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Original Article

Detection of delayed hypersensitivity to *Fonsecaea pedrosoi* metabolic antigen (chromomycin)

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Abstract

An experimental study was conducted between January 2002 and April 2003 for the detection of delayed hypersensitivity to *Fonsecaea pedrosoi* metabolic antigen (chromomycin) in skin tests. A total of 194 subjects were attended by spontaneous demand at the Infectious and Parasitic Diseases outpatient clinic of the Federal University of Maranhão-UFMA and at the Department of Microbiology, Federal University of Minas Gerais-UFMG and classified into three groups: patients with chromoblastomycosis caused by *F. pedrosoi* (n=20), healthy subjects (n=86) and patients with other diseases (n=88). For the skin test, 0.1 ml of the antigen was applied to the anterior side of the right forearm and 0.1 ml Smith medium was applied to the anterior side of the left forearm as control. The results were analyzed 48 h after inoculation of the antigen and an induration ≥ 5 mm was considered to indicate a positive test. A cellular immune response to chromomycin was detected in 18 (90.0%) of the 20 patients with chromoblastomycosis caused by *F. pedrosoi*, and one of the patients with a negative test had reactional leprosy. Eighty-five (98.8%) of the 86 healthy subjects presented a negative reaction and only one reacted positively to the antigen. The skin test was negative in all 88 (100%) patients with other diseases, such as dermatophytosis, paracoccidioidomycosis, pulmonary aspergilloma, candidiasis, pityriasis versicolor, tuberculosis, leprosy, tegumentary leishmaniasis and syphilis, and one case of chromoblastomycosis caused by *Rhinocladiella aquaspersa*. Chromomycin was effective in detecting delayed hypersensitivity in patients with chromoblastomycosis caused by *F. pedrosoi*, with a sensitivity and specificity of 90.0% and 98.8%, respectively. These results suggest that this antigen can be used in the auxiliary diagnosis of the disease and also in epidemiological studies for determination of the prevalence of chromoblastomycosis infection in endemic areas.

Key words: chromoblastomycosis, *Fonsecaea pedrosoi*, chromomycin, skin test, metabolic antigen

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Introduction

Chromoblastomycosis is a chronic fungal disease affecting the skin and subcutaneous tissue, which results from the traumatic transcutaneous inoculation of propagules of a variety of dematiaceous fungi of the family *Herpotrichiellaceae*. The disease occurs in regions with tropical and subtropical climates and predominates among male rural workers aged 30 to 50 years[1-6]. In most reports, the fungus *Fonsecaea pedrosoi* has been identified as the causal agent of the disease, accounting for 95.5% of all cases reported in Brazil[7-10].

Although skin tests for the detection of delayed hypersensitivity have been used for the routine diagnosis of other mycoses such as sporotrichosis, paracoccidioidomycosis, histoplasmosis, dermatophytosis and cryptococcosis, they are not applied to the diagnosis of chromoblastomycosis probably because of the lack of an antigen with good sensitivity and specificity[11-15].

Studies using antigen preparation obtained from the culture filtrate of *F. pedrosoi* have shown that patients infected with this fungus present a skin hypersensitivity reaction[16-18]. Other studies have used skin tests in asymptomatic subjects living in areas where the disease occurs and demonstrated the presence of infection in certain population groups[19-21].

In Brazil, the first studies using skin tests with *F. pedrosoi* metabolic antigen (chromomycin) were carried out in the state of Minas Gerais (southeast region of the country). In these studies, the diluted and non-diluted antigen was inoculated into patients with chromoblastomycosis and patients with other diseases and a positive reaction was only observed to the non-diluted antigen in patients with chromoblastomycosis[16].

Since chromoblastomycosis frequently occurs in the Amazon region of Maranhão[8], it has become important to have an antigen with good sensitivity and specificity that can be used in the auxiliary diagnosis of the disease, as well as in epidemiological studies to determine the prevalence of infection. The objective of the present study was to evaluate the cellular immune response to crude *F. pedrosoi* metabolic antigen in skin tests of patients with chromoblastomycosis, subjects without a history of the disease and patients with other infectious and parasitic diseases common in the region.

