

## Binding and Uptake of *Trichophyton Rubrum* Mannan by Human Epidermal Keratinocytes: A Time-course Study

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*Trichophyton rubrum* infects skin. This fungus or its products might affect the function of epidermal cells. We previously reported that *T. rubrum* mannan (TRM) exhibits a suppressive effect on proliferation of human lymphocytes. The goal of the present study was to investigate the possibility of direct interaction of TRM with cultured normal human epidermal keratinocytes (EK). Mannan, a cell wall glycoprotein, was extracted from *T. rubrum* by precipitation with cetyltrimethylammonium bromide and conjugated with fluorescein isothiocyanate (FITC-TRM). After incubation of EK with 50 µg/ml FITC-TRM for 30 min, the surface of EK showed bright fluorescent staining. EK cultures pretreated with non-labelled TRM remained unstained. The fate of TRM bound to EK surface was determined in a time-course study. After pulse exposure to FITC-TRM, EK cultures were washed and incubated for various time periods. The EK moved surface mannan to the one area of cell membrane, so that at 4-6 h, the homogeneous staining of the entire cell surface was replaced by staining in a "cap" pattern. By 12 h, FITC-TRM was taken up into the cell, brought to the nuclear area and concentrated in the EK nucleoli. During the next 3 days nucleolar and cytoplasmic staining of the cells was observed. The intensity of fluorescence gradually diminished. On the 4th day, the sharp staining of organelles disappeared; instead, a large number of small fluorescent granules were seen intra- and extracellularly. By the 6th day after exposure, no EK staining remained. Thus, EK specifically bound, internalized and apparently catabolized TRM. These findings indicate that EK take up mannan and suggest that this could influence their function. Excreted catabolites could have biological effects on other cells. **Key words:** Fungal cell wall glycoprotein; Endocytosis; Fluorescence.

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Many factors probably elicit inflammation in skin infected by *Trichophyton rubrum*. The broad spectrum of the host defense includes both a specific immune response and non-immune responses that inhibit the growth of the fungus. Circulating antibodies (1), sensitized T lymphocytes (2), polymorphonuclear leukocytes (3), activated components of complement (4), serum inhibiting factor (5) and fungicidal products of professional phagocytes (6, 7) resist the infection. We recently reported that *T. rubrum* mannan (TRM), a fungal cell wall glycoprotein, inhibits antigenic and mitogenic responses of lymphocytes (8). By inhibiting the lymphocyte response to antigen, the fungus might be able to persist on the skin surface and establish a more chronic infection. If so, TRM must leave the stratum corneum and interact with immunocompetent cells

in the viable epidermis or dermis. Microconidia of *T. rubrum* can adhere to human epidermal keratinocytes (EK) and epidermal cells can phagocytize fungi (9, 10). This suggests that keratinocytes may directly participate in host defense, perhaps by processing *T. rubrum* products at the early stage of fungal invasion. We present herein data to show that EK can take up, concentrate and degrade or excrete mannan.

