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THE MORPHOLOGY OF CRYPTOCOCCUS NEOFORMANS IN HUMAN CRYPTOCOCCOSIS A LIGHT-, PHASE-CONTRAST AND ELECTRON-MICROSCOPIC STUDY

by

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ABSTRACT

As we found no comprehensive descriptions with reference to the morphology of *Cryptococcus neoformans* in the relevant literature, a light-, phase- contrast and electron-microscopic study on this blastomycete seemed advisable. The postmortem material was taken from two fatal cases of cryptococcosis. This paper gives a detailed presentation of the morphological findings and a schematic illustration which clearly reveals the morphological overall structure of the suitably stained *Cryptococcus neoformans* as compared to the usual microscopical sections and smears.

INTRODUCTION

In the relevant literature the morphology of *Cryptococcus neoformans* (subsequently termed Cr. n.) is described only incompletely and somewhat contradictorily. Even recent monographs, textbooks and contributions to handbooks (LITTMAN & ZIMMERMAN, 1956; EMMONS, BINFORD & UTZ, 1963; BADER, 1965; SCHIEFER, 1967; SEELIGER & WERNER, 1967; SYMMERS, 1968; GEDEK, 1968) give no detailed cytological representation of *Cr. n.* and we felt that a morphological study on this blastomycete is desirable.

MATERIALS AND METHODS

The material was taken from two autopsy cases; i.e., one pathogenic and one opportunistic infection. First the tissue was fixed in formalin (or, for methyl green-pyronin staining, in a mixture of formalin and alcohol). Small samples of tissue were then taken from the formalin-fixed material and (either with or without follow-up treatment by $0s0_4$ solution) embedded in Vestopal W. $0s0_4$-treated thin sections were stained with lead citrate and examined in a SEM 3-1 electron microscope (VEB Werk für Fern-

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sehelektronik, Berlin). Paraffin section of 4 to 5μm thickness had to be prepared for light microscopic studies which were subjected to the following staining techniques or cytochemical reactions: HE, Giemsa, methyl green-pyronin, hematoxylin according to R. HEIDENHAIN, methenamineargentic nitrate according to GOMORI, GOLDNER, FEULGEN, PAS, HALE and acridine orangefluorochromation with various pH-stages (either with or without previous sulphation). Paraffin sections and thin sections of 0.08 μm thickness - prepared by means of an ultramicrotome - were used for phase-contrast microscopy.

RESULTS

Nucleus

Light microscopy proved to be successful with HE stain (Fig. 1), FEULGEN reaction (after 2 hours hydrolyzing with 10 % $HCLO_4$)

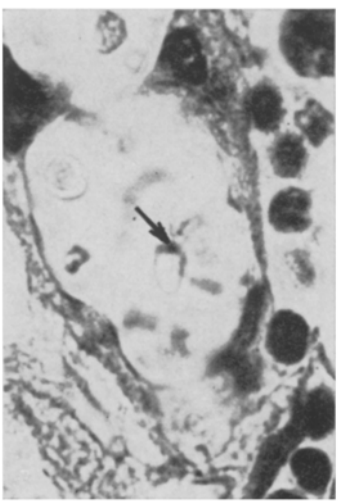

Fig. 1. (HE, x 1,300): Centre — Cryptococcus in the network of the Arachnoides encephali with large central vacuole, small protoplasmic margin and internal nucleus (arrow). Cell wall vanished and cytoplasm stained eosin-red in the original. Upper left — Capsule-containig Cryptococcus. Right — Lymphocytes.

and sulphation-acridine orange-fluorochromation. Usually the nucleus is not greater then 0.2 μm. Only when a nucleus is flattened due to the effects of the central vacuole - which results in a 'cap-to-disk-like' configuration - its diameter in longitudinal direction will be greater than 0.2 μm. - Phase-contrast studies have shown that part of the fungus cells also have luminescent organelles (conventional paraffin sections and ultrathin sections) which - depending on location, size and shape - agree well with the nuclei.