Materials and Methods

Characteristics of the population: An experimental study was conducted between January 2002 and April 2003 at the Infectious and Parasitic Diseases outpatient clinic of the Federal University of Maranhão-UFMA and at the Department of Microbiology, Federal University of Minas Gerais-UFMG, Brazil, for the detection of delayed hypersensitivity to *Fonsecaea pedrosoi* metabolic antigen (chromomycin) in skin tests. The sample consisted of 194 subjects attended by spontaneous demand, who were classified into three groups: patients with chromoblastomycosis and two control groups as described below.

Group 1 consisted of 20 (10.3%) patients with chromoblastomycosis caused by *F. pedrosoi* who were from the Baixada Ocidental Maranhense, the main area of occurrence of the disease in the state. A chart containing demographic and clinical data was used in this group. For confirmation of the diagnosis, a lesion biopsy was obtained for the following exams: direct mycological and histopathological exams and culture on mycobiotic agar (Difco, Detroit, MI, USA). The fungus was identified based on micromorphological features of conidiogenesis on potato agar according to Riddell[22].

Control group 1 consisted of 86 (44.3%) apparently healthy subjects without a present or past history of chromoblastomycosis from São Luís, Maranhão (n=71, 82.5%) and Belo Horizonte, Minas Gerais (n=15, 17.5%), including students, teachers, doctors, biologists, biochemists, nurses, laboratory technicians, and individuals performing general services.

Control group 2 consisted of 88 (45.4%) patients, including 87 with other infectious diseases (tuberculosis, tegumentary leishmaniasis, leprosy, pulmonary aspergilloma, syphilis, paracoccidioidomycosis, candidiasis, dermatophytosis, pityriasis versicolor), and one patient with chromoblastomycosis caused by *Rhinocladiella aquaspersa*. All diseases in this study were confirmed by clinical and laboratory diagnosis.

Preparation of the *F. pedrosoi* metabolic antigen (chromomycin): The metabolic antigen was prepared at the Laboratory of Mycology, Department of Microbiology, UFMG, according to the technique described by Oliveira[23]. The *F. pedrosoi* strain was deposited in the American Type Culture Collection (ATCC 46428). The microorganism was maintained on Sabouraud dextrose agar and replated at 3-month intervals. Partial chemical analysis of the antigen performed by Barros & Resende[6] had shown that the antigen consists of 0.23 mg/m*l* lipids, 0.57 mg/m*l* protein and 10.73 mg/m*l* carbohydrates.

Skin test: The skin test consisted of the intradermal injection of 0.1 m*l* antigen (57 μg/m*l* protein) into the anterior side of the right forearm of each subject. As a control, 0.1 m*l* Smith medium[33] was injected into the anterior side of the left forearm. The results were ana-

Table 1. Demographic and clinical characteristics and the results of skin tests using chromomycin obtained for patients with chromoblastomycosis caused by *F. pedrosoi*.

Patient	Gender	Age (years)	Occupation	Lesion		Skin test (*)	
				Type (**)	Location	Chromomycin (induration, mm)	Control (Smith) (mm)
B.G.S	M	70	Laborer	2	Lower limbs	15 × 13	0
F.C.S.C	M	67	Laborer	1,2,3	Lower limbs	10 × 11	0
H.G.M	F	60	Laborer	1	Lower limbs	30 × 27	0
A.A	M	59	Laborer	1	Lower limbs	0 × 0	0
B.L.S	M	58	Laborer	1,5	Lower limbs	11 × 13	0
E.J.D	M	71	Laborer	1,4	Lower limbs	10 × 12	0
R.S.L	M	67	Laborer	1,2,3	Lower limbs	6 × 8	0
V.S	M	56	Cart-driver	2,3,4,5	Lower limbs	6 × 7	0
F.C.J	M	44	Laborer	1,2	Lower limbs	5 × 6	0
M.I.B	M	37	Laborer	2	Lower limbs	25 × 23	0
L.A	M	70	Laborer	1,6	Lower limbs	7 × 6	0
O.J.C	M	64	Laborer	1,3	Lower limbs	15 × 13	0
E.S	M	58	Laborer	1,2	Lower limbs	8 × 6	0
J.R.M	M	56	Laborer	1,2	Trunk/nose	15 × 13	0
M.J.C	F	42	Laborer	1	Lower limbs	12 × 15	0
R.S	M	67	Laborer	1,3,5	Lower limbs	16 × 14	0
V.S	M	37	Laborer	1,4,6	Lower limbs	10 × 11	0
S.S	M	50	Laborer	2	Upper limbs	12 × 13	0
P.C	M	63	Laborer	2	Upper limbs	0 × 0	0
V.G	M	42	Laborer	2,6	Lower limbs	11 × 10	0

