

Possible Role of Trichophytin Antigen in Inducing Impaired Immunological Clearance of Fungus in Onychomycosis

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Abstract The immunology of onychomycosis is poorly understood. Th1 and Th17 are the principal effector cells responsible for protective immunity against fungi, while it is assumed that Th2 responses are associated with deleterious effects. The study was conducted to appraise the role of interleukin-6 (IL-6), transforming growth factor β (TGF- β) and immunoglobulin E (IgE) in onychomycosis patients and to study skin reactivity to trichophytin antigen in them. Serum samples of 60 cases of chronic onychomycosis and 30 healthy controls were assayed for serum IgE, IL-6 and TGF- β levels using specific immunoassay kits; 0.01 ml of trichophytin antigen, *Candida* antigen and phosphate-buffered saline using separate syringes were injected intradermal at three independent sites of the forearm in cases and controls. Serum IL-6 levels were significantly lower in cases as

compared to controls, while serum TGF- β levels in both cases and controls were comparable. Serum IgE levels in cases were significantly higher when compared with controls. Thirty-eight patients showed immediate hypersensitivity response to trichophytin antigen, while none showed delayed hypersensitivity reaction to trichophytin antigen. Constant fungal antigenic stimuli induce a state of anergy as indicated by low serum IL-6 levels and the absence of delayed hypersensitivity reaction to trichophytin antigen in cases, leading to chronicity of infection. High total IgE may indicate a high probability of prior fungal sensitization.

Keywords Onychomycosis · Dermatophytes · Cytokines · ELISA · Hypersensitivity

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Introduction

“Onychomycosis” is traditionally referred to a non-dermatophytic infection of the nail but is now used as a general term to denote any fungal nail infection. Dermatophytes are the most frequently implicated causative agents in onychomycosis; however, yeasts and non-dermatophytic molds (NDM) are now increasingly recognized as pathogens in nail infections [1].

It is a well-known fact that immune response elicited against any infection depends on factors such as host defense against microbes, virulence of infecting species and local environmental factors. Innate immunity is essential to trigger and modulate the adaptive immune response against infection. Despite being restricted to superficial layers, dermatophytes can elicit severe inflammation. The pattern recognition receptors (PRRs), e.g., Toll-like receptors (TLRs), lectin and lectin-like receptors, dectin-1 and dectin-2, which are present on keratinocytes, dendritic cells, Langerhans cells, macrophages, T and B cells recognize conserved pathogen-associated molecular patterns (PAMPs) expressed by microbes and participate in controlling activation of acquired immune response [2, 3]. Humoral immune response has been found to be variable as compared to the healthy controls. Chronic dermatophytosis patients are frequently observed to demonstrate atopy and a poor cell-mediated cutaneous response [2]. The nature of offending antigens and the specific repertoire of cytokines produced lead to the polarization of effector T cells. A change in the balance between Th1 and Th2 responses has also been implicated in chronic dermatophyte infection [3, 4]. Th1 and Th17 response may be responsible for protective immunity against fungi, while it is assumed that Th2 responses are associated with deleterious effects [5]. Recently, the role of regulatory T (Treg) cells determining the failure to clear fungal infections and allowing persistence of infection has been identified [6]. Induced Treg and Th17 cells may arise from the same precursor cell and selective differentiation would be favored in the presence of transforming growth factor β (TGF- β), which would determine the predominance of either Treg cells with suppressor activity or Th17 cells with inflammatory activities, determining the outcome of the disease. The immunology of onychomycosis is poorly understood. The understanding of immune

responses to the disease may provide new insights on its pathogenesis and may reveal novel targets for therapy. Keeping this in mind, we conducted a pilot study with the aim to appraise the role of immunoglobulin E (IgE), interleukin-6 (IL-6) and TGF- β in onychomycosis patients and to determine and correlate skin reactivity to trichophytin and *Candida* antigens in onychomycosis.

Materials and Methods

The study included subjects attending Department of Dermatology Outpatient, of a tertiary care hospital during a 1-year period from March 2013 to March 2014. Sixty microscopically confirmed patients of chronic onychomycosis suffering for more than 6-month duration (hyphae/yeast positive on KOH mount) aged between 15 and 75 years were included. Patients who had taken systemic antifungal within last 3 months on topical antifungal/systemic corticosteroids/immunosuppressant within last 1 month, and topical corticosteroid within last week or antihistamines within last 5 days were excluded from the study. Patient with widespread dermatitis or extensive skin eruptions, psoriasis, lichen planus, any other nail disorder or who had suffered from any systemic disease/autoimmune disorder/immunodeficiency/malignant disease or pregnant females were also excluded from the study. Thirty age- and sex-matched healthy controls were also included. Detail clinical history and examination of study population relevant to the present study was noted. The study was conducted after obtaining institutional ethical clearance and written informed consent from the study participants.

