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THE ANTIGENICITY OF TRICHOPHYTON RUBRUM: *IN SITU* STUDIES BY AN IMMUNOPEROXIDASE TECHNIQUE IN LIGHT AND ELECTRON MICROSCOPY

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Abstract. The antigenic sites of cultured and invading *Trichophyton rubrum* can be located by using an immunoperoxidase technique in light and electron microscopy. The cell wall is identified as a major antigenic determinant in both cultured and invading *T. rubrum* and, in the cultured dermatophyte, other intracellular structures are also shown to be antigenic. The keratin immediately surrounding invading hyphae shows positive staining, suggesting the diffusion of dermatophyte-derived antigens, possibly keratinolytic enzymes.

Key words: Trichophyton rubrum; Immunoperoxidase techniques; Electron microscopy.

Dermatophytosis is a common infection, but the mechanisms of dermatophyte pathogenicity and host immunity are unclear.

Chemically extracted components of dermatophytes will induce specific antibodies when injected into animals (6) and both immediate and delayed hypersensitivity when injected intradermally into human skin (1). One component, a glycoprotein, has been localized by an immunofluorescence technique to the cell wall of *Trichophyton mentagrophytes* (12). However, other antigens have not been demonstrated in situ in the living dermatophyte, whether in culture or in tissue.

Immunofluorescence studies on cultured fungi by light microscopy have substantiated the existence of marked cross-reactivity between *Trichophyton rubrum* and *Trichophyton interdigitale* and weaker cross reactivity with other dermatophyte species (10). Such a cross reaction obviously hampers immunological differentiation of dermatophyte species but does not invalidate the identification by antisera of antigenic sites within a particular species. Miura (1963) also highlighted the difficulties caused by the autofluorescence of some species of fungi. Recently, immunofluorescent labelling of invading dermatophytes in patients with chronic *T. rubrum* has been demonstrated by light microscopy (7). However, the sites of antigenicity on the fungus and the relationship of the invading dermatophyte to the surrounding corneocytes are more precisely determined by electron microscopy.

MATERIALS AND METHODS

Antisera

(*a*) Rabbit anti *T. rubrum* serum used in a dilution of 1 : 30. This antiserum was highly active against dermatophytes, but was shown to cross-react with *T. mentagrophytes* by crossed immunoelectrophoresis. Its preparation and properties have been described previously by Holden, Hay and MacDonald (8).

(*b*) Peroxidase-conjugated sheep anti-rabbit immunoglobulin used in a dilution of 1 : 30 (Institut Pasteur, Paris).

Skin specimens

Skin biopsies of hands and feet chronically infected with *T. rubrum* were obtained under local anaesthesia from 6 patients.

Light microscopy

(*a*) Small amounts of a 7-day-old culture of *T. rubrum* were heat-fixed onto a glass slide.

(*b*) 4 μm cryostat sections of infected skin prefixed with 2% glutaraldehyde in 0.1 mol/l sodium cacodylate were mounted on glass slides and air-dried for half an hour.

After gentle washing the immunohistochemical reaction was carried out as previously described (8) by incubating with rabbit anti *T. rubrum* antiserum followed by peroxidase-conjugated sheep anti-rabbit immunoglobulin. The peroxidase activity was revealed by the addition of Graham-Karnovsky medium (5) and the specimens were viewed with a Zeiss photomicroscope.

Electron microscopy

(*a*) Aliquots of a 7-day-old shaker culture of *T. rubrum* were centrifuged at 4000 rpm for 10 min. After removal of the supernatant, the resultant pellet was resuspended in 2% glutaraldehyde in 0.1 mol/l sodium cacodylate and fixed at 4°C for 3 hours.

After washing in isotonic buffer, the final pellet was thoroughly mixed with OCT compound before freezing in hexane in a bath of carbon dioxide and methanol slush (2). 15-μm sections were mounted on slides.

