Detection of Antigens in Mycelial and in Arthroconidial Phases of *Trichophyton mentagrophytes*

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ABSTRACT. Protein pattern changes were investigated in the filamentous fungus *Trichophyton mentagrophytes* during the morphological transition induced by increased temperature and higher CO_2 partial pressure in cultivation atmosphere. The differences between the mycelial and the arthroconidial phase were characterized by SDS-PAGE and by immunodetection with mouse polyclonal antibodies. The components found by Western blotting in mycelia were 88, 86, 32, 29, 19.5, 18.5 kDa, in arthroconidia 108, 92, 88, 66, 56, 41, 39, 19.5 kDa. The results suggest the participation of some heat shock associated proteins of *T. mentagrophytes* in host immune response against mycotic infection.

Reversible transitions between mycelial and yeast phases were descibed in many mycopathogens, *e.g. Candida albicans, Paracoccidioides brasiliensis* and *Histoplasma capsulatum* (Szaniszlo 1985). Heat shock proteins (HSP) take part in the phase transitions of dimorphic fungal pathogens (Plesovski-Vig 1996). Increased expression of HSP was observed both in the microorganism and in the host during infection. HSP are immunodominant antigens of many pathogens (De Nagel and Pierce 1993; Suzue and Young 1996). In view of the high evolutionary conservation of HSP, cross-reactivity may occur between the HSP of the microorganism and the HSP of the host (Al-Dughaym *et al.* 1994). With *Candida albicans* the monoclonal antibody is presumed to have a protective effect against conservative epitope of HSP90 during systemic candidosis (Matthews *et al.* 1991). Homology was found between HSP70 and the 80 kDa protein from *H. capsulatum* (Gomez *et al.* 1992; Jeavons *et al.* 1994), which induces immune response in mice. Four proteins (80, 66, 54, 32 kDa) from the cell wall and from the plasma membrane of *H. capsulatum* reacted with two monoclonal antibodies against HSP70 (Gomez *et al.* 1992). The immune response that could be set against specific epitopes of such proteins offers possibilities for diagnosis and for vaccine development (Kaufmann 1990; Suzue *et al.* 1996). Although dermatophytes are not ranked among typical dimorphic fungi, the morphological transition occurs in these fungi even during pathogenic process. While the saprotrophic (mycelial) phase is characterized by filamentous mycelium with microconidia and macroconidia, arthroconidia, or arthroconidia-like forms are differentiated during the parasitic (arthroconidial) phase. This phenomenon was described even for *in vitro* conditions in dermatophytes (Bibel *et al.* 1977; Weigl and Hejtmánek 1979). Morphological transition was induced by enhanced temperature (37 °C) in air atmosphere with increased CO_2 (10 %). These conditions correspond with those in pathogens' preferred locality on the skin surface (Weigl 1981). It can be hypothesized that morphological transition in dermatophytes is accompanied by modification of the fungal metabolism analogous to that seen in typical dimorphic pathogens (Szaniszlo 1985). Monitoring of previously described metabolism alterations is important for determining the parallel changes, which occur in the range of antigen of the pathogen and in the immune response of the host.

This work is focused on the monitoring of the protein pattern changes during morphological transition of *T. mentagrophytes* and on the interactions of the expressed fungal proteins with polyclonal antibodies from immunized mice. Some of these proteins presumably belong to HSP, especially those that appear only in the arthroconidial form.

MATERIAL AND METHODS

Strain. Strain TM-10 of *Trichophyton mentagrophytes* from the dermatophyte culture collection of *Department of Biology, Medical Faculty, Palacký University* in Olomouc was used. This strain has been isolated from a cutaneous lesion in cattle and maintained on preservative agar media. It forms granular colonies with abundant microconidial sporulation on plates with Sabouraud glucose agar.