### MATERIAL AND METHODS

#### *Isolation and preparation of mannan*

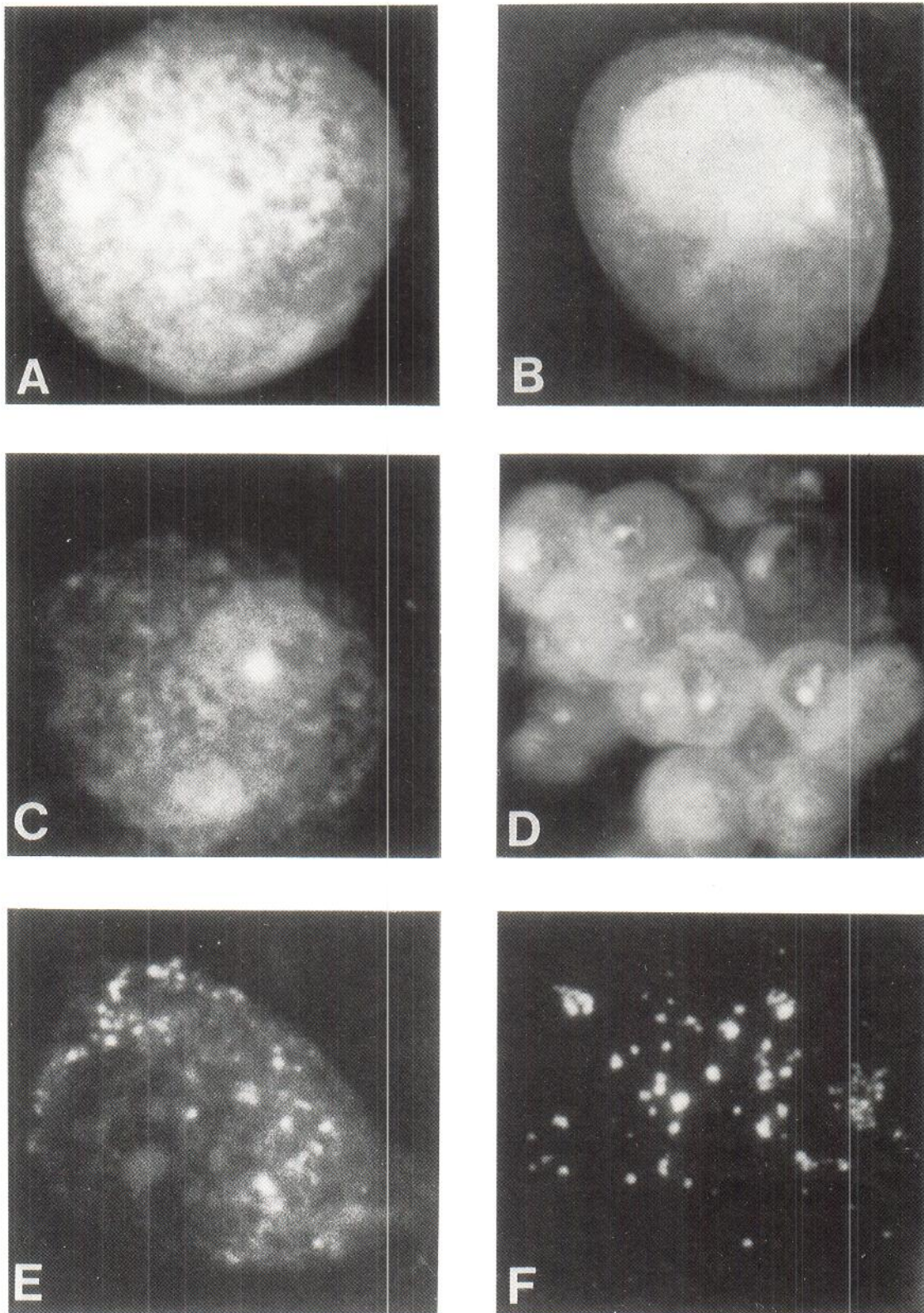
*T. rubrum* mycelia were obtained by the method of Blake et al. (8) by growing fungi for 3 weeks in 15-l carboys containing fluid Sabouraud medium (Difco, Detroit, MI) inoculated with a dehydrated *T. rubrum* isolate (No. 28188 American Type Culture Collection, Rockville, MD). Crude glycoprotein was extracted from lyophilized mycelia by addition of methanol, and mannan was further precipitated with cetyltrimethylammonium bromide (11). Purified *T. rubrum* mannan (TRM) was 75% carbohydrate by weight with a 4:1:1 ratio of mannose to glucose to galactose and contained 17% protein and 2.4% phosphate.

TRM was conjugated with fluorescein isothiocyanate (FITC) using a standard technique (12). Briefly, 2 mg FITC (Sigma Diagnostics, St Louis, MO) in 100 µl dimethyl sulfoxide was added to 10 mg TRM in 2.0 ml bicarbonate buffer, pH 9.5; and incubated for 2 h at 25°C. FITC-labelled TRM (FITC-TRM) was separated from superfluous FITC by chromatography on G-10 Sephadex (Pharmacia Fine Chemicals, Inc., Piscataway, NJ) and eluted using phosphate-buffered saline (PBS). A stock solution of FITC-TRM was prepared by dissolving the conjugate in PBS to a concentration of 5 mg/ml.

