Original article

Dermatophyte—host relationship of a murine model of experimental invasive dermatophytosis

James Venturini a,b, Anuska Marcelino Álvares c, Marcela Rodrigues de Camargo a,b, Camila Martins Marchetti a, Thais Fernanda de Campos Fraga-Silva a, Ana Carolina Luchini d, Maria Sueli Parreira de Arruda a,*

a Faculdade de Ciências, UNESP – Univ Estadual Paulista, Departamento de Ciências Biológicas, Laboratório de Imunopatologia Experimental, Av. Eng. Luiz Edmundo C. Coube 14-01, 17033-360 Bauru, SP, Brazil
b Faculdade de Medicina de Botucatu, UNESP – Univ Estadual Paulista, Distrito de Rubião Junior s/n, 18618-970 Botucatu, SP, Brazil
c Departamento de Microbiologia, Imunologia e Parasitologia, UNIFESP – Universidade Federal de São Paulo, R. Botucatu 862, 04023-900 São Paulo, SP, Brazil
d Instituto de Ciências Biológicas e Naturais, UFTM, Av. Frei Paulino 30, 38025-180 Uberaba, MG, Brazil

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Abstract

Recognizing the invasive potential of the dermatophytes and understanding the mechanisms involved in this process will help with disease diagnosis and with developing an appropriate treatment plan. In this report, we present the histopathological, microbiological and immunological features of a model of invasive dermatophytosis that is induced by subcutaneous infection of Trichophyton mentagrophytes in healthy adult Swiss mice. Using this model, we observed that the fungus rapidly spreads to the popliteal lymph nodes, spleen, liver and kidneys. Similar to the human disease, the lymph nodes were the most severely affected sites. The fungal infection evoked acute inflammation followed by a granulomatous reaction in the mice, which is similar to what is observed in patients. The mice were able to mount a Th1-polarized immune response and displayed IL-10-mediated immune regulation. We believe that the model described here will provide valuable information regarding the dermatophyte—host relationship and will yield new perspective for a better understanding of the immunological and pathological aspects of invasive dermatophytosis.

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Keywords: Dermatophytes; Invasive dermatophytosis; Trichophyton mentagrophytes; Cellular immune response; Experimental dermatophytosis

1. Introduction

Dermatophytes are a group of fungi that are able to utilize keratin as a food source. These fungi are universally distributed and may be categorized as geophilic, zoophilic or anthropophilic based on their natural habitats [1]. Dermatophytes are among the most common zoonotic agents. Dermatophytosis affects approximately 20–25% of the world’s population and are responsible for 30% of all skin fungal infections; in addition, it is considered to be the third most common skin disorder among children younger than 12 and the second most common skin disorder in adults [2,3].

Dermatophytosis is not an opportunistic infection and frequently infects the skin, hairs and nails of healthy humans and animals, causing superficial infections [4,5]. The clinical manifestations of dermatophytosis range from mild to severe based on the host reaction to the fungal metabolic products, the species of the fungus, the virulence of the fungus and the anatomic site of infection [6]. Rarely, in immunocompromised hosts, the infection can disseminate into deeper skin layers and other organs, resulting in invasive dermatophytosis [7–10].

Experimental models of dermatophytosis have played a crucial role in assessing the efficacy of antimicrobial agents [11,12] and in obtaining a better understanding of disease...
pathogenesis [13–15]. Guinea pigs and rabbits have been the most frequently employed model animals in studies of dermatophytosis [16]. However, several points remain to be elicited. One of them is that these experimental models have not been used to explore the mechanisms involved in immune response against dermatophytes, mainly due to the limited specific tools for these species such as species-specific recombinant protein and monoclonal antibodies targeting to these species. The other one refers the question of fungal dissemination to internal organs. According to Hay & Baran [17], ‘the mechanisms limiting spread of dermatophyte fungi, under normal circumstances, to the epidermis remain elusive. The hypothesis linking a specific clinical form of dermatophyte nail infection with lymphatic spread, although intriguing, needs verification’. Cutaneous dermatophytosis in guinea pigs and rabbits are self-limiting, which restricts their usefulness in the investigation of the aspects of the disseminated infection. In order to overcome these claims, this study presents a model of invasive dermatophytic infection using Swiss mice inoculated subcutaneously with *Trichophyton mentagrophytes*. The model mimics the human condition by presenting the following aspects: 1) spread of the fungus from prior subcutaneous lesion, 2) involvement of various internal organs, 3) resolution of infection associated with development of cellular immune response, 4) in the deep layer of skin, the acute inflammatory lesion progresses to granulomatous formations. Once it is well characterized, this model will allow studies of underling conditions that exhibit increased susceptibility to dermatophytosis such as diabetes.

