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Granulomatous reaction and tissue remodelling in the cutaneous lesion of chromomycosis

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Abstract. The cell-mediated immune reaction was studied in the cutaneous lesion of chromomycosis, using monoclonal antibodies against polymorphonuclear neutrophils, macrophage and lymphocyte subsets, endothelial and fibroblast cells. In addition, immunostaining of the main degradative enzymes (neutrophil elastase and interstitial collagenase) and certain important cytokines (transforming growth factor-β, tumour necrosis factor-α and interferon-γ) suggested an explanation for the granulomatous reaction and the associated tissue remodelling. The distribution pattern of neutrophils and macrophage subsets, observed by computer-aided image analysis, suggests that the in situ persistence of fungi is the main pathological factor.

Key words: Chromomycosis – Skin – Granuloma – Immunohistochemistry – Electron microscopy

Introduction

Chromomycosis is a chronic fungal disease due to various pigmented fungi from the Dematiacae family, leading to spectacular verrucous skin lesions. The only available information concerning this disease describes the clinical and histological features (Binford and Dooley 1976; Carrion 1975; Cespedes 1971; Salfelder 1990) or the tissue form of the fungi (Rosen et al. 1980; Walter et al. 1982).

We have studied the relative proportions and the topography of the different cell subsets in the skin lesions by immunohistochemistry on frozen and paraffin-embedded sections and computer-aided image analysis. Eleven patients infected with *Phialophora pedrosoi* were biopsied before and, in three cases, after specific antifungal treatment. The aim of this study is to determine the composition and the tissue distribution of the cell-mediated immune response, adding to the data on the previously described distinctive features in the associated extracellular matrix (Esterre et al. 1991b, 1992a).

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Materials and methods

We investigated six patients from Cayenne, French Guiana, who had been followed by the Department of Dermatology for more than 10 years. The diagnosis was easily made by observation of thick-walled septate pseudo-yeasts in scraped smears, and confirmed by histological examination and in vitro cultures of the pathogenic fungi (*P. pedrosoi*). No antifungal treatment had been given to them for 1 year prior to obtaining the first biopsy specimen. For three patients, two supplementary biopsies were taken after 6 months and after 1 year of therapy with itraconazole.

Five millimetre punch biopsies were taken from each patient, one part of the specimen being fixed in formalin and then embedded in paraffin for histological examination, the remaining part being immediately frozen in liquid nitrogen then cut into 5 µm cryostat sections for immunoenzymatic studies. We used three biopsies from the forearms of healthy individuals as controls. Five paraffin blocks, corresponding to reference cases studied in the Histopathology Unit, Institut Pasteur, were added for investigations on routinely embedded material. A second biopsy, also taken from the edges of the cutaneous lesion, was immediately immersed in 2% glutaraldehyde in cacodylate buffer, followed by osmium tetroxide fixation and embedding in epoxy resin.

A section from each biopsy was stained with haematoxylin and eosin (H & E) to study the lesion architecture. Serial sections were deparaffinized before being immunohistochemically stained with a streptavidin-labelled biotin immunoperoxidase system (Dako, Denmark). For this routine material, the panel of monoclonal antibodies (mAbs) used included the following: CD3, OPD4 (CD4), L26 (CD20), JC7 (CD31), KP1 (CD68), HLA-DR, Mac 387, S-100 and PCNA (Dako); ION1 (CD15), UCHL-1 (CD45RO) and BNH9 (Immunotech, Marseille-Luminy, France); and HECA-452 (donated by L.J. Picker, Stanford University). Serial cryostat sections, fixed in cold acetone, were studied using the reference avidin-biotin immunoperoxidase technique (Vector, Burlingame, Calif., USA) with an extended panel of mAbs to cell surface antigens (Table 1). A semi-quantitative estimate of the different cell subsets in each biopsy was obtained by counting the number of positive cells with the respective antibodies in five high-power fields in the inflammatory infiltrates (Esterre et al. 1992b), and particularly in

Table 1. Characteristics and specificity of the monoclonal antibodies used on frozen material