M=male; F=female.
(*) Positive skin test: induration ≥ 5 mm.
(**) Type of lesion: 1=vegetating plaque, 2=infiltrating plaque, 3=verruciform, 4=nodular, 5=tumor-like, 6=cicatricial.

lyzed 48h after inoculation of the antigen by measuring the diameter of the induration in two directions. An induration ≥ 5 mm[17] in at least one direction was considered to indicate a positive test.

Statistical analysis: The sample size necessary to determine the sensitivity and specificity of the antigen was calculated to be 194 subjects according to Snedecor & Cochran[24], based on a prevalence of the disease of 25% reported by Baquero[19]. Statistical analysis was performed with the Epi-Info program, version 6.0 (Centers for Disease Control, 2000), calculating the sensitivity, specificity, positive and negative predictive values, and their respective 95% confidence intervals (95% CI).

Results

In group 1, consisting of 20 (10.3%) patients with chromoblastomycosis, there was a predominance of males (n=18, 90%) with a mean age of 56.9 years, most of them laborers (n=19, 95%). Analysis of the clinical findings showed lesion polymorphism, with a predominance of vegetating and infiltrating plaques mainly located on the lower limbs (Table 1). All patients underwent the exams for a definitive diagnosis of chromoblastomycosis and *F. pedrosoi* was isolated in all cases. Eighteen (90%) patients presented a positive reaction in the skin tests using chromomycin, whereas the reaction was negative in 2 (10%) (Table 1). One of the patients with a negative result had the reactional form of leprosy.

Control group 1 consisted of 86 (44.3%) apparently healthy individuals, 42 (48.8%) females and 44 (51.2%) males ranging in age from 17 to 56 years. The skin tests using chromomycin were negative in 85 (98.8%) of the subjects; however, one 21-year-old female subject presented a positive reaction, with an induration measuring 6 × 6 mm. Table 2 shows the age, gender and occupation of the healthy subjects.

Control group 2 consisted of 88 (45.4%) patients with 10 different types of infectious diseases, including tuberculosis in 18 (20.4%), dermatophytosis in 15 (17,1%), syphilis in 13 (14.8%), leprosy and candidiasis

Table 2. Demographic characteristics of healthy subjects undergoing a skin test using chromomycin (control group 1).

Profession	Number of subjects (%)	Gender		Mean age (years)
		Male N (%)	Female N (%)	
Students	35 (40.6)	19 (43.1)	16 (38.1)	25.2
Teachers	6 (6.9)	3 (6.8)	3 (7.1)	46.3
Doctors	3 (3.5)	2 (4.6)	1 (2.4)	40.3
Biologists	2 (2.3)	1 (2.2)	1 (2.4)	33
Biochemists	8 (9.3)	4 (9.2)	4 (9.6)	39.1
Nurses	4 (4.7)	2 (4.5)	2 (4.7)	38.3
Laboratory technicians	19 (22.2)	8 (18.2)	11 (26.1)	28.6
General service workers	9 (10.5)	5 (11.4)	4 (9.6)	30.7
Total	86 (100)	44 (100)	42 (100)	35.1

Table 3. Demographic characteristics of patients with other infectious diseases undergoing a skin test using chromomycin (control group 2).

Disease	Number of patients (%)	Gender		Mean age (years)
		Male N (%)	Female N (%)	
Tuberculosis	18 (20.4)	8 (14.8)	10 (29.4)	44
Dermatophytosis	15 (17.1)	8 (14.8)	7 (20.5)	32.4
Syphilis	13 (14.8)	10 (18.5)	3 (8.9)	27.9
Leprosy	10 (11.3)	8 (14.8)	2 (5.8)	48.7
Candidiasis	10 (11.3)	7 (12.9)	3 (8.9)	31.5
Pityriasis versicolor	9 (10.3)	4 (7.4)	5 (14.7)	28.8
Tegumentary leishmaniasis	6 (6.8)	3 (5.6)	3 (8.9)	31.8
Paracoccidioidomycosis	4 (4.6)	4 (7.4)	−	39
Pulmonary aspergilloma	2 (2.3)	1 (1.9)	1 (2.9)	58
Chromoblastomycosis (*R. aquaspersa*)	1 (1.1)	1 (1.9)	−	52
Total	88 (100)	54 (100)	34 (100)	39.4