2. Material and methods

2.1. Mice

Two-month-old male Swiss mice from the UNESP Animal House at the Laboratorio de Imunopatologia Experimental (LIPE) of UNESP – Univ Estadual Paulista, Bauru, SP, Brazil were housed in groups of three to five mice and were provided food and water *ad libitum*. All protocols used were in accordance with the ethical principles for animal research adopted by the Brazilian College of Animal Experimentation (COBEA). This study was approved by the Ethical Committee of Faculdade de Ciências, UNESP – Univ Estadual Paulista.

2.2. *T. mentagrophytes* inoculum

The *T. mentagrophytes* strain (2118/99-ILSL) was originally obtained from the fungal collection of the Lauro de Souza Lima Institute, Bauru, SP, Brazil. The fungi were maintained on Mycosel® agar slants (Difco Laboratories, Detroit, Michigan, USA) and cultured in the same media for 10 days at 25 °C. The fungi were washed carefully by sterile saline solution. Afterward, the suspension was mixed twice for 10 s on a vortex-mixer and decanted off for 5 min. The supernatants were collected and washed twice. Fungal viability was determined by cotton blue staining, and the concentrations were adjusted to $5 \times 10^8$ viable conidia of *T. mentagrophytes*/ml.

2.3. Experimental design

In the present study we employed the Swiss mice because this strain is more susceptible to dermatophytes [18], particularly to *T. mentagrophytes* [19]. Thus, the mice were injected in the footpad, subcutaneously, with 0.04 ml of *T. mentagrophytes* inoculum (TM group) or sterile phosphate-buffered saline (PBS, pH 7.4, alone (CTL group). The mice were sacrificed at 6, 24 and 48 h and 7, 14 and 30 days after the inoculation.

2.4. Collection of the biological material

Mice were sacrificed via CO₂ asphyxiation. Fragments of the footpad, popliteal lymph nodes, liver, spleen and kidneys were dissected and submitted to microbiological analyses. The footpads were submitted to histological analyses by routine procedures for embedding in paraffin.

2.5. Colony-forming unit (CFU) determination and frequency of fungal dissemination

Fragments of the footpad, popliteal lymph nodes, liver, spleen and kidneys were weighed and homogenized in 1 ml of PBS, and 0.1 ml of the homogenate was cultured on 15 × 90 mm Mycosel® agar plates at 25 °C for 14 days. Each sample was assessed in duplicate. Total colonies were counted, and the results were expressed as the number (log₁₀) of *T. mentagrophytes* per gram of tissue.

The frequencies of fungal dissemination were also reported as the frequencies of *T. mentagrophytes*-positive mice which had one or more internal organ affected and exclude footpad/total number of mice analyzed.

2.6. Histological procedures

Histological sections (4 μm) of mouse footpads were stained with hematoxylin-eosin (HE) and periodic acid-Schiff staining (PAS).

2.7. Spleen cell culture

Fragments of the spleen were collected and homogenized in ice-cold sterile PBS. The red blood cells were lysed with 0.15 M ammonium nitrate. After washing, the cell suspension was centrifuged, and the cells were resuspended in 1 ml RPMI-1640 (Nutricell, Campinas, SP, Brazil) containing 10% heat-inactivated fetal calf serum (Gibco BRL, Grand Island, NY, USA). The cell concentrations were adjusted to 2.0 × 10⁶ cells/ml, as determined by 0.1% trypan blue staining. A total of 2.0 × 10⁵ spleen cells were placed into each well of 96-well flat-bottom microtiter plates (Costar, Cambridge, MA, USA) and incubated at 37 °C in a 5% CO₂ humidified chamber. The cells were cultured with or without 10 μg/ml Concanavalin A (Sigma, St. Louis, MO, USA) as an internal control (data not shown). After 48 h, the cell-free
supernatants were harvested and stored at $-80\,^\circ\text{C}$ for cytokine analysis.