Antibody	Cluster differentiation[a]	Isotype	Principal reactivity and distribution	Source
LCA	CD45	IgG1	All leucocytes	Dako
IOT11	CD2	IgG1	>95% E-rosette$^+$ cells (T)	Imm
IOT3	CD3	IgG2a	95% Mature peripheral T	Imm
IOT4	CD4	IgG2a	Mature peripheral helper T	Imm
UCHL1	CD45RO	IgG2a	Granulocytes, monocytes, CD4$^+$ T cell subset	Dako
IOT8	CD8	IgG1	Mature peripheral suppressor-cytotoxic T	Imm
IOT9	CD71	IgG1	Transferrin receptor	Imm
IOT14	CD25	IgG2a	IL-2 receptor	Imm
IOT2a		IgG2b	Class II major histocompatibility molecules (HLA-DR)	Imm
IOM2	CD14	IgG1	Monocytes (>90%),	Imm
LeuM3	CD14	IgG2b	Macrophages	BD
IOB1	CD37	IgG1	B lymphocytes	Imm
Leu7	CD57	IgM	Resting NK cells, some CD8$^+$ T cells and tissues	BD
Leu11b	CD16	IgM	Resting NK, some granulocytes	BD
Leu19	CD56	IgG1	Resting and activated NK	BD
ION3	CD65	IgG1	Granulocytes	Imm
IOT6a	CD1a	IgG1	Epidermal Langerhans and	Imm
IOT6b	CD1b	IgG2a	dermal dentritic cells	Imm

[a] CD clusters established at the 4th workshop on human leucocyte differentiation antigens, Vienna, Austria, 1989
BD, Becton-Dickinson, Mountain View, Calif., USA; Dako, Dako, Santa Barbara, Calif., USA; Imm, Immunotech, Marseille-Luminy, France

the granulomas. The results are expressed as a percentage of positive cells on total leucocyte scoring [estimated by leucocyte common antigen (CD45) immunostaining: 91.1 ± 43.5 CD45+ cells/mm^2]. In addition, the distribution pattern of the most important cell populations together with the main degradative enzymes [neutrophil elastase, with NP57 (Dako), and interstitial collagenase, with a mAb from our laboratory] and certain cytokines [tumour necrosis factor-α (TNF-α) and interferon-γ (IFN-γ), respectively] with mAbs IP-300 and IP-500 (Genzyme, Cambridge, USA); and transforming growth factor-β (TGF-β), with BDA-5 (British Biotechnology, Oxon, UK) were studied using an image analysis workstation (Biocom 500, Les Ulis, France). The study of Langerhans and dendritic cells was performed on six frozen lesions, compared with two control (healthy skin) biopsies and 11 paraffin-embedded lesions. The material embedded in epoxy resin was studied by standard electron microscopy technique.

Results

Sections of biopsies taken at the edges of verrucous lesions or in the psoriasis-like plaque, stained with H & E, showed a characteristic downgrowth of the rete pegs. This pseudo-epitheliomatous hyperplasia may be confused with skin carcinomas (of the squamous or basal cell types; Paul et al. 1991) by inexperienced practitioners. Papillomatosis and marked hyperkeratosis, often associated with parakeratosis and acanthosis, were found regularly. Typical brown fungus cells, often associated in clusters, were identified in intraepidermal abscesses. We observed dense inflammatory infiltrates in the dermis (Fig. 1), rich in granulocytes and often surrounded by

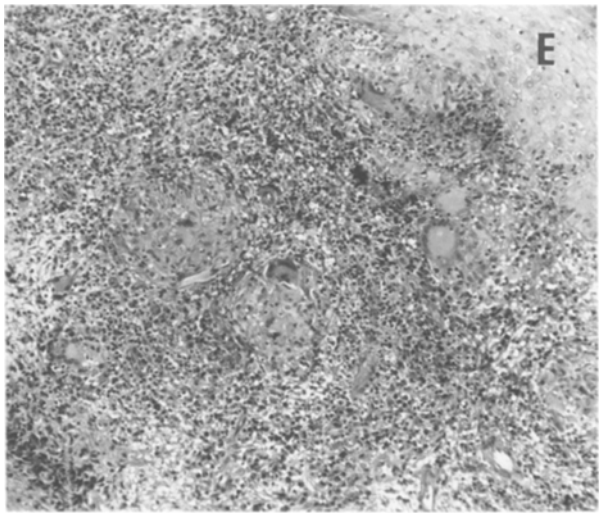


Fig. 1. A typical granulomatous pattern observed in the upper dermis in the inflammatory edges of the lesion. *E*, Epidermis. H & E, ×250

