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Paracoccidioides brasiliensis-gp43 used as paracoccidioidin

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A purified glycoprotein of 43 000 daltons from *Paracoccidioides brasiliensis* (gp43) was tested as paracoccidioidin in delayed-type hypersensitivity (DTH) tests in both experimental animals (guinea pig and mice) and patients with paracoccidioidomycosis (PCM). The gp43 paracoccidioidin was compared with the traditional Fava Netto antigen (AgFN). In guinea pigs, the intradermal injection of 2 μg of gp43 showed a similar response to those obtained with AgFN, showing in histological sections a population of lymphoid cells that participate in DTH. In mice, gp43 at a dose of 3·75 μg showed positive DTH response. The use of gp43 as paracoccidioidin in humans showed that this molecule can be used to evaluate the DTH response in patients with PCM. Of 25 PCM patients studied, 48% were positive to gp43 while only 28% were positive to AgFN; 12 PCM patients were completely anergic to both antigens. Considering only those 13 PCM patients who were responsive to gp43 and/or to AgFN, 92·3% reacted against gp43 and 53·8% reacted against AgFN ($P < 0{\cdot}05$). Gp43 skin test responses (13·67 ± 9·56 mm) were significantly larger than those obtained with AgFN (8·43 ± 3·69 mm). Immunohistochemical study of the human skin showed a perivascular inflammatory response constituted predominantly by T lymphocytes, macrophages and polymorphonuclear leukocytes.

Keywords gp43, paracoccidioidin, paracoccidioidomycosis, *P. brasiliensis*

Introduction

Paracoccidioidomycosis (PCM) is a mycotic disease caused by the dimorphic fungus *Paracoccidioides brasiliensis*. Primary infection is admittedly acquired by inhalation of mycelial-phase propagules, which reach the lungs and convert to the yeast phase. Manifestations of PCM range from a benign, self-limited pulmonary infection to a severe, progressive and fatal mycosis, involving the lungs and other internal organs [1].

Several studies suggested that there is a positive correlation between the depression of cell-mediated immunity and the severity of the disease [1,2]. High anti-*P. brasiliensis* antibody levels associated with a lack of cell-mediated immunity have been observed frequently in PCM [1]. Mota *et al.* [2], in a comprehensive study, classified patients according to the clinical forms of PCM and demonstrated by different *in vivo* and *in vitro* immunological tests that in PCM there is a wide spectrum of immunological reactivity.

In past decades many investigators have employed different crude antigenic preparations to study delayed-type hypersensitivity (DTH) by skin reactivity. Mycelial-phase [3,4] or yeast-phase culture filtrates [5], polysaccharide extracts [6], mycelial and yeast-phase ethanol-precipitated fractions [7] of *P. brasiliensis* have been applied either to patients or to normal individuals during epidemiological studies as well as in experimental investigations [8]. Moreover, each antigen was prepared from cells cultured in different growth conditions, such as culture medium, incubation time, growth temperature, shaken or stationary cultures, yeast or mycelial growth. It

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is possible that different antigenic preparations vary considerably in their activity and quality as a result of the lack of proper production standards even in the same laboratory. For these reasons, it is not surprising that there is considerable disagreement regarding the sensitivity and specificity of skin tests in PCM.

The *P. brasiliensis* antigen usually employed for skin tests is called paracoccidioidin and the most used preparation is known as 'Fava Netto's antigen' [6]. Its exact composition and the components responsible to elicit T cell responses have not yet been established. This antigen gives positive cutaneous reactions in 87% of proven cases of PCM, but cross-reactions have been observed among patients with other types of mycoses.

Paracoccidioidins are preparations containing mixtures of antigens (proteins, glycoproteins, polysaccharides and glycolipids) and the precise identity of the *P. brasiliensis* proteins that elicit T cell responses is unknown.