Cultivation. Microconidial suspension prepared by filtering homogenized mycelium through cotton-wool (Bibel *et al.* 1977) was spread on Sabouraud glucose agar plates (Vanbreuseghem *et al.* 1978) with thiamine (50 mg/L media). The mycelial phase was grown at 26 °C in the dark with standard atmosphere for 12 d. The arthroconidial phase was cultivated on the same media at 37 °C with 10 % CO_2-in-air atmosphere for 12 d.

Protein isolation. Mycelia or arthroconidia were harvested and washed three times with cold 0.8 % NaCl (4 °C) in glass tubes. Then 10 mL of buffer (0.8 % NaCl, 2 mmol/L EDTA, 20 mmol/L Tris, 0.4 mmol/L PMSF) was added and the tubes were agitated with the glass beads (5 mm diameter) for 10 min. Ballotini beads (100 μm) were added to the resulting suspension and the fungi were disintegrated (Desintegrator-S, *Biomatik GmbH*) at 75 Hz for 10 min at 0 °C. After centrifugation (6000 *g*, 10 min, 0 °C), both supernatants from the mycelial and the arthroconidial phases were lyophilized.

Electrophoresis. Lyophilized samples were dissolved in 50 μL sample buffer (5 mg/mL) containing: 1 % SDS, 10 % glycerol, 5 % 2-mercaptoethanol, 125 mmol/L Tris-HCl (pH 6.8) and 200 ppm bromophenol blue. Before loading in duplicate, the samples were heated to 95 °C for 5 min (Laemmli 1970). SDS-PAGE was done on 7.5–15 % gradient acrylamide gel at 250 V for 3 h. Proteins were visualized by silver staining (Hochstrasser *et al.* 1988).

Mice immunization. Ten female Balb/c mice about 25–30 g were employed for the immunization. These mice were immunized 3 times in 14 d periods. Each mouse received subcutaneously up to 0.2 mL of conidial suspension (10^6 conidia per mL H_2O). The full Freund adjuvans was used for the first immunization. The mice were bled 14 d after the application of the third dose of *T. mentagrophytes* conidia. Blood was centrifuged and the sera were pooled.

Immunoblotting. After electrophoresis, the proteins were transferred onto nitrocellulose membrane (NC), pore size 0.45 μm. Western blotting was done in buffer (25 mmol/L Tris, 192 mmol/L glycine, 20 % methanol, 0.1 % SDS) (Towbin *et al.* 1979) at a constant voltage of 0.8 V/cm^2 for 2 h. The membrane was cut into two identical parts. The first part was stained with 500 ppm aminoblack in 2-propanol–acetic acid–water (5 : 2 : 13, *V/V/V*) to verify the transfer. The second part of the membrane was used for immunodetection. The membrane was blocked in 5 % skimmed milk in PBS for 1 h, then placed into a solution of diluted mouse serum in 5 % skimmed milk (1 : 100) and incubated at room temperature for 3 h. Washing (3 × 5 min) in skimmed milk followed. The membrane was then incubated in a solution of pig antibodies against mouse immunoglobulins with peroxidase tag (1 : 4000) (*Sevac* — SwaMPx) in 5 % skimmed milk and washed three times in 0.1 % Tween-20 in PBS (3 × 5 min) and twice in PBS (2 × 5 min). Solution of 300 ppm 3,3'-diaminobenzidine in 50 mmol/L Tris-HCl (pH 7.5) + 90 ppm H_2O_2 was used for the visualization. The reaction was terminated by washing in 0.5 % NaN_3 (5 min). The detected bands were converted to a digital form and their mobility was evaluated by specialized software (Elfoman 2.0).

RESULTS

The transition of the cells from the mycelial to the arthroconidial phase was observed after a 12-d cultivation at 37 °C in a 10 % CO_2 atmosphere. The differences in the morphology of the mycelial and the arthroconidial phase were recognized at both macroscopic (Fig. 1) and microscopic (Fig. 2) levels.

The molar mass of proteins isolated from arthroconidia (A) differed markedly from that of mycelial-phase (M) proteins. Bands detected by silver staining of both vegetative phases generated characteristic patterns (Fig. 3).