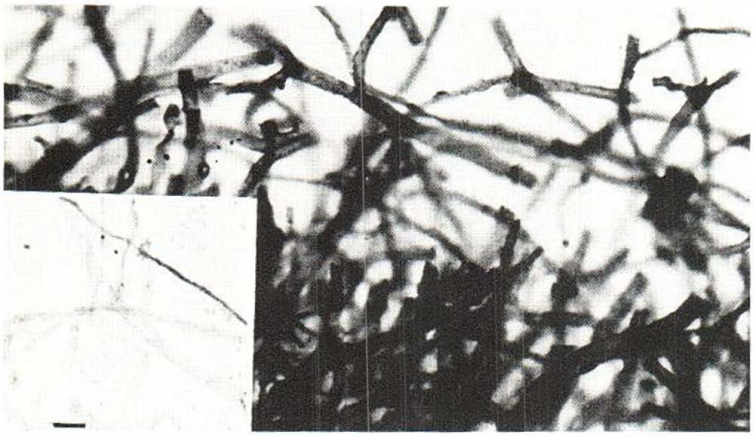

Fig. 1. Cultured *T. rubrum* (I. Po. ×500). Positive labelling of the hyphal walls by the linear deposition of reaction product. *Inset:* Control; no deposition of reaction product.

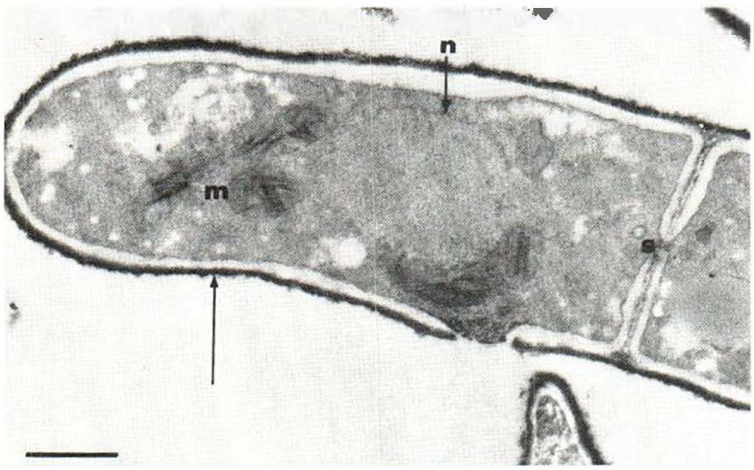

Fig. 2. Cultured *T. rubrum* (I. Po. ×30000). Linear deposition of the electron-dense reaction product along the cell wall (↑). *m*, mitochondria; *n*, labelling along the nuclear membrane; *s*, septal pore.

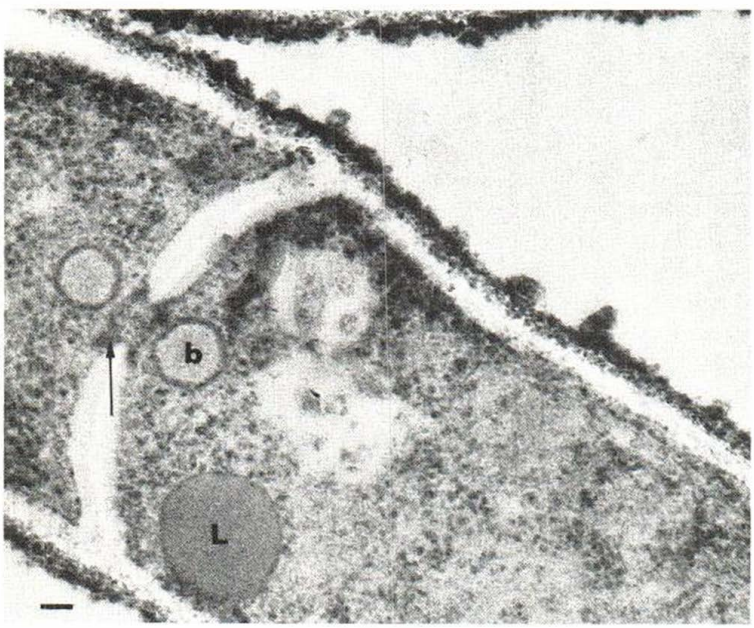

Fig. 3. Septal pore (×52000). Two linear condensations of the reaction product can be seen on either side of the septal pore (↑). *b*, septal bodies ringed in peroxidase; *L*, osmophilic lipid granule.