#### *Keratinocyte cultures*

EK were cultured from normal foreskins obtained by infant circumcision. The foreskin specimens were freed from fat and clotted blood, rinsed in Ca<sup>2+</sup>- and Mg<sup>2+</sup>-free PBS (Life Technologies, Inc, Grand Island, NY; Gibco) and placed, epidermis up, into 35 mm Petri dishes (Falcon 3001, Becton Dickinson Labware, Lincoln Park, NJ). The specimens were incubated overnight in a solution of trypsin (0.125%) in Eagle minimal essential medium (EMEM) containing Earle's salts and L-glutamine (Biologos, Inc, Naperville, IL) and supplemented with 10 µl gentamicin and 20 µl fungizone (Gibco) at 37°C in a humidified atmosphere with 5% CO<sub>2</sub> in air. Epidermis was separated from the dermis and individual EK disrupted by rapid pipetting. EK were resuspended in wash medium consisting of EMEM supplemented with 20% pooled decomplexed newborn calf serum (Biologos) and centrifuged for 10 min at 200 × g. The pellet was resuspended in growth medium (GM) consisting of keratinocyte serum free medium supplemented to contain 5 ng/ml recombinant epidermal growth factor and 50 µg/ml bovine pituitary extract (all from Gibco) and grown in 75 cm<sup>2</sup> Falcon tissue culture flasks at 37°C in CO<sub>2</sub> incubator. GM was changed every 3 days until keratinocytes covered 2/3 of the flask bottom. EK were released from the flasks surface by a 2-min incubation in 0.05% trypsin-0.02% EDTA solution (Biologos), spun 10 min at 200 × g in wash medium, resuspended in GM and passed to new T-flasks for further growth. After 2-3 passes EK were used in experiments.

Immunoperoxidase staining of cell smears employing DAKO-CK



*Fig. 1.* The patterns of EK fluorescence at different time periods after 30 min exposure to FITC-TRM. (A) Homogeneous staining of the entire cell surface immediately after exposure ( $\times 1500$ ); (B) "capping" pattern of surface staining at 6 h ( $\times 1500$ ); (C) clustered granular and nucleolar staining at 12 h ( $\times 1500$ ); (D) homogeneous cytoplasmic and sharp nucleolar fluorescence 48 h after exposure ( $\times 1000$ ); (E) punctate pattern of cytoplasmic staining at 96 h ( $\times 1500$ ); (F) extracellular fluorescent granules in EK cultures on the 5th day of incubation ( $\times 1250$ ).

monoclonal mouse anti-human cytokeratin antibody (MNF116, Dako-patts, Carpinteria, CA) revealed not less than 90% cytokeratin-positive keratinocytes in tested EK cultures.

The viability of EK at various stages of the experiments was never less than 85% as assessed by the trypan blue exclusion test.

#### Fate of mannan

EK were suspended in GM to a concentration of  $1 \times 10^6$ /ml, plated onto 8 chamber Lab-Tek tissue culture units (Nunc, Inc, Naperville IL) in a total volume of 100  $\mu$ l/chamber, and cultivated at 37°C in a humid CO<sub>2</sub> incubator. On the 3rd day of cultivation, 100  $\mu$ l GM containing FITC-TRM were added to each chamber (final concentration 50  $\mu$ g/ml), and incubated for 30 min. Then chambers slides were transformed into slides by removing bonding material, washed in PBS, air-dried and examined with a fluorescence microscope. In control experiments, EK cultures were preincubated for 30 min with 50  $\mu$ g/ml unlabelled TRM before being exposed to FITC-TRM. For extended incubation, EK (attached to tissue culture units) were thrice washed with prewarmed PBS and incubated for additional time periods in GM at 37°C in the humid CO<sub>2</sub> incubator. A phase-contrast microscope was employed in order to distinguish cellular organelles exhibiting fluorescence.

## RESULTS

Exposure of EK cultures to 50  $\mu$ g/ml FITC-TRM for 30 min resulted in the bright fluorescence of the entire keratinocyte surface (Fig. 1A). Pretreatment of EK cultures with unlabelled TRM prevented fluorescent staining of the cells. Extended incubation of EK after the unbound FITC-TRM had been removed from the cultures allowed us to determine the fate of mannan bound to epidermal cells. At 6 h, we observed a focal cell surface "capping" staining pattern of EK (Fig. 1B). At 12 h, FITC-TRM induced both granular cytoplasmic and nuclear fluorescence of the cells (Fig. 1C). Later, 24–72 h after exposure, mannan was seen in the EK nucleoli (Fig. 1D). At this time we also observed homogeneous cytoplasmic staining of the cells. The intensity of fluorescence gradually diminished during this period of observation. The pattern of fluorescent staining dramatically changed after 3 days of incubation. At 96–120 h, the cellular organelles were almost invisible. Instead, a large number of small intra- and extracellular fluorescent granules appeared in EK cultures. At 96 h, most of the cells exhibited a punctate cytoplasmic staining pattern (Fig. 1E). At 120 h, fluorescent granules were observed extracellularly (Fig. 1F). No fluorescent staining of EK remained after 5 days of incubation.