After the transfer of proteins from the gel to the NC membrane, the separated proteins with molar mass of 88, 86, 32, 29, 19.5 and 18.5 kDa reacted strongly with polyclonal mouse antibodies against *T. mentagrophytes* proteins from the mycelial phase (M). Detected arthroconidial (A) proteins had molar mass of 108, 92, 88, 66, 56, 41, 39 and 19.5 kDa (Fig. 4). The most intensive bands on the membrane were due to 32 and 19.5 kDa mycelial phase antigens. The 32 kDa band was missing and the intensity of the 19.5 kDa band was reduced in arthroconidial phase. In line (A), the 32 kDa band was absent and the 19.5 kDa band was attenuated also on the gel. On the other hand, bands visualized by immunodetection in arthroconidia only (108, 92, 66, 56, 41, 39 kDa) were intensive also on the silver stained gel.
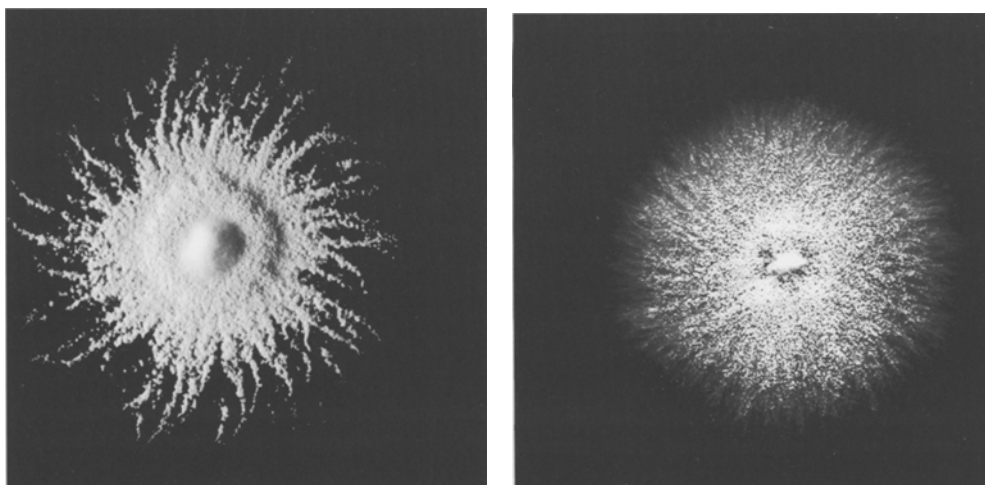
Fig. 1. *T. mentagrophytes* after 9 d of cultivation; *left*: mycelial phase formed characteristic white granular mycelium with 44 mm colony diameter, *right*: arthroconidial phase formed fine granular mycelium with 28 mm colony diameter.
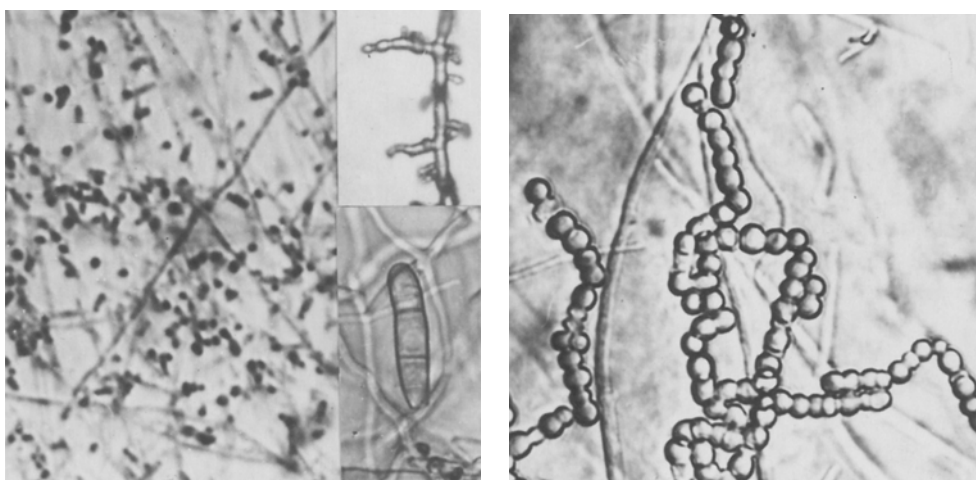

