Rev. Inst. Med. trop. São Paulo
34 (3):243-249, maio-junho, 1992

EVALUATION OF DIFFERENT IMMUNIZATION PROTOCOLS WITH P. brasiliensis ANTIGENS IN GUINEA PIGS

Amélia KAMEGASAWA (1) Maria Tereza REZKALLAH IWASSO (2), Rosa VIERO (3) & Marcello FRANCO (3)

SUMMARY

The objective of the present study was to develop an efficient and reproducible protocol of immunization of guinea pigs with **P. brasiliensis** antigens as an animal model for future studies of protective immunity mechanisms. We tested three different antigens (particulate, soluble and combined) and six protocols in the presence and absence of Freund's complete adjuvant and with different numbers of immunizing doses and variable lenght of time between the last immunizing dose and challenge.

The efficacy of the immunizing protocol was evaluated by measuring the humoral and cellular anti-**P. brasiliensis** immune response of the animals, using immunodiffusion, skin test and macrophage migration inhibition test. It was observed that: 1. Three immunizing doses of the antigens induced a more marked response than two doses; 2. The highest immune response was obtained with the use of Freund's complete adjuvant; 3. Animals challenged a long time (week 6) after the last immunizing dose showed good anti-**P. brasiliensis** immune response; 4. The particulate antigen induced the lowest immune response. The soluble and the combined antigens were equally efficient in raising good humoral and cellular anti-**P. brasiliensis** immune response.

KEY WORDS: Paracoccidioides brasiliensis antigens; Guinea pigs; Immunization protocols.

INTRODUCTION

Paracoccidioidomycosis is a systemic mycosis caused by **Paracoccidioides brasiliensis** and characterized by granulomatous lesions. The disease is endemic in various Latin American countries, particularly Brazil, Venezuela, Colombia and Argentina[9].

Paracoccidioidomycosis has been reported to affect the immune response, as to cause hyperreactivity of humoral immunity[1] and depression of cellular immunity[10, 12, 16]. Protective immunity mechanisms in populations of endemic areas are not fully known. The existence of paracoccidioidin reactive individuals without clinical evidence of disease suggests that protective immunity against **P. brasiliensis** may occur after exposure to the fungus[9].

(1) Department of Ophthalmology, Faculty of Medicine, UNESP, Botucatu, São Paulo, Brasil.
(2) Department of Immunology, Institute of Biosciences, UNESP, Botucatu, São Paulo, Brasil.
(3) Department of Pathology, Faculty of Medicine, UNESP, Botucatu, São Paulo, Brasil.
Correspondence address: M. Franco, Dept. Patologia, Fac. Medicina, UNESP. 18610 Botucatu, São Paulo, Brasil.

The most common strategy for studying protective immunity to infectious agents has involved the use of animal models. Small animals susceptible to **P. brasiliensis** will remain a valuable tool for vaccine studies if the effects of the immunity induced by immunological manipulations can be identified and the relevant protective epitopes isolated and synthesized.

We recently worked with a guinea pig experimental model of paracoccidioidomycosis, first described by BRITO et al.[2], which frequently causes ocular involvement. Since these lesions can be easily monitored "in vivo" by ophthalmologic examination, the model appears to be useful in studies of the efficacy of protective immunization protocols.

Since the first step in animal vaccination experiments is to standardize an adequate immunization schedule, the objective of this study was to develop an efficient and reproducible protocol of immunization of guinea pigs with **P. brasiliensis** antigens.

MATERIAL AND METHODS

Animals

Healthy, male guinea pigs (**Cavia porcellus**) weighing 250-300 g, obtained from the Animal House of the Botucatu Campus, UNESP, were used in this study.

Fungus

P. brasiliensis strain 18 from the Department of Microbiology and Immunology of the Biomedical Science Institute, USP, cultured in Fava Netto medium at 37°C in 20 x 220 mm tubes, was used to prepare the antigens[5].

Antigens

Particulate antigen: Obtained from a suspension of **P. brasiliensis** yeast cells, cultured as described above. The cell suspension in sterile saline solution (SSE) was centrifuged for 10 min at 1500 rpm, washed 3 times in SSE, resuspended in 2% formalin solution and maintained at 37°C for 48 h. The cells were then washed with SSE 3-5 times, and counted in a Neubauer hemocytometric chamber, adjusting the final concentration to 4 x 10^6 cells/ml. The death of the fungus was proven by culture.