The major and specific antigenic component of *P. brasiliensis* is a concanavalin A (ConA)-binding glycoprotein of apparent molecular mass of 43 000 daltons [9], gp43, which is very useful for serodiagnosis [10,11]. It is recognized by 100% of sera (IgG) from patients with PCM by immunoblotting assay [12,13]. This glycoprotein can be purified from culture supernatant fluids by affinity chromatography with IgG from patient's serum, protein A-purified rabbit anti-gp43 IgG and with anti-gp43 monoclonal antibody. There is evidence that the molecule is a peptide chain with N-linked high mannose oligosaccharide side chains with the presence of galactopyranose units [9].

Fava Netto's paracoccidioidin has proven its usefulness in the study of cellular immunity in PCM, but the availability of purified antigen such as gp43 led us to evaluate the efficacy of this purified molecule in DTH assays.

Materials and methods

Fungal strains

P. brasiliensis strain 18 (Pb-18) and *P. brasiliensis* B-339 (Pb-339) were kindly provided by Dra. Vera Calich, ICB, USP, Brasil, and Dra. Angela Restrepo, CIB, Medellin, Colombia, respectively. The fungi were converted to the yeast form on Sabouraud dextrose agar (Difco) supplied with 0·01% Tiamine and 0·14% Asparagine, at 35 °C, and has been maintained in this form.

Gp43 purification

The exoantigen from *P. brasiliensis* B-339 was used as the antigen source. Supernatant fluids, from 7-day-old cultures were filtered on paper, concentrated and dialysed, according to our previous report [11]. Purification of gp43 was performed by affinity chromatography in a column of Affi-gel 10 (Bio-Rad) coupled with an IgG anti-gp43 mouse monoclonal antibody. Gp43 was eluted with 0·1 M acid citric buffer, pH 2·8, neutralized with 1 M Tris, pH 9·0, and concentrated in an Amicon 10 K cell. Protein contents were determined by the Bradford method [14]. All purification steps of gp43 were monitored by SDS-PAGE.

Preparation of gp43 for intradermal use

Solutions of gp43 in saline containing 10, 20, 40, 50 and 75 $\mu g\ ml^{-1}$ were sterilized by filtration in 0·22 μ disposable filters (Sigma) and stored at −20 °C before use.

Fava Netto's paracoccidiodin (AgFN)

Paracoccidioidin was kindly prepared and provided by Professor Celeste Fava Netto (Instituto de Ciências Biomédicas, Universidade de São Paulo, Brazil) [15].

Sensitization assays

Guinea pigs

Ten albino male guinea pigs, weighing 500–600 g, were inoculated intratesticularly with an aqueous suspension of 6×10^6 viable yeast cells of the virulent Pb-18 strain in a single dose of 0·1 ml. Three other guinea pigs were inoculated with *Histoplasma capsulatum* (EPM-41) by intraperitoneal inoculation of 2×10^6 viable yeast cells in a single dose of 0·5 ml.

Mice

Three groups of Swiss mice were actively sensitized by three weekly subcutaneous injections of 1·0, 2·5 and 5·0 μg of crude *P. brasiliensis* (Pb-18) exoantigens in 100 μl of PBS, respectively. This crude exoantigen was prepared as described for Pb 339 crude preparation in previous report [11]. Animal controls were not previously sensitized. Sensitivity was assayed at week 4. All animals were housed in accordance with the NIH Guide for Care and Use of Laboratory Animals.

Sensitivity assays

Guinea pigs

Ten animals infected with *P. brasiliensis* were skin tested in the fourth week after inoculation by intradermal injections of 0·1 ml solution of gp43 containing 1 and 2 μg of protein, and of 0·1 ml of Fava Netto's paracocciodioidin (AgFN), used as control. The injections were made into the previously shaven back skin. The animals infected

with *H. capsulatum* were skin tested with 1 and 2 μg of purified gp43 (in 100 μl) and AgFN (100 μl). Controls consisted of five uninoculated guinea pigs tested with 1 μg (100 μl) and 2 μg of gp43 (100 μl), and AgFN (100 μl). The diameter of induration was measured with a millimetre ruler 24 h after injection.

