Laboratory study

IMMUNOGENIC PROTEINS IN DERMATOPHYTE MYCELIAL EXTRACTS

M. Drobnič-Košorok, P. Zrimšek and I. Zdovc

ABSTRACT

Background. Dermatophyte cytosolic proteins and cell wall proteins as well as different metabolites secreted by fungi are known to be involved in eliciting humoral and cellular immune response in invaded host.

Methods. SDS-PAGE and Western blot techniques were used to study the imunogenic nature of the cytosolic proteins extracted from two dermatophyte species *Microsporum canis* and *Trichophyton mentagrophytes* and two non dermatophyte fungal species *Malassezia pachydermatis* and *Aspergillus fumigatus.* Crossreactivity was confirmed with ELISA technique.

Results. SDS-PAGE analysis of cytosolic proteins revealed several protein bands, which are common to the dermatophytes examined, together with some distinctive bands, which enabled us to discriminate between the dermatophyte species. However, comparison of the cytosolic protein profile obtained from dermatophytes and nondermatophyte fungi studied demonstrated a much higher extent of species-specific proteins. The crossreactivity nature of the investigated fungal antigens was observed in ELISA, and developed for the detection of specific IgG in cats infected with *Microsporum canis*.

Conclusion. Besides strongly crossreacting antigenic protein bands, Western blot analysis, using the same serum samples as in ELISA, also disclosed species-specific immunogenic proteins, which may be the candidates for further investigation of cellular immune response and development of protective vaccines.

KEY WORDS

Microsporum canis, dermatophytes, cats, SDS-PAGE, immunoblotting, antigens

INTRODUCTION

Microsporum canis is an important fungal pathogen of the skin of man and several animal species. Among domestic animals *Microsporum canis* infection in cats accounts for nearly all of dermatophyte infections (1). However besides the direct involvement of *Microsporum canis* in feline skin diseases, an infected cat represents a possible carrier of the infection, the cat population in general can therefore be assumed as a reservoir of human infection (1,2).

Although dermatophytosis is a superficial infection of keratinized tissue by a species of fungus, the appearance of circulating antibodies as well as cellular immune response to dermatophyte antigens have been demonstrated (2,3,4,5,6,7,8,9,10,11).

As has been reported, feline dermatophytosis shares several clinical features in common with human Trichophyton infection, such as the minimal host inflammatory responses observed during infection and the apparent chronic nature of some infections. Therefore, we believe it beneficial to study the cat as a potential model of human dermatophyte infection and to determine similarities between the feline and human immune response in dermatophytosis (2).

In other animal species such as cattle, investigation of the immunological response to dermatophyte fungi has led to the development of effective vaccines (12). Several attempts have been made to obtain a vaccine with good immunoprophylactic properties against infection with *Microsporum canis*, however none of the preparations developed showed satisfactory effects in carnivores or man (13,14,15).

The relative importance of the various antigenic proteins in the immune response is still poorly investigated, though it is supposed that this may represent a key factor in understanding the pathogenesis of the disease and could be important in the development of protective vaccine (16).

The activation of the immune system is thought to be due to the action of proteolytic enzymes, bound to the cell surface, present intracellularly, or secreted by the fungus.

It was found that antibodies against keratinolytic proteinase were detected in 75% of the sera of guinea pigs infected with *Microsporum canis* as well as in 55% of patients with dermatophytosis (17).

In our study we tried to determine the protein pattern, typical for different dermatophyte and non-dermatophyte fungi isolated from domestic animals and to analyze the immunogenic potential of the proteins representing possible antigens for the host.

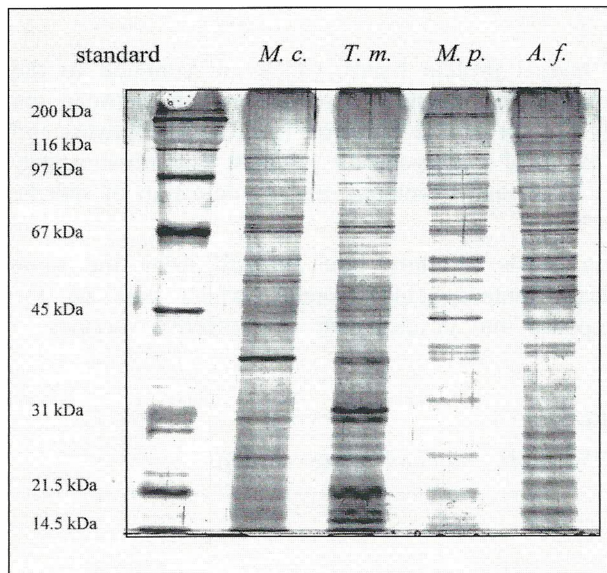

Figure 1. SDS-PAGE of standard proteins and cytosolic protein extracts of Microsporum canis (M.c.), Trichophyton mentagrophytes (T.m.), Malassezia pachydermatis (M.p.) and Aspergillus fumigatus (A.f.).

MATERIALS AND METHODS

PREPARATION OF ANTIGENS

The fungal protein extracts were prepared as described by Zdovc (18,19).