in 10 each (11.3%), pityriasis versicolor in 9 (10.3%), tegumentary leishmaniasis in 6 (6.8%), paracoccidioidomycosis in 4 (4.6%), pulmonary aspergilloma in 2 (2.3%), and chromoblastomycosis caused by *R. aquaspersa* in 1 (1.1%). Fifty-four (61.4%) patients were males and 34 (38.6%) females, with a mean age of 39.4 years (Table 3). The skin test using chromomycin was negative in 100% of cases, a finding calling attention to the fact that the patient with chromoblastomycosis caused by *R. aquaspersa* presented a negative reaction to the *F. pedrosoi* antigen.

To evaluate the validity of the skin test using *F. pedrosoi* metabolic antigen (chromomycin), the sensitivity, specificity and positive and negative predictive values were determined, comparing patients with *F. pedrosoi* chromoblastomycosis with the subjects of control groups 1 and 2 (Tables 4 and 5). Comparison of patients with chromoblastomycosis and control group 1 showed a prevalence of *F. pedrosoi* of 18.9%. The antigen presented 90.0% sensitivity (CI 95% 66.9-98.2%) and 98.8% specificity (CI 95% 92.8-99.9%). The positive and negative predictive values were 94.7 and 97.7%, respectively. Compared to control group 2, sensitivity was 90.0% (CI 95% 66.9-98.2%) and specificity was 100.0% (CI 95% 94.8-100.0%), with a positive and negative predictive value of 100.0 and 97.8%, respectively. The prevalence of infection was 18.5%.

Discussion

The laboratory diagnosis of chromoblastomycosis can be made based on direct microscopic examination of the collected biopsy material, which reveals the presence of fungal structures with characteristic morphology and staining, but the detection of these

Table 4. Sensitivity and specificity of chromomycin in skin tests of patients with chromoblastomycosis caused by *F. pedrosoi* compared to healthy subjects (control group 1).

Skin test	Patients with chromoblastomycosis	Control group 1	Total
Positive	18	1	19
Negative	2	85	87
Total	20	86	106

Sensitivity=90.0%	(66.9 − 98.2)
Specificity=98.8%	(92.8 − 99.9)
Positive predictive value=94.7%	(71.9 − 99.7)
Negative predictive value=97.7%	(91.2 − 99.6)

Table 5. Sensitivity and specificity of chromomycin in skin tests of patients with chromoblastomycosis caused by *F. pedrosoi* compared to patients with other infectious diseases (control group 2).

Skin test	Patients with chromoblastomycosis	Control group 2	Total
Positive	18	0	18
Negative	2	88	90
Total	20	88	108

Sensitivity=90.0%	(66.9 − 98.2)
Specificity=100.0%	(94.8 − 100.0)
Positive predictive value=100.0%	(78.1 − 100.0)
Negative predictive value=97.8%	(91.4 − 99.6)

microorganisms is not always easy. In addition, these exams do not permit immunological surveys, thus impairing data collection for a better understanding of the epidemiology of this infection[16, 18].

Skin tests employing antigen preparations obtained from the culture filtrate of *F. pedrosoi* have been used in some studies for the detection of delayed hypersensitivity[6,16 − 18,27]. A delayed hypersensitivity reaction was detected in patients with chromoblastomycosis caused by this fungus, but these studies did not evaluate the sensitivity or specificity of the antigens used.

According to Kurita[25], serum precipitin levels are very low in patients with chromoblastomycosis, a fact impairing their detection by laboratory methods. However, cell-mediated immunity (granuloma formation) plays an important role in the defense of the organism against tissue invasion by this fungus[29]. On the other hand, studies investigating the humoral immunity in patients with chromoblastomycosis using enzyme-linked immunosorbent assays (ELISA) were unable to detect antibodies in patients with a single lesion, revealing negative serology when this test was used[26]. This agrees with Iwatsu *et al.*[27] who reported that the cellular immune response is more effective than the humoral response in chromoblastomycosis.