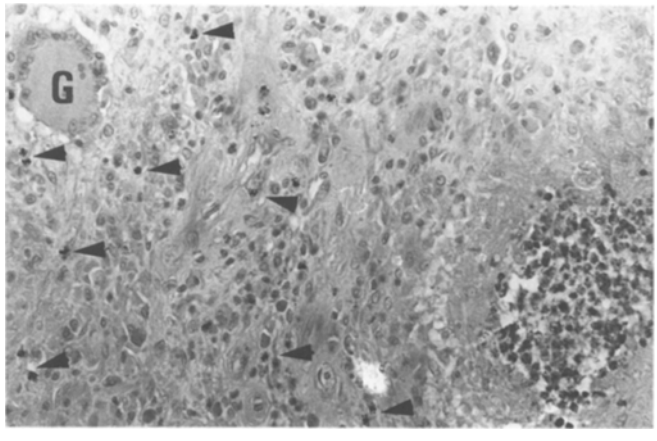


Fig. 2. Infiltrating neutrophils, identified by the immunostaining of elastase (NP57+ cells) in the granulomatous reaction and in a sub-epidermal micro-abcess (on the right). *G*, Giant cell. Immunohistochemistry, ×250

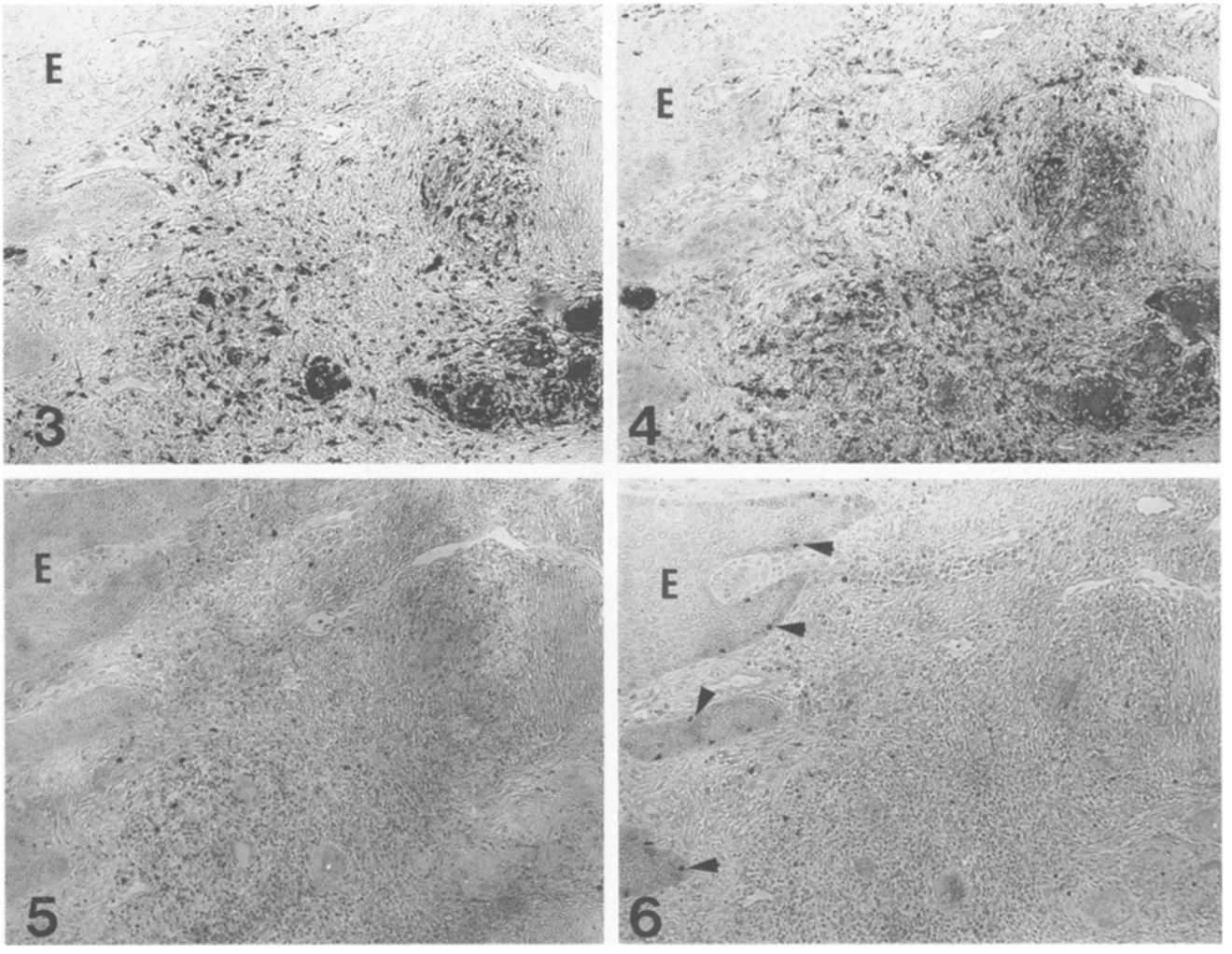


Fig. 3. The macrophages present are obviously displayed using the CD68 marker. *E*, Epidermis. Immunohistochemistry, ×250

Fig. 4. A few T (CD3+) lymphocytes can be revealed in the same area. *E*, Epidermis. Immunohistochemistry, ×250

Fig. 5. HLA-DR+ cells observed in the same area as Fig. 3. *E*, Epidermis. Immunohistochemistry, ×250

Fig. 6. Demonstration of some proliferating (PCNA+) macrophages in the infiltrate. Note the presence of positive cells (*arrowheads*) in the basal layer of the epidermis (*E*). Immunohistochemistry, ×250

a strong granulomatous reaction; this was sometimes organized with a follicular epithelioid pattern. A more diffuse arrangement of the inflammatory infiltrate was also observed around the vessels of the fibrotic areas.

In sections stained immunohistochemically with various mAbs, the majority of the cellular infiltrate is composed of neutrophils (ION3+, elastase+; Fig. 2). Most of the mononuclear cells belong to the monocyte/macrophage lineage [CD14+, CD68+ (Fig. 3), Leu M3+ and Mac387+]. Only a few samples contain isolated B cells (CD22+ on frozen sections, CD20+ on paraffin ones) and T lymphocytes [stained with CD2, CD3 (Fig. 4) or CD45R] are only observed in perivascular areas. The bulk of the cell population in organized granulomas (Table 2) is made up of macrophages and polymorphonuclear neutrophils, with a very low participation of T lymphocytes and NK phenotypes. The local CD4:CD8 ratio, examined on frozen sections (in the lack of any CD8 marker useable on paraffin material), is estimated at 0.87 ± 0.36. Most inflammatory macrophages in organized granulomas and in perivascular infiltrates expressed an HLA-DR+ phenotype (Fig. 5). An anomalous expression of HLA-DR (but not HLA-DQ) molecules on a consistent number of fibroblasts (about 15%) was detected in sections with dense tissue fibrosis. Other non-immune DR+ cells (endothelial cells and keratinocytes) are rarely observed to give a weak surface expression of the marker. The image analysis confirms the histological description of concentric zones (epithelioid and giant cells in the centre, frequently with fungal elements; activated macrophages and neutrophils in a distal zone; histiocytes and few lymphocytes in the periphery). A few cells were positive for proliferating cell nuclear antigen in the periphery of the granuloma, in addition to some cells of the basal layer in the epidermis. This gave us an internal control for the experiment (Fig. 6).

