

Report

Sensitization and cross-reactions of dermatophyte and *Candida albicans* allergens in patients with chronic urticaria

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Abstract

Background Chronic fungal infections are known to exacerbate allergic symptoms, including those of asthma and chronic urticaria (CU). We applied four prepared fungal antigens of *Trichophyton rubrum*, *Trichophyton mentagrophytes*, *Epidermophyton floccosum*, and *Candida albicans* to examine sensitization to each in subjects with CU and onychomycosis and in healthy subjects, and to evaluate the etiologic role of dermatophytic infection in CU and observe any cross-reactions among these four antigens.

Methods Participants were divided into four groups, including those with CU with onychomycosis (experiment group), those with onychomycosis without allergic diseases (control group 1), those with CU without fungal infections (control group 2), and a healthy group (control group 3). In all subjects, skin prick tests with the four fungal antigens were performed. Subjects in the experiment group and control group 1 were also submitted to mycologic investigations.

Results The experiment group showed significantly higher rates of positivity than the three control groups to *T. rubrum*, *E. floccosum*, and *T. mentagrophytes* antigens. Control group 1 showed rates higher than those in control groups 2 and 3; no significant difference emerged between control groups 2 and 3. Positivity to the *C. albicans* antigen did not differ among the four groups. In control group 1, rates of positivity to the three dermatophytic antigens did not differ significantly but did for *C. albicans*.

Conclusions Fungal infection seems to be an important determinant of trichophyton hypersensitivity. Cross-reactions among *T. mentagrophytes*, *T. rubrum*, and *E. floccosum* antigens were obvious, but none emerged between the antigens of the three dermatophytes and that of *C. albicans*.

Introduction

Fungi have worldwide distribution and grow in a wide range of natural habitats. Some fungi are pathogenic and are able to cause infection, teratogenesis, toxicosis, carcinogenicity, or allergic reactions. As they represent some of the most common and important inhalant allergens, fungi can induce many allergic diseases, including bronchial asthma, anaphylactic rhinitis, and allergic bronchopulmonary aspergillosis (ABPA); of these fungi, species of the genera *Aspergillus* and *Penicillium* have been most studied.¹ In line with the increasing attention paid to the etiologic roles of dermatophytic infections in asthma and other allergic diseases in recent studies,^{2–4} previous clinical investigations have shown that subjects with chronic urticaria (CU) and dermatophytic infections demonstrate higher rates of positivity for *Trichophyton* spp. in skin prick tests (SPTs) than those with only CU, indicating that dermatophytic infections may be one of the causes of CU.

Another discovery is that different dermatophytic infections can give positive results in trichophyton SPTs, which suggests that different dermatophytes may induce common antigenic substances and cause allergic cross-reactions.⁵ Therefore, we prepared our own antigens for *Trichophyton rubrum*, *Trichophyton mentagrophytes*, *Epidermophyton floccosum*, and *Candida albicans*, and tested them in subjects with CU to identify the possible etiologic role of dermatophytic infection in people with CU who also acquire dermatophytic infections and to explore the possible cross-reactions between dermatophytic antigens.

Materials and Methods

Subjects

Outpatients treated for CU and onychomycosis at our Department of Dermatology between July 2011 and February 2012 were selected and divided into four groups according to their medical histories, clinical symptoms, and the findings of

physical examinations and mycologic studies.^{6,7} The groups were designated as the experiment group (subjects with CU and onychomycosis), control group 1 (subjects with onychomycosis without allergic diseases), control group 2 (subjects with CU without fungal infection), and control group 3 (healthy subjects). The diagnosis of CU was defined by the presence of recurrent episodes of hives with or without angioedema of ≥ 6 weeks in duration and excluded special subtypes including factitious urticaria, cold urticaria, cholinergic urticaria, solar urticaria, and pressure urticaria. Exclusion criteria prevented the participation of patients taking antihistamines, glucocorticoids, mast cell stabilizers, or immunomodulators within 2 weeks of the study period, patients who were pregnant or lactating, patients with epinephrine contraindications, and patients with severe cardiovascular, cerebrovascular, and other systemic diseases. Patients who had used oral antifungal agents within 1 month or topical antifungal agents within 2 weeks of the study period were also excluded. Particular exclusion criteria denied the inclusion of female patients with candida vaginitis, underage patients with mycotic stomatitis, and male patients with candida balanitis. Control group 1 excluded patients with other allergic diseases, such as asthma and anaphylactic rhinitis. Control group 2 excluded patients with other fungal infections, such as tinea corporis and tinea cruris. This experiment was approved by the Institutional Review Board of Nanjing General Hospital of Nanjing Military Command (approval no. 2011NKY-002). All patients agreed to their participation and provided signed informed consent.

