fig. 15). As an apparent last step the two cells separate entirely by the splitting of the cell wall material along the dense line (or splitting plate) just described and the bud becomes a complete cell.

*Histoplasma duboisii.* The budding process is similar to that described for *H. capsulatum.* A few micrographs of budding cells of *H. duboisii* are presented (Pl. 6, figs. 17–19) to illustrate this similarity. There is, however, a difference in electron density between the mother and the daughter cells of *H. duboisii.* The cytoplasmic and nuclear matrices of the bud are of higher density and therefore appear darker in the micrograph, so that its fine structure is difficult to observe (Pl. 6, fig. 19). This finding suggests that the distinction between the light and dark cells may be associated with differences in age and metabolic activity.

**DISCUSSION**

A capsule, or thin layer of slime, is not present on the outside of the cell of either species of *Histoplasma.* These findings are in agreement with those of Kligman & Baldridge (1951), Salvin & Ribi (1955) and Ribi & Salvin (1956) who used different electron microscopic techniques. It could perhaps be argued that the procedure of fixing and dehydrating washed off the capsular material from the cells. Such criticism, however, would not seem justified in view of the fact that other micro-organisms such as *Cryptococcus neoformans* (unpublished data) and *Chlamydomonas pyriforme* (Sager & Palade, 1957), known to possess a capsule, when studied by the same technique were not completely deprived of their capsular material.

In both species of *Histoplasma* the cell walls are laminated, appearing to be comparable in density and structure to the basement membrane of some insect...
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cells (cf. Pl. 134, fig. 4, in Edwards, Ruska & de Harven, 1958). It seems of interest to mention here that the basement membrane of insect cells is composed of chitin and protein, and that the framework of the fungi causing systemic diseases has also been found to be composed of chitin (Blank, 1954). A similar laminated cell wall has been described in Saccharomyces cerevisiae in which chitin was also mentioned as present (Agar & Douglas, 1955). The correlation between the laminated structure of these cell walls and their chitinous framework, however, remains to be proven.

The comparative cytological investigation of the two species of Histoplasma in culture revealed no significant differences. The size of the cells of H. duboisii was very close to that of H. capsulatum, and in both species the nucleus and the cytoplasmic organelles showed the same fine structure. However, the younger cells of H. duboisii were generally denser to the electron beam than the older cells, a distinction not found in H. capsulatum, under the conditions of this study. This difference alone would not seem to be of significance. A much more striking distinction was found in the study of the two species in mouse spleen (Edwards, Edwards & Hazen, 1959), in which the cells of H. duboisii were, on the average, 5-5 times larger than the cells of H. capsulatum.

REFERENCES


EXPLANATION OF PLATES

Symbols used in figures: CW, cell wall; PM, plasma membrane; Nu, nucleus; Nc, nucleolus; Mi, mitochondrion; er, endoplasmic reticulum; G, reticular apparatus of Golgi; L, lipid body.

Plate 1

Fig. 1. Portions of two cells of Histoplasma capsulatum. Each cell is bounded by a relatively thick, layered cell wall and a smooth to irregular plasma membrane. The cytoplasm contains scattered granules, membrane-bound profiles and relatively large mitochondria. The nucleolus of the left-hand cell displays a prominent nucleolus. ×50,000.

Fig. 2. Histoplasma capsulatum. The plasma membrane shows considerable pinocytosis. The nucleus has a dense nucleolus. Lipid bodies occur near the few membranes of the Golgi apparatus. The mitochondrion possesses few, incomplete cristae. Membrane bound profiles of the endoplasmic reticulum appear as vesicles or tubules. ×40,000.

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**Plate 2**

Fig. 3. *Histoplasma capsulatum*. The plasma membrane shows intense pinocytosis and appears connected to the endoplasmic reticulum. The external nuclear membrane is also connected with membranes of the endoplasmic reticulum. $\times 50,000$.

Fig. 4. *Histoplasma capsulatum*. The outer membranes of mitochondria often appear continuous with the outer nuclear membrane or with the plasma membrane. $\times 45,000$.

Fig. 5. *Histoplasma duboisii*. Dark cell whose nucleus is considerably less dense than is the cytoplasm. Two small mitochondria and several elements of the Golgi apparatus may be discerned in the cytoplasm. $\times 35,000$.

Fig. 6. Light cell of *Histoplasma duboisii* with all organelles. The cell wall is thick and layered. The plasma membrane is relatively smooth. The mitochondria show few cristae. The endoplasmic reticulum comprises both sacs and vesicles. The nucleus possesses wide pores (arrow). $\times 35,000$.

**Plate 3**

Fig. 7. *Histoplasma duboisii*. A mitochondrion may possess long, complete, parallel cristae, usually in young cells. In upper portion of the cell there appear elements of the Golgi apparatus in cross section. $\times 45,000$.

Fig. 8. *Histoplasma duboisii*. Young, dark cell with extreme pinocytotic activity of the plasma membrane. The cytoplasm contains a fat body, scattered granules and endomembranes, and two large mitochondria. Nuclear pores (arrows) are indicated. $\times 35,000$.

Fig. 9. *Histoplasma duboisii*. Light cell showing mitochondrion with circular cristae. $\times 45,000$.

Fig. 10. *Histoplasma capsulatum*. Early stage of the budding process. The cell wall is thickened around the bud and the plasma membrane in the bud area is extremely pinocytotic. $\times 45,000$.

**Plate 4**

Fig. 11. *Histoplasma capsulatum*. In early budding the nucleus is seen near the bud area. Mitochondria usually follow nucleus. $\times 32,000$.

Fig. 12. *Histoplasma capsulatum*. As the bud increases in size, mitochondria appear in both mother cell and bud. $\times 32,000$.

Fig. 13. *Histoplasma capsulatum*. The neck of the bud is constricted. The nucleus of the mother cell is located close to the neck of the bud and nuclear material seems to be present also in the bud. $\times 30,000$.

Fig. 14. *Histoplasma capsulatum*. A definitive nucleus is present in the bud (at right) as well as in the mother cell. Mitochondria, lipid bodies, and endomembranes are present in both cells. In the neck region, the two cells are separated by their plasma membranes. In the common cell wall between plasma membranes, a dark line (the splitting plate) and partial furrowing (arrow) may be seen. $\times 30,000$.

**Plate 5**

Figs. 15, 16. *Histoplasma capsulatum*. Last steps of the budding process. In both figures the bud shows all the elements of an adult cell, but still remains connected to the mother cell by the common cell wall. In fig. 15, the splitting plate (arrow) along which the two cells will part, appears as two intimately apposed, thickened lines. These, on separation, will presumably be the independent, dense and thin, outer layers of the cell walls of the respective cells. Fig. 15, $\times 40,000$; fig. 16, $\times 35,000$.

**Plate 6**

Fig. 17. *Histoplasma duboisii*. Early stage of the budding process. The cell wall around the bud is considerably thickened, the bud proper has a high electron density and its plasma membrane shows pinocytotic vesicle formation. $\times 30,000$.

Fig. 18. *Histoplasma duboisii*. Intermediate stage of the budding process. A protrusion of the nucleus of the mother cell runs towards the bud. A small amount of nuclear material appears also in the bud itself. $\times 27,500$.

Fig. 19. *Histoplasma duboisii*. The bud has a high electron density and its mitochondria are large with parallel cristae. Note the double plasma membrane formed in the splitting zone (double arrows) of the neck of the bud. $\times 35,000$.

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