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Intraepithelial Parasitism as an Infection Mechanism in Human Paracoccidioidomycosis (South American Blastomycosis)

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Summary. Eight typical clinical and laboratory proved human cases of Paracoccidioidomycosis, presenting lip lesions rich in parasites, were studied by light and electron microscopy. Besides producing a typical pathological picture in the corium, *Paracoccidioides brasiliensis* was seen parasitizing epithelial cells of the lip mucosa. Intraepithelial abcess formation was established through the detachment of the parasitized epithelial cells from their connections with the neighbouring cells and a further local invasion of inflammatory cells, mainly granulocytes and large mononuclears.

In few instances, structures with no wall, tentatively inter preted as spheroplasts of *Paracoccidioides brasiliensis* were also present inside the epithelial cells cytoplasm.

Intraepithelial parasitism is regarded as an important mechanism of penetration and spread of the parasite. The possible relation of this phenomena to immunogenic defects is discussed.

Introduction

Knowledge of the penetration mechanism of the *Paracoccidioides brasiliensis* in humans is still reduced to mere speculation. A previous study of the chorio allantoic membrane (CAM) of chick embryo, experimentally inoculated with suspensions of the fungus *Paracoccidioides brasiliensis* (Splendore) Almeida and Lacaz, revealed the presence of fungal elements within the proliferating ectodermal cells of the membrane model (Brito *et al.*, 1972).

At later stages of infection the fungus cells appeared in the mesoderm where necrosis occured, accompanied by secondary ulceration of the ectodermal layer. Some fungal cells with no walls, found intracellularly in ectodermal components of the CAM, were then tentatively interpreted as fungal spheroplasts (Brito *et al.*, 1972). An electron microscopic study of the lip mucosa of patients with paracoccidioidomycosis was undertaken to investigate aspects of fungus penetration and development in human tissue.

Materials and Methods

Material was collected from eight male patients, all clinical and laboratory proved cases of paracoccidioidomycosis. Some of the patients had several previous hospital admissions. All of them showed active manifestations of the disease at the time when biopsies were performed. Examination of direct microscopic preparations from lip lesions in all patients revealed numerous *P. brasiliensis* cells, no other fungi being detected. General patient data is included in Table 1.

Table 1. Distribution of patients according to age, duration, clinical course and treatment of the disease

Case number	Age (years)	Main clinical manifestations	Illness duration	Previous relapses	Treatment
1	30	mucous	1 year	none	Amphotericin and sulfa
2	41	mucous and pulmonary	5 months	none	Amphotericin and sulfa
3	24	mucocutaneous	1 year and 4 months	one	Amphotericin and sulfa
4	40	cutaneous and lymphatic	21 years	four	Amphotericin and sulfa
5	54	mucocutaneous and pulmonary	3 years	none	Sulfa
6	43	mucocutaneous	13 years	eight	Amphotericin and sulfa
7	71	mucocutaneous and pulmonary	8 years	two	Amphotericin and sulfa
8	62	mucocutaneous and pulmonary	3 months	none	Amphotericin and sulfa

Biopsies were performed in the inner side of the lip mucosa, close to an ulceration, in seven patients; in one, the biopsy included only the tongue. The fragments were divided in two halves with razor blades: one was fixed in 10% formalin or Zenker's fluid, embedded with paraffin and routinely stained with hematoxilin-eosin, Gridley's and methenamine silver stains. The second halve was cut further into smaller pieces and immediately processed for electron microscopy as follows: fixation for at least two hours with 2% glutaraldehyde in phosphate buffer at pH 7.2 at 5° C; washed with buffer and postfixed with ice-cold 1% aqueous OsO_4 for 2 hours; rinsed with buffered saline and immersed in aqueous 0.5% uranyl solution for 12 hours at room temperature; dehydrated with a graded ethanol series followed by immersion in propylene oxide and finally embedded in Araldite. Sections were made with an ultramicrotome equipped with either a glass or a diamond knife. Two microns thick sections were stained with toluidine blue for light microscopy as a means to select blocks. Thin sections from selected blocks were double stained with uranyl acetate and lead citrate. Examination was performed with a Zeiss EM-9 electron microscope.