Serum Separation

Blood samples from all cases and controls were collected, centrifuged, serum separated and stored at -80°C till further tested for cytokines and IgE assays.

Cytokines and IgE Assay

Quantitative ELISA was performed to determine the levels of IL-6 and TGF- β in serum of patients and healthy control groups using commercially available human IL-6 and TGF- β enzyme immunoassay kits

(Genprobe Diaclone, France). Serum IgE levels were determined using commercially available two-site sandwich ELISA kit according to manufacturer's specifications (Calbiotech, California). Each test was performed according to manufacturer's specifications. Values less than the sensitivity of the kit were taken as zero for statistical analysis.

Skin Prick Test

Trichophytin antigen and Candida antigen (each with protein concentration 2.3 mg/ml) derived from anthropophilic *Trichophyton rubrum* and *Candida albicans* strains, respectively, were commercially procured from All Cure Pharma Pvt. Ltd., Delhi (India); 0.01 ml each of trichophytin antigen, Candida antigen (recall antigen) and phosphate-buffered saline (PBS) (negative control) was injected intradermal using separate syringe at independent sites of skin of the forearm in all cases and controls. The test was read after 15 min for immediate hypersensitivity (IH) response and 48 h for delayed type hypersensitivity (DTH) response. Any reaction with mean wheal diameter at least 3 mm greater than negative control was taken as positive [7].

Statistical Analysis

Data were analyzed by SPSS version 20 statistical software. Mann–Whitney *U* test was applied to compare cytokines and IgE levels between cases and control groups. Pearson correlation coefficient was calculated to establish correlation between two cytokines. *p* value less than 0.05 was taken as significant. The prevalence of positive SPT tests for each individual antigen was computed using simple percentages.

Results

The mean age of cases and controls was 36.3 ± 13.7 and 32.8 ± 10.3 years, respectively. Of 60 cases, 39 (65 %) were males and 21 (35 %) were females.

Significantly lower levels of serum IL-6 were observed in cases than in controls (*p* value 0.007), whereas serum TGF- β levels were comparable among cases and controls (*p* value 0.7). No significant correlation between serum IL-6 and TGF- β levels

(coefficient of correlation $r (-) 0.03$, *p* value 0.8) was observed among the cases. In contrast, serum IgE levels were significantly higher in cases than in controls (*p* value 0.007) (Table 1).

Of patients, 63.3 % had IH response to trichophytin antigen, 43.3 % had IH response to Candida antigen, while 40 % showed IH response to both trichophytin antigen and Candida antigen (Table 2; Fig. 1). None of the patients showed DTH to any of the antigens. Among the control group, two subjects showed positive IH to trichophytin antigen, while only one elicited both IH and DTH. All three of them had positive history of atopy.

Discussion

The delineation of the immune mechanisms which determine distinct immune responses against the causative fungus in onychomycosis may be pivotal in understanding the host determinants of protective immunity. Innate immune system acts instantly upon exposure to invading pathogens and generates signals to activate appropriate effector molecules and cells of the adaptive immune system. Dendritic cells bridge innate and adaptive immunity by shaping the T cell response following PRR-dependent cytokine production. In the present study, we assayed the role of IL-6, TGF- β and IgE in patients with onychomycosis.

We observed lower serum IL-6 in cases as compared to controls. The lower serum levels of IL-6 may probably be explained on the basis of long-term exposure and fungal induced immunosuppressive mechanisms. In a study, the expression of TLRs was markedly inhibited when keratinocytes were stimulated with viable whole conidia of *T. rubrum* possibly due to overexpression of proteolytic enzymes (mannan) by the fungus during infection process. Another view suggests that certain fungal agents can induce an IL-10-mediated tolerogenic T cell response, thus explaining the low proinflammatory response. It has also been shown in an experiment that the interaction of conidia of *T. rubrum* with macrophages may induce prostaglandins which can inhibit the other inflammatory response and facilitate tolerance by inducing T cell anergy and also increase the development of CD4+ CD25+ Treg cells [8, 9]. We anticipate a similar phenomenon in our subjects. It was speculated that TGF- β being anti-inflammatory and

Table 1 Serum cytokines (IL-6 and TGF- β) and IgE levels (mean \pm SD) in cases and healthy controls