Mycologic studies

Direct microscopic examination of the material was performed in the experiment group and control group 1. Skin samples were obtained by scratching with a sterile surgical knife and were observed directly under the microscope after clarification with 20% potassium hydroxide. Skin samples were inoculated on to Sabouraud medium in plates containing chloramphenicol and cycloheximide. The culture plates were incubated at 25 °C for 2 weeks.

Dermatophytic antigen preparations and SPTs

Fungus sources

Separated and purified *T. rubrum* CMCC(F)T1a, *T. mentagrophytes* CMCC(F)T5a, *E. floccosum* CMCC(F)E1d, and *C. albicans* CMCC(F)T5a were provided by the Fungus Preservation Center of the Institute of Dermatological Medicine of the Chinese Academy of Medical Science (CAMS).

Preparation procedure

Separation

The separated and purified fungi were inoculated aseptically into liquid Sabouraud medium (by funnel) and cultivated for

2 weeks at 27 °C. When the mycelium had propagated through the entire substrate layer, mycelium suspensions were sampled and centrifuged at 7104 g for 10 minutes. Precipitations were collected at the bottom of the substrate.

Inactivation

After moisture content had been removed, the cultures were submerged in 95% alcohol for 4 hours to deactivate and kill spores.

Desiccation

The deactivated cultures were spread on laboratory paper to allow them to dry naturally, after which the blocks of fungi were sheared into pieces and ground into fine grain in mortars.

Degreasing

The cultures were degreased with diethyl ether three times and stored.

Alkaline extracts

Active ingredients (sodium chloride 5 g, sodium bicarbonate 2.75 g, phenol 4 g, distilled water 1000 ml) were extracted according to a 5% ratio for 48 hours, filtrated with filter paper, tested, and corrected to reach a pH value of 7.0.

Aseptic filtration

The first filtration was performed coarsely using four layers of gauze filter, after which filtration was conducted using filter paper to sieve out insoluble impurities and a non-porous container to eliminate aseptic microorganism contamination.

Sterile verification

Quantities of 0.5 ml of antigen concentrates were inoculated into Sabouraud dextrose medium and cultivated for 5–8 days at 25 °C to observe any signs of bacteria and fungi.

Virulence test

Intraperitoneal injections of each 0.5-ml antigen concentrate were administered to five laboratory rats per group, which were fed for 7 days to maintain healthy conditions in order to confirm the clinical readiness of the antigens.

Preservation

The clinic-ready fungal antigens were then stored in a refrigerator at 4 °C.⁸

Skin prick tests

The skin of the flexor side of the forearm was chosen as the area for test sites spaced 4 cm apart. The histamine positive control solution, saline negative control solution, and allergens were applied with disposable SPT needles. Subsequently, skin reactions were observed and recorded after 15–20 minutes.

Reactions were considered positive if they produced a mean weal size equal to or greater than that produced by histamine (given a negative reaction to saline).

Statistical analysis

Statistical analyses were performed using SPSS for Windows Version 13.0 (SPSS, Inc., Chicago, IL, USA). Quantitative data conformed to a normal distribution and are shown as the mean $\pm$ standard deviation (SD), whereas qualitative data are shown as the number and percentage of cases per group. Rates of positive responses to antigens in the four groups were compared using a tabulated chi-squared test. A P -value of ≤ 0.05 was considered to indicate a difference of statistical significance. Intergroup pairwise comparisons of positive responses to antigens were compared using a random four-fold chi-squared test or Fisher's exact test. A P -value of ≤ 0.008 was set as the variance threshold of statistical significance (The pair comparison of the number of sample rate in the table has a probability of error of $\alpha = \frac{0.05}{k(k-1)}$ where k is the number of sample. In this test, $k = 4$, $\alpha = 0.008$.) Similar random four-fold chi-squared tests or Fisher's exact tests were applied in the comparison of rates of positivity to the four fungal antigens in the experiment group; a P -value of ≤ 0.05 was set as indicating statistical significance.

Results

Patients

The study included 174 participants (95 males, 79 females), of whom the oldest was aged 83.0 years and the youngest 11.0 years. The mean $\pm$ SD age of the participant group was 35.7 ± 13.5 years (median age: 33.0 years) (Table 1).