## DISCUSSION

We demonstrated that mannan can interact with epidermal cells. The abrogation of FITC-TRM binding to EK by unlabelled TRM suggests binding by a receptor/ligand type of interaction. Mannose (13) and mannose-6-phosphate (14) receptors have been previously found on mammalian cells, and TRM contains this sugar. If mannan interacts with these receptors or some other EK receptor(s), this may affect cell-to-cell attachments and interactions in infected skin.

After binding to the keratinocyte membrane, FITC-TRM was first clustered and then internalized by the cells. The time-course study revealed that FITC-TRM was transported to EK nuclear area where it accumulated in the cell's nucleoli.

Glycoproteins can bind to the fibrillar and granular components of nucleolus (15). Since TRM is a glycoprotein, and since the mannan-binding sites of mammalian cells are in the nucleoli where 45 S rRNA is synthesized (16), TRM may interfere with preribosomal RNA synthesis. This may affect cell function. For example, this may be how TRM decreases EK proliferation (17).

A homogeneous cytoplasmic staining was observed 24–72 h after pulse exposure to FITC-TRM. In EK cytoplasm, mannan could interact with mannose-6-phosphate lectin similar or identical to those that target hydrolytic enzymes to Kupffer cell lysosomes (18).

The homogeneous cytoplasmic staining by FITC-TRM in epidermal cells lasted 3 days. The disappearance of this type of staining and the appearance of small fluorescent granules extracellularly in EK cultures on the 4th day of incubation suggest that the processing of FITC-TRM by EK was concluded by the excretion of mannan or mannan catabolites. This observation is important since it provides evidence that epidermal cells may directly participate in the processing of fungal products.

Mannan inhibits host defense. Its pathogenic effects include inhibition of the immune response (8) and reduction of phagocytic ability (19). TRM did not kill epidermal cells but its uptake could affect their function. EK proliferation (17), desquamation (20), phagocytosis (10), killing of fungi (21), and production of cytokines and other mediators of inflammation (22, 23) could be affected by mannan to promote the survival of *T. rubrum* on the skin.

Thus, we have shown that human keratinocytes can bind, internalize and excrete TRM or its metabolites. These findings indicate that epidermal cells may process mannan. Since EK have some properties of antigen presenting cells (24) and since TRM is antigenic (25), this processing could be an event helping the host to rid the fungus by inducing a specific immune response. On the other hand, the uptake of TRM by keratinocytes might foster survival of fungus on the surface of the skin by altering normal EK proliferation and desquamation.

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## REFERENCES

1. Calderon RA, Hay RJ, Shennan GI. Circulating antigens and antibodies in human and mouse dermatophytosis: use of monoclonal antibody reactive to phosphorylcholine-like epitopes. *J Gen Microbiol* 1987; 133: 2699–2705.
2. Calderon RA, Hay RJ. Cell-mediated immunity in experimental murine dermatophytosis. II. Adoptive transfer of immunity to dermatophyte infection by lymphoid cells from donors with acute and chronic infection. *Immunology* 1984; 53: 465–472.
3. Dahl MV, Carpenter R. Polymorphonuclear leukocytes, complement, and *Trichophyton rubrum*. *J Invest Dermatol* 1986; 86: 138–141.
4. Swan JW, Dahl MV, Coppo PA, Hammerschmidt DE. Comple-