The results obtained with the two CD1 mAbs (Table 3) are in line with our controls on paraffin sections

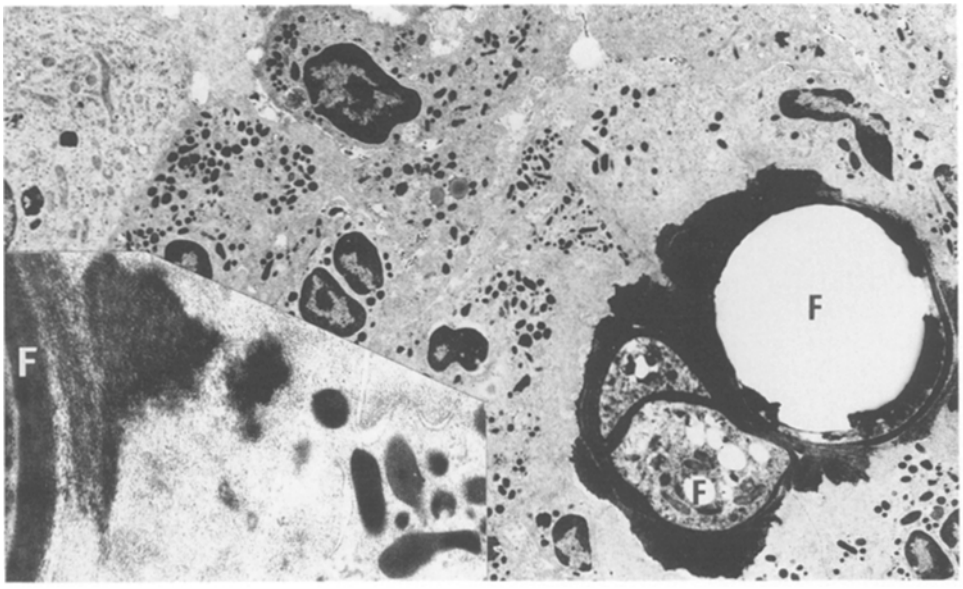
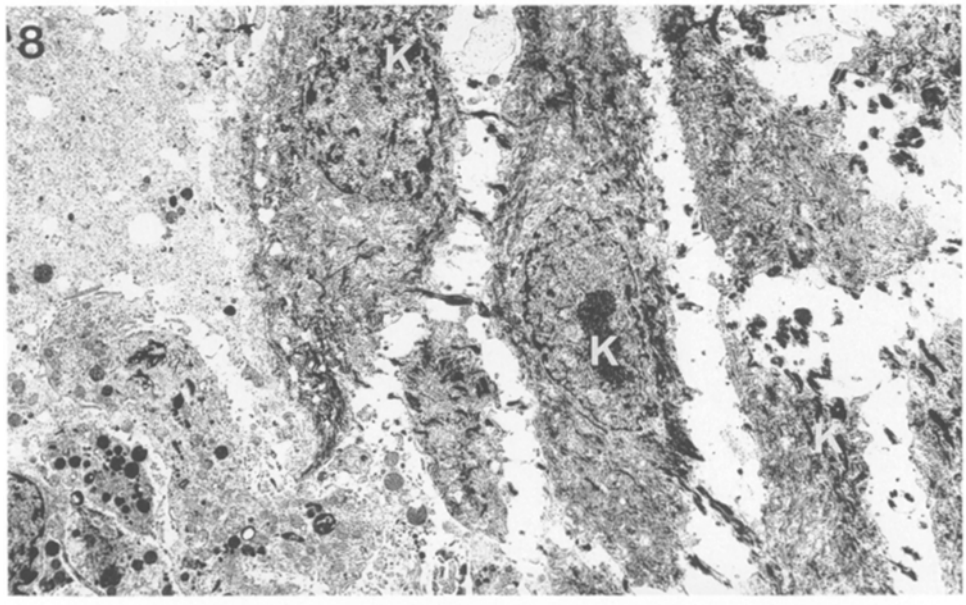


Fig. 7. Ultrastructural observation of polymorphonuclear neutrophils surrounding fungal elements (*F*). The inset shows a close contact between a yeast, with a partially destroyed wall, and a neutrophil, with typical elongated granules. Electron microscopy, ×1700; *inset* ×22000