Control material for light and electron microscopy was taken from unaffected lip zones of patients undergoing surgery for carcinoma.

Results

Light Microscopy

Both paraffin embedded and thick sectioned Araldite infiltrated material from all eight patients revealed the presence of fungus cells and typical paracoccidioidomycosis lesions. The buccal mucosa showed evidences of either keratinization or epithelial proliferation (mainly through the basal and deep elements of the squamous layer), or both phenomena.

Inflammatory cells were predominantly represented by histiocytes, neutrophils and eosinophils which were observed in areas permeating epithelial cells.

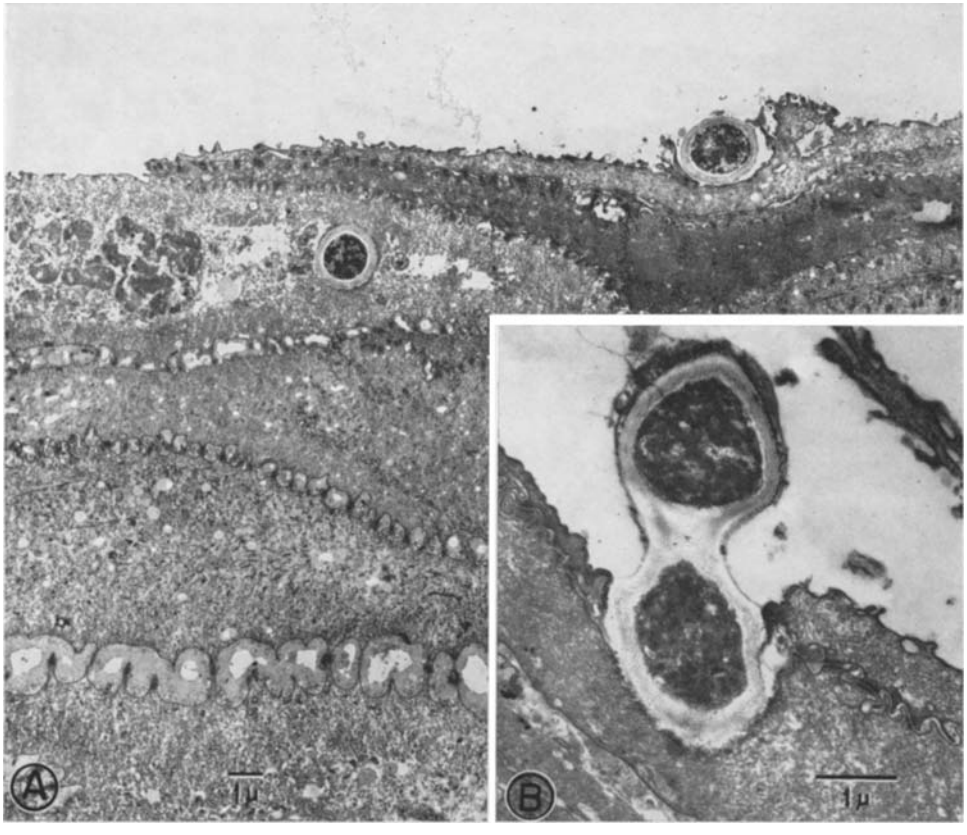

Fig. 1. Epithelium of the lip mucosa showing small forms of *Paracoccidioides brasiliensis* over and inside cells of the surface layer. The so called "laking effect" is seen in the epithelial cell with an intracytoplasmatic located parasite. Insert shows a reproduction form of the fungus

Intercellular spaces in the epithelium appeared enlarged due to edema. Foci located usually in upper epithelial layers, derived from accumulations of the above mentioned inflammatory cells, were also observed. These constituted the intra-epithelial abcesses commonly detected in paracoccidioidomycosis. No epithelial ulceration was observed in the examined areas. In a few instances small intra-epithelial granulomata were also encountered.