2.8. Cytokine analyses

The levels of TNF-$\alpha$, IFN-$\gamma$ and IL-10 were measured in the cell-free supernatants of the spleen cell cultures using a cytokine Duo-Set Kit (R&D Systems, Minneapolis, MI, USA), according to the manufacturer’s instruction. Each sample was analyzed in duplicate.

2.9. Footpad test

The development of a specific cellular immune response was evaluated using the footpad test (FPT) as described by Arruda et al. [20]. We used the exoantigens produced from the same strain of *T. mentagrophytes* inoculated in the mice according Venturini et al. [21]. On the 6th, 13th and 29th days after inoculation, the mice were injected with the specific antigen (50 $\mu$g of protein /ml, previously standardized — data not showed) in the opposite footpad. After 24 h, the footpads were collected and evaluated histologically. Mice were considered FPT-positive if they showed mononuclear cell infiltration.

2.10. Ouchterlony double immunodiffusion

The humoral immune response was evaluated based on the protocol described by Camargo et al. [22] using the *T. mentagrophytes* exoantigens [21].

2.11. Statistical analyses

The microbiological analyses and cytokine production data were analyzed using the non-parametric Kruskal–Wallis test and Dunn’s post-test. All statistical tests were conducted using GraphPad InStat version 3.0 for Windows (GraphPad Software, San Diego, California, USA), and a two-tailed $P$-value of less than 0.05 was considered to be statistically significant.

3. Results

3.1. Microbiological evaluation

All TM-mice contained fungi at their sites of inoculation until day 7. The fungal load began to decrease on day 7, culminating in fungal clearance by day 30 (Fig. 1A–C).

The data regarding the frequency of mice with fungal dissemination are summarized in Table 1. Six hours after fungal inoculation, we observed dissemination of the fungus to the popliteal lymph nodes, spleen, liver and kidneys. The fungal dissemination was more frequent in the popliteal lymph nodes and spleen. The number of mice with affected organs decreased with time until complete clearance was observed on day 14 after fungal inoculation.

3.2. Histopathological evaluation

Changes in the footpad inoculation sites were observed as early as the first few hours after fungal inoculation. These lesions were characterized by acute inflammatory reactions leading to suppurative abscess formation, edema and lymphohistiocytic infiltrates, with polymorphonuclear leukocytes being observed at the limit between the abscess and the upper layer of the skin (Fig. 2B). In the abscesses, hyphae and conidia were detected by PAS staining (Fig. 3A).
Table 1

Fungal dissemination frequencies in mice inoculated with *T. mentagrophytes*. Microbiological analyses of the popliteal lymph nodes, spleen, liver and kidneys from 6 h to 30 days after fungal inoculation. The values are reported as the frequencies of *T. mentagrophytes*-positive mice which had one or more internal organ affected and exclude footpad/total number of mice analyzed. (*n* = 6).

<table>
<thead>
<tr>
<th>Organ</th>
<th>Infection progress</th>
<th>Hours</th>
<th>Days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Popliteal lymph nodes</td>
<td>6/6</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>Spleen</td>
<td>4/6</td>
<td>24</td>
<td>3/6</td>
</tr>
<tr>
<td>Liver</td>
<td>2/6</td>
<td>48</td>
<td>2/6</td>
</tr>
<tr>
<td>Kidneys</td>
<td>1/6</td>
<td>6</td>
<td>6/6</td>
</tr>
</tbody>
</table>

After 24 h, the interstitial infiltration was more intense, and the epidermis exhibited hyperkeratosis (Fig. 2B). During this time period, the hyphae and conidia decreased (Fig. 3B).

At 48 h, the reaction had spread, and the perilesional infiltrates were composed of fibroblasts and mononuclear cells (Fig. 2C). The fungal pattern at this time point was the same as that observed at 24 h.

