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Histochemical Studies of Rhinosporidium seeberi*

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Summary. The generative cycle of Rhinosporidium seeberi is described with some new results and interpretations. With several routine staining methods and histochemical reactions it was possible to study especially the: a) germinative zone of the sporangium, b) structure of the spore, c) implantation form.

Detailed histochemical studies of fungi causing visceral mycoses included also Rhinosporidium seeberi (Bader and Rohde, 1970; Bader, 1970). These results supplement and clarify previous findings (Ashworth, 1923; Karunaratne, 1964; Narayana Rao, 1966).

Zusammenfassung. Beschreibung des Entwicklungscyclus von Rhinosporidium seeberi mit einigen neuen Ergebnissen und Interpretationen. Mit zahlreichen Routinefärbungen und mit histochemischen Reaktionen wurden besonders untersucht: a) Germinativzone des Sporangium, b) Aufbau der Spore, c) Struktur der Implantationsform.

Material and Methods

Polypous tissue material from three cases of fully developed nasal mucosal rhinosporidiosis was used for the study.

The tissue was embedded in paraffin and sections were subjected to the following staining methods: hematoxylin-eosin (HE); Van Gieson stain; azan (Heidenhain); Weigert's resorcin-fuchsin; reticulumstain (Gömöri); methylviolet; congo red; Giemsa; Goldner's connective tissue stain; trichrome stain (Ladewig's modification); modified Weigert's technique for myelin sheaths; Romanes silver method for neurofibrils; Weigert's fibrin-stain; iron stain; Kossa's stain (calcium); periodic acid Schiff reaction (PAS); alcianblue-stain; PAS-alcianblue; Hale-Müller-method (colloidal iron); Hale-PAS; methylation-alcianblue and demethylation-alcianblue; methylation-PAS and demethylation-PAS; paraformic acid-PAS, paraformic acid-alcianblue; chromic acid-methylenamine-silvernitrate reaction (Grocott-Gömöri [G-Ag], after $^1/_2$, 1, 2 hours); Gram; Ziehl-Neelsen (decolourisation with hydrochloric-acid-alcohol and with hydrochloric acid only); aldehydfuchsin; aldehydfuchsin-alcianblue; ninhydrin — Schiff reaction; acrolein — Schiff reaction; alloxan — Schiff; diazo reaction; tryptophane-reaction (Adams); DDD-method; amidoblack 10 B; histidine reaction; fastgreen stain; gallocyanin-chromalum; methylgreen-pyronin; cresylviolet (Nissl); ribonuclease fermentation; Feulgen-reaction; test of basophilia (pH 1—10); thionin-reaction; toluidin-reaction.

Structures investigated histochemically are:

1. Implantation form, a) capsule, b) cell wall, c) content of cell;
2. Early trophocyte (30 to 50 μm ⌀), a) cell wall, c) cytoplasm, c) nucleus;
3. Mature trophocyte (50 to 100 μm ⌀), a) cell wall: inner, middle and outer zone, b) cytoplasm: granulary and filamentous structures, c) nucleus, d) nucleolus;
4. Germinative cell (up to 2 μm ⌀), a) capsule, b) cell wall, c) nucleus;
5. Germinative cell (up to 6 μm ⌀), a) capsule, b) cell wall, c) content of cell, d) nucleus;
6. Early spore, a) capsule, b) cuticule, c) cell wall, d) nucleus;
7. Mature spore, a) capsule, b) cuticule, c) cell wall;
8. Thread-like substance of sporangium (between the spores);
9. Sporangial wall: inner, middle and outer zone;
10. Degenerate implantation form, cell wall;
11. Degenerate trophocyte, a) cell wall: inner, middle and outer zone, b) content of cell;
12. Degenerate sporangium, cell wall, a) early phase, b) late phase.

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Results

In contrast to most of the other pathogenic fungi, the tissue forms of Rhinosporidium can be adequately demonstrated by common staining methods including hematoxylin-eosin staining.