Soluble antigen: Obtained by a previously described technique[14]. Briefly, after the addition of one volume of buffered saline solution (pH 7.2) to one volume of compact yeast-like forms, the fungi were sonicated in an ultrasound sonicator (Thornton) for 15 cycles at 75 A for 3 min in an ice bath. Fungal rupture was evaluated microscopically. The suspension was then centrifuged at 12,000 rpm for 20 min, at 4°C. The supernatant was sterilized by passage through a 0.22 μm Millipore filter and submitted to a sterility test in simple broth, blood agar and simple Sabouraud agar. Protein was measured by the method of LOWRY et al.[8].

Compound antigen: Prepared using equal proportions of the particulate and soluble antigens.

Polysaccharide antigen: Prepared according to FAVA NETTO[5]. This antigen was used for the cutaneous tests.

Evaluation of the immune response

Humoral immunity: Performed by the double immunodiffusion reaction on agar gel (ID) according to a technique previously described[14].

Cellular immunity: Performed by "in vivo" and "in vitro" tests. For the cutaneous test, each animal received intradermally 0.1 ml polysaccharide antigen in the dorsal region. Readings were taken 24 h later, measuring the two largest diameters of induration. A biopsy of the induration papule was performed for histological examination. A method previously described was used for the inhibition of macrophage migration (IMM)[12].

Histological study

Fragments from the cutaneous test sites were fixed in 10% formalin and embedded in paraffin. Sections with 4 μm were stained with hematoxylin and eosin (HE) and examined under a light microscope.

Experimental groups

Animals were divided into 6 groups according to the type of **P. brasiliensis** antigen inoculated, dose, and week of sacrifice to optimize the immunization protocol.

Group A: Six guinea pigs were injected subcutaneously (sc) particulate antigen (2 x 10^6 fungal cells) emulsified in an equal volume of Freund's complete adjuvant, followed one week later by a 1.0 ml sc injection of antigen (4 x 10^6 fungal cells). Immunological evaluation was performed one week later.

Group B: Six animals were submitted to weekly immunization. The 1st and 3rd doses were 0.5 ml particulate antigen and 0.5 ml Freund's complete adjuvant and the 2nd dose was 1.0 ml antigen. The immunological evaluation was performed 1 week after the 3rd dose.

Group C: Ten animals were submitted to the same schedule as the previous group, however, with double the antigen concentration of these. Five (subgroup C1) were submitted to immunologic evaluation one week after the 3rd dose and 5 (subgroup C2) two weeks after the 3rd dose.

Group D: Eight animals were submitted to weekly immunizations. The 1st and 3rd doses were 0.5 ml soluble antigen (2.5 mg/protein) and 0.5 ml Freund's complete adjuvant and the 2nd dose was 1.0 ml antigen (5.0 mg/protein). This group was used for histologic examination of the cutaneous tests 1 week after the 3rd dose.

Group E: Six animals were submitted to one dose weekly, sc, of 0.5 ml soluble antigen, emulsified in an equal volume of Freund's complete adjuvant, for 3 weeks. Two (subgroup E1) and 6 (subgroup E2) weeks after the 3rd dose, the two subgroups were evaluated immunologically.

Group F: Six animals were submitted to one dose per week, sc, of 0.5 ml (1 x 10^6 fungal cells; 1.25 mg/protein) emulsified in an equal volume of Freund's complete adjuvant for 3 weeks. Two weeks and 6 weeks after the 3rd dose, each of two subgroups of 3 animals were evaluated immunologically (F1 and F2).

The different protocols described above are shown in Table 1. Each immunizing dose (1.0 ml) was applied divided into 4 equal parts (0.25 ml) on the ventral side of the root of each leg.

TABLE 1
Immunization protocols.

Group	PbAg*	Protocol		Challenge	
		Dose	FCA**	Day	No. of animals
A	Particulate	1st dose (day 0)	with		
		2nd dose (day 7)	without	14	6
B	Particulate	1st dose (day 0)	with		
		2nd dose (day 7)	without		
		3rd dose (day 14)	with	21	6
C	Particulate	1st dose (day 0)	with		
		2nd dose (day 7)	without		
		3rd dose (day 14)	with	21	5 (Group C1)
				28	5 (Group C2)
D	Soluble	1st dose (day 0)	with		
		2nd dose (day 7)	without		
		3rd dose (day 14)	with	21	8
E	Soluble	1st dose (day 0)	with		
		2nd dose (day 7)	with		
		3rd dose (day 14)	with	28	3 (Group E1)
				56	3 (Group E2)
F	Combined	1st dose (day 0)	with		
		2nd dose (day 7)	with		
		3rd dose (day 14)	with	28	3 (Group F2)
				56	3 (Group 2)

* PbAg = **P. brasiliensis** antigen
** FCA = Freund's complete adjuvant

RESULTS

Humoral and cellular immunity — Table 2 presents an evaluation of the humoral and cellular immune response of the different groups. The main results observed were:

Group A (particulate Ag, 2 doses): Only one animal presented a positive cutaneous test. None of the animals showed a humoral immune response.