DETERMINATION OF PROTEIN CONCENTRATIONS

Protein concentration was determined according to the method of Bradford using Bio-Rad Assay II (Bio-Rad, Germany) with bovine serum albumin as a standard (20).

SODIUM DODECIL SULFATE-POLYACRYLAMIDE ELECTROPHORESIS (SDS-PAGE) ANALYSIS

Electrophoretic separation of fungal antigens and standard protein samples was carried out under discontinuous, denaturing conditions according to modified method of Laemmli (21). The cytosolic extracts were mixed in a ratio of 1:4 with sample buffer, containing 2% sodium dodecil sulphate (SDS) and 5%b-mercaptoetanol and applied on 4% stacking gel followed by separation on 12% resolving gels in TRIS/HCl buffer, pH 8.3. Electrophoresis was performed at 200V for 45 min at room temperature.

The protein bands were visualized by silver staining, using the Bio-Rad silver staining kit and scanned by GS-700 Imaging Densitometer, Bio-Rad.

SERUM SAMPLES

Sera from cats with naturally occurring microsporosis, and dermatophyte free animals as identified by fungal culture, were taken for immunochemical studies.

WESTERN BLOTTING

The separated proteins were electrophoretically transferred to PVDF membrane (Immobilon-P, Millipore USA) at 100V for 45 min in 0.025 M Tris buffer, containing 0.192 M glycine, and 20% of methanol. The membrane was incubated by shaking over night at 4°C in PBS, pH 7.2, containing 2% $Tween_{20}$. After washing three times with 0.05% $Tween_{20}$ in PBS, the membrane was incubated 1.5h at room temperature with feline sera, diluted 1:400 with PBS, containing 0.05% $Tween_{20,}$ followed by three 5 min cycles of washing with the same buffer. The membrane was further incubated for 1 h at room temperature with horseradish peroxidase labeled antifeline goat IgG in a ratio 1:1500 in PBS, containing 0.05% $Tween_{20}$, washed, and the complexes were detected with aminoethilcarbazol in the presence of 30% H_2O_2. Protein standards applied to SDS gels were transferred to the membrane by the same procedure as described above and afterwards stained by Coomassie Brilliant Blue (R-250).

ELISA

Indirect enzyme linked immuno assay, developed for detection of specific feline IgG in cats with naturally occurring microsporosis, was used to study the crossreactivity nature of different fungal antigens (22).

RESULTS

Water soluble protein antigens, liberated from mechanically disrupted mycelium of two dermatophyte species and two non-dermatophyte fungal species isolated from cats were analyzed by SDS-PAGE.

From Fig 1 it is evident, that cytosolic dermatophyte extracts, belonging to the two different species analyzed (*Microsporum canis* and *Trichophyton mentagrophytes*), exhibit similar protein profiles. They share nearly 50% of the protein bands observed in the range from 14.5 to 200 kD. Both species expressed a clearly visible species-specific protein band at 35 kD.

The protein pattern of the cytosolic extracts obtained from *Malassezia pachydermatis* and *Aspergillus fumigatus* which were selected as non-dermatophyte fungi can easily be discerned from the dermatophytes on the basis of SDS-PAGE, demonstrating differences at low molecular weights.

The reactivity of the investigated protein samples with the sera, obtained from cats with naturally occurring microsporosis was studied by Western blot analysis and the results were compared with the data obtained with ELISA developed for detection of humoral immune response in cats with naturally occurring microsporosis (22). It is evident that ELISA

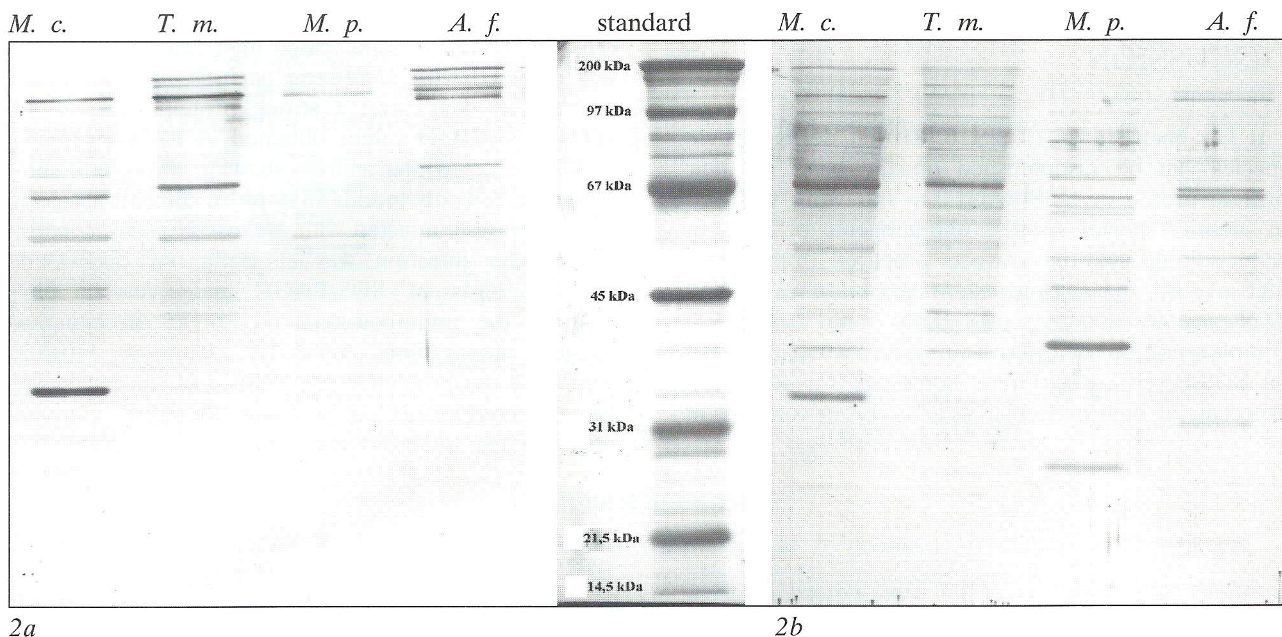

Figure 2. Two examples of Western blot analysis of cytosolic protein extracts of Microsporum canis *(M.c.),* Trichophyton mentagrophytes *(T.m.),* Malassezia pachydermatis *(M.p.) and* Aspergillus fumigatus *(A.f.) with sera taken from cats with naturally occurring microsporosis.*

showed no statistically significant differences (P=0,9226) in the reaction of feline sera with different dermatophyte antigens. However, these results differ significantly from the ELISA OD values, obtained with *Malassezia pachydermatis* (P=0,0005) or *Aspergillus fumigatus* (P<0,0001) antigens respectively.

The same feline sera were used for the Western blot analysis (Fig 2a). As expected, several common immunoreactive bands were detected in dermatophyte protein extracts, mainly in the range of 50-200kDa. A species-specific immunoreactive protein band was observed at 35 kD in the case of *Microsporum canis* cytosolic extract, which was absent in the case of *Trichophyton mentagrophytes* cytosolic extracts, although a strong protein band is present on SDS-PAGE gels at a similar molecular weight.

The crossreactivity with the nondermatophyte fungal antigens was considerably less expressed. Only a few *Microsporum canis* positive feline sera, which were highly reactive in ELISA as well, showed a specific positive reaction band at 40kD (Fig. 2b) in the reaction with *Malassezia pachydermatis* cytosolic proteins. Most of the sera gave only weak positive reactions with *Malassezia pachydermatis* and *Aspergillus fumigatus* high molecular weight proteins in the antigenic preparation which is in good correlation with the results obtained using ELISA, mentioned above.

DISCUSSION

It has been shown (23,25,16,18) that dermatophyte species can be distinguished on the basis of their protein profile by SDS-PAGE. In our work we tried to identify species specific immunogenic proteins in water-soluble cytosolic extracts prepared from fungal mat of two dermatophytes, *Microsporum canis* and *Trichophyton mentagrophytes.* They both revealed a complex pattern of cytosolic proteins when analyzed by SDS-PAGE gels. Great homology was observed especially at high molecular weights. From comparison with SDS-PAGE protein profiles of analyzed nondermatophyte fungi it is evident, that besides protein bands present in all fungal species analyzed, a protein, clearly expressed as a band at 35 kD, was not detected in nondermatophyte fungi. According to its molecular weight it can be correlated to keratinase described by Ferreiro (25). Keratinolytic enzymes are supposed to enable the penetration of the fungal metabolites into the epidermal tissue (14). Antibodies against them had already been detected in sera of patients with dermatophytosis (17). Humoral and cellular immune response to different dermatophytes had been demonstrated in man (3,4,5,20,24) and different animal species (2,6, 7,9,10,11 and 22) by intradermal testing, lymphocyte blastogenesis assay or serological tests.

Immunoblotting using *Microsporum canis, Trichophyton mentagrophytes* and *Microsporum gypseum* derived antigens was used to investigate the humoral immune response in cats by Sparkes et all. (16).

In the present study the crossreactivity among two dermatophyte and two nondermatophyte fungal antigens was studied by immunoblotting. It is evident that *Microsporum canis* and *Trichophyton mentagrophytes* antigens showed much higher crossreactivity than non-dermatophyte fungal antigens when reacted with sera obtained from cats with naturally occurring microsporosis. Differences in immunoreactive proteins can be found mainly below 50 kD. These findings correlate well with the observations of Sparks et al. (16). However, in some cases high immunoreactivity was detected with *Malassezia pachydermatis* antigens, which is evident from a strong protein band at 40 kD and high OD values obtained in ELISA (22). It may be assumed that animals showing strong immunoreactivity towards nondermatophyte fungal antigens were previously infected with *Malassezia pachydermatis,* though the infection was clinically not diagnosed. On the basis of SDS-PAGE and Western blot analysis, the immunodominant proteins in complex antigen preparations could be identified. Further studies of the protective significance of the particular protein component in eliciting an host immune response can then be conducted in the future.

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