Fig. 2. Microphotographs of *T. mentagrophytes* after 12 d of cultivation; *left*: mycelial phase with the typical hyphae and microconidia, *right*: arthroconidial phase with differentiated arthroconidia; magnification 560×.

DISCUSSION

Dermatophytes exhibit morphological plasticity which is demonstrated also in other genera of pathogenic fungi. Various morphs are distinguished especially during the transition from the saprotrophic to the parasitic phase (Bibel *et al.* 1977; Weigl and Hejtmánek 1979). It can be assumed that the morphological transition changes not only the metabolism of the fungi, but also the antigen spectra of the fungal pathogen. Some antigen-effective components, especially polysaccharides, induce humoral immune response. Fungal antigens activate the cellular effector system (Calderon 1989; Jones 1989; Matthews *et al.* 1988; James 1990). The principle of this phenomenon is not yet clear. A number of experimental results indicate that proteins, which are presented in both mammalian MHC class II and MHC class I pathway, are necessary for the development of antifungal vaccines. Thus, both humoral and cellular components of the immune system are activated (Romani 1977; Calderon 1989; Buc and Ferenčík 1994). However, only some among the presented fungal antigens may serve as protective
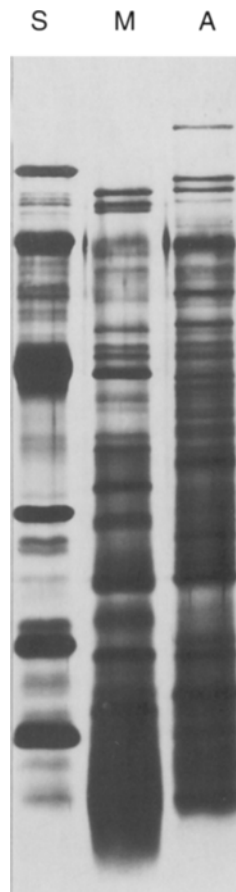
S   M   A

M   A

Fig. 3. SDS–PAGE, gradient gel 7.5–15 % stained by silver; S — standards of molar mass (kDa), M — sample from the mycelial phase, A — sample from the arthroconidial phase.
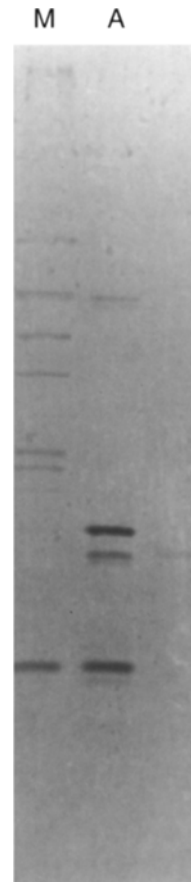
Fig. 4. Immunoblot detected by polyclonal mouse antibodies against *T. mentagrophytes*; M — sample from the mycelial phase, A — sample from the arthroconidial phase.

antigens which activate an effective defense mechanism against fungal pathogens. Literature data confirm that in mycopathogens such as *C. albicans* and *H. capsulatum*, such antigens include heat shock proteins (HSP) or products originating by their cleavage (Smith and Griffin 1995; Gomez *et al.* 1992; Gomez *et al.* 1995). The detection of arthroconidial proteins of molar mass 108, 92, 66, 56, 41, 39 kDa by polyclonal mouse antibodies against *T. mentagrophytes* point to the possibility of analogous involvement of heat shock proteins in the immune response of the host against *T. mentagrophytes* infection.

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