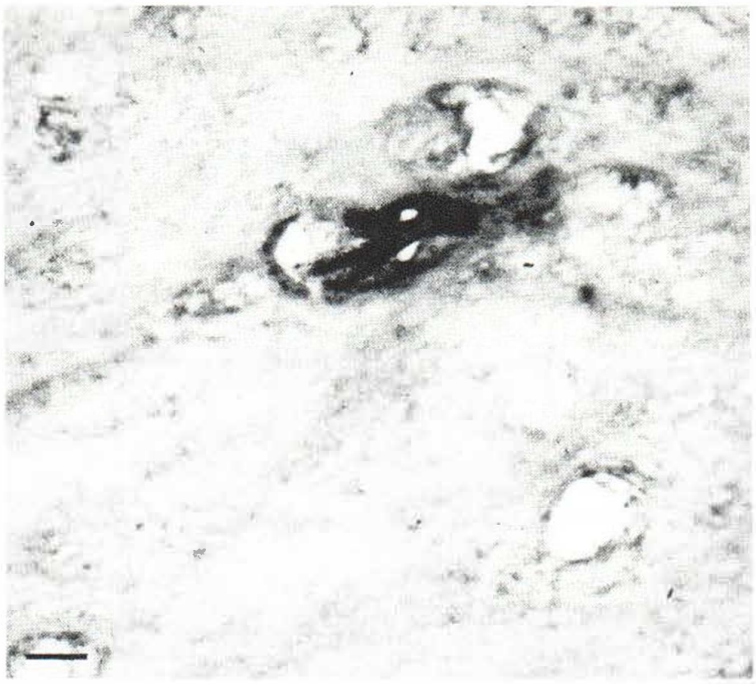

Fig. 4. Invading *T. rubrum* (I. Po. ×400). Characteristic labelling of the cell wall of the dermatophyte. Some labelling of the sorrounding keratin can be seen.

(*b*) 15-μm sections of prefixed skin were air-dried onto glass slides coated with formal gelatin to aid adhesion. The immunohistochemical reaction was performed and the tissue was post-fixed with 1% osmium tetroxide. The sections were dehydrated, embedded in resin, and areas suitable for ultrastructural examination were chosen from semithin sections. Ultrathin sections were studied without counterstaining.

Negative controls were employed as follows:

1. Non-immunized rabbit sera to replace specific anti-dermatophyte sera.
2. 0.1 M phosphate-buffered saline (PBS) (ph 7.3) to replace both sera.
3. Peroxidase-conjugated sheep anti-mouse immunoglobulin to replace peroxidase-conjugated sheep anti-rabbit immunoglobulin.

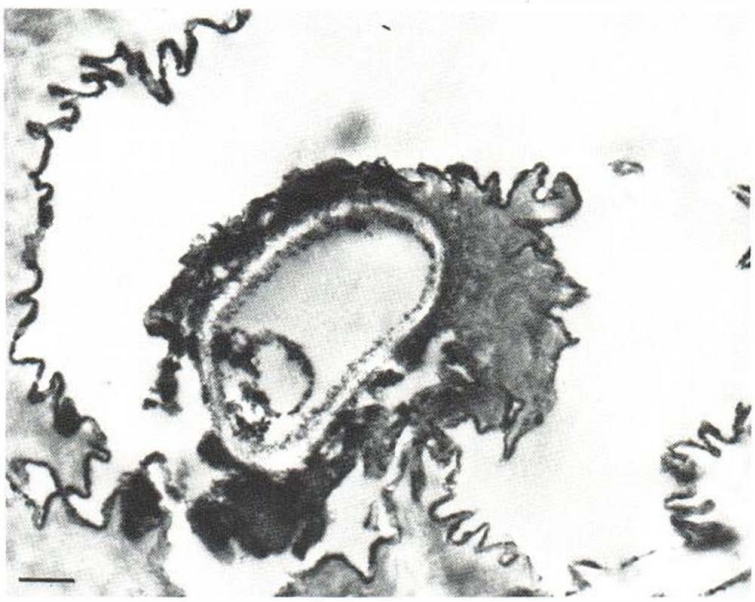

Fig. 5. Invading *T. rubrum* (I. Po. ×22 500). A dermatophyte hypha lies in a lacuna in the keratin. The margins of the lacuna are positively labelled.