- ment activation by *Trichophyton rubrum*. *J Invest Dermatol* 1983; 80: 156–158.
5. King RD, Khan HA, Foye JC, Greenberg JH, Jones HE. Transferrin, iron, and dermatophytoses. I. Serum dermatophyte inhibitory component definitively identified as unsaturated transferrin. *J Lab Clin Med* 1975; 86: 204–212.
  6. Calderon RA, Hay RJ. Fungicidal activity of human neutrophils and monocytes on dermatophyte fungi, *Trichophyton quinckeanum* and *Trichophyton rubrum*. *Immunology* 1987; 61: 289–295.
  7. MacCarthy KG, Dahl MV. Inhibition of growth of *Trichophyton rubrum* by the myeloperoxidase-hydrogen peroxide-chloride system. *J Invest Dermatol* 1989; 92: 639–641.
  8. Blake JS, Dahl MV, Herron MJ, Nelson RD. An immunoinhibitory cell wall glycoprotein (mannan) from *Trichophyton rubrum*. *J Invest Dermatol* 1991; 96: 657–661.
  9. Zurita J, Hay RJ. Adherence of dermatophyte microconidia and arthroconidia to human keratinocytes *in vitro*. *J Invest Dermatol* 1987; 89: 529–534.
  10. Csato M, Bozoky D, Hunyadi J, Dobozy A. *Candida albicans* phagocytosis by separated human epidermal cells. *Arch Dermatol Res* 1986; 279: 136–139.
  11. Lloyd KO. Isolation, characterization, and partial structure of peptidogalactomannans from the yeast form of *Cladosporium werneckii*. *Biochemistry* 1970; 9: 3446–3453.
  12. Godin JW. Monoclonal antibodies: principles and practice. Production and application of monoclonal antibodies in cell biology, biochemistry and immunology. New York: Academic Press, 1983: 208–249.
  13. Pohlmann R, Nagel G, Hille A, Wendland M, Waheed A, Bräulke T, Figure K von. Mannose-6-phosphate receptors: structure and function. *Biochem Soc Trans* 1989; 17: 15–16.
  14. Shepherd VL, Campbell EJ, Senior RM, Stahl PD. Characterization of the mannose/fucose receptor on human mononuclear phagocytes. *J Reticuloendothel Soc* 1982; 32: 423–431.
  15. Bourgeois CA, Seve AP, Monsigny M, Hubert J. Detection of sugar-binding sites in the fibrillar and the granular components of the nucleolus: An experimental study in cultured mammalian cells. *Exp Cell Res* 1987; 172: 365–376.
  16. Fakan S, Puvion E. The ultrastructural visualization of nucleolar and extranucleolar RNA synthesis and distribution. *Int Rev Cytol* 1980; 65: 255–299.
  17. Cabrera RM, Blake JS, Dahl MV, Herron MJ, Nelson RD. Inhibition of keratinocyte proliferation by a mannan glycoprotein isolated from *Trichophyton rubrum*. *J Invest Dermatol* 1991; 86: 616A.
  18. Kiess W, Thomas CL, Greenstein LA, Lee L, Sklar MM, Rechler M, Sahagian GG, Nissley SP. Insulin-like growth factor-II (IGF-II) inhibits both the cellular uptake of beta-galactosidase and the binding of beta-galactosidase to purified IGF-II/mannose 6-phosphate receptor. *J Biol Chem* 1989; 264: 4710–4714.
  19. Felipe I, Bim S, Loyola W. Participation of mannose receptors on the surface of stimulated macrophages in the phagocytosis of glutaraldehyde-fixed *Candida albicans*, *in vitro*. *Braz J Med Biol Res* 1989; 22: 1251–1254.
  20. Sohnle PG, Kirkpatrick CH. Epidermal proliferation in the defense against experimental cutaneous candidiasis. *J Invest Dermatol* 1978; 70: 130–133.
  21. Csato M, Kenderessy ASZ, Dobozy A. Enhancement of *Candida albicans* killing of separated human epidermal cells by ultraviolet radiation. *Br J Dermatol* 1987; 116: 469–475.
  22. Partridge M, Chantry D, Turner M, Feldmann M. Production of interleukin-1 and interleukin-6 by human keratinocytes and squamous cell carcinoma cell lines. *J Invest Dermatol* 1991; 96: 771–776.
  23. Pentland AP, Needleman P. Modulation of keratinocyte proliferation *in vitro* by endogenous prostaglandin synthesis. *J Clin Invest* 1986; 77: 246–251.
  24. Dupuy P, Bagot M, Hesland M, Dubertret L. Synthetic retinoids inhibit the antigen presenting properties of epidermal cell *in vitro*. *J Invest Dermatol* 1989; 93: 455–459.
  25. Holden CA, Hay RJ, MacDonald DM. The antigenicity of *Trichophyton rubrum*: *in situ* studies by an immunoperoxidase technique in light and electron microscopy. *Acta Derm Venereol (Stockh)* 1981; 61: 207–211.