Serum levels	Cases <i>n</i> -60	Controls <i>n</i> -30	<i>p</i> value
IL-6 (pg ml ⁻¹)	7.08 \pm 31.14	37.94 \pm 74.91	0.007*
TGF- β (ng ml ⁻¹)	33.45 \pm 55.00	31.50 \pm 51.01	0.7
IgE (IU ml ⁻¹)	389.75 \pm 309.25	239.73 \pm 314.83	0.007*

* *p* value significant

Table 2 Skin testing response to trichophytin and *Candida* antigen in cases and healthy controls

Antigen	Cases <i>n</i> = 60 (%)		Controls <i>n</i> = 30	
	IH (at 15 min)	DT (at 48 h)	IH (at 15 min)	DTH (at 48 h)
Trichophytin	38 (63.3)	0	2	1
<i>Candida</i>	26 (43.3)	0	0	0

**Fig. 1** Immediate hypersensitivity response to trichophytin and *Candida* antigen

immunosuppressive would be high under these conditions. Surprisingly, serum TGF- β levels were comparable in both cases and controls. This may be explained as TGF- β being a house-keeping anti-inflammatory cytokine is constitutively expressed and not responsive to proinflammatory stimuli, thus inhibiting initiation of inflammation, as opposed to other anti-inflammatory cytokines such as IL-10 and IL-35 that are induced by proinflammatory stimuli and suppress full-blown inflammation [10]. When the immune response is not activated, TGF- β favors the generation of induced Treg cells, which suppress

inflammation; however, when an infection is established, IL-6 synthesized during the innate immune response inhibits the generation of Treg cells and acting synergistically with TGF- β induces the differentiation of Th17 cells which provide antifungal host defense [11, 12]. However, in our study, patients with low IL-6 are indicative of poor acute inflammatory response (Th1) indicative of persistence of infection.

It is a well-known fact that proteins derived from fungus affecting the skin and nails can contribute to allergic disease [13]. We also studied total IgE response in onychomycosis and observed significantly higher total IgE levels in cases than in controls. Our findings are in agreement with previous studies by Leibovici et al. [14] who also observed increased total IgE levels in chronic dermatophytosis. However, atopy is a complex phenomenon and sustained serum levels of IgE also depend on persistent allergen exposure, given the brief half-life of IgE and the immune proclivity of the affected individual. Thus, high total IgE, although non-specific, may indicate a probability of early or prior fungal sensitization.

The trichophytin antigen has the ability to induce distinct immune response in different individuals. In the present study, 63.3 % patients elicited IH response to trichophytin antigen indicating a Th2 pathway, while none of the patients had DTH to trichophytin antigen, reflecting immunological unresponsiveness or anergy. Several reasons have been hypothesized for the absence of DTH in chronic onychomycosis. IgE antibodies may deplete the antigen by either combining with it or by forming complexes that could block receptors on the surface of cells abrogate the production of chemical mediators involved in eliciting cell-mediated immune (CMI) responses [15]. Also, the fungal antigens could bind to epidermal cells become cryptic antigens inaccessible to immune system or protracted antigen exposure may depress CMI [16]. Immunosuppressive properties of fungal mannan or

chitin could be another reason [5]. It has been seen that the chronically infected patients who failed to show delayed responses to trichophytin antigens respond normally to other antigens on skin testing [5]. In contrast, none of the patients in our study mounted positive delayed response to recall *Candida* antigen. These patients were not known to suffer from any disorder or on medication which could immunosuppress them, leading to generalized defect in CMI responses. The anergic response to fungal antigens in skin testing clearly indicates a broad lack of T cell responsiveness which might be due to sharing of antigenic epitopes.

In conclusion, constant fungal antigenic stimuli induce a state of anergy as suggested by low IL-6 levels in the present study, leading to chronicity of onychomycosis. Observation of TGF- β levels, being comparable in both cases and controls, was inconclusive to associate its role in immunopathogenesis of the disease. IgE serves as a non-specific marker to indicate early or prior sensitization to fungal antigens. Moreover, the skin response to trichophytin antigen can be considered as a marker of immune status in fungal nail infections and assists in prognosticating treatment outcome. Our study provides important pilot data to understand some immunological aspects in onychomycosis and due to resource limitations, we could not do follow-up study after treatment; hence, it is difficult to comment whether IL-6 and IgE return to baseline level after treatment. The apparent immunological disengagement between IL-6, TGF- β , or Th17 and Treg coupled with elevated IgE, but the absence of DTH response in afflicted patients may conceal a more complex immunoregulatory circuit than what is apparent through cytokine measurements. However, such complex immune regulation circuit can better be comprehended, with a bigger sample size spanning a longer duration perhaps in combination with other immune markers and sequential monitoring of patients before and after specific antifungal treatment.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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