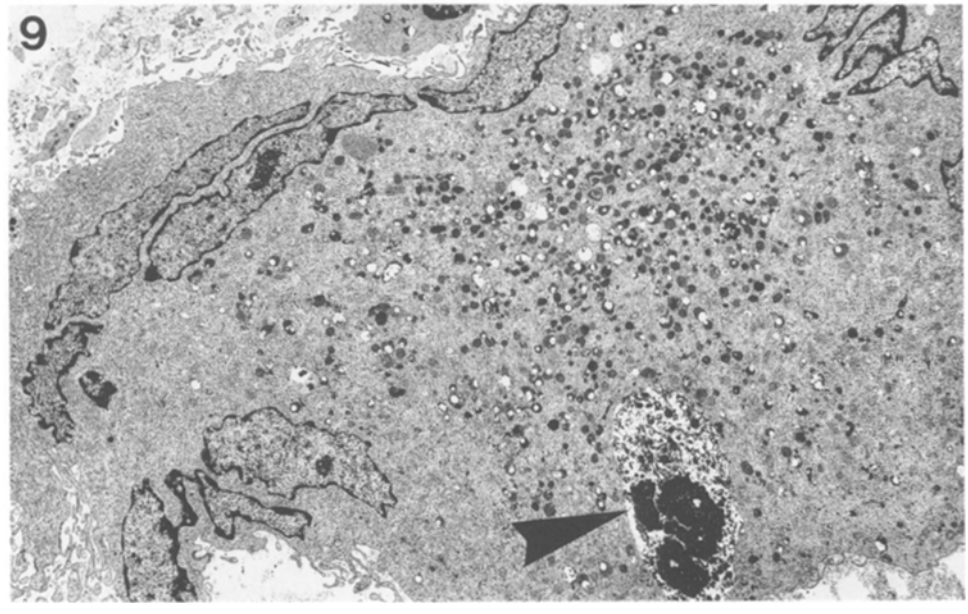
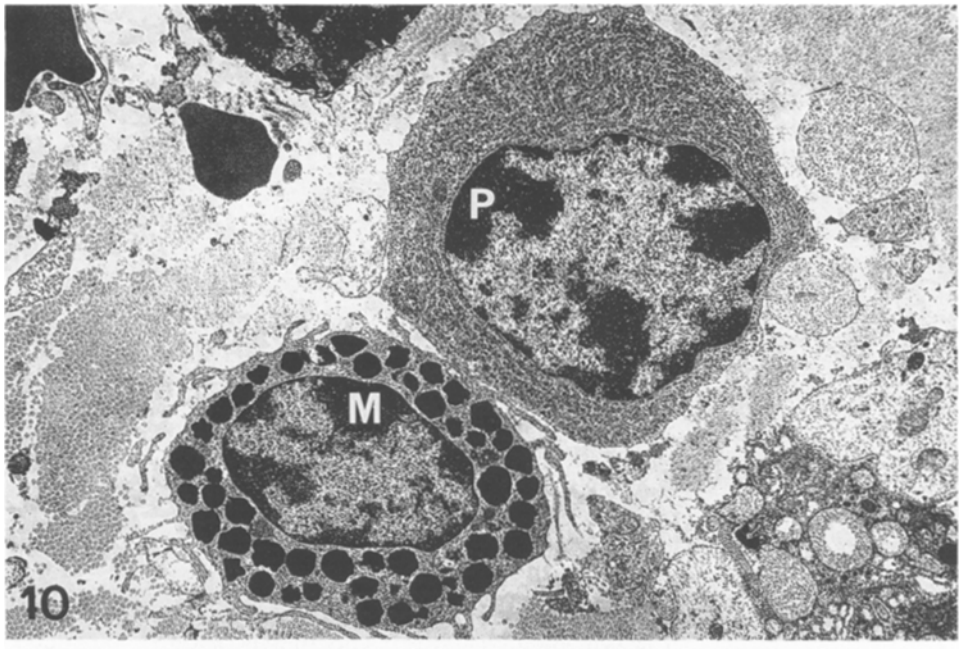


Fig. 8. Keratinocytes (*K*), observed in a peri-suppurative (neutrophil-rich) area, show features of necrosis. Marked intercellular oedema is responsible for the distended pattern observed in this epidermal hyperplasia. Electron microscopy, ×1700

Fig. 9. A giant cell of the central part of a granuloma shows multiple nuclei gathered in the periphery and a residual body (*arrowhead*) in the cytoplasm. Electron microscopy, ×1700