On day 7 and 14, acanthosis was observed in the epidermis, and the infiltrated area was enlarged with central necrosis and a peripheral granulomatous reaction (Fig. 2D), which was composed of lymphocytes, plasma cells and a small number of modified macrophages (Fig. 2F). Fungal fragments were seen inside the modified macrophages on day 14 (Fig. 3C).

On day 30, the lesions showed well-formed granulomas composed of modified macrophage and rare epithelioid cells surrounded by mononuclear cell infiltrates (Fig. 2E and F). No fungal elements were detected by PAS at this time point (Fig. 3D).

3.3. Specific cellular and humoral immune responses

TM-mice showed positive FPTs to specific antigens at 7, 14 and 30 days after infection. Sera from these mice were non-reactive for the IDD test, and both specific cellular and humoral responses were negative in the CTL-mice.

3.4. Cytokine determination by spleen cells

In the TM-mice, the TNF-α and IFN-γ levels were only lower at 48 h after fungal inoculation (Fig. 4A and Fig. 4B). Interestingly, the levels of IFN-γ were higher after fungal clearance than during earlier periods. Conversely, we observed high levels of IL-10 soon after introduction of the fungus, and over time, the levels of this cytokine decreased to baseline levels (Fig. 4C).

4. Discussion

Several recent studies have reported that superficial dermatophytic infection may result in complications such as bacterial superinfection in diabetic patients [23] and in invasive dermatophytosis [review in 8]. Although rare, the incidence of invasive dermatophytic infection is increased in immunocompromised patients; however, the infection is often undiagnosed, and thus its occurrence may be underestimated. Consequently, little is known about the pathophysiology of invasive dermatophytosis and its associated immune events.

Because there are considerable difficulties associated with conducting studies in humans, the use of experimental animal models has facilitated researchers’ understanding of the phenomena involved in infectious diseases. The experimental fungal spread in dermatophytosis was documented by Cutsen and Jensen [15], and these authors reported that guinea pigs and rabbits inoculated intravenously with *T. mentagrophytes* contained fungi in their lungs, kidneys and liver. In contrast with these findings, the spread seen in humans stems from a pre-existing superficial dermatophyte infection, and there are no published data indicating unexpected fungemia due to dermatophytes [8,17]. In order to better mimic the human condition, we studied the behavior of the fungus in Swiss mice after introduction into the subcutaneous footpad tissue. We observed that the fungus reached the popliteal lymph nodes, spleen, liver and kidneys soon after *T. mentagrophytes* footpad inoculation. In a review of 41 human cases with invasive dermatophytosis, the lymph nodes were the most infected sites (17%); however, several other organs and tissues were also affected by invasive dermatophyte infection, including the bones (7.3%), brain (7.3%), liver (4.9%) and other sites, such as spleen, muscle, testis and vertebral joints (2.4% each) [8]. Thus, our model more closely mimics what occurs in patients with invasive dermatophytosis.

Histologically, the introduction of subcutaneous *T. mentagrophytes* in Swiss mice initiates an acute inflammatory process followed by a granulomatous reaction. In the human disease, approximately 44% of the histopathological findings included granuloma formation [8]. In our experimental model, the number of mice with affected tissues decreased with time, culminating in complete clearance by day 14 after fungal inoculation. While spontaneous clearance of fungi from the organs has not been observed in the human disease, this clearance cannot be ruled out because little is known regarding the biology of the dermatophytes in the deeper tissues. Interestingly, several authors have suggested that internal organs could act as reservoirs of fungi in recurrent dermatophytic infections [15]. Our results do not confirm this premise, as the internal organs were the first ones to exhibit fungal clearance in our model. Also, it is important to highlight that the normal body temperature is one of the factors involved in the death of dermatophytes in deep tissue.