Dermatophytic antigen preparations and SPTs

The experiment group showed higher rates of positive reactions to *T. rubrum*, *T. mentagrophytes*, and *E. floccosum* antigens than did control groups 1, 2, and 3; the differences were identified as statistically significant. Control group 1 showed higher rates of positivity to all three of these dermatophytic antigens than did control groups

2 and 3; no significant difference was found between control groups 2 and 3 (Table 2, Fig. 1). In the experiment group, paired comparisons of rates of positive reactions to the three antigens showed no statistically significant difference. However, comparisons of rates of positive reactions to the three dermatophyte antigens with that to *C. albicans* did show statistically significant differences. Paired comparisons of rates of positive reactions to the *C. albicans* antigen among the four groups showed no significant differences.

Mycologic results

Of cultures performed in the experiment group, 66.0% were positive and 52.8% were positive for *T. rubrum*. Of cultures performed in control group 1, 57.8% were positive and 48.9% were positive for *T. rubrum* (Table 3).

Cross-reactions to dermatophytic antigens in the experiment group

In 35 cases of positive fungal culture in the experiment group, the numbers of SPTs showing positivity to dermatophytic antigens were 22 (78.6%) for *T. rubrum*, 26 (92.9%) for *T. mentagrophytes*, 22 (78.6%) for *E. floccosum*, and none for *C. albicans*. The comparison of rates of positivity for the three dermatophytic antigens showed no statistically significant difference. Comparisons of positivity to *C. albicans* and the antigens for *T. mentagrophytes*, *T. rubrum*, and *E. floccosum* showed statistically significant differences.

Discussion

As increasing incidences of dermatophytic infection-induced onychomycosis, tinea pedis and tinea manuum have become worldwide healthcare concerns, so has the focus on the etiologic role of dermatophytic infections in allergic diseases (such as CU) in various studies.⁹⁻¹¹

We performed SPTs in patients in particular groups using prepared antigens. The results showed that rates of positivity to the three dermatophytic antigens were higher in the experiment group than in control groups 1, 2, and 3. Control group 1 showed higher rates of positivity than did control groups 2 and 3. In this experiment, some patients with dermatophytic infection without allergic diseases were found to react positively to the three dermatophytic antigens. This indicates dermatophytic infection may be a determining factor for trichophyton hypersensitivity. We hypothesized that metabolites mediate type I hypersensitivity during fungal infection, which is important to fungi antigens detection. In a study in which intracutaneous antigen tests were performed in 86 asthma patients using dermatophytic extracts, Mungan *et al.*¹² found fungal infection to be a factor determining der-

Table 1 Group and patient data

Group	Patients, <i>n</i>	Gender, F/M, <i>n</i>	Age, years, mean $\pm$ SD
Experiment group	53	22/31	39.6 $\pm$ 15.8
Control group 1	45	16/29	37.1 $\pm$ 11.5
Control group 2	42	25/17	39.0 $\pm$ 11.9
Control group 3	34	16/18	33.5 $\pm$ 12.2

F, female; M, male; SD, standard deviation.

Table 2 Rates of positive skin prick tests by group

Group	<i>T. rubrum</i> n (%)	<i>T. mentagrophytes</i> n (%)	<i>E. floccosum</i> n (%)	<i>C. albicans</i> n (%)
Experiment group (n = 53)	40 (75.5)	45 (84.9)	38 (71.7)	2 (3.8)
Control group 1 (n = 45)	15 (33.3)	10 (22.2)	11 (24.4)	0
Control group 2 (n = 42)	4 (9.5)	1 (2.4)	1 (2.4)	0
Control group 3 (n = 34)	0	0	0	0
χ^2	69.45	102.77	76.13	4.62
P-value	0.000	0.000	0.000	0.202

T. rubrum, *Trichophyton rubrum*; *T. mentagrophytes*, *Trichophyton mentagrophytes*; *E. floccosum*, *Epidermophyton floccosum*; *C. albicans*, *Candida albicans*.