Electron-optically the nucleus appears as an electron dense and heterogeneous structure (Fig. 2). The nuclear membrane remains still undefined. In many Cryptococci, the nucleus is located in the electron dense region of the protoplast and could not be identified with certainty.

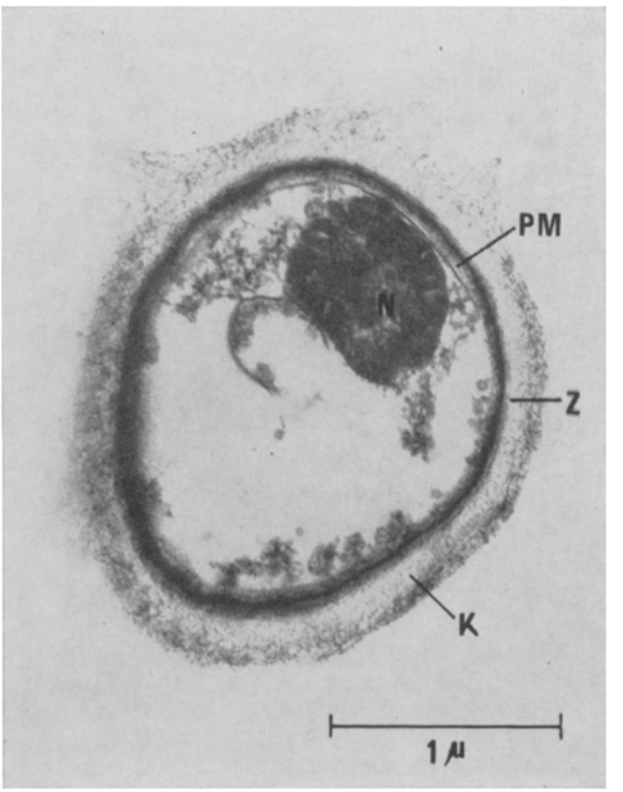

Fig. 2. (x 30,000): Part of a flattened nucleus (N) in contact with the protoplast membrane (PM). Granular aggregates, membranous structures and small vesicles are also visible in the protoplast. Relatively thin cell wall (Z). K = capsule.

Protoplast

With the aid of light microscopy the cytoplasm could be depicted through HE and GOLDNER stains only in cases where the wall of the *Cr. n.* was partly destroyed. Such fungus cells are characterized by a ring-like or halfmoon-shaped cytoplasm margin around the central vacuole (Fig. 1). Depending on the method and the selected pH-value, the central vacuole shows a secondary fluorescence which is orange to lemon-coloured. As for the presentation of *Cr. n.* with other stains it is assumed that the often

visible bright inclusions in the frequently (non-characteristic) homogeneous and weakly stained fungus cell are the substrate of the central vacuole. (With sprout formation spread rectangular to the tissue section the germinating daughter cell may appear as an inclusion in the parent cell; however, with the 'inclusion' limited by a cell wall.)