The corium displayed an edema with the presence of both an acute and chronic inflammation with granulomata formation. Small and large cells of *P. brasiliensis* were found inside either the epithelioid or the foreign body and Langhans giant cells of the granulomata, or in them all. A halo composed of lymphocytes, eosinophils and large mononuclears was seen in the periphery of many granulomata. In few instances neutrophils were depicted both in the periphery and in the center of the granulomata.

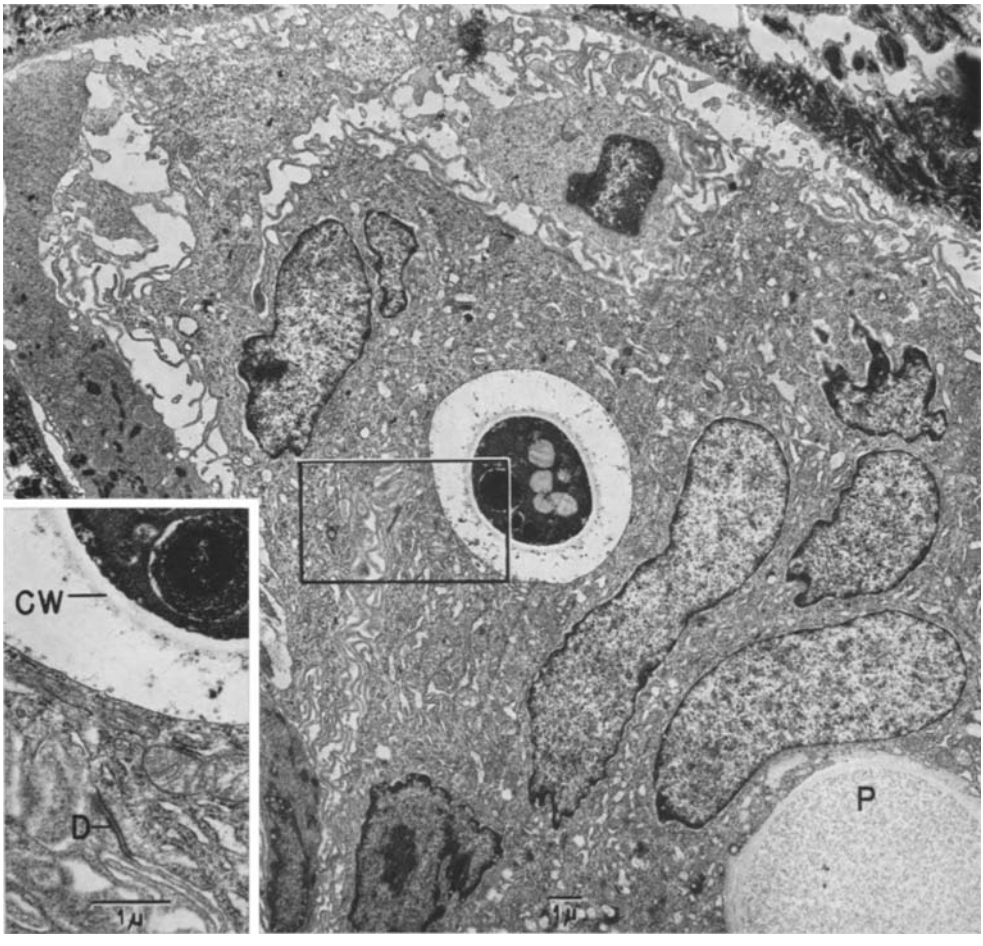

Fig. 2. Intraepithelial parasitism of cells of the upper prickle layer of the lip mucosa. The fungus has a well definite wall *CW* with a grumous and slightly electron dense deposit over it. The host unit membrane is also seen, delimiting a vacuole which contains the parasite. The insert shows in detail the above findings plus a desmosome *D* between the epithelial cells. Also observed, a degenerated large form of the fungus *P*, with a definite cell wall, located inside the cytoplasm of an epithelial cell

Electron Microscopy

Small cells of *P. brasiliensis* (Furtado *et al.*, 1967), were found over and inside the epithelial host cells of the surface layer, which (Fig. 1) displayed the "laking-effect". Large fungus cells were also found inside the epithelial elements of the upper prickle cell layer. Such invading fungus cells were surrounded by host delimiting unit vacoular membrane. In some cases, the fungus cell wall was covered with an electron dense, granulous deposit (Fig. 2). In addition to small and large fungus cells epithelial elements of the host also contained sporulating fungus components.

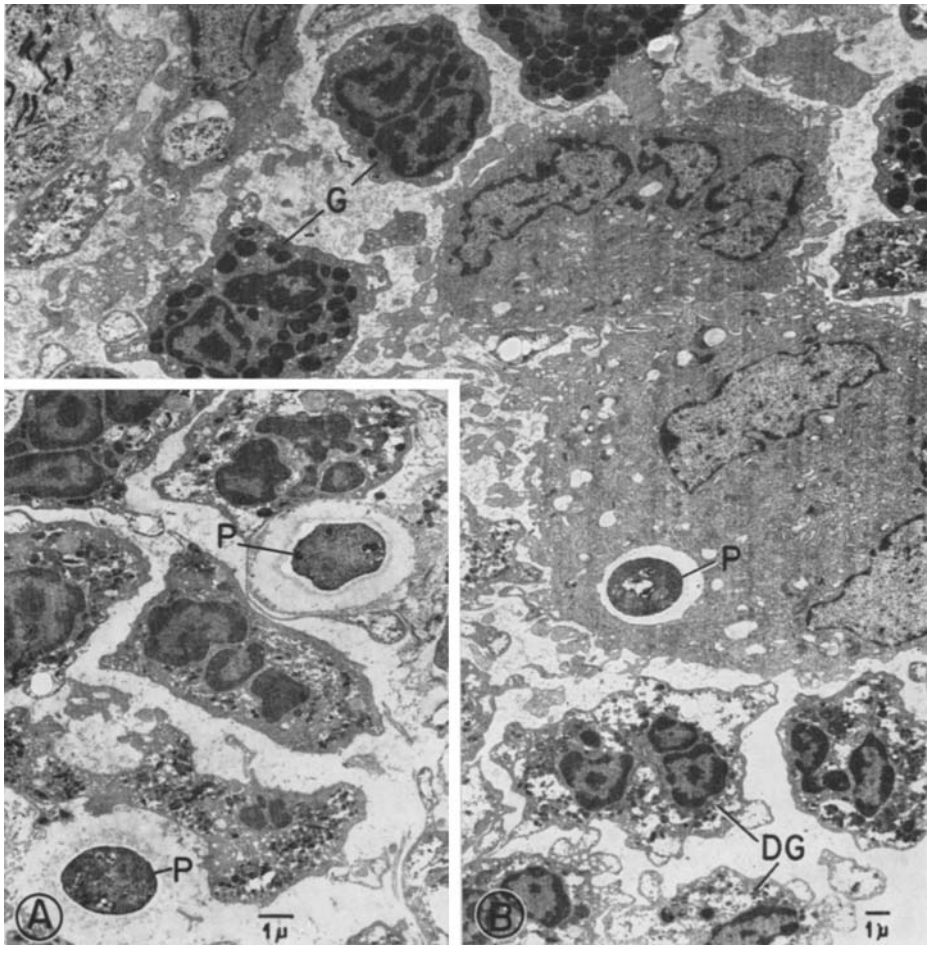

Fig. 3. Intraepithelial abcess showing groups of epithelial cells free from their connections with the neighbouring cells, one of them parasitized *P*. They are among the granulocytes *G*, most of them exhibiting cytoplasmatic regressive changes *DG*. Insert shows small forms of the fungus *P* in contact and being engulfed by the granulocytes

Remarkable alteration in tonofilament arrangement, in overall cytoplasm structure or organelle organization was not observed at the beginning of infection. Keratinized epithelium revealed an accumulation of microgranules with a pronounced striation (Frithof and Wersall, 1965). At the later stages of parasitism, epithelial cells lost their neighbouring contact and groups of two or three of them were found inside intraepithelial abcesses composed of granulocytes and histiocytes (Fig. 3). The granulocytes showed both small and large fungus cells inside their cytoplasm, probably engulfed by phagocytosis. This aspect was also seen in neutrophils of the granulomata formed in the corium.

Control preparations revealed the normal buccal mucosa, the structure of which is similar to that previously described (Hashimoto *et al.*, 1966; Zelickson and Hartmann, 1962).

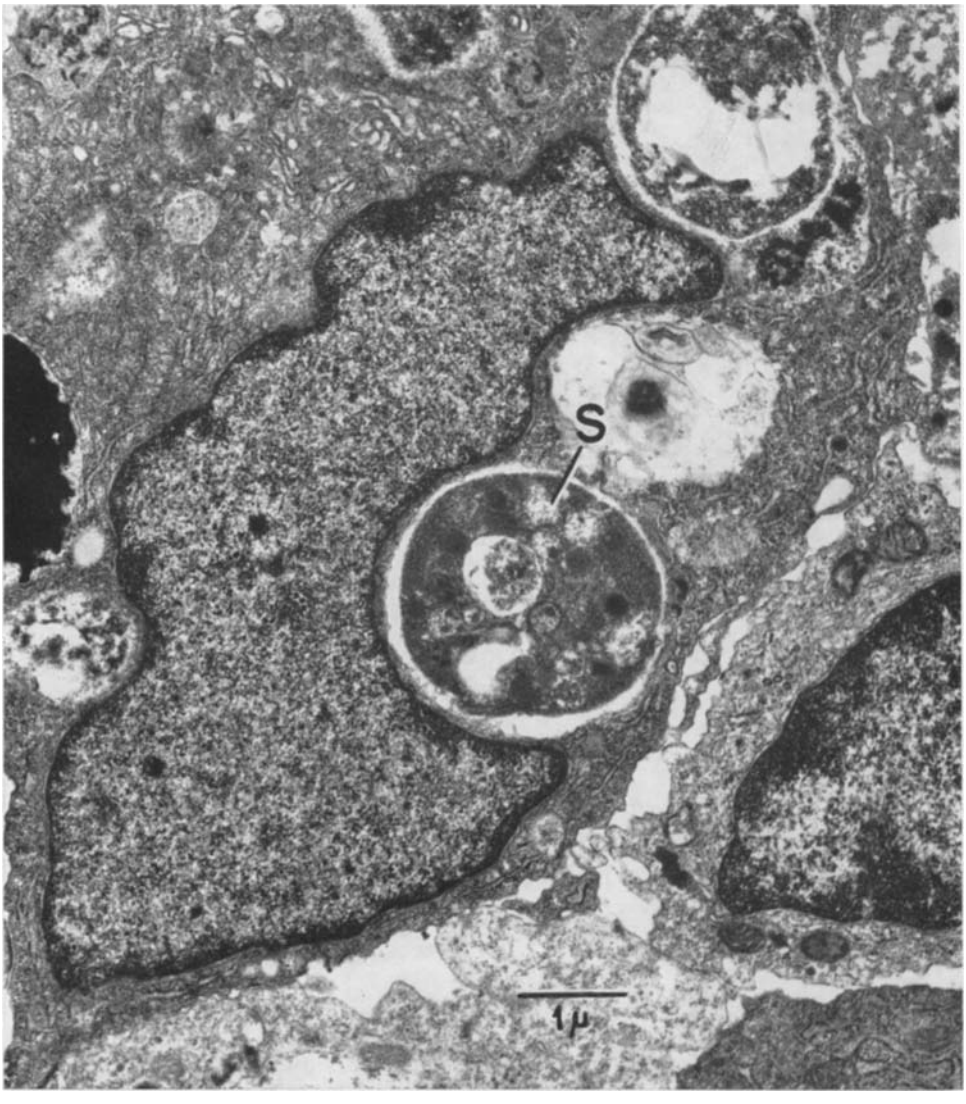

Fig. 4. Round structure without a definite wall, *S* seen inside an epithelial cell and tentatively interpreted as a spheroplast of the *Paracoccidioides brasiliensis*

In two instances structures approximately 2.5–3.5 μ in diameter were found inside the epithelial cells of the upper prickle layer, with a delimiting unit membrane immediately followed by another surrounding host vacuolar unit membrane (Fig. 4). These contained ribonuclein, protein and possible glycogen material, associated with centrally located vacuoles and mitochondria. These organelles showed a smaller diameter as compared with those seen in the cytoplasm of the epithelial elements, though containing a larger number of cristae (Fig. 6). In some cases small nuclei with their reduced nucleoli were clearly seen (Figs. 4 and 5).

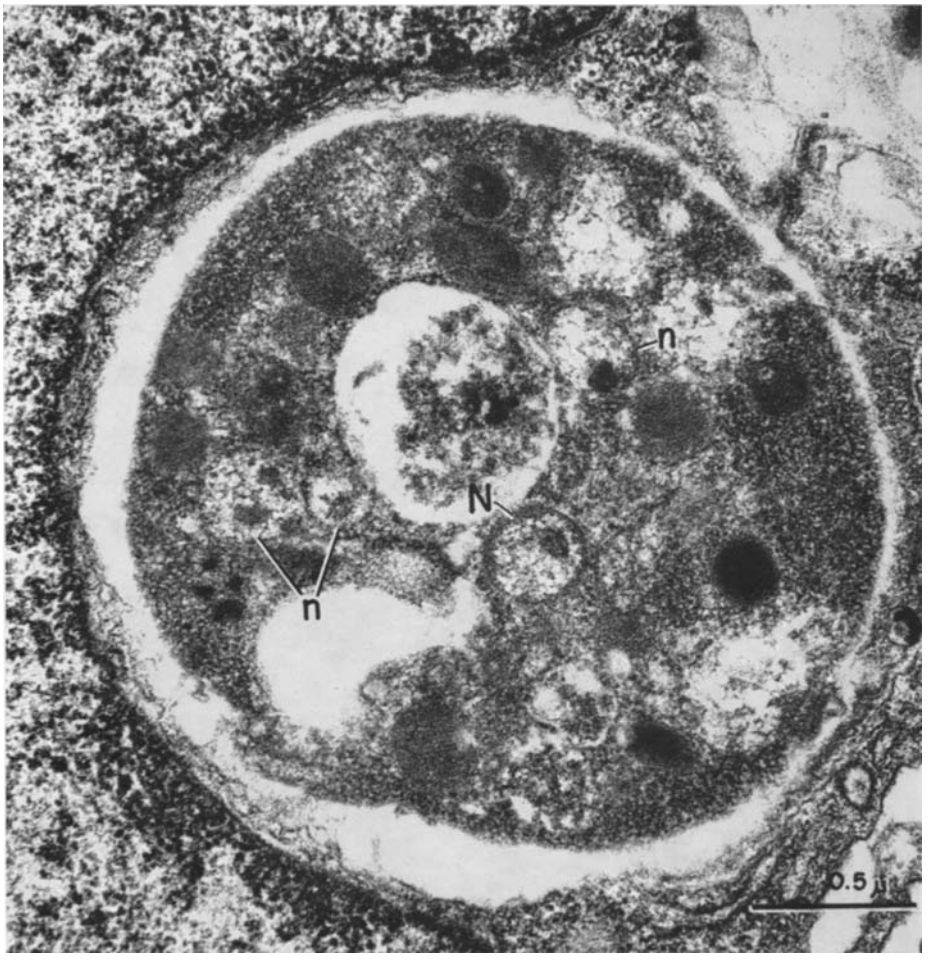

Fig. 5. Detail of the intracytoplasmatic structure showing the absence of a definite wall and the presence of nuclei (*n* and *N*) with small nucleoli. One of the nuclei *N* has a more definite nuclear membrane and nucleoli. Vacuoles, ribonuclein granules and round electron dense structures, probably lipidic, are also seen in the cytoplasm

Usually, however, nuclei were not seen in most preparations, certainly due to insufficient preservation. In some cases, such intracellularly located structures showed in contact with their delimiting membrane, whorls which assumed the aspect of myelin figures. These structures were also tentatively identified as spheroplasts of *P. brasiliensis*. Golgi organized into dictyosomes were not found in such structures.

Discussion

Lesions in buccal mucosa of patients with paracoccidioidomycosis contained typical fungus cells which were found inside the epithelial cells of both surface

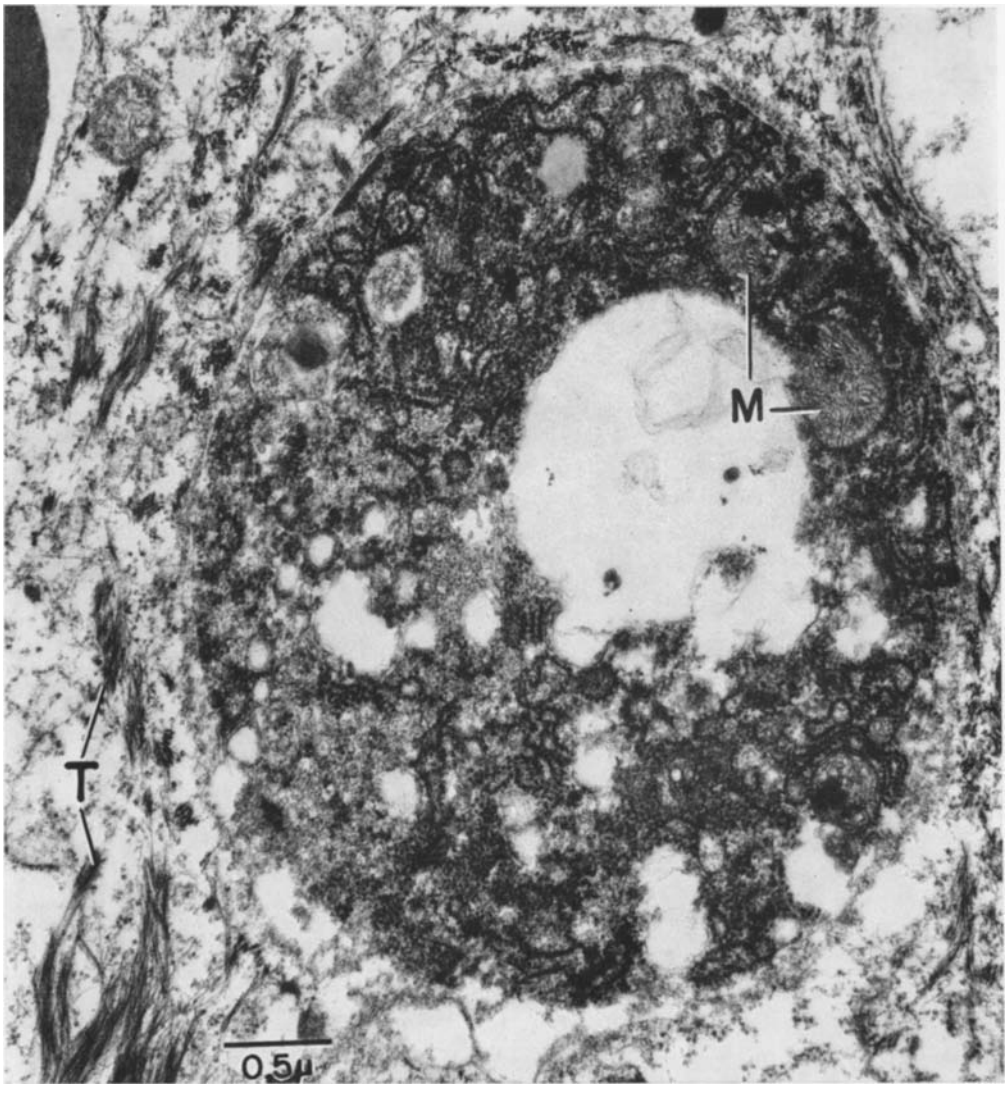

Fig. 6. Another structure, also deprived of a definite wall, inside the cytoplasm of an epithelial cell of the prickle layer. *T* designates the tonofibrils of the parasitized cell. Inside the structure, ribonuclein granules and vacuoles are seen. *M* designates mitochondria with many cristae

and upper prickle layers. The intimáte mechanism of fungus penetration is unknown. It has been mentioned that several dermatophytes and a few subcutaneous and deep invasors elaborate elastases, the production of which is associated with more inflammatory types of infection (J. W. Rippon, 1968; Rippon and Garber, 1969; Rippon and Varadi, 1968).

Nonetheless, no elastase has been demonstrated for *P. brasiliensis* (Rippon and Varadi, 1968). Therefore, the possible enzymatic system accounting for

invasion of this fungus and other deep-seated agents of mycosis throughout the epithelium is, so far, merely speculative. Invasion of cells of *P. brasiliensis* into epithelial elements is a form of infection mechanism in paracoccidioidomycosis. This parasitism leads to detachment of the epithelial cells from their neighbouring contacts and possibly, the liberation of parasite forms in the interstitium. This is then followed by local invasion by mononulears and granulocytes which phagocytize both the parasites and host cell debris. This may represent the pathogenesis of the intraepithelial abcesses seen under light microscopy in South American Blastomycosis.

Structures located in the cytoplasm of epithelial cells and deprived of wall were tentatively identified with spheroplasts of *P. brasiliensis* based on the relative dimension of nuclei and mitochondria as compared to counterparts present in host epithelial cells. Preliminary report (Brito *et al.*, 1972) and experimental inoculations of CAM of chick embryo with suspensions of *P. brasiliensis* in progress, have stressed the correlation between such structures with no wall and their possible fungus nature. They may represent a fungus stage related to the mechanism of parasitism.

The invasion of the epithelial components of the lip mucosa by the fungus cells could be related to an individual susceptibility to the agent of paracoccidiodomycosis. In this respect it is worthwhile mentioning that E. Mendes and Raphael (1971) demonstrated a significant depression of delayed hipersensitivity in patients with paracoccidioidomycosis to intracutaneous tests to tuberculin, oidomycin and trichophytin, as well as a significant decrease in dinitrochlorobenzene sensitization, even when the results were compared to those obtained from a study of a group of patients with lepromatous leprosy. N. Mendes *et al.* (1971) showed reduced lymphocyte transformation stimulated by phytohaemaglutinin, which points towards a defect in the mechanism of delayed hypersensitivity in patients with paracoccidioidomycosis, similarly to lymphoproliferative diseases such as sarcoidosis and Hodgkin's disease.

A significant alternative is whether the involvement of the delayed hypersensitivity mechanism precedes or is a consequence of the disease. For paracoccidioidomycosis such a question remains open. However, Fava Netto *et al.* (1965) reported cases of this disease among relatives, and suggested, among other hypothesis raised as an explanation of such occurence, the possibility of a genetic immunogenic defect.

How much congenital or acquired immunological deficiency does facilitate penetration and development of *P. brasiliensis* inside epithelial cells of buccal mucosa remains in the realm of speculation. It is worth mentioning, however, that a similar parasitism in epithelial cells by candidiasis is also prone to occur in patients with acquired or congenital immunological defects (Pontes and Wilborn, 1968; Cawson and Rajasingham, 1972).

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