Figure 1 Six skin prick tests were performed on each subject's forearm for, respectively: saline; 1% histamine; *Epidermophyton floccosum*; *Trichophyton rubrum*; *Trichophyton mentagrophytes*, and *Candida albicans*. (a) Negative for all four fungal antigens. (b) Positive for *T. mentagrophytes*. (c) Positive for *T. mentagrophytes* and *T. rubrum*. (d) Positive for *T. mentagrophytes*, *T. rubrum* and *E. floccosum*

Table 3 Culture-positive subjects by group

Group	Fungal antigen positive				
	Any antigen n (%)	<i>T. rubrum</i> n (%)	<i>T. mentagrophytes</i> n (%)	<i>E. floccosum</i> n (%)	<i>C. albicans</i> n (%)
Experiment group (n = 53)	35 (66.0)	28 (52.8)	2 (3.8)	1 (1.9)	4 (7.5)
Control group 1 (n = 45)	26 (57.8)	22 (48.9)	3 (6.7)	0	1 (2.2)
Control group 2 (n = 42)	—	—	—	—	—
Control group 3 (n = 34)	—	—	—	—	—

T. rubrum, *Trichophyton rubrum*; *T. mentagrophytes*, *Trichophyton mentagrophytes*; *E. floccosum*, *Epidermophyton floccosum*; *C. albicans*, *Candida albicans*.

matophytic hypersensitivity, which is consistent with the present experimental findings. We speculated that metabolites of intense local inflammation induced by the dermatophytic infection entering into the blood circulation will stimulate immunoglobulin E antibodies (IgEab).

IgEab bind to Fc receptors on the surface of tissue mast cells. Later exposure to the same antigen cross-links the bound IgEab on the surface of tissue mast cells, resulting in degranulation of mast cells and release of histamine and other inflammatory mediators.¹³ Most patients

showing this reaction probably carried high titers of allergen-specific IgEab such as those that appear in vasculitis and urticaria. This gave us the opportunity to further examine dermatophytic allergen-specific IgE in these patients in order to confirm our speculations.

The results of our experiment showed no statistical significance in comparisons of rates of positive responses in the experiment group to *T. rubrum*, *T. mentagrophytes*, and *E. floccosum* antigens. Comparisons of rates of positive responses to the three dermatophytic antigens in 28 *T. rubrum* culture-positive patients in the experiment group showed no significant differences, indicating cross-reactions among dermatophytic antigens. This probably reflects similarities in protease structure among the dermatophytic antigens. Currently, researchers have extracted eight dermatophytic antigens of enzyme homolog from *T. rubrum* (Tri r2, Tri r4), *Trichophyton tonsurans* (Tri t1, Tri t4), *T. mentagrophytes* (Tri me2, Tri me4), and *Trichophyton schoenleinii* (Tri sc2, Tri sc4). The antigens of genera of dermatophytes contain serine proteases (such as Tri r2, Tri r4, Tri me2, and Tri me4), similar to bacterial secretory or vacuolated subtilisin. These four antigens separated from *T. rubrum* and *T. mentagrophytes* contain two vacuolated serine proteases, Tri r2 and Tri me2, and two alkaline serine proteases, Tri r4 and Tri me4.^{13,14} None were extracted from *E. floccosum*; however, from its evident cross-reaction with *T. rubrum* and *T. mentagrophytes*, the constituents of its antigen may include the same serine protease. Most antigens extracted from *C. albicans* are alcohol dehydrogenase (Cand a1), enolase (Cand a enolase), cyclophilin (Cand a CyP), acid proteinase (Cand a CAAP), peroxidase body protein (Cand a3), HSP90 (Cand a HSP90), and other antigens with unknown functions.¹⁴ In the present study, the pair comparisons of rates of positivity to antigens of *C. albicans* between groups and with those of the dermatophytic antigens showed no significant difference. All *C. albicans* culture-positive patients showed negative reactions to dermatophytes and to *C. albicans* antigens.

Some clinicians may prescribe oral corticosteroids in refractory CU, which can suppress immunoreaction but will induce fungus overgrowth, causing persistent infection in patients with fungal infection complications. It is recommended that clinicians who are engaged in diagnosing and treating patients with persistent CU should request a detailed medical history, perform a thorough clinical examination, and conduct some laboratory examinations. In allergic diseases, the application of a standardized desensitization preparation can increase the efficacy of treatment and reduce the allergic reaction to the maximum extent. Therefore, the possible use of fungi desensitization therapy in some refractory CU patients with positive responses to dermatophytic antigens deserves further inves-

tigation. However, because of the multitude of factors influencing the cultivation of fungi and causes of sensitization, the standardization of the fungal antigen preparation currently presents some difficulties. Medium components and ambient factors including light, temperature, duration, and other environmental conditions all have influence on the culture. In the present experiment, the dermatophytic antigens prepared by ourselves were coarse. The preparation of standardized fungal antigens is a challenge that requires interdisciplinary effort by specialists in mycology, immunology, functional genomics, proteomics, and bioinformatics in order to find a feasible resolution.

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