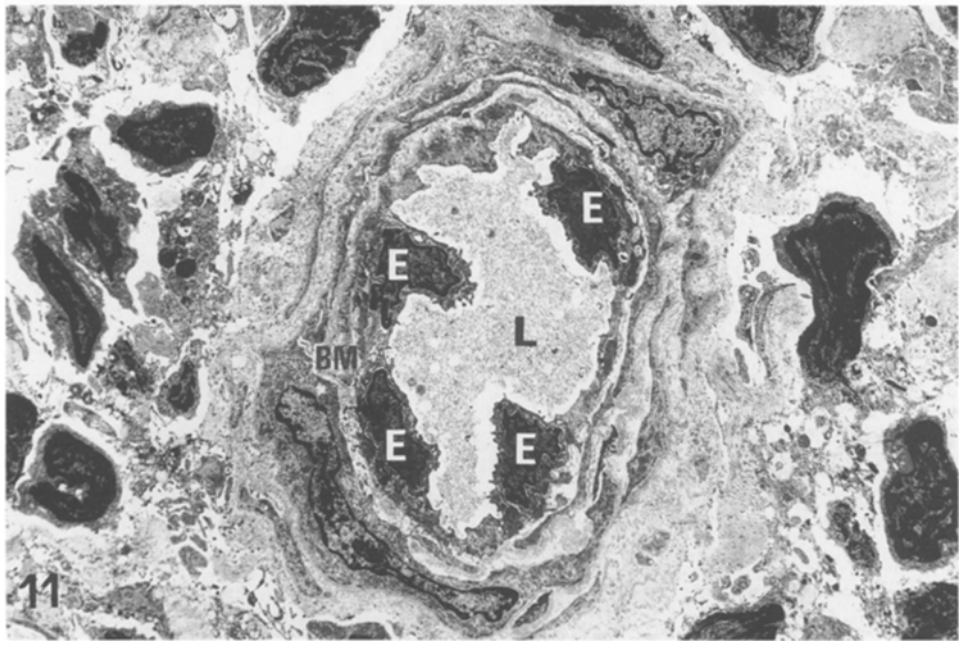


Fig. 10. Numerous mast cells (*M*), frequently in close contact with other cells here a plasma cell (*P*), are observed in the perigranuloma fibrosis. Electron microscopy, $\times 5000$

Fig. 11. A dense inflammatory infiltrate in a perivascular localization. Note the presence of activated endothelial cells (*E*), protruding into the lumen (*L*) of the vessel, and the thickening of the basement membrane (*BM*). Electron microscopy, $\times 1700$

(580.8 ± 165.2 S100+ cells in the epidermis and 21.9 ± 9.5 S100+ in the dermis). As with a more acute inflammatory infiltrate (Esterre et al. 1992b), we found some dendritic CD1b+ (S-100−) cells in the dermis. Elastase was easily detected with the NP57 marker within the neutrophils, but no extracellular form of elastase or interstitial collagenase was observed.

Some macrophages are immunostained with the anti-TNF-α mAb in the granulomatous reactions. The presence of TGF-β is also observed within some vessels (probably in platelets) and in the fibrotic areas (macrophages), but no IFN-γ secreting cells were detected on the same frozen sections.

In the dense connective matrix described in the centre of the verrucous lesion (Esterre et al. 1992a) and in the inflammatory edges studied here, undamaged fungal elements are observed by electron microscopy in close proximity to neutrophils and macrophages. The infiltrating neutrophils seem to be very active, with an expressive "frustrated" phagocytosis picture (Fig. 7 and Bainton et al. 1989). Some macrophages, with a relatively quiescent morphology, are regularly observed in close association with the abscesses. Keratinocytes look distended by intense intercellular oedema, and many of them have developed characteristic features of necrosis (Fig. 8). This is found only in epidermis presenting a pseudo-epitheliomatous hyperplasic reaction histogically and seems related to neutrophil cytotoxic activity.