Few epidemiological studies investigating the prevalence of chromoblastomycosis in the general population are available, probably because of the lack of an antigen with good sensitivity and specificity[6,19 − 21,27]. In a study carried out in Cuba, asymptomatic individuals from urban and rural areas were submitted to skin tests using an antigen prepared from *F. pedrosoi* cultures. The results showed 100% negativity in the urban area, whereas in the rural area 25% of the reactions were positive. These findings demonstrate the occurrence of infection in certain population groups[19]. The skin sensitivity to antigens of agents causing chromoblastomycosis has also been studied in Medellin and Sahagun (Colombia), where *F. pedrosoi* and *Cladosporium carrionii* antigens were used in both populations. The sample consisted of healthy subjects from the urban area of Sahagun and of plant nursery workers from Medellin. In the two areas, positivity was higher for *F. pedrosoi* compared to *C. carrionii*[21]. Skin tests have also been carried out in Venezuela, showing 44% positivity for *C. carrionii* and only 4.5% for *F. pedrosoi*. In this region, the prevalence of chromoblastomycosis caused by *C. carrionii* was higher than that caused by *F. pedrosoi*[20].

In Brazil, the first study for the detection of delayed

hypersensitivity to *F. pedrosoi* metabolic antigen (chromomycin) was conducted by Oliveira[16] in the state of Minas Gerais (southeastern Brazil). Inoculation of non-diluted antigen induced a positive response in 13 patients with chromoblastomycosis, whereas the reaction was negative in patients with paracoccidioidomycosis, leprosy and syphilis and in healthy subjects, in agreement with our findings.

Analysis of our results showed that chromomycin was effective in detecting delayed hypersensitivity in patients with chromoblastomycosis caused by *F. pedrosoi*. The reaction was considered to be positive in 90% of cases and a negative result was obtained for two patients. One of these patients presented extensive lesions on the lower limbs classified as the severe form of the disease according to Queiroz-Telles *et al.*[5], and the other had the reactional form of leprosy and was taking steroids. In addition, one patient with chromoblastomycosis caused by *R. aquaspersa* presented a negative skin test, demonstrating that the *F. pedrosoi* metabolic antigen (chromomycin) was not cross-reacting with antigen determinants of *R. aquaspersa*, a finding suggesting good specificity of this antigen. Similar results have been reported by Iwatsu *et al.*[17]; however, the EP-1 antigen (antigen precipitated with ethanol and prepared from *F. pedrosoi* culture filtrate) used by these authors showed cross-reaction with *Exophiala jeanselmei*[17,27].

Lacaz *et al.*[18] submitted patients with chromoblastomycosis to skin tests using the EP-1 antigen of Iwatsu *et al.*[17] and observed 66.6% positivity. A higher positivity rate (90%) was obtained in the present study using chromomycin. In this group of patients with chromoblastomycosis, one 37-year-old male patient, who was clinically cured after one year, presented a positive reaction, indicating that delayed hypersensitivity persists even after clinical cure, in agreement with the findings of Lacaz *et al.*[18].

In the present study, a negative reaction was observed in 98.8% of subjects of control group 1, but one 21-year-old woman presented a positive skin test. This finding suggests that the patient had probably come into contact with the fungus since this microorganism is ubiquitous in nature[30]. However, 100% of the healthy subjects studied by Oliveira[16] and Lacaz *et al.*[18] presented a negative reaction. In contrast, in control group 2, 100% of the skin tests were negative, in agreement with the findings of Oliveira[16] and Iwatsu *et al.*[17].

The present skin test using *F. pedrosoi* metabolic antigen (chromomycin) showed good sensitivity and specificity, demonstrating its efficacy in the detection of skin sensitivity in patients with chromoblastomycosis caused by this fungus. In addition, this study confirms the results observed by Oliveira[16] in Minas Gerais, employing the same test in another area of occurrence of the disease and involving a larger sample. These facts suggest that *F. pedrosoi* metabolic antigen (chromomycin) might be used in the immunodiagnosis of chromoblastomycosis caused by this fungus and in epidemiological surveys for the determination of the prevalence of this infection in areas of occurrence of the disease.

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