The immunological mechanisms involved in the initiation and clearance of systemic dermatophytosis remain poorly understood. Several studies have suggested that the cellular immune response participates in modulating the disease, as this response could cause increased epidermal proliferation and facilitate dermatophyte elimination [review in [24]]. The data from our study support this premise once that we observed epidermal hyperplasia in the early stages of infection and became more expressive with the development of the infection and delayed-type hypersensitivity (DTH).
Despite our findings, clinical and experimental data have suggested that the immune response to dermatophytes is not exclusively DTH-dependent. Among patients with AIDS, for example, the characteristic decrease in CD4⁺ T lymphocytes was expected to lead to higher incidences of invasive dermatophytosis, but in reality, the frequency is not increased in these patients. Conversely, invasive dermatophytosis is more frequently observed in non-AIDS immunocompromised patients, such as those suffering from atopic disease, leukemia, diabetes, systemic lupus erythematosus, psoriasis and myasthenia gravis [8,17], suggesting that the immunological mechanisms underlying invasive dermatophytosis are different. Furthermore, patients with chronic dermatophytosis may present DTH against trichophytin [25]. These findings have also been confirmed experimentally. While athymic nude BALB/C mice were resistant to T. mentagrophytes infection [26], in others strains of mice, the ablation of T-cell mediated pathways leads to chronic and extensive but not internally disseminated dermatophytic infection [27].

In order to expand the discussion about this question, in our experimental model we carried out other immunological approaches. Thus, we investigated the production of the Th1
cytokine IFN-γ, the Th2/regulatory IL-10, and the pro-inflammatory cytokine TNF-α in the cell-free supernatants of cultured splenocytes. We did not observe altered IFN-γ levels during the early phase of the infection, but it is possible that these results may be associated with the high levels of IL-10, which is a downregulator of the Th1 response. Over time, the response changed, and IL-10 production decreased, while the levels of IFN-γ increased. In the human disease, IFN-γ-positive cells were observed in the upper dermis in lesions in situ [28]. Interestingly, in our study, the levels of IL-10 were notably high during the early phase of the infection when the fungus was present in the viscera, including the spleen. These results are similar to the findings of Campos et al. [29], who reported that the interaction of Trichophyton rubrum with phagocytic peritoneal cells induced production of IL-10. This finding was also supported by our microbiological analyses of the infections in our mice, which showed that when the fungal load decreased, the levels of IL-10 returned to basal levels. The role of IL-10 in this process remains unclear. In addition to inducing a Th2-type response, this cytokine plays an important role in both innate immunity and the regulation of the immune response. By preventing excessive production of TNF-α and cytotoxic metabolites, IL-10 prevents the development of a destructive inflammatory response and facilitates the proper development of a specific immune response [30].

With respect to the humoral immune response, we were unable to determine the presence of specific antibodies in the sera of our mice. Even considering the low sensitivity of the method used, the presence of IgG and IgM antibodies specific for the fungus had been previously demonstrated [30—33]. Similar to other pathologies, the consensus among most researchers is that specific humoral immunity does not play a large role in the resistance to fungal infections. According to the authors, the humoral response is weak and directed predominantly against polysaccharide antigens. The fact that antibody titers were higher in chronic infections suggests that these antibodies are not effective at controlling these infections [33—35].

Taken together, the subcutaneous inoculation of Swiss mice with T. mentagrophytes triggers fungal dissemination to the internal organs, where the fungus remains until approximately the seventh day. During this period, the mice assembled a Th1-type immune response and IL-10-mediated immune regulation. Our lab has initiated studies using this experimental model to evaluate dermatophytic infection during a hypoinsulinemia-hyperglycemic (HH) state, a model for diabetes, as this metabolic disease is associated with a high incidence of dermatophytic infection [36]. We report that HH mice exhibited disseminated dermatophytosis following a delay in the infection outcome and that this dermatophytosis was associated with a lower percentage of peripheral blood CD4+ T cells [36]. The data obtained from our experimental model may provide more information regarding immunological mechanisms to help researchers better understand the immunological and pathological aspects of invasive dermatophytosis.
inoculated with supernatants of spleen cells maintained in culture for five days. Swiss mice experimental time points. Kruskal sterile saline. The various letters indicate the statistical differences between the fungal infection. The CTL group was composed of animals inoculated with References

Fig. 4. Evaluation of TNF-α (A), IFN-γ (B) and IL-10 (C) levels in the supernatants of spleen cells maintained in culture for five days. Swiss mice inoculated with T. mentagrophytes were evaluated from 6 h to 30 days after fungal infection. The CTL group was composed of animals inoculated with sterile saline. The various letters indicate the statistical differences between the experimental time points. Kruskal–Wallis test; P < 0.05, n = 6.

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