In the dermal granulomatous reaction, numerous histiocytes are mixed with activated macrophages. Those

Table 2. Cell composition of the granuloma in chromomycosis

Antibodies	Cluster differentiation	Percentage of positive cells[a]
LCA	CD45	100.0
UCHL1	CD45RO	65.8±35.8
IOT11	CD2	18.1±14.2
IOT3	CD3	16.8±13.1
IOT4	CD4	7.8±4.9
IOT8	CD8	9.0±7.1
IOT9	(Transfer+)	9.4±5.1
IOT2a	(HLA-DR)	100.0
IOT14	(IL-2r)	6.5±5.9
LeuM3	CD14	59.1±26.3
IOM2	CD14	55.1±23.2
IOB1	CD37	0.86±0.8
Leu7	CD57	16.2±7.1
Leu11b	CD16	11.7±5.1
Leu19	CD56	10.2±4.8
ION3	(Granulocytes)	38.8±21.1

[a] Results expressed as mean percentage ± standard error

Table 3. Dentritic cells in the lesion of chromomycosis

Antibody	Cluster differentiation	Positive cells (cells/mm^2)[a]	
		in the epidermis	in the dermis
IOT6a	CD1a	520.4±154.1	37.9±28.2
IOT6b	CD1b	0.0	16.8±12.5

[a] Results are expressed as mean count ± standard error. The sample was composed of six biopsies

are closely associated with giant cells of Langhans type. In the most organized granulomas, multinucleated giant cells are observed in the central zone sometimes containing yeasts in different stages of degradation (Fig. 9). Lymphocytes and fibroblasts are present in the infiltrates. Numerous mast cells, isolated or in close contact with other cells (fibroblasts or lymphocytes; Fig. 10) are observed in the surrounding fibrosis. Important vascular changes [increased tortuosity of dermal capillary vessels, "high endothelial" morphology (Duijvestijn et al. 1988) and multiplication of the basal membrane] are frequently observed in the same areas (Fig. 11).

For the three patients whose lesions (two verrucous and one psoriasis-like) were biopsied after long-term (1 year) chemotherapy with itraconazole, two presented an obvious clinical improvement while the third did not show any cicatrization. In the intermediate (6 months) and the last (1 year) biopsies, histopathological examination revealed tissue remodelling and some signs of inflammatory reaction (epidermal and dermal microabscesses, with presence of apparently normal fungi in two cases; granulomatous reaction or, in one partially healed lesion, non-specific lympho-histiocytic infiltrates). Ultrastructurally, fungal elements which sometimes appeared viable, were observed in suppurative areas. The activated neutrophils and the ultrastructural alterations of the vascular system associated with the adjacent dermal fibrosis (Esterre et al. 1992a) were identical to observations made before the treatment.

Discussion

Information on the host defence mechanism against mycotic agents is very limited (Diamond 1989; Fromtling and Shadomy 1986; Lagrange and Hurtrel 1984), with the exception of standard histopathology (Salfelder 1990). Immunohistochemical techniques have been rarely used in order to define the cellular infiltrate in situ in lesions of paracoccidioidomycosis (Moscardi-Bacchi et al. 1989), lobomycosis (Esterre et al. 1991a) and, as preliminary results, in chromomycosis (Esterre et al. 1991b, 1992a).

The granulomatous reaction observed in chromomycosis must be differentiated from that described for other fungal diseases (Salfelder 1990), in particular sporotrichosis and American blastomycosis, and, to a lesser degree, in localized cutaneous leishmaniasis (Esterre et al. 1992b; Salfelder 1990). This fungal lesion is controlled, as in many other mycoses (Salfelder 1990), by phagocytes (polymorphonuclear neutrophils and tissue macrophages). These cellular defence mechanisms are inefficient in eliminating the fungi fully, as confirmed by the frequent observation of apparently viable yeast cells in an intracellular (macrophage phagolysosome or giant cell cytoplasm) or extracellular (neutrophil abscess) microenvironment. The alterations of the architectural pattern of the tissue seem to depend upon a cytokine-activated fibroblast subset responsible for the dermal fibrosis (Esterre et al. 1992a), and cytotoxic neutrophil activity (linked to the keratinocyte necrosis). The functional pertinence in vivo of mast cells in tissue fibrosis (Broide et al. 1990; Esterre et al. 1992a; Stevens and Austen 1989) remains to be established.

Further investigations on the host-parasite interactions in chromomycosis and on the biochemistry of the fungi are needed before developing novel therapeutic strategies such as combined antifibrotic immunotherapy and antifungal treatment to reduce the morbidity associated with this tropical disease.

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