Group B (particulate Ag, 3 doses): None of the animals developed a humoral or a cellular immune response.

Group C (concentrated particulate Ag, 3 doses): None of the animals sacrificed one week after the 3rd immunizing dose presented a positive immunologic response (Group C1). Two of the 5 animals challenged 2 weeks after the 3rd dose were skin-test reactive (Group C2).

Group E (Soluble Ag, 3 doses): All of the animals challenged 2 weeks after the last immunizing dose (Group E1) showed the presence of antibodies. Positive skin test was detected in 2 animals of these animals. A more intense cellular immune response and reduced antibody levels were observed in animals challenged 6 weeks after the 3rd dose (Group E2).

Group F (compound Ag, 3 doses): The humoral immune response was positive in all animals and the cellular immune response was positive in only one animal challenged 2 weeks after the last dose of the antigen. However, the humoral and cellular immune responses were positive in all of the animals challenged after 6 weeks.

Histology

Cutaneous test: Immunization with particulate antigen induced paracoccidioidin characterized by mild to moderate (1 −2 +) edema and an inflammatory infiltrate composed of lymphocytes, monocytes and macrophages. The number of neutrophils was variable, but, never greater than the number of monocytes. The response was most intense with three antigen doses.

TABLE 2
Humoral and cellular immune response of guinea pigs immunized with **P. brasiliensis** antigens.

Group	Antigen	No of immunizing doses	Time between last dose and challenge	Test (medians)		
				CT	MMI	ID
A	Part	2	1	Neg (16%)	Neg (0%)	Neg (0%)
B	Part	3	1	Neg (0%)	Neg (0%)	Neg (0%)
C1	Part	3	1	Neg (0%)	Neg (0%)	Neg (0%)
C2	Part	3	2	Neg (40%)	ND*	ND*
E1	Sol	3	2	6 (66%)	12 (33%)	16 (100%)
E2	Sol	3	6	10 (100%)	40 (66%)	4 (100%)
F1	Comb	3	2	Neg (33%)	13 (0%)	16 (100%)
F2	Comb	3	6	9 (100%)	33 (100%)	4 (100%)

ND — Not done; CT = cutaneous test; MMI = macrophage migration inhibition test; ID = immunodiffusion; Part = particulate; S = soluble; Comb. = combined.

Inflammation was more intense (3 −4 +) and neutrophilic in the animals immunized with soluble Ag. The inflammatory cellular infiltration was diffuse and/or perivascular and located mainly in the papillary dermis. It was frequently found deep in the subcutaneous and muscular layers.

TABLE 3
Intensity of the histological inflammation at the skin test sites (0 −4 + scale) of guinea pigs immunized with **P. brasiliensis** antigens.

Group		Inflammation (median)		
Antigen	No. of immunizing doses	Edema	Mononuclear cells	Total index
Particulate	2	1 +	2 +	3 +
Particulate	3	2 +	2 +	4 +
Particulate	3	2 +	2 +	4 +
Soluble	3	2 +	2 +	4 +
Soluble	3	2 +	3 +	5 +
Combined	3	2 +	2 +	4 +

DISCUSSION

The objective of the present work was to standardize an efficient and reproducible protocol for the immunization of guinea pigs with **P. brasiliensis** antigens. We tested three different antigens and six protocols.

In animals immunized with a suspension of dead fungi (particulate antigen), a dissociation between the gross and the histological reading of the cutaneous tests was observed. Grossly the tests were negative, whereas histological examination showed edema and inflammatory cellular infiltration. The histological alterations were more marked in guinea pigs immunized with three doses. The results are in agreement with data in the literature which report dissociation between gross and histological readings of cutaneous tests with **P. brasiliensis** antigens, both in man[1] and in animals[4].

The soluble and compound antigens induced a better humoral and cellular responses both macro- and microscopically. To explain this result, one can reason as TUESTA[17] who affirmed that the more immunogenic fractions of pathogenic fungi are located in the cytoplasm of the yeast-like forms, being released with the breaking down of phospholipid and polysaccharide components of the cell wall.

The immunization schedule which induced the best indices of cellular immune response in the animals was three doses of soluble antigen emulsified in Freund's complete adjuvant, with challenge 2 or 6 weeks after the last immunizing dose.