Electron-microscopic findings (Fig. 3) have shown an electron-

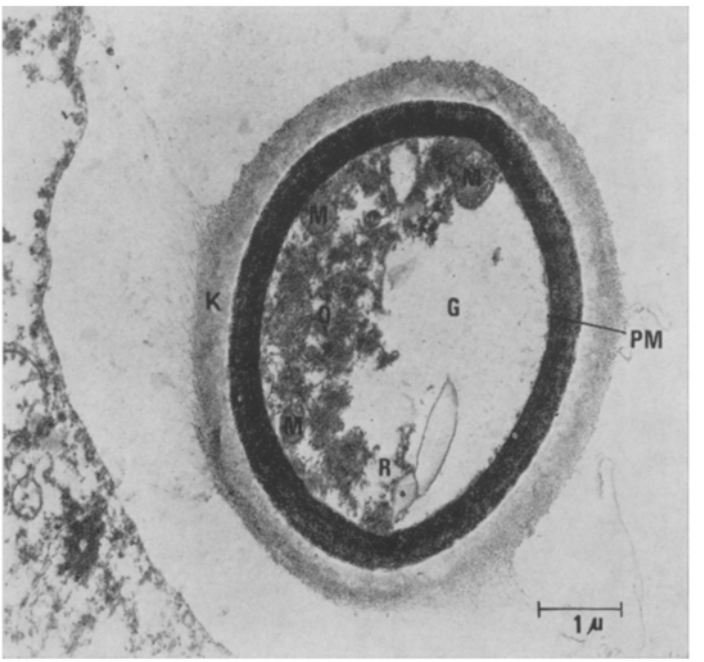

Fig. 3. (x 12,000): Indications within the cell body that, obviously due to its higher turgor, the ground plasma (G) has displaced the other cell constituents toward the periphery in a sickleshaped manner (mitochondria (M) with electron dense matrix; vacuolar deformed reticulum (R); osmiophil granular zones (O)). Partly well defined protoplast membrane (PM). (K = capsule).

lucent, spherical region of plasm as a correlate to the central vacuole, frequently without a membranous boundary against the environment. Size and shape of this vacuole is variable; in many fungus cells the central vacuole seems to be missing, and the major part of the cell interior is filled with a homogeneous ground plasm. - In *Cr. n.* the mitochondria are the most frequent organelles (max. up to 10 per cell; mean diameter of the mitochondria 0.6 to 0.7 μm; max. diameter 0.9 μm). The matrix of the mitochondria is homogeneous to fine-granular and electron dense. Internal structures consist of short cristae and small vesicles. Often a single membrane is found as outer boundary; sometimes even a

double membrane. The endoplasmic reticulum essentially consists of vesicles and, in most cases, of stratified and ring-shaped or bulbous membranes. Moreover, we found in the fungus cells fat droplets, small compact sections of plasm and pinocytotic vesicles. The outer membrane of the protoplast generally is in direct contact with the cell wall.

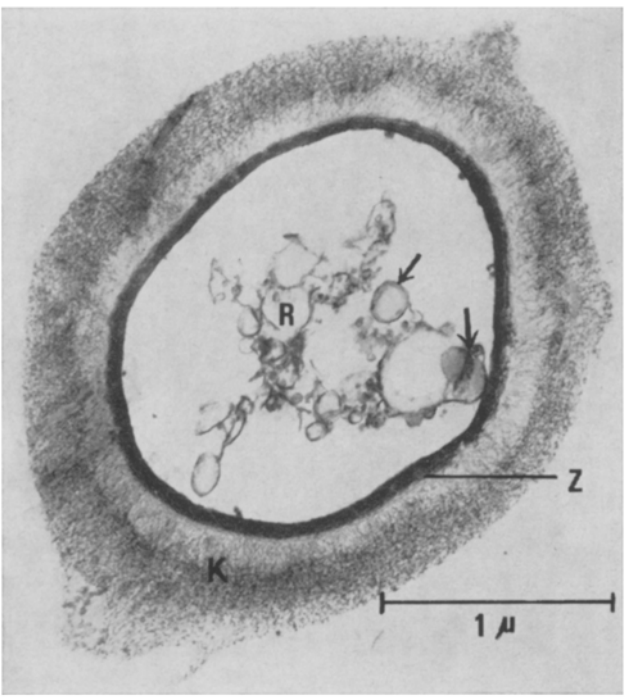

Fig. 4. (x 30,000): Besides the optically void ground plasm the protoplast often shows a vesicular or vacuolar shaped endoplasmic reticulum (R). Some vacuoles (→) are characterized by a low to medium osmiophil content. Relatively thin cell wall (Z). K = capsule.

Cell Wall

Composed of two layers, the mean cell wall thickness is 250 to 300 μm (500 μm, max.; 60 μm, min.). The inner layer is thinner and more electron dense than the outer. According to our electron-microscopic findings both wall layers consist of circular arranged membranes. It is assumed that the varying electron density stems from the difference in the also visible homogeneous ('cementing') substance. From the electron micrographs it can be seen that it is always the outer wall layer which bursts during germination while the inner layer grows continuously on the sprout.

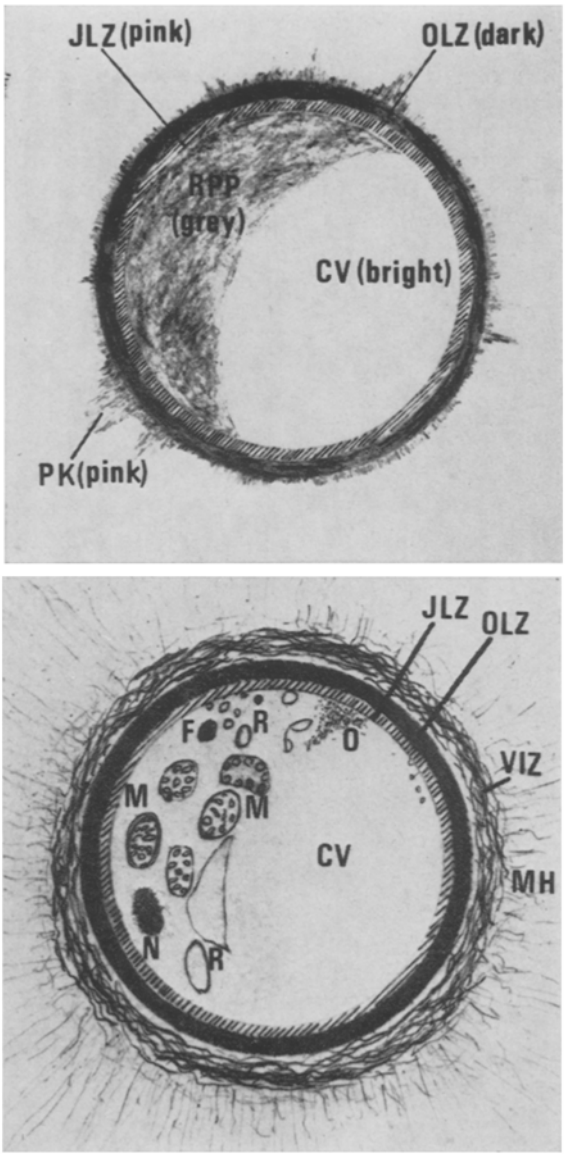

Fig. 5. Schematic presentation of the morphology or the *Cryptococcus neoformans* based on usual microscopic preparation (A) and on electron-microscopic presentation (B). The stainability in Fig. 5 A (given in parenthesis) refers to the diagnostically ample PAS reaction.

CV	= central vacuole	O	= osmiophil granular section
F	= fat droplet	OLZ	= outer layer of the cell wall
ILZ	= inner layer of the cell wall	PL	= portions of the capsule,
M	= mitochondria	R	= endoplasmic reticulum
MH	= mucous halo of the capsule	RPP	= remaining portions of the protoplast
N	= nucleus	VIZ	= viscous inner zone of the capsule

Light microscopic findings revealed the following staining properties of the cell wall (outer layer mentioned first) : HE: deep violet/light tint of red; PAS dark/pink; methyl green-pyronin: dark/red; methenamine-argentic nitrate: black/brownish; acridine orange-fluorochromation (pH 7): green/none; sulphation-acridine orange-fluorochromation (pH 2.7): orange/none. With other stains the cell wall appears as a uniform structure; for example, black with HALE, green with GOLDNER, and black with hematoxylin according to R. HEIDENHAIN.

Cell Capsule

We were able to define capsules with a width of up to 2.1 μ. Similar to TOMCSIK (1956), the cell capsule had to be subdivided in a viscous inner zone which is hard to separate from the cell wall, and in a less viscous outer mucous halo. The latter preferably showed up in cases where the growh of *Cr. n.* was not inhibited and where colonies were formed. But there are also many Cryptococci without any indication of such a mucous halo. The representation of the inner capsule region of *Cr. n.* always was successful with the HALE reaction (blue), often with the PAS reaction (pink) and occasionally with HE (violet) or hematoxylin stain according to HEIDENHAIN (transparent black). It should be noted, however, that even positive results of the last three staining techniques failed to give a complete presentation of the capsule. With light microscopy, however, proper staining of the mucous halo was obtained solely by means of the HALE reaction (sometimes a less intensive blue than found with the inner region of the capsule). With all other stains the mucous halo appears as an optical void around the *Cr. n.* Electron-optical findings revealed that the inner region of the capsule has a 'granular-filamentous-reticular' structure of different density. The outer mucous halo of electron-microscopic preparations remained intact with only a few Cryptococci and is much more electron-lucent than the capsule. It consists of loosely arranged and often reticular meshed filaments, and exhibits a continuous contact with the capsule.

DISCUSSION

Fig. 5 shows a schematic presentation of the typical morphological structure of *Cr. n.* as found in our studies. To our knowledge, the contributions to the morphology of *Cr. n.* found in the relevant literature give no complete display of its optically detectable structure and, in part, deal with the individual organelles of this fungus in quite another context (FEDER & SIDMAN, 1958; EMMONS, 1959; BADER, 1965; BADER & ROHDE, 1970).

Possible autolytic alterations need not be considered because Cryptococci transposed by vaccination from postmortem material to cultures or experimental animals showed a propagation on the

latter. In addition, electron-microscopic control tests with cells of the apathogen species Cryptococcus diffluens - which were fixed in isotonic osmium tetroxide solution immediately after being taken from the culture - principally resulted in pictures of analogous nature. The presence of the central vacuole in our test material also speaks against any artificial alteration as the central vacuole is a highly sensitive organelle which, in consequence, may be taken as an indicator with reference to the state of maintenance of the individual fungus cells (MUNDKUR, 1964; BADER, 1965). As is generally known, degenerating fungus cells will loose their central vacuole to form a so-called bell.

In our opinion an additional discussion seems feasible with regard to central vacuole, cell wall and cell capsule.

The central vacuole is devoid of a distinguishing morphological structure. According to our electron-microscopic findings the central vacuole represents a cell region enriched with fluid which can be associated to the ground plasm, and which features a higher turgor then that of the remaining cell sections. Occasionally, however, this region may be limited by membranes of the endoplasmic reticulum or by (deformed) membranes of other organelles.

Our findings have shown that the cell wall generally consists of two layers. Solely during germination the wall of the newly created fungus cell may temporarily consist of a single layer. Here it is always the outer layer - mainly consisting of chitin - which is missing. As already described, the thickness of the capsule, which mainly consists of acid mucopolysaccarides, can differ to quite an extent. Newly formed Cryptococci - which frequently are still in contact with the parent cell - can be free from such a capsule. Moreover, the capsule is often missing in the case of degenerated species, especially with Cryptococci situated in human macrophages.

Zusammenfassung

Da wir in der Literatur keine umfassende Darstellung der Morphologie des *Cryptococcus neoformans* finden konnten, hielten wir eine licht-, phasenkontrast- und elektronenmikroskopische Untersuchung dieses Sproßpilzes für angezeigt. Das Material hierfür stammt von zwei menschlichen Obduktionsfällen mit tödlich verlaufender Cryptococcose. Die morphologischen Befunde werden detailliert dargestellt. In einer schematischen Abbildung wird die morphologische Gesamtstruktur des *Cryptococcus neoformans* noch einmal veranschaulicht und dem morphologischen Bild, welches dieser Pilz mit geeigneten Färbungen in den in der üblichen Weise angefertigten mikroskopischen Schnitt- und Ausstrichpräparaten zeigt, gegenübergestellt.

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