RESULTS

(a) Cultured Trichophyton rubrum

Light microscopy revealed tangled fungal mycelia whose walls showed positive staining in the form of linear deposition of the brown reaction product (Fig 1). Control specimens showed absence of specific labelling (Fig. 1, inset).

Ultrastructurally, positive labelling of antigenic sites was evidenced by deposition of the electron-dense reaction product. Cell wall labelling, the major feature, was seen to consist of continuous deposition of the electron-dense reaction product on both inner and outer surfaces. Some labelling of other cytoplasmic organelles could be seen, such as mitochondria and the nuclear membranes (Fig. 2). The septal bodies were ringed by positive labelling and linear condensation of the electron-dense reaction product was also seen on either side of the septal pore (Fig. 3). The specific labelling was absent in control specimens.

(b) Invading Trichophyton rubrum

In light microscopy, the dermatophytes were located by focusing on the areas of deposition of brown reaction product seen at low magnification. These areas were absent in control specimens and no fungus could be found.

At high magnification of these diffusely stained areas, dermatophytes were found centrally, evidenced by the characteristic positive linear deposition along the cell wall (Fig. 4).

Ultrastructurally, the labelled *T. rubrum* hyphae were found lying in lacunae surrounded by corneocytes whose margins also showed positive labelling by the electron-dense reaction product (Fig. 5). The pattern of peroxidase labelling on the hyphae was similar to that seen in vitro. The cytoplasmic content of the fungal cells was often vacuolated and disorganized and the cell wall was the main site of antigenic labelling. Control specimens and areas not containing fungi were not labelled.

DISCUSSION

The immunoperoxidase technique easily demonstrates the site of the dermatophyte infection and under the electron microscope the relationship of the hyphae to the corneocytes is more easily seen. The same method may be used to study other mycotic infections such as candidasis or pityriasis versicolor.

Comparable studies may be undertaken using fluorescein-labelled conjugates but, unlike peroxidase-stained specimens, the immunofluorescent sections cannot be viewed with a conventional microscope or stored indefinitely. In addition the peroxidase technique allows ultrastructural examination of the tissue.

The cell wall was shown to be a major antigenic site in all studies, probably because of powerful and direct stimulation of antibody production by the constituent polysaccharide. However, the antigen used for raising antiserum in rabbits was a soluble extract of disrupted cells. Labelling of cell wall antigens, therefore, may suggest some of the cell wall components to be soluble, or the presence in cytoplasm of subunits of cell wall polysaccharide. The latter finding has been demonstrated with *Cryptococcus neoformans* (4). In this study, the cell membrane and the specialized structures around the septal pore were also highlighted as areas of antigenicity.

Intra- and extracellular keratinases have been isolated from *T. mentagrophytes* (13, 14), and the enzyme has been demonstrated around infected guinea pig hairs (3). *T. rubrum* has keratinolytic activity in vitro (11) and our finding of the hyphae of *T. rubrum* in the lacunae, whose margins were coated with dermatophyte-derived antigens, may suggest that a diffusible substance such as lytic enzyme is secreted by the dermatophyte.

It has been suggested that dermatophytes are limited to the stratum corneum by the presence of soluble serum factors, such as unsaturated transferrin (9). Diffusion across the epidermis is two-directional and it is likely that only the diffusible antigenic components such as secreted enzymes or products released from damaged hyphae are capable or reaching and stimulating the immunocompetent cells of the dermis or epidermis.

Further study with specific antisera against components of dermatophytes such as a keratinase, would elucidate this question and may further declarify the mechanism of dermatophyte pathogenicity and host immunity.

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