Several critical points for obtaining a good immunizing protocol ought to be cited. One of these points is the time between the challenge and the last immunizing dose. Guinea pigs challenged 1 week after the last dose presented weak cellular and humoral immune responses, which became more intense after 2 or more weeks from the last dose. Another important point is the use of Freund's complete adjuvant. Pilot experiments (data not shown) showed that the use of antigen without adjuvant always induced less intense immune responses.

Time of paracoccidioidin reading was an important aspect in standardizing the protocol. Pilot experiments with readings at 24 and 48 h showed better results after 24 h, as also reported in human[5] and animal paracoccidioidomycosis (MF Franco, personal communication), as well as in other deep mycosis[7, 13].

It is important to note that the protocols tested, especially those using soluble antigen, showed agreement between the results of the two cellular immune response tests: animals reactive to the cutaneous test were also positive for the "in vitro" test of inhibition of macrophage migration.

These tests may show a positive correlation or may be dissociated both in man and in experimental animals[4, 11]. There are multiple variables which influence this type of phenomenon, such as immunization route, type and dose of antigen, animal, time of challenge after immunization, and function of different lymphocyte subpopulations involved in the tests[15].

However, under the experimental conditions of the present study, particularly in the protocols using the soluble antigen, stimulation of lymphocyte subsets occurred in both tests of immune cellular response evaluation.

The immunization protocols using soluble and compound antigens were equally efficient in the induction of the anti-**P. brasiliensis** humoral imune response, inducing specific antibody response of moderate intensity.

As the protocol with the soluble antigen showed good results and the procedure is practical and easily performed we have already tested it in a vaccination experiment against **P. brasiliensis** infection. This immunization protocol was effective in protecting the ocular lesions of guinea pigs infected by the intracardiac route with yeast forms of a virulent isolate of **P. brasiliensis**[6].

RESUMO

Avaliação de diferentes protocolos de imunizaçao em cobaias utilizando antígenos de P. brasiliensis.

O objetivo deste trabalho foi desenvolver protocolo eficiente e reprodutível de imunização em cobaias com antígenos de **P. brasiliensis**, visando a obtenção de modelo experimental para futuros estudos de mecanismos de proteção imunológica.

Testaram-se três diferentes antígenos (particulado, solúvel e composto) e seis protocolos nos quais foram avaliadas as influências dos seguintes fatores: presença ou ausência de adjuvante completo de Freund, número de doses imunizantes e intervalo de tempo entre a última dose imunizante e o desafio.

A eficiência do protocolo de imunização foi estudada pela avaliação da resposta imune celular e humoral anti-**P. brasiliensis**, utilizando teste cutâneo e teste de inibição da migração do macrófago, e imunodifusão, respectivamente.

Observou-se que: 1. Três doses imunizantes de antígeno induziram melhor resposta do que duas doses; 2. Maior resposta imune foi conseguida com a utilização de adjuvante completo de Freund; 3. Animais desafiados depois de longo tempo (6 semanas) da última dose imunizante mostraram melhor resposta imune anti-**P. brasiliensis**; 4. Os antígenos solúvel e composto foram igualmente eficientes induzindo maior resposta imune humoral e celular anti-**P. brasiliensis** enquanto que o antígeno particulado provocou menor reatividade.

REFERENCES

1. BIAGIONI, L.; SOUZA, M. J.; CHAMMA, L. G.; MENDES, R. P.; MARQUES, S. A.; MOTA, N. G. S. & FRANCO, M. — Serology of paracoccidioidomycosis. II. Correlation between class-specific antibodies and clinical forms of the disease. **Trans. roy. Soc. trop. Med. Hyg., 78**: 617-621, 1984.

2. BRITO, T. & FAVA NETTO, C. — Disseminated blastomycosis of the guinea pig. A pathologic and immunologic study. **Path. et Microbiol. (Basel), 26**: 28-43, 1963.

3. CARVALHO, T. P.; VIERO, R. M. & FRANCO, M. F. — Paracoccidioidina com antígeno polissacarídico de **P. brasiliensis** em pacientes com paracoccidioidomicose; leitura macro e microscópica e correlação clínica. In: ENCONTRO SOBRE PARACOCCIDIOIDOMICOSE, 1, Barra Bonita, 1979. **Anais**. Barra Bonita, Faculdade de Medicina, Universidade Estadual Paulista, 1979. p. 19.

4. DEFAVERI, J.; REZKALLAH-IWASSO, M. T. & FRANCO, M. F. — Experimental pulmonary paracoccidioidomycosis in mice: morphology and correlation of lesions with humoral and cellular immune response. **Mycopathologia (Den Haag), 77**: 3-11, 1982.

5. FAVA NETTO, C. — Estudos quantitativos sobre a fixