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Rhinosporidiosis: immunohistochemical and electron microscopic studies

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Abstract

Sixteen biopsies of rhinosporidiosis (15 nasal and one conjunctival) from 16 Southern Indian male immigrant workers showed mucosal lymphoplasmacellular infiltrates together with transepithelial elimination of nodular bodies and destruction of some late stage nodular bodies in histiocytic granulomata with central neutrophilic microabscesses. Early nodular bodies were immunohistochemically positive for alpha$_1$-AT, alpha$_1$-ACT, CEA, S100, fibronectin, amyloid-*p*-component, IgG, IgA, C_{1q} and C_3. Electron microscopy showed organized concentric lamellated bodies in early nodular bodies and not in end-stage nodular bodies which contained mostly amorphous electron dense materials. Structures formerly regarded as 'sporangia' and 'spores' are believed to be lysosomal bodies loaded with indigestible residues to be cleared via transepithelial elimination or segregated/destroyed by secondary immune/granulomatous responses.

Key words: Rhinosporidiosis; Lysosomes; Immunohistochemistry; Microscopy, electron

Introduction

Rhinosporidiosis is a chronic polypoid granulomatous disease of the anterior nares and nasopharynx. Other uncommon sites of involvement include the conjunctiva, lacrimal sac, skin, oral cavity, larynx, vagina, rectum, urethra, bone and brain (Mohapatra, 1971; Sasidharan *et al.*, 1987). It is seen in the younger age group (20 to 40 years) and is more frequent in males than females. The disease is reported most commonly from India and Sri Lanka but smaller series and sporadic cases have been described from other geographical areas. Rhinosporidiosis occurs naturally in man and animals including horses, mules, cattle, goats, dogs, wild ducks and geese, and the parrot *Psittacus ondulatus*, (Ramachandra *et al.*, 1971), but, the 'organism' has never been successfully, transmitted to experimental animals.

The unique round structures histologically diagnostic for rhinosporidiosis were first described by Seeber (1900) as a protozoan, later by Ashworth (1923) as sporangium of a fungus he designated *Rhinosporidium seeberi* and recently as lysosomal bodies loaded with indigestible residue somewhat reminiscent of lysosomal storage diseases. 'Sporangia', therefore, have been redesignated nodular bodies and 'spores' as spheres of cellular waste (Ahluwalia, 1992).

In this article we report immunohistochemical and electron microscope findings and describe histopathological features of potential immunological interests in rhinosporidiosis. We also underline transepithelial elimination which in addition to its significance as a biological phenomenon may account for the usefulness of cytological preparations in diagnosis and the large numbers of nodular bodies and spheres of cellular waste in the nasal secretions described in several papers (Jimenez *et al.*, 1984; Van Der Coer *et al.*, 1992).

Materials and methods

Formalin-fixed paraffin embedded tissue blocks of 16 cases of rhinosporidiosis in the files of the Department of Pathology, Hamad General Hospital, Doha, State of Qatar, diagnosed between 1984 and 1993 were available for examination.

Sections (5 μm thick) were stained with haematoxylin and eosin (H&E), periodic-acid-Schiff with and without diastase (PASD and PAS), alcian blue/PAS pH 2.5 (AB/PAS) and Congo red methods.

Immunohistochemical staining was performed by a peroxidase-anti-peroxidase (PAP) procedure (Filipe and Lake, 1990) using a panel of monoclonal and polyclonal antibodies raised against human antigens (except where otherwise specified); all antibodies were obtained from DAKOPATTS, Denmark except for anti-chromogranin A (from Enzo Diagnostics, U.S.A.). Antibodies (dilutions) included mouse monoclonals to cytokeratin (DAKO-Ck$_1$, LP$_{34}$, 1:10, which recognizes keratin 18 and 6 but not 1, 8 or 19), swine vimentin (1:10), desmin (1:10), epithelial membrane antigen (EMA 1:10) and chromogranin A (1:50), and also rabbit polyclonals to bovine glial fibrillary acedic protein (GFAP 1:100), myoglobin (1:100), carcinoembryonic antigen (CEA 1:10), cow S100 protein (1:50), neuron-specific enolase (NSA 1:100), Von Willibrand factor (VIII related antigen 1:100), fibronectin (1:200), amyloid-*p*-component (1:50), alpha$_1$ anti-trypsin

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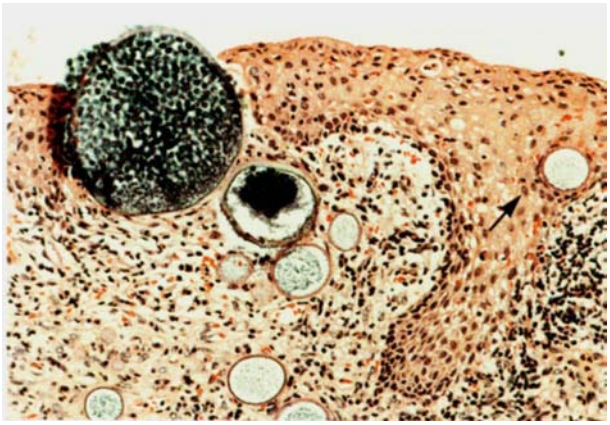

FIG. 1

Transepithelial elimination in nasal rhinosporidiosis showing 'mature' nodular bodies on the way out. Note a small 'early' nodular body (arrowed) in transit in the squamous epithelium. (H & E; × 67).

(α_1-AT 1:100), alpha$_1$ anti-chymotrypsin (α_1-ACT 1:100), immunoglobulin heavy chains IgG (1:500), IgA (1:400), IgM (1:400), immunoglobulin light chains Kappa (1:300), lambda (1:300), complement components C_{1q} (1:10) and C_3 (1:10).

Secondary antibodies were rabbit anti-mouse (1:25) for monoclonals and swine anti-rabbit (1:25) for polyclonals; tertiary antibodies were mouse PAP (1:25) and rabbit PAP (1:25) respectively.

Endogenous peroxidase activity was blocked with three per cent H_2O_2 in Tris-buffer for 30 minutes. Enzymatic digestion was carried out by 0.025 per cent protease (incubation at 37°C) for 5 to 20 minutes prior to treatment with primary antibodies except for EMA, vimentin, chromogranin and NSE. All other incubations were performed at room temperature (about 20°C). Appropriate positive controls were included. Attention was also paid to those tissue elements that could act as built-in positive controls. Incubation of slides with omission of primary antibodies were included as a negative control.

Electron microscopy was performed on six cases. Pieces of tissue from paraffin blocks in five cases were removed, deparaffinized in xylene and rehydrated in graded ethanol. In the sixth case fresh tissue (cut into 1 mm pieces) was immediately preserved in Zamboni's solution. The tissue from all six biopsies was fixed secondarily in glutaraldehyde, post-fixed in osmic acid and embedded in Epon. Ultra-thin sections stained with uranyl acetate and lead citrate were examined under a Philips CM_{12} electron microscope.

Results

Sixteen biopsies (15 nasal and one lower eye lid conjunctival) were available from 16 Southern Indian male immigrant workers aged 22 to 36 (mean 27.5) years.

Histology

Unique round nodular bodies (formerly known as 'sporangia') were seen in sections of all cases. Large nodular bodies, arbitrarily classified as 'mature', had a relatively thin cellulose-like wall and contained numerous spheres of cellular waste (formerly known as 'spores'). Small to medium-sized 'early' nodular bodies had a thick cuticle-like wall and contained an eosinophilic structureless matrix.

'Mature' nodular bodies showed a tendency to be located close to the mucosal surface. Transepithelial elimination of nodular bodies was observed in several zones in all cases, predominantly in association with extensive squamous metaplasia and hyperplasia. This was also seen less frequently in respiratory-type epithelium unaffected by metaplastic changes. The process of transepithelial elimination appeared to occur in stages by the nodular bodies moving towards the surface, pressing against it and then breaking through the basement membrane to become surrounded by epithelial cells. Progressive atrophy of the overlying epithelial cells caused eventual opening of a 'window' and expulsion of the nodular bodies (Figure 1). Polymorphonuclear infiltrates in the epithelium or around sporangia in transit were observed in some foci but ulceration of adjacent mucosa was rarely noticed. Lymphoplasmacellular infiltrates in the mucosa had an inverse relationship with the numbers of nodular bodies, to the extent that abundant nodular bodies with very few inflammatory cells had apparently produced an 'anergic' response in some zones (Figure 2) while in other parts of the same specimen dense lymphoplasmacellular infiltrates were associated with few or no nodular bodies. Foamy macrophages were abundant in some areas and a few Touton's giant cells were occasionally present. Disorganized histiocytic granulomata including multinucleated giant cells usually with central neutrophilic microabscesses were seen in a few foci incorporating 'mature' nodular bodies in different stages of disintegration ranging from partially to severely ruptured 'mature' nodular bodies to almost totally destroyed spheres of cellular waste (Figure 3) but were very rarely associated with 'early' nodular bodies. Eosinophilic granulocytes were not often seen.

All nodular bodies (wall and contents) irrespective of size stained red with PAS and retained most of their red colour following diatase digestion (PAS+, DPAS+). 'Mature' nodular bodies stained predominantly blue (Figure 4) in AB/PAS but red PAS positive cores were seen in the centre of most blue-stained spheres of cellular waste (AB+, PAS+). There was no alcian blue staining seen in 'early' nodular bodies (AB−, PAS+).

Congo red (Figure 5) stained only the walls of the nod-

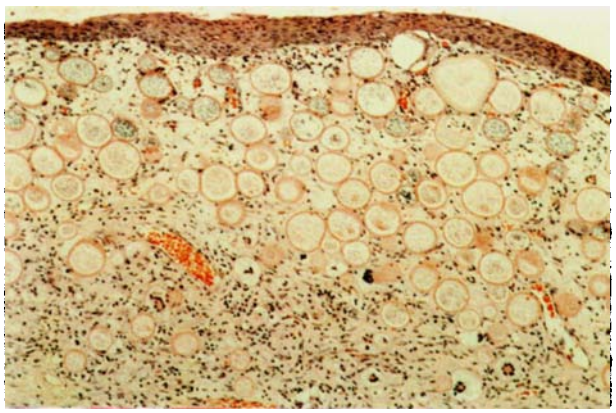

FIG. 2

'Anergic' response in nasal rhinosporidiosis characterized by numerous 'early' nodular bodies; associated with sparse lymphoplasma cellular infiltrates. Also note Touton's giant cells. (H & E; × 55).

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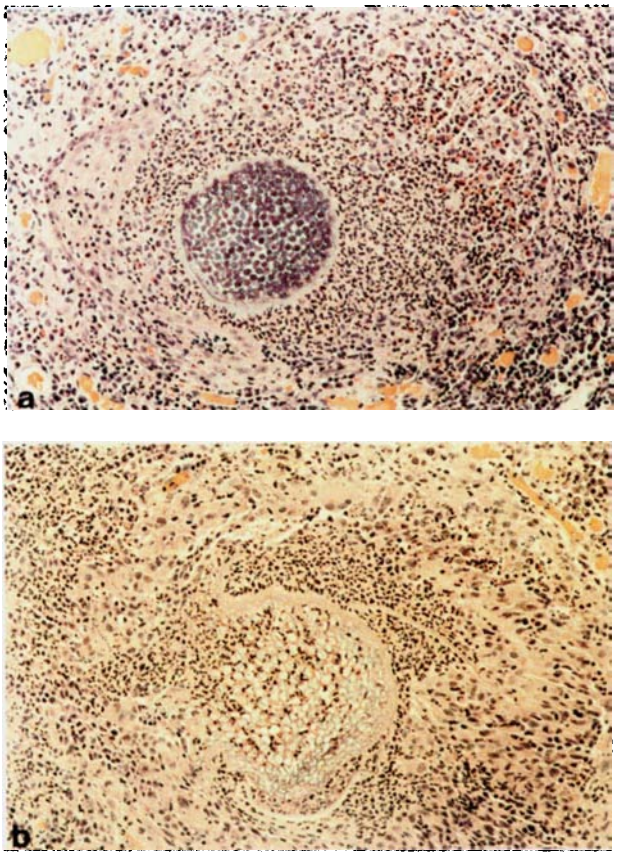

FIG. 3

Stages in destruction of a mature nodular body and sphere of cellular waste in disorganized histiocytic granulomata with central neutrophilic microabscesses: (a) early; (b) late stages. (H & E; × 105).

ular bodies which, under polarized light, especially in ideally oriented 'early' nodular bodies appeared to consist of three concentric layers: a red thick central loop (lamina densa) between two very thin yellowish green loops (lamina rara interna and externa). Various overlapping of these three loops were seen but convincing and characteristic greenish birefringence of amyloid was not noticed.

Immunohistochemistry

Immunohistochemical findings are listed in Table I and partly illustrated in (Figures 6 to 8). The wall and matrix of the 'early' nodular bodies were positive for more antibodies when compared to 'mature' nodular bodies. Where there was positive staining of the wall it was almost always limited to the internal and external lamina with a thick unstained loop in between. All 'mature' nodular bodies (walls and spheres of cellular waste) which were intact in the tissue or ruptured outside were generally negative for most antibodies except for unequivocal fairly strong staining for CEA and rather weak staining for S100 protein and for fibronectin. However, those 'mature' nodular bodies undergoing degradation by an inflammatory and granulomatous process in the tissue as well as spheres of cellular waste released in the connective tissue were also positively stained for vimentin, alpha$_1$-ACT, IgG, IgA, kappa, lambda, C_{1q} and C_3. Plasma cells were polyclonal as judged by staining for kappa and lambda light chains; about two-thirds were IgG class and one third IgA class with a very few IgM plasma cells seen in occasional foci. Macrophages stained positively for alpha$_1$-AT and alpha$_1$-ACT.

Electron microscopy

'Early' nodular bodies had a fibrillar wall (about 3.5 μm thickness) and numerous spherical to elliptical, organized, ring-tree-like lamellated structures in the matrix interpreted as lysosomal bodies (Figures 9 and 10). Some lamellated structures were much larger, less well organized and contained large vacuoles. A 'halo' was present at the interface of the inner aspect of the wall with the matrix. Numerous fibrils were seen apparently incorporated into the outer surface of the wall (Figure 10).

'Mature' nodular bodies showed a honeycomb appearance because they were packed with multiple oval to spherical compartments (4 to 5 μm in diameter) containing multiple vacuoles and numerous coarse black granules in the central cores (corresponding to the PAS-positive cores of the spheres of cellular waste). Each compartment was surrounded by a thick wall, which appeared to be formed by condensation of granules present in the background which were grey in colour, uniform in size and intermingled in varying proportions with extremely thin fibrils. Intervening spaces (between spheres of cellular waste) were occupied by large vacuoles and grey to black granules (Figure 11). End-stage spheres of cellular waste released in the tissue were somewhat larger, had predominantly fibrillar but comparatively thinner walls, contained large dense black globules in radial arrangements with sparse vacuoles and no lamellated bodies; some showed a very dense, smudged, charcoal-like texture with fragmentation and a folded periphery possibly

TABLE I

FINDINGS FROM IMMUNOHISTOCHEMICAL STAININGS OF NODULAR BODIES AND SPHERES OF CELLULAR WASTE (SCW) IN RHINOSPORIDIOSIS

	Intact 'mature' nodular body		Ruptured 'mature' nodular body		'Early' nodular body	
Antibody	Wall	SCW	Wall	SCW	Wall	Matrix
Cytokeratin	–	–	–	–	–	–
EMA	–	–	–	–	–	–
CEA	+	+	+	+	+	(+)
Vimentin	–	–	–	+	–	–
Desmin	–	–	–	–	–	–
Myoglobin	–	–	–	–	–	–
GFAP	–	–	–	–	–	–
NSE	–	–	–	–	–	–
S100	(+)	(+)	(+)	(+)	(+)	(+)
Chromogranin	–	–	–	–	–	–
F VIII RAg	–	–	–	–	* (+)	–
Fibronectin	(+)	–	+	–	+	+
Amyloid-*p* component	–	–	–	–	(+)	+
Alpha$_1$-AT	–	–	–	–	–	+
Alpha$_1$-ACT	–	–	+	+	+	+
IgG	–	–	+	+	+	2+
IgA	–	–	(+)	+	+	+
IgM	–	–	–	–	–	–
Kappa	–	–	(+)	(+)	+	2+
Lambda	–	–	(+)	(+)	+	2+
C_{1q}	–	–	+	+	+	+
C_3	–	–	(+)	(+)	+	(+)

Staining scores: – none; (+) faint; 1+ strong; 2+ very strong; *outer lamina only.

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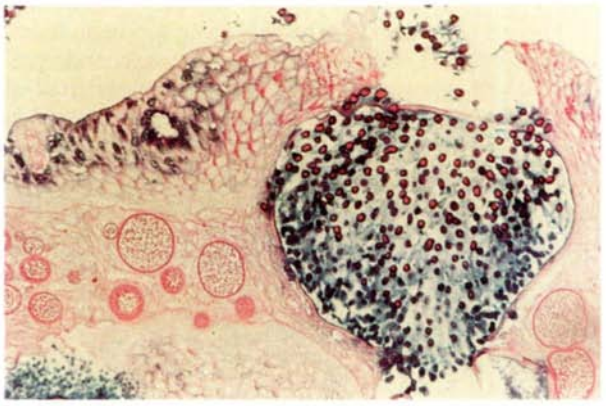

FIG. 4

'Early' nodular bodies stained red with PAS but mature nodular bodies stained predominantly blue with alcian blue except for PAS-positive red-staining cores seen in some spheres of cellular waste (AB/PAS; × 105).

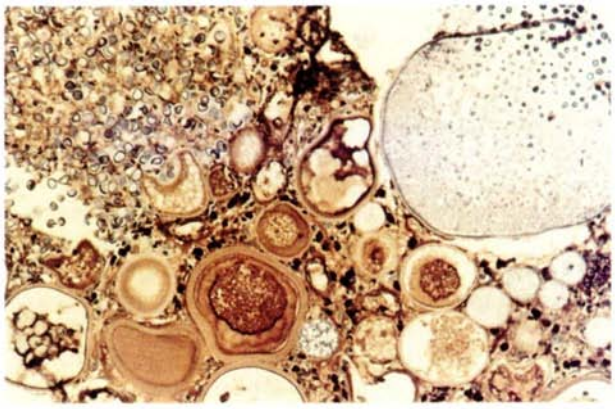

FIG. 6

Immunostaining for the IgG heavy chain demonstrating positive staining of 'early' nodular bodies and many of the spheres of cellular waste released into the tissue while spheres of cellular waste within an intact 'mature' nodular body have not stained. (PAP; × 105).

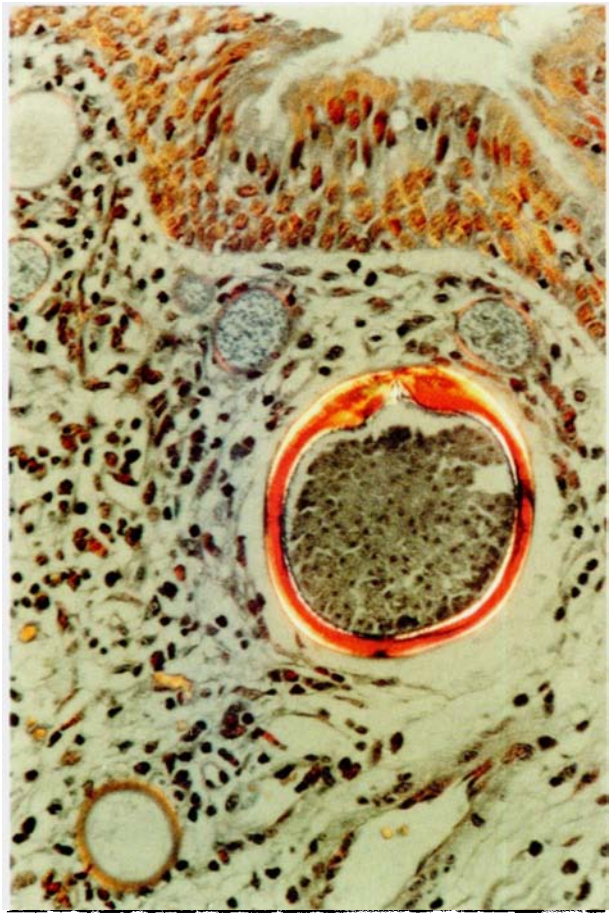

FIG. 5

Polarized light has differentiated the wall of a large 'early' nodular body into a thick, dense, red central loop between thin, transluscent, bright internal and external lamina. Greenish birefringence is not visible. (Congo red; × 170).

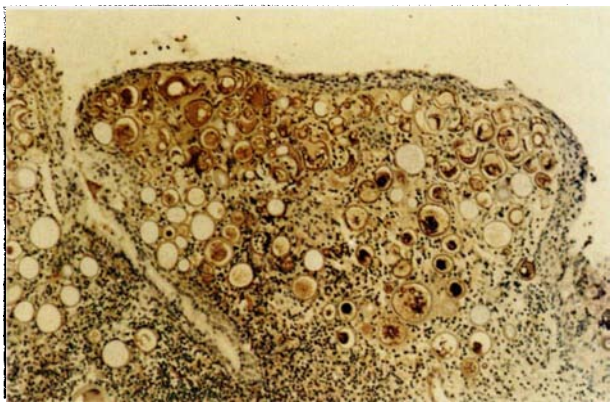

FIG. 7

Immunostaining for alpha$_1$-AT showing positive staining of the matrix of 'early' nodular bodies. The walls except for the inner lamina are generally negative. (PAP; × 52).

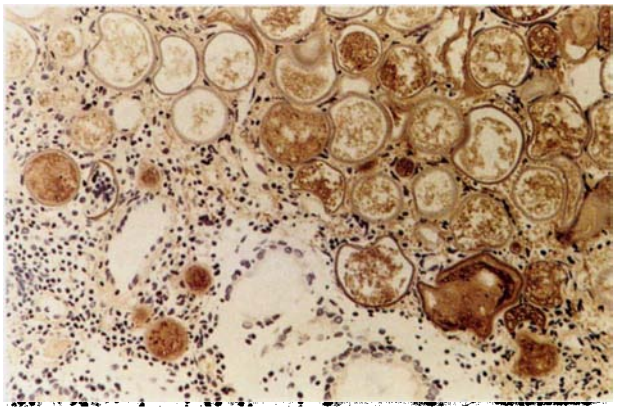

FIG. 8

Immunostaining for amyloid-*p*-component decorating the matrix and wall of 'early' nodular bodies. (PAP; × 105).

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reflected terminal degradation caused by inflammatory cells (Figure 12).

Discussion

The wall of nodular body is fibrillar and chemically different from that of fungi (Stoddart, 1984; Thianprasit and Thagerngpol, 1989), in that it contains beta$_1$-4 linked D-manosyl residues which are supposed to be widespread in plants: it also contains cellulose and a polysaccharide demonstrated to be plant starch (Kaprova, 1964; Rao, 1966; Thianprasit and Thagerngpol, 1989). Earlier ultrastructural studies (Kannan-Kutty and Teh, 1974; Savino and Margo, 1983) have interpreted single accumulations within trophocytes as nuclei containing chromatin. Savino and Margo (1983) described laminated bodies within more mature trophoblasts but were unable to confirm their presence in sporoblasts; these authors accepted the proposal of Kannan-Kutty and Teh (1974) that the laminated body consists of chromatin and that it gives rise to the sporoblast because of its transient appearance during the late trophic and early endosporulating stages. Structures formerly regarded to be nuclei of spores (spheres of cellular waste) are now believed to be lysosomal storage bodies loaded with indigestible residues (Ahluwalia, 1992). Two carbohydrates, namely an endogenous defective proteoglycan and an exogenous polysaccharide presumed to be ingested through a diet of tapioca (root of *Manibut esculenta*) constitute the indigestible material in the nodular body and sphere of cellular waste. The beta$_1$-4 glycosidic bonds between mannose residues in these poly-

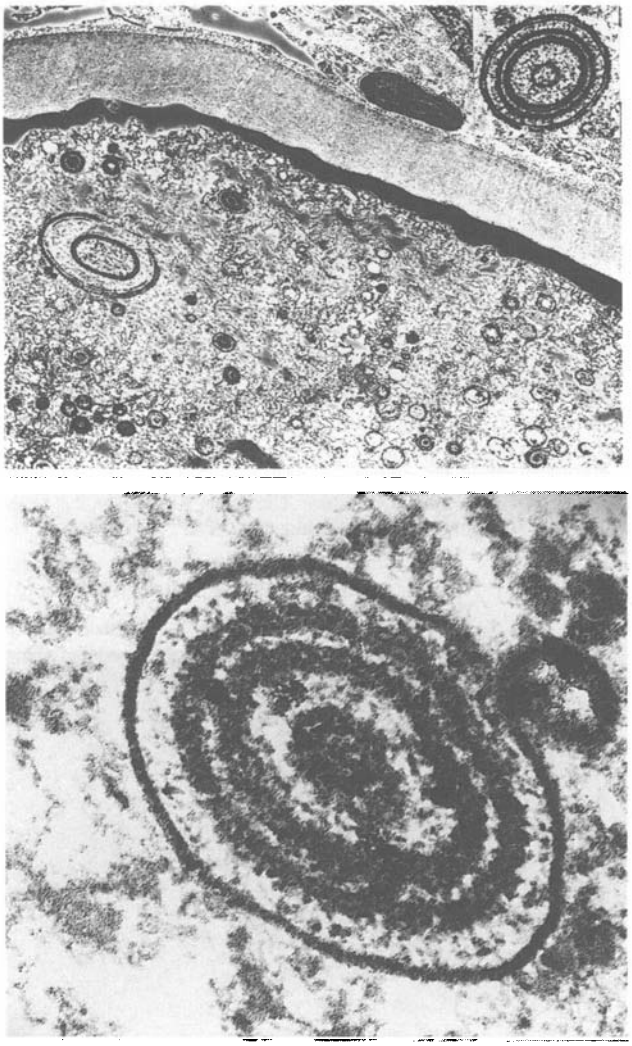

FIG. 9

Ultrastructural view of an 'early' nodular body with a 'halo' at the interface of the compact fibrillar wall and the matrix containing numerous lamellated bodies. (× 5500). Insets: top right × 22 000; bottom × 45 000).

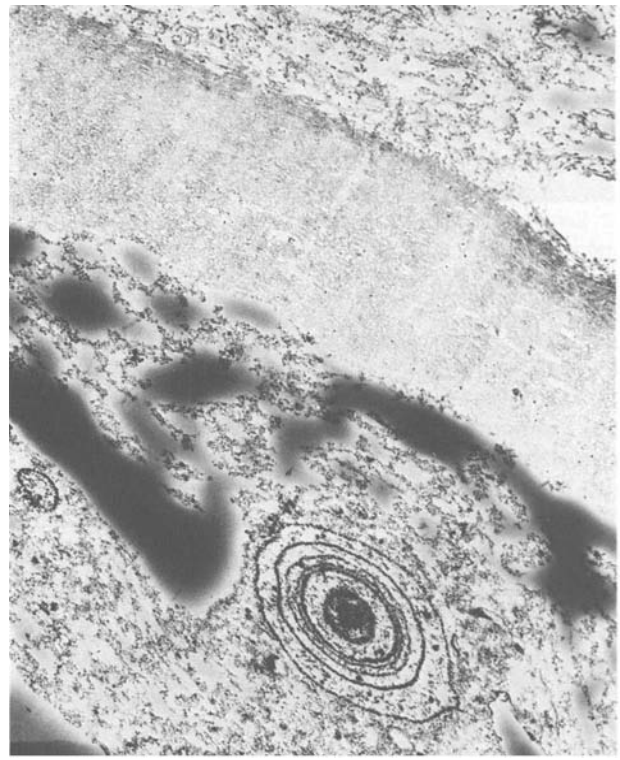

FIG. 10

An elliptical lamellated body in the matrix of an 'early' nodular body. Also note apparent incorporation of fibrils into the outer surface of the wall. (× 14 500).

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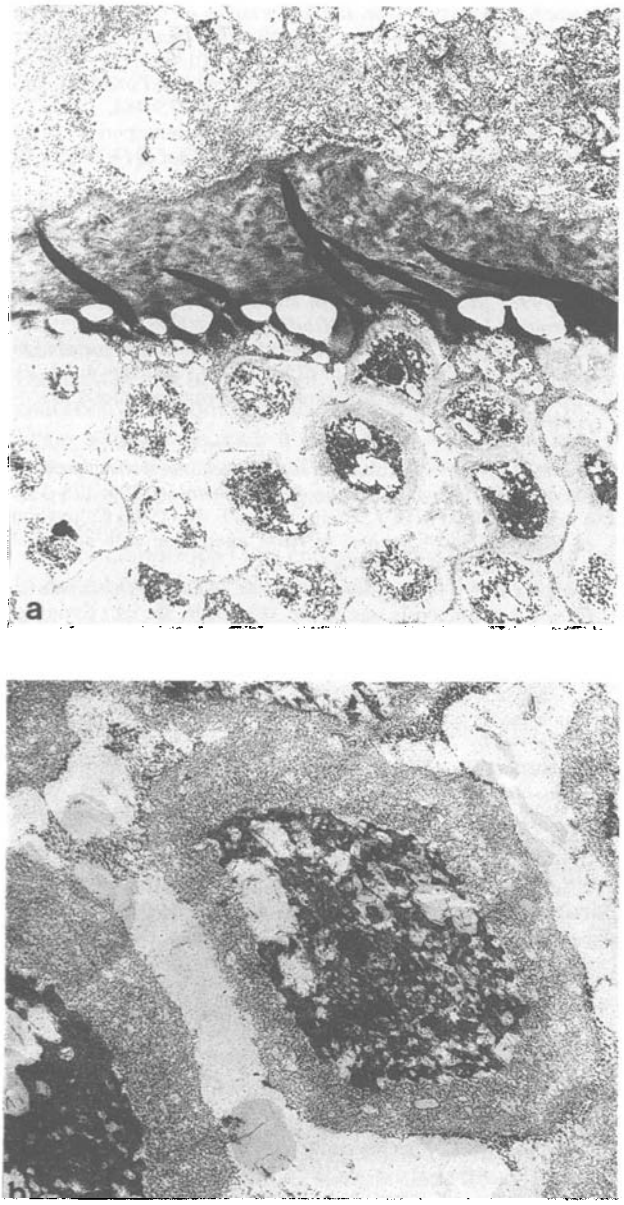

FIG. 11

A 'mature' nodular body with multiple spheres of cellular waste containing multiple vacuoles and coarse granules. (a: × 5500; b: × 18 000).

saccharides are not degradable by gastrointestinal enzymes nor in intercellular lysosomes which break only alpha–glycosidic bonds (see Ahluwalia, 1992: for references). We are not convinced of the presence of amyloid in nodular bodies. Positive stainings observed for amyloid-*p*-component, like those of CEA, S100 and fibronectin, may merely reflect endogenous components originating from plasma or local secretory activities or both.

Levy *et al.* (1986) have reported growing fungi interacting with a human neoplastic epithelial cell line *in vitro* which has stimulated these cells to form polypoid excrescences suggesting that *R. seeberi* may elaborate an epithelial growth factor. In view of earlier ultrastructural interpretations, apparent propagation in the tissue culture experiment of Levy *et al.* (1986) and a recent report of some success in culture in standard media (Krishnamoorthy *et al.*, 1989) the possibility of *R. seeberi* being an infectious organism should not be disregarded with certainty. However, our immunohistochemical and electron microscopic findings, on the whole, support the hypothesis of nodular bodies and spheres of cellular waste as residue-loaded lysosomal bodies. Positive staining of 'early' nodular bodies for acid phosphatase has been reported to become negative in residual bodies owing perhaps to exhaustion of hydrolases and consequent piling up of residues (Ahluwalia, 1992; Thianprasit and Thagerngpol, 1989). It is conceivable that nodular bodies in early stages contain both digestible and indigestible materials in the matrix, but, as digestion proceeds lysosomal components are depleted and indigestible materials prevail in 'mature' nodular bodies. Positive stainings for $alpha_1$-AT, $alpha_1$-ACT and most immunoreactants observed in 'early' nodular bodies become subsequently negative in intact end-stage 'mature' nodular bodies which are ready to be eliminated from the system.

We believe positive stainings observed in a few ruptured and disintegrating 'mature' nodular bodies for

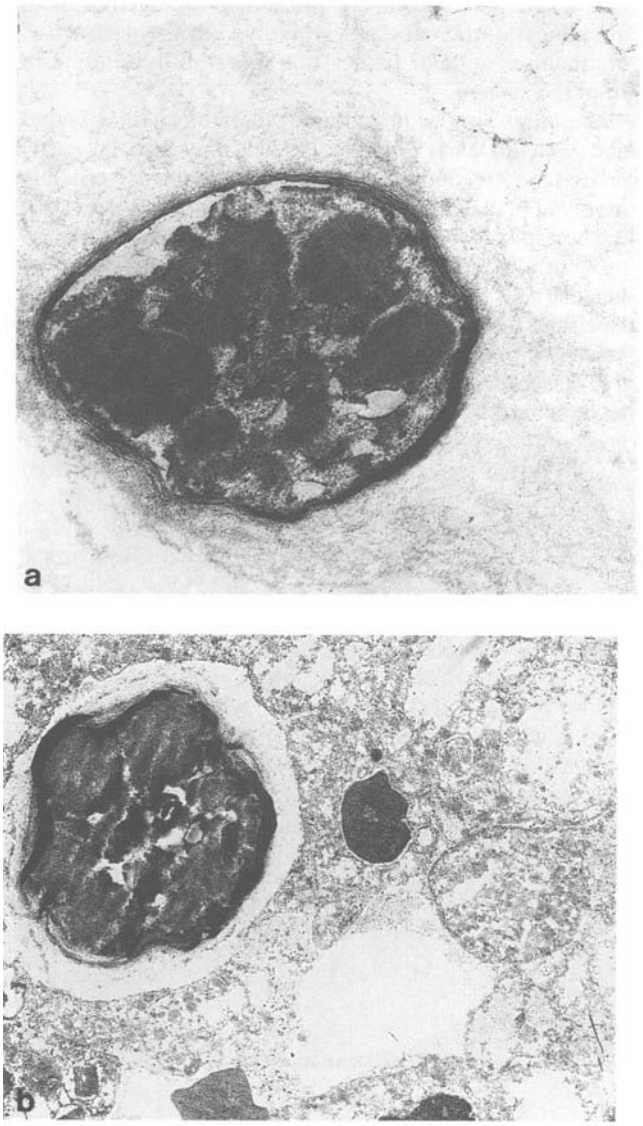

FIG. 12

(a) An intact end-stage sphere of cellular waste released into tissue showing a fibrillar wall surrounding large dense globules, sparse vacuoles and no lamellated body. (× 14 500); (b) end-stage sphere of cellular waste released into tissue showing a dense and fragmented content and a folded periphery possibly caused by destructive inflammatory reactions. (× 11 500).

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immunoglobulins, complement components and alpha$_1$-ACT are due to secondary events related to inflammatory/granulomatous reactions involved in their degradation. Granulocytic microabscesses are also seen at some stage in disorganized histiocytic granulomata and appear to be engaged in enzymatic digestion of nodular bodies. Goihman-Yahr (1986) who compared rhinosleroma and rhinosporidiosis through an historical approach has postulated lack of digestive capacity of phagocytes in patients affected by these diseases. However, there is no clinical or experimental evidence to confirm a local or systemic deficiency of this kind. Disorganized histiocytic granulomata which are also seen in other conditions (e.g. acute cutaneous leishmaniasis) should be distinguished from those seen in tuberculosis, sarcoidosis, berylliosis or chronic lupoid leishmaniasis which are well organized epithelioid granulomata and are equated with delayed cell-mediated hypersensitivity responses. Disorganized histiocytic granulomata in rhinosporidiosis appear to be a late stage and secondary phenomenon together with granulocytic microabscesses involved in the destruction of 'mature' nodular bodies not successfully eliminated from the system.

Stainings similar to ruptured 'mature' nodular bodies and seen in 'early' nodular bodies may also be partly related to secondary immune mechanisms possibly involved in their segregation. It is tempting to assume that the 'anergic' responses observed, characterized by numerous 'early' nodular bodies associated with very few mononuclear cells, confer a temporary advantage to the host in shielding tissue against toxic products in 'early' nodular bodies or potentially destructive immune responses.

Transepithelial elimination in rhinosporidiosis appears to be a significant but unsuccessful biological phenomenon of the host attempting to clear the lesion and control the inflammatory processes. However, this and other mechanisms discussed in the final analysis are ineffective. Rhinosporidiosis remains a chronic inflammatory disease with no medical treatment and only amenable to surgical excision with risks of recurrence and occasional widespread and even fatal dissemination (Rajam *et al.*, 1955; Sharan, 1979). Remission of infection within one year of therapy with dapsone has been reported in three patients (Job *et al.*, 1993). Aetiology and pathogenesis are unknown and it is not clear what pathobiological demands or influences motivate assembly of waste products in such an organized and apparently unprecedented fashion.

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