Mice

DTH was evaluated by testing mice in the footpad with gp43 (1·0, 2·0 and 3·75 μg of protein). Mice were injected in the right hind footpads with 50 μl of antigen in pyrogen-free phosphate-buffered saline (PBS) and with PBS alone in the left hind footpads. Just before and 24 h after injection, footpad thickness was measured with a dial calliper (Mytutoyo, Tokyo, Japan). The results were calculated as the difference in thickness of antigen and PBS-injected pads at 24 minus the footpad thickness of antigen and PBS-injected pads before footpad challenge. Mice controls were tested with similar doses in the same schedule.

PCM patients and healthy volunteers

Skin test were carried out in 25 patients using 7·5 μg of gp43 in 0·1 ml (gp43 concentration was previously determined; data not shown) and 0·1 ml of AgFN. Antigens were injected in both forearms employing standard techniques. Skin tests were considered positive when, after 24 h, an induration larger than 5 mm was observed (diameter measured with millimetre ruler). The mean and standard deviation was done only of the positive skin tests. Twenty clinically normal adults who had never been skin tested with paracoccidioidin and serologically negative to PCM were admitted as controls, and skin tested with gp43 and AgFN. Patients and healthy people previously agreed to submit to the skin test. Protocol of gp43-skin test was approved by the Commission for medical ethics of Hospital São Paulo, Escola Paulista de Medicina.

Histopathology

Guinea pigs

Skin specimens were taken 24 h after from the sites of antigen injection. An area of tissue approximately 10 mm in diameter was excised with fine scissors and a scalpel, with particular attention to preserve the underlying loose connective tissue. Specimens were fixed in 10% buffered formalin, sectioned, and stained with haematoxylin and eosin by routine methods.

Mice

The material obtained from the swelling footpad was immediately frozen in liquid nitrogen. The footpad was then sectioned in a cryostat and the sections were immunohistochemically stained by the avidin-biotin-peroxidase method [16]. The monoclonal antibodies used were 30-H 12 (T cells), GK-1·5 (T helper cells), 53-6·7 (T cytolytic cells) and MK-D 6 (B cells and dendritic cells). All monoclonal antibodies were provided by Becton Dickinson.

Humans

Biopsies were obtained from three patients after their consent. The biopsies were fixed in 10% buffered formalin for 24 h and embedded in paraffin for immunohistochemical studies. Immunohistochemical staining of skin specimens was performed according to the avidin-biotin-peroxidase method [16]. Briefly, sections were deparaffinized; endogenous peroxidase was blocked with 0·3% hydrogen peroxide in methanol, and the sections were incubated successively with the appropriate non-immune serum, the appropriate primary antiserum overnight at 4 °C, biotinylated secondary antibody for 30 min at 27 °C followed by the avidin-biotin-peroxidase complex reagent for 30 min at 27 °C before incubation with diaminobenzidine in a Tris HCl buffer. The antibodies used in this study were UCLH 1 (T cells, macrophages and myeloid cells), L 26 (B cells and follicular dendritic reticulum cells), HLA-DR/Alpha (B cells, activated T cells, macrophages, antigen presenting cells and some endothelial and epithelial tissues), Dako-M 1 (clone C3D-1#granulocytes and dendritic reticulum cells), Mac 387 (macrophages and myeloid cells), S-100 protein and HAM 56 (macrophages and monocytes). All monoclonal antibodies were obtained from Dakopatts S/A, Denmark, except HAM 56 that was from Enzo Biochem, NY.

Immunodiffusion (ID) test

Sera from PCM patients were titrated by ID assay as previously described [11].

Results

Dermal reactions to gp43 at 1 or 2 μg and to AgFN were observed in all guinea pigs previously sensitized with *P. brasiliensis*, showing a well-defined zone of redness and induration at 24 h. Similar intensity of the reactions were also observed at 48 h. Results of skin tests in guinea pigs showed that there were no differences of the reactivity between the doses of 1 and 2 μg of gp43. However, the dose of 2 μg gp43 evoked a positive reaction (induration) similar to that obtained by the Fava Netto's paracocidioidin. Positive reactions, measured by induration, were 8·5 mm, 9·5 mm and 9·7 mm in diameter to 1 μg gp43, 2 μg gp43 and AgFN, respectively. In the non-infected

Table 1 Footpad swelling responses of Swiss mice to different concentrations of gp43, after active subcutaneous sensitization with different doses of *P. brasiliensis* exoantigen

Sensitizing dose (Pb-18 exoantigen) (mg/100 μl)	Challenge* (gp43)		
	1 μg	2 μg	3·75 μg
1	1·5 ± 0·7	1·5 ± 0·7	1·2 ± 0·6
2·5	1·7 ± 0·3	1·7 ± 0·3	2·0 ± 0·4
5	1·5 ± 0·7	1·7 ± 0·3	1·0 ± 0·5

Values are in mm.
*Results are the mean ± standard deviation of the responses in groups of 18 mice each.

guinea pigs (control group) there was no measurable response to the test antigens (gp43 or AgFN). Guinea pigs inoculated with *H. capsulatum* reacted with 2 μg gp43 and AgFN with a 4 mm induration (response considered as negative, based on protocols used in tuberculosis [17]).

Histological evaluation of the positive skin tests evoked by gp43 in guinea pigs showed a population of cells that usually participate in delayed-type hypersensitivity (DTH), such as polymorphonuclear leukocytes, histiocytes and lymphocytes around blood vessels, sometimes with characteristic epithelioid cells suggesting a granulomata. The histological features of the response to the AgFN paracoccidioidin were essentially identical to those obtained with gp43.

In mice, optimal sensitization antigen concentration and optimal sensitivity dose to develop DTH were analysed. For sensitization purposes, 25 μg ml^{-1} was found to be the best dose. On the other hand, for sensitizing assay, the dose of 3·75 μg of gp43 developed significant DTH at 24 h (mean ± standard deviation of pad swelling = 2·0 ± 0·4 mm) (Table 1). Histopathological examination of the footpad of mice inoculated with gp43 showed an intense, predominantly perivascular inflammatory infiltrate consisting of mononuclear cells, most of them CD4 T lymphocytes, histiocytes in the superficial dermis. Rare B cells were found.

The results of the skin tests of the PCM patients ($n = 25$) with gp43 and AgFN were positive in 12 (48%) and in seven (28%) patients, respectively. On the other hand, complete cutaneous anergy was verified in 13 (52%) patients to gp43 and in 18 (72%) patients to AgFN parcoccidioidin (Table 2). However, if considering only those 13 patients who reacted positively to one or two antigens, 12 (92·3%) patients were reactive to gp43 and seven (53·8%) patients to AgFN. The intensity of the DTH response in the PCM patients tested with gp43 (13·67 ± 9·56 mm) was significantly larger ($P < 0·05$) than those obtained with AgFN paracoccidioidin

Table 2 Results of skin tests of patients with PCM challenged with 7·5 μg gp43, and AgFN

Patients	gp43 (7·5 μg/100 μl)	AgFN (100 μl)
C.G.S.	5	12
O.P.S.	10	—
A.T.	15	—
J.P.B.	10	5
S.R.O.	17	—
M.A.F.	41	—
J.G.A.S.	18	—
H.C.	5	5
P.C.	—	5
G.F.S.	10	10
L.P.R.	14	14
L.C.S.	10	8
O.M.	9	—
O.B.	—	—
A.B.	—	—
M.P.P.	—	—
A.C.	—	—
M.R.S.	—	—
G.G.S.	—	—
C.G.	—	—
V.J.M.	—	—
A.H.C.S.	—	—
L.O.D.	—	—
L.S.S.	—	—
D.S.	—	—
Mean ± SD (mm)	13·67 ± 9·56	8·43 ± 3·69

— = negative.

(8·43 ± 3·69 mm). Among the control group (healthy people) only one reacted positively to gp43 (5%) and one (5%) reacted against AgFN.

The immunohistochemical study of the human skin showed that gp43 elicited a perivascular inflammatory response constituted predominantly by T lymphocytes (identified with the UCHL1 antibody; Fig. 1a), followed by macrophages (identified with the HAM 56 and MAC387; Fig. 1b) and polymorphonuclear leucocytes. B lymphocyte cells were absent. Rare S-100 positive cells were found in the inflammatory infiltrate.

Discussion

PCM is a disease in which T cell-mediated immunity has been shown to play a critical role in host defense either in humans or in experimentally infected animals [1,2,18]. PCM is characterized by a large spectrum of clinical and immunological manifestations but two main polar forms were defined, namely: (i) anergic or malignant form,

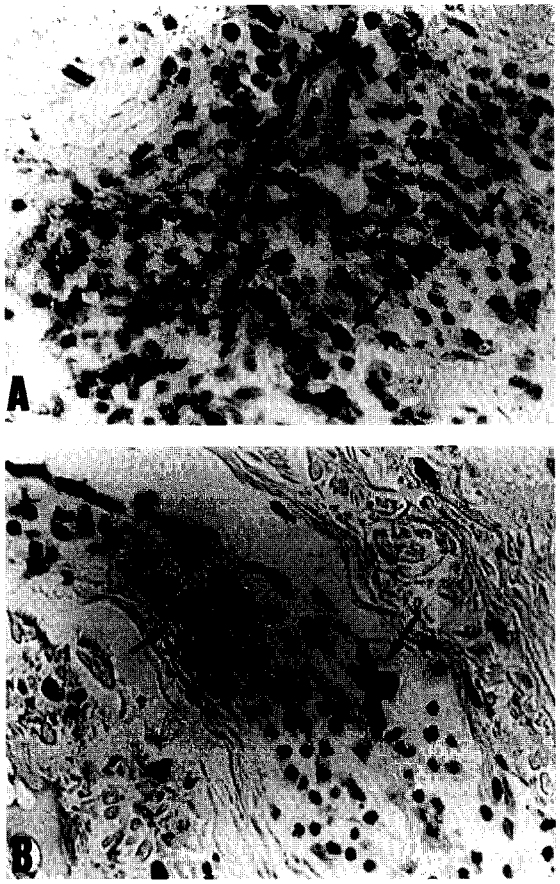

Fig. 1 DTH after gp43 skin test. (a) Perivascular inflammatory response constituted predominantly by T lymphocytes (identified with the UCHL 1 antibody). (b) Macrophages (identified with the HAM 56 and MAC 387 antibodies) and polymorphonuclear leucocytes. Avidin–biotin–peroxidase technique, × 330.

characterized by disseminated disease, impaired cellular immune response, high levels of specific antibodies, loose granulomatous inflammation with necrosis and large numbers of fungi in the lesions, and (ii) hyperergic or benign form, characterized by localized disease, adequate cellular immune response, low levels of antibodies, compact epithelioid granulomata and the absence of or few fungi in the lesions [1].

Many *P. brasiliensis* antigenic preparations obtained from the broth culture filtrate of the mycelial or yeast phase, aqueous lysate or yeast delipidized extracts have been shown to elicit cellular immune response in both patients with PCM and experimentally-infected animals. However, these are non-homogenous preparations containing mixtures of antigens, and the precise identity of the *P. brasiliensis* components that elicit T cell response is unknown. The determination and understanding of which *P. brasiliensis* antigen stimulates cellular immune responses at the level of individual molecules or epitopes constitute the basis for the ultimate development of a vaccine against the paracoccidioidomycosis. Although these paracoccidioidins have been used for a long time, none of their components has been purified and assayed in DTH tests. We chose to use the gp43 purified component as the selective antigen in skin tests because this component is early excreted by the yeast cells in broth cultures, is the major component excreted and elicits early antibody response in active disease. All PCM patients sera have IgG anti-gp43, as shown by Western blotting [12,13].

On the basis of the results of skin tests presented in this study, we have shown that gp43 can elicit cellular immunity in animals and humans. Comparatively, 2 μg of gp43 evoked the same response ($\sim$9·5 mm of induration) as the traditional Fava Netto's paracoccidioidin ($\sim$9·7 mm of induration) in guinea pigs experimentally infected. The histological findings of the positive skin tests evoked either by gp43 or AgFN in guinea pigs showed similar cells involved in the DTH process, such as polymorphonuclear leucocytes, lymphocytes, monocytes and histiocytes in the superficial dermis.