The vegetative phase is formed by the *Trophocyte* (Karunaratne, 1964), which is a spherical or oval cell with the diameter of 15—100 μm and a cell wall of about 1—3 μm thickness. The cell wall is laminated. Histochemical investigation suggests that it consists of a chitinous membrane incorporating PAS-positive neutral polysaccharides of a hemicellulose character, possibly of low molecular weight. On both sides there is a layer of acid compounds, the inner layer probably representing a membrane of condensed cytoplasma (Fig. 4). The trophocyte shows small to coarse granular cytoplasmic particles, 1—2 μm in diameter. They are composed of acid polysaccharides; some thread-like structures appear to contain protein. Small granules, mainly seen in young trophocytes frequently also consist of neutral polysaccharides. These granules as well as inclusions of fatty material may function as storage globules (Karunaratne, 1964). During maturation of the trophocyte, these Hale- and alcianblue-positive cytoplasmic granules grow lager, become more numerous and occupy the whole cell when it has reached a diameter of about 50 μm. They may still be found in the periphery of the germinative zone of sporangia in connection with membranous structures.

The nucleus is a vesicular structure with a diameter of 4—13 μm. No condensations of chromatin are present in it. Brief, mild acid — hydrolysis decomposes the DNA. Slightly acid fixatives are sufficient to cause this effect. RNA or DNA are not stained specifically in these fungus cells by methylgreen — pyronin, even if the surrounding tissue showes positive reaction (however, a non-specific, though definite pyronophilia is seen in coarse granules of trophocytes, as well as in spores and in cell walls of sporangia). Each nucleus has a solitary nucleolus of 2—4 μm diameter. The margin is often slightly refractile. It shows frequently one or occasionally several small vacuolated areas. The nucleus shows light, the nucleolus strong basophilia with hematoxylin and eosin. The following diameters (in micron) of nuclei and cells of trophocytes were found:

Cell size	10	15	25	40	60	80	up to 100
Nuclear size	4	5	7	8	10	11	13
Nucleolar size	—	2	2.5	3	3.5	3.5	4

Ashworth (1923), quoted by Karunaratne (1964), has stated that mitotic nuclear divisions commence in trophocytes of about 50 μm diameter. 4 chromosomes pro nucleus are supposed to appear attached to the nucleolus. In our material extensively examined, 4 nuclei with nucleoli were found as a maximum in a single transverse section of trophocytes. In spite of a multitude of staining methods and of intensive search we did not find mitoses in the granular cytoplasm. At least in the later stages of nuclear division, we presume, that an amitotic mechanism is operative which is supposed to produce up to 16,000 nuclei without visible nucleolus (Karunaratne, 1964).

During a transitional stage toward the generative phase, a semi-lunar generative zone is found near the margin of the cell (see later). At the same time, the width

of the cell wall doubles in cells of 50—100 μm diameter. Initially there is also a lamination of the membrane which disappears subsequently. The cell wall of the early *Sporangium* contains acid compounds, the amount of which increase from the inner to the outer part of the cell wall. At the same time the aldehyde groups, as demonstrated by PAS-reaction, disappear gradually, though zones of PAS-positive material remain visible for some time in the inner layers. It remains a cell wall with a fairly constant width about 2 μm, which store up increasing acidic groups, probably sulfated polysaccharides (Narayana Rao, 1963, 1966; Sood and Narayana Rao, 1967). There is a small inner blue zone with fringes as demonstrated by the Hale — PAS-reaction; peripheral to this, a broad layer of light pink chitinous material is surrounded outside by a small PAS-positive shell (Karunaratne, 1964).After brief application of the G-Ag-method, a dense network of circularly arranged, slightly wavy threads is visible (Fig. 2a). These may represent a cellulose scaffolding within the chitinous wall. Results of the G-Ag-method and PAS-stain, however, are not always parallel. Homogenous, PAS-positive masses without structure are often poorly stained by G-Ag. On the other hand, silver deposition is particularly strong in some structures, like cellulose and hemicellulose (see Bader, 1970). It is therefore not surprising that the PAS-reaction is only very weak or absent in the walls of sporangia during maturation, in spite of a positiv silver reaction. Also there are differences between Hale-Müller-reaction and alcian-blue. An argyrophilic coarse network, which is arranged in a predominantly circular fashion is Gram-positive and alcianphilic, but the Hale-reaction is negative. Similar discrepancies are found in staining the mucous capsule of spores in early development (see later). The system of canaliculi, described by Karunaratne (1964) could not be shown in the wall of sporangia.

A previously neglected area is the *Germinative Zone* of sporangia in which a constant formation of *Spores* takes place (Fig. 1a—c). This is possibly not only due to a maturation process but due to a constant nuclear division. The structural processes within the germinative zone have been investigated by a fractionated G-Ag-method. Immediately inside the cell wall, only separated by a few Hale — and alcianblue — positive threads, the most immature cells forms appear. They are flat cellular units, initially of about 1.5—2 μm diameter. After prolonged application of the silver method marginal structures resembling cell walls can be shown (Fig. 1c). At the same time, there are PAS-positive bodies in the interior, which have initially a diameter of up to 2 μm are oval and resemble immature stages of spores. Hematoxylin-eosin stain shows most of the cell forms of this fungus exceptionally well. These stages consist of a small violet membrane with an eccentric round, considerably basophilic central body, which probably resembles the nucleus (Fig. 1b). With advancing maturation the central part stains better. The cells are larger and their margins — similar to cell walls — are arranged polygonally (mainly hexagonally) in a honeycomb fashion (Fig. 1a). The substance around the spores shows a positive Hale- and alcian-blue-reaction at this developmental stage and stains red with Gram. This substance has its origin in the marginal areas of the spore-forming cell. It appears to be more probable that the perisporular "mucoid cell wall", inspite of its boundary resembling a cell-membrane, is formed by rough granulations of the trophocytes which reacts histochemically in a similar fashion (the cuticula has probably to be considered

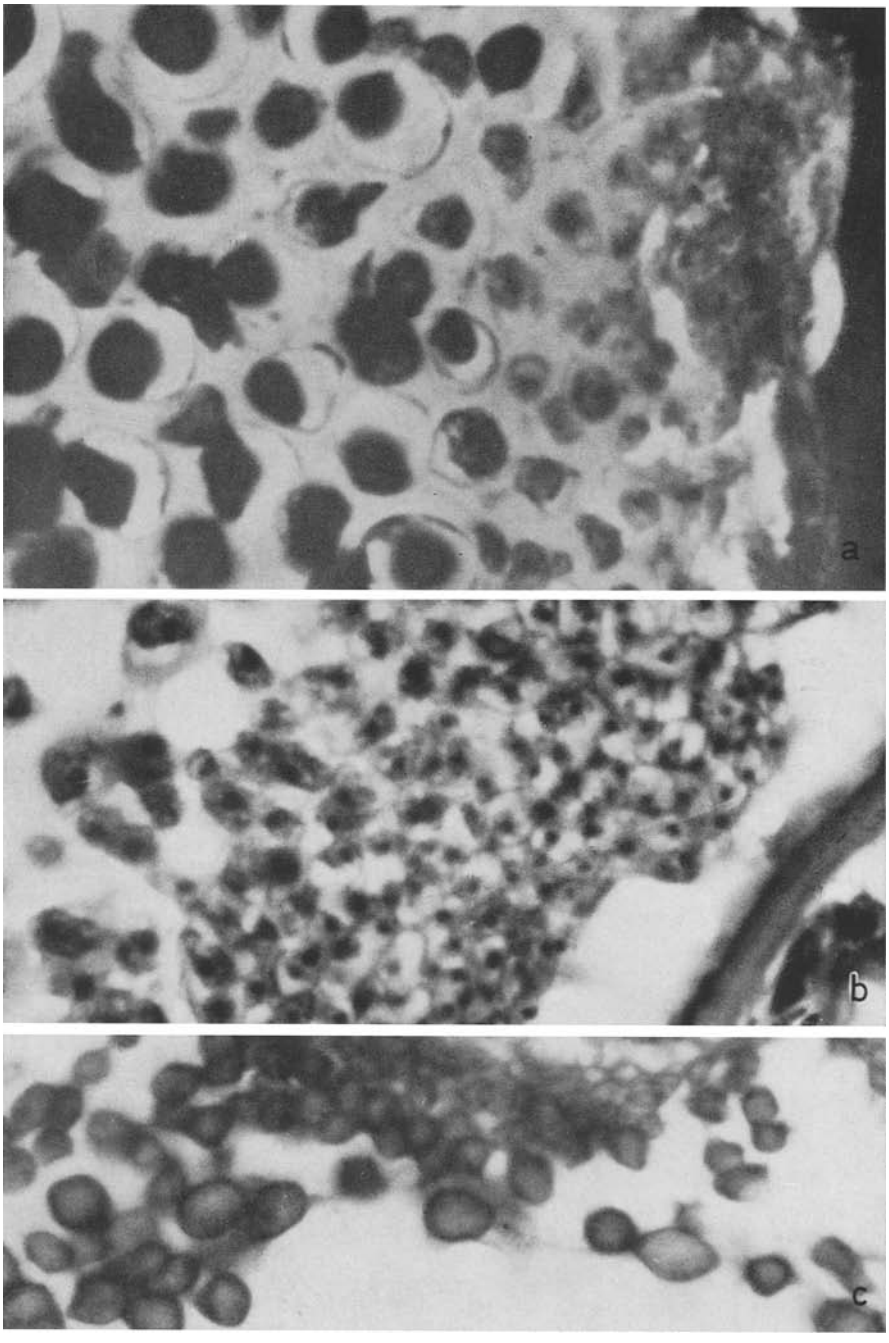

Fig. 1 a—c. Germinative zone of sporangium. a) Black stained cell wall, spores and praespores with black thin cuticula and gray mucoid capsule in a honeycomb fashion. G-Ag-stain. b Praespores and spores with nucleus like inclusions, sometimes visible capsules. Cresylviolet stain. c Early spores with smooth surface. G-Ag-stain. Magnification: Fig. a—c 1,900 ×

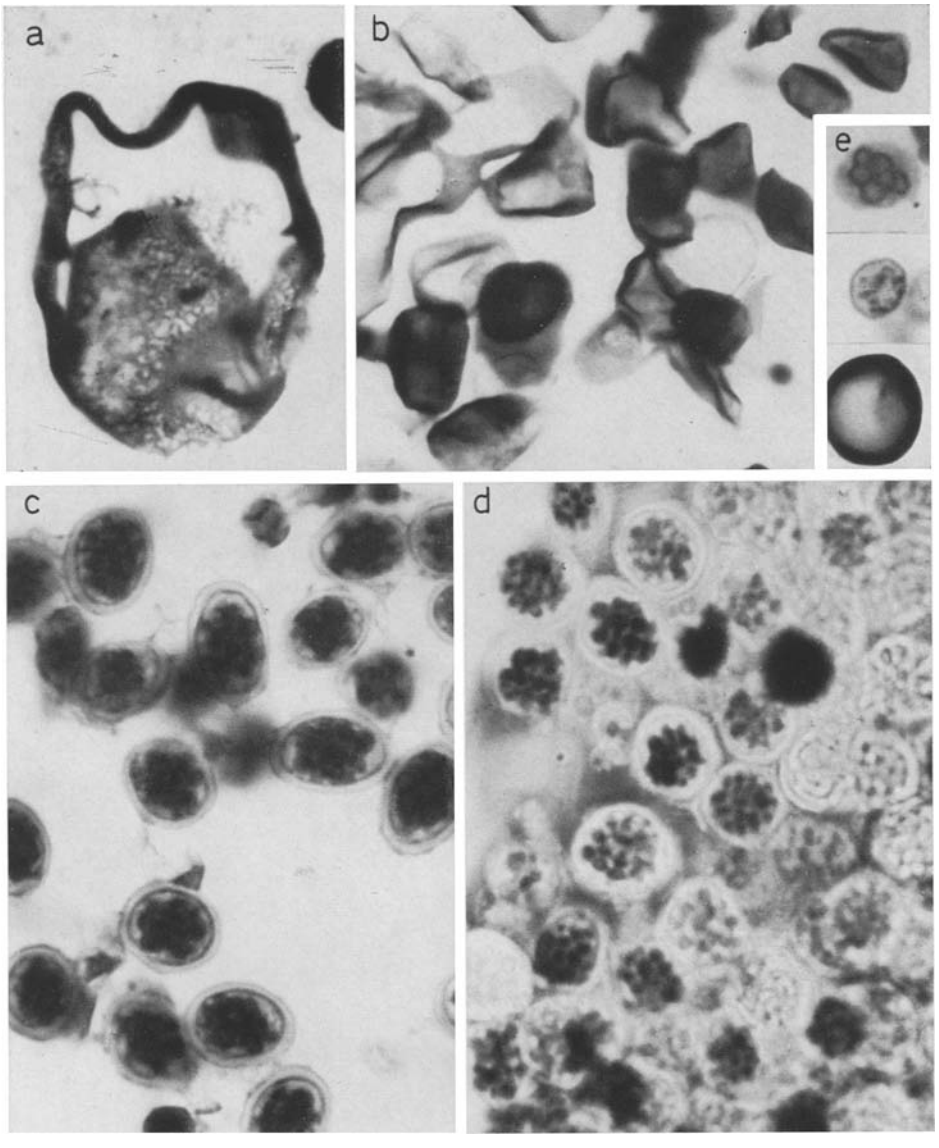

Fig. 2. a Network of G-Ag-positive threads in cell wall of sporangium. G-Ag-stain. b Degenerate forms of implantation cells and young trophocytes. G-Ag-stain. c Morula-like spores with well visible cuticulae and outside distinct lined capsules. Carbolfuchsin stain. d Maturate spores lose proteins (refractile cells) — black spores in a morula fashion still have proteins in the cell wall. Amido-black 10 B stain. e Evolution of morula-like spore with capsule (G-Ag-stain) into early implantation form with PAS-positive granules (PAS-stain) and late implantation form (Gram stain). Magnification: Fig. a 900 ×, Fig. b—d 2,000 ×, Fig. e × 1,500

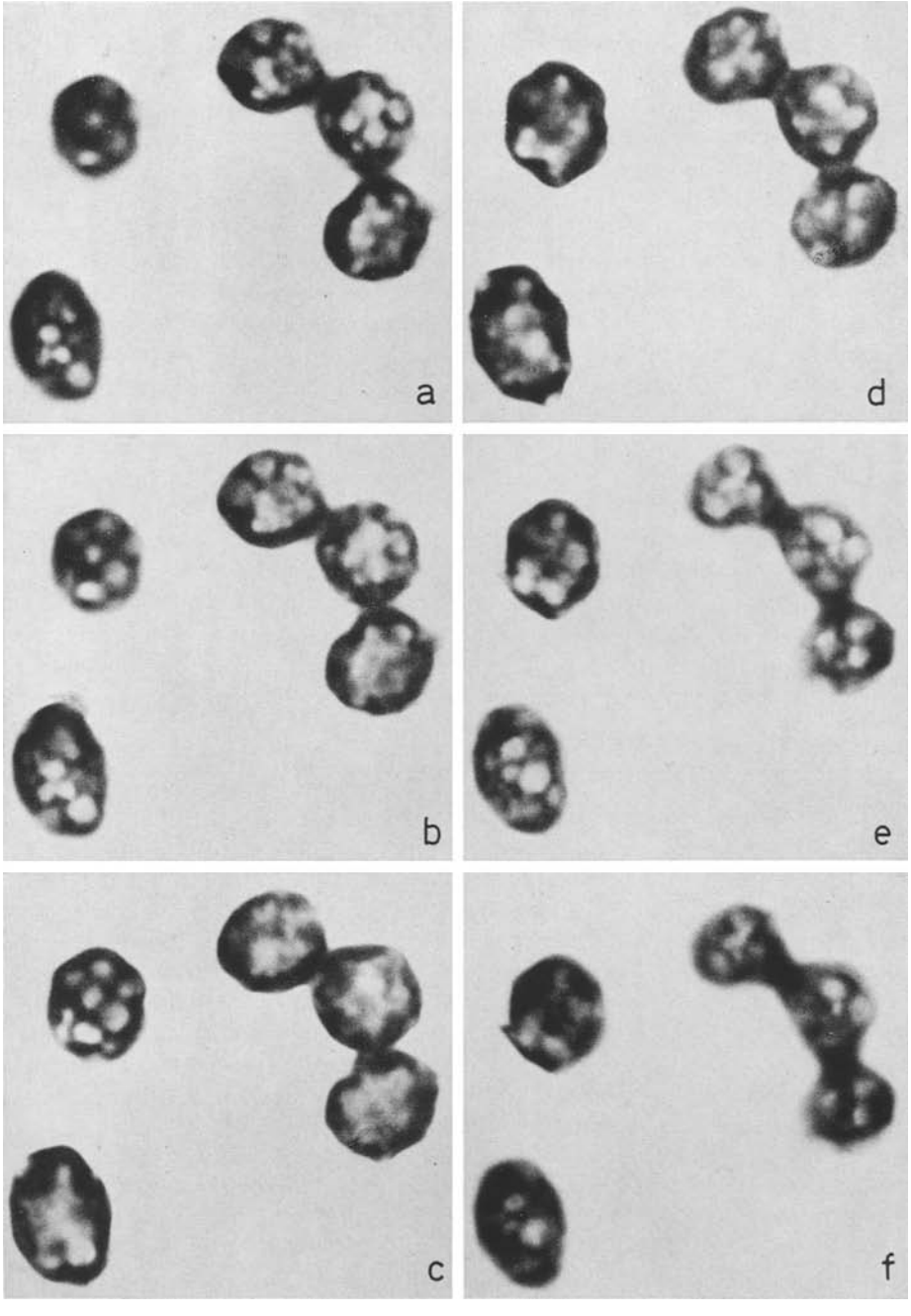

Fig. 3a—f. Tomography of maturate spores demonstrate in Fig. c and d a single spore cell in each group of "spherules". "Spherules" are the morula-like surface of the spores — see Fig. a and b, e and f. Magnification: Fig. a—f × 1,725

to be the wall of the sporulating cell, see below). During this process of maturation, the PAS-positive immature spores undergo also changes: at size of 2—4 μm there is only little argyrophilia of the wall, even after prolonged G-Ag-staining. Further details of sporulation can be demonstrated with this method. The centre of the spores shows small argyrophilic granules, which are accumulating in one area, usually in the centre. At a spore size of 4—6 μm, circular figures appear and the cell wall stains more intensely with silver but remains smooth at the surface (Fig. 1c). Only from 6 μm cell diameter do the walls appear intensely black with a mulberry surface (Fig. 2c and d; Fig. 3). PAS and other stains show circular structures of 2—4 μm diameter. These previously called spherules (Ashworth, 1923; Narayana Rao, 1966). The thick morula-like wall of the spore is refractile and shows in flat, silverstained sections small subunits, 12—16 spherules per spore (Fig. 3). During further maturation the diameter of spore increases finally up to 10—12 μm. Further structures become visible with various staining methods. Hematoxylin-eosin stain shows increasing basophilia of the cell wall up to 6 μm diameter. However, the stainability decreases during "morulation". They lose their proteins in amido-black 10B stain (Fig. 2d). For a short time-immediate before maturation — the spore wall contains very much sulfhydril groups. The excentric nucleus, which is free of nucleoli, is often still visible in mature spores. It increases in diameter, whilst the basophilia remains the same.

The following relations between nucleus and cytoplasm of the spores were observed:

Size of spores (in μm)	2,0 to 3,0	3,0 to 4,0	4,0 to 5,0	about 10,0
Size of nuclei (in μm)	1,0 to 1,5	1,5 to 1,9	1,9 to 2,2	about 3,5

The polygonal zone surrounding the spore undergoes a particular transformation during the maturation process. The sharp boundary of this substance around the spore is lost after separation of the spore from the germinative zone (Fig. 1b). A firmly adherent capsular substance appears. It is increasingly Hale- and alcian blue-positive, but PAS-negative. The G-Ag-reaction is only slightly positive. The mature spore is surrounded by a kind of cuticula (Fig. 2c) which often protrudes in one, or occasionally in 2 or 3 places in a vesicular fashion. This is probably the genuine wall of the sporulating cell. The space between the spore and the wall (cuticula), shows hardly any staining by any of the histochemical methods used. The capsule almost always remains connected with the spore up to the implantation stage. The cuticula stains strong with carbolfuchsin (Fig. 2c). At full maturation of the spore (10—12 μm diameter) the capsule consists of strong acidic (sulfated) polysaccharides (Ashworth, 1923, already believed that it were mucin). Spores which lose their capsule and cuticula are possibly degenerating (the majority of the spores?). There is no change in composition of the wall of the spore (of neutral polysaccharides), sometimes acidic groups cover the red staining of the wall in combination stains (Hale-PAS, alcian-blue-PAS). Between spores with their capsules, there are threadlike masses, showing the same histochemical reactions as the capsular substance.

With continuing growth, the wall of the sporangium stretches and becomes thinner due to the increasing numbers and size of spores and their capsules. At

early stages (100—125 μm diameter of the sporangium) the width of the wall is 3—6 μm. At the maximal diameter (300—400 μm) the wall width is constant at 2 μm.

Ashworth (1923) and Karunaratne (1964) found a temporary circular thickning of the sporangial wall up to 12 μm. This is supposed to be the site where rupture will take place ("pore"). However, the present authors could not find this structure anywhere in mature sporangia. Our investigations show frequently variations in the width of sporangial walls which are often due to tangential sections. We also found folds in the wall. We can only state that the site of rupture is distant from the germinative zone.

Rupture of the sporangium is probably due to exogenous mechanical pressure. Ashworth (1923) mentions the possibility of suddenly increasing internal pressure. It is possible that the increasing infiltration of the surrounding tissue with polymorphonuclear leucocytes causes a swelling of mucous substances within the sporangium.

The *Implantation* is initiated by disappearance of the "spherules". The rough tubercular wall of the spore becomes smooth as the cell takes up fluid. Cytoplasmic granules appear which are PAS- and strongly silver-positive (Fig. 2e). At this stage, the diameter of the smallest trophocyte is not below 10 μm. This is one of the facts which suggests that the "spherules" are not spores. Soon, the cuticula disappears and the small trophocyte loses its capsule. With increasing size, the cytoplasm becomes granular, but remains PAS-positive. Only later, acid groups are present within the granules (see above). Already earlier (at 20 μm) a nucleus becomes apparent in which a nucleolus is formed. At this developmental stage the cell wall is clearly PAS-positive. The G-Ag-reaction which is initially strongly positive, however, decreases to a grey and even brown appearance in large trophocytes, even after prolonged silver impregnation. The growth cycle of the organisms is completed at this developmental stage (Fig. 4).

The frequency of the various stages of development is difficult to assess (see also Ashworth, 1923) apart from the fact that the implantation stage is rarely found; probably it is a shortlived transition. The larger the organisms, the more frequently they must appear in the section.

Cells of the vegetative as well as the generative phase are frequently found in clusters. One can assume that in a circumscribed area of the tissue several spores are implanted at the same time and that a synchronous development takes place.

Ashworth (1923) did not believe in a transepithelial reinfection. We, however, frequently found implanted spores between the cells of the surface epithelium of nasal mucosa. This confirms Karunaratne's observations (1964).

In contrast at a diameter of 20 μm all organisms are found in the subepithelial connective tissue (lymphogenic transport and deposition of Rhinosporidium, Ashworth, 1923). Smaller spores are only rarely found in macrophages. After rupture of a sporangium, spores are occasionally found intraepithelially in large numbers mixed with polymorphs ("pseudocysts").

The walls of empty sporangia remain in the tissue for considerable periods, though some of them are expelled by leucocytic inflammatory processes. As compared to mature sporangia the walls become thicker due to shrinkage and are

often folded and kinked. Calcification sometimes occurs. Occasionally there are remnants of the germinative layer inside of the wall.

Degenerating organisms can also be found at all other stages of development. They are particularly frequently seen as remains of degenerate trophocytes and of implantation forms (Fig. 2b). The cell walls of degenerated implantation forms are clearly demonstrated with G-Ag stain. They are frequently found in clusters in the tissue, appearing as bell shaped or creased remains of the cell walls. Degenerate forms of large trophocytes are recognisable by coarse vacuolation of the cytoplasm, which stains homogeneously PAS-positive and eosinophilic with H. E. The nucleus disappears and the cell wall becomes thicker, particularly in its external Hale- and alcian-blue-positive zone.

Reproduction of Rhinosporidium outside its sporulation organs was not found, for instance cell divisions of trophocytes were never seen. Development of trophocytes already within a sporangium, postulated by Ashworth (1923) and Karunaratne (1964), were not observed. We would like to stress that artefacts in histological sections due to brittleness of spores and sporangial walls (for instance displacement of fungal cells) are easily possible.

Protein reactions are seen in cytoplasmic and nuclear structures (nucleolus), are also possible in the inner zone of the cell walls of sporangia and the inner and outer layers of large trophocytes.

Positive Feulgen-reaction has been in condensed DNS of nucleus in germinative cells and spores.

Discussion

The present histochemical investigation of the composition of various developmental forms of Rhinosporidium seeberi allows some fresh or more detailed conclusions about their structural nature (cp. Fig. 4).

1. Histochemically the chitinous cell wall shows multiple layers similar to those described for the sporangial wall of Aspergillus and Mucor (Bader and Stiller, 1965). During growth (trophocyte) the wall still contains hemicellulose-ike low molecular polysaccharides which are PAS-positive. These disappear in the sporangium after maturation while many free (probably sulfated) acid groups are now present. With the G-Ag-method a dense network of threads can be demonstrated in the cell wall, presumably consisting of cellulose. After methylation additional acid groups are set free. Our results of some histochemical methods in spores and sporangia correspond to studies of Narayano Rao, 1966, but our interpretation is different.

2. It is possible that the final nuclear divisions in trophocytes are amitotic and that amitotic division is continuing in the germinative zone of the sporangia.

3. A detailed description of the germinative zone of the sporangium, which has attracted little attention previously, is given in the text above. Rupture of the sporangium takes place opposite the germinative zone. At this site no preceeding alteration of the cell wall was observed in contrast to the observations of previous investigators (Ashworth, 1923; Karunaratne, 1964).

4. The "spherules" of Ashworth (1923) are not spores but simplyprotrusions of a morula — like cell wall of the spore. This could definitely be demonstrated by the G-Ag-method (Fig. 3). This supposed in his text also Narayano Rao (1966).

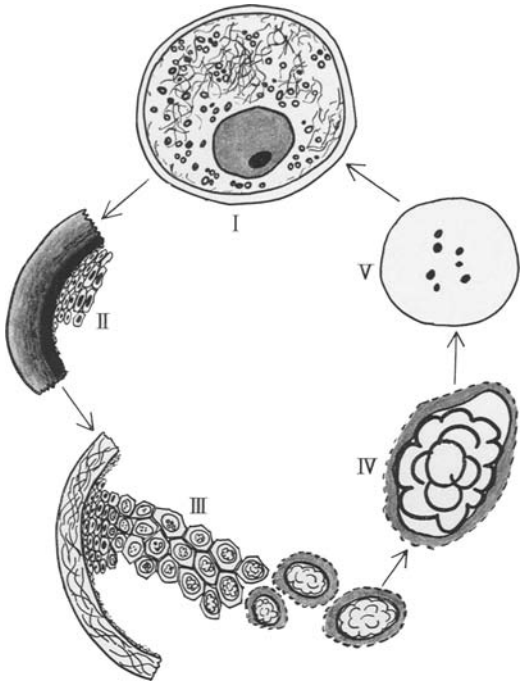

Fig. 4. Cyclus of Rhinosporidium seeberi. *I* Trophocyte. *II* Early sporangium with germinative zone; increasing acid groups in the inner layers of cell wall. *III* Maturate sporangium with spores; network of cellulose in the cell wall. *IV* Maturate spore with cuticula and capsule *V* Implantation form

The smallest implantation forms are never smaller in size than mature spores of 10—12 μm diameter (see also paragraph 5). The mature spore is surrounded by a delicate membrane (cuticula) which shows histochemically little activity. By means of the aldehyde-fuchsin and aldehyde-thionin methods it was shown that the cuticula includes sulphur-containing compounds, probably precursors of sulphate radicals in the capsular substance (further details see Bader and Rohde, 1970). This cuticula is probably a remnant of the cell wall of the germinative cell and of the immature spore.

5. Structural alterations of the spore during its implantation stage are described in the preceeding text.

6. Mature sporangia have considerable leucotactic properties. Other fungi, e.g. Coccidioides immitis, show a similar developmental cycle. It has been questioned whether the acid groups of the tissue forms may alter the acidity of the tissue and cause a purulent inflammatory reaction. On the other hand, the capsule of Cryptococcus neoformans consists of sulfated polysaccharides (Bader, 1965) with the opposite effect i.e. suppression of leucocytosis in the tissue. It is possible that the capsular substance of the spores of Rhinosporidium (implantation form) has a similar protective function despite its strongly acid reaction. The capsular substance may possibly also be important for initial implantation processes in the tissue.

7. Results of PAS- and G-Ag-methods do not always show parallel results (details see Bader, 1970). The silver method is considerably more sensitive and shows also cellulose and hemicellulose, even PAS-negative degenerative forms of the fungus. Higher sensitivity of the G-Ag-reaction is probably due to a more efficient interaction with periodic structures to which the silver ion attaches. Similarly divergent results have also been found between Hale-Müller-reaction and alcian-blue-stain.

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