The DTH reaction in mice may show a striking variation in size and histological features, depending on the dose and schedule of immunization. In previous experiments we have observed a greater DTH response in mice immunized by *P. brasiliensis* cytoplasmic antigen in complete Freund's adjuvant (data not shown). However, this approach did not produce a positive DTH reaction in all animals tested and in both inbred and outbred mice the same pattern of reactivity was observed. The response herein observed seems to be similar to the tuberculin's reaction, as it reaches its peak in 24 h and remains for 48 h [17]. In the present study, a maximal response, as seen by footpad thickness, was obtained when the mice were challenged with the higher dose of gp43 (3·75 μg). In histological sections, mononuclear cells predominated and were placed near the epithelium. Naive mice displayed a non-specific response before 18 h, characterized by an evident oedema, but this reaction could no longer be found after 18 h while typical DTH response was observed only in sensitized animals at 24 h. This finding is in agreement with previous studies [19,20]. As emphasized by Marchall and Milon [19] the early inflammatory response is very important for the development of the DTH and may be demonstrated in non-immunized mice. The initial response is a component of the natural immunity. Although gp43 contains epitopes that are immunodominant for humoral response, it became clear that this molecule also contains epitopes that stimulate T lymphocyte proliferation, responsible for DTH in skin tests.

In the present study, 13 (52%) of the PCM patients were reactive to skin test with paracoccidioidin (gp43 and/or AgFN) and 12 (48%) of them showed complete anergy to *P. brasiliensis* antigens. This anergy might be related to a

generalized depression of cellular immunity in the patients, and these results are in agreement with others [2,5]. Regarding all the patients studied ($n = 25$) and comparing both types of antigens, gp43 showed positivity in 48% of them whereas for AgFN the positivity was seen only in 28%. However, considering only the responsiveness of the patients to gp43 and/or AgFN ($n = 13$), gp43 showed 92·3% of positivity and AgFN, 53·8%. Patients with gp43 positive skin test reactivity had an induration of 5–41 mm (13·67 ± 9·56 mm) whereas for AgFN positive reactions produced an induration of 5–14 mm (8·43 ± 3·69 mm). AgFN positive reactions were in agreement with gp43 in all cases but one. In six cases where AgFN was negative, gp43 was positive and in only one case gp43 was negative and AgFN positive. On other hand, the results of skin tests in normal people showed that gp43-paracoccidioidin was able to detect 5% of normal people sensitized with *P. brasiliensis* antigens and AgFN detected 5% of them.

Restrepo and Schneidau [7], studying DTH responses in PCM with a *P. brasiliensis* ethanol-precipitate fraction, postulated that the glycopeptide is the material primarily responsible for the skin reactivity. On the other hand, Barker *et al.* [21] showed that a degradation of the peptide moiety of a glycoprotein of *Trichophyton mentagrophytes* led to a loss of delayed-type reactivity. Similarly, gp43 is a glycoprotein and we have observed that the degradation of its proteic content by alkaline hydrolyses produce a molecule that does not evoke DTH (data not shown).

Fava Netto's paracoccidioidin is an antigenic preparation that has been intensively tested since the 1960s by various researchers in Latin America, mainly in epidemiological surveys. It was also tested to verify the cellular competence of numerous PCM patients during the period of active disease as well as in follow-up studies. It is routinely used in PCM patients, and its positivity reflects a good prognosis.

Our study did not propose the substitution of the Fava Nettos' paracoccidioidin by the gp43-based paracoccidioidin. The results reported here are a preliminary study to evaluate the potential of purified gp43 in skin tests. More detailed studies are needed to better establish the gp43 potential as paracoccidioidin. Currently we are investigating the gp43 paracoccidioidin in epidemiological studies to verify whether unresponsiveness to gp43 in patients with PCM is associated with the impairment of cell-mediated immunity and severity of the disease.

The use of purified molecules as antigenic preparations in skin tests is particularly important because of the possible introduction of contaminants such as endotoxins. Gp43 seems to be a promising molecule for this kind of study in PCM as evidenced by the adequate or impaired cellular immunity as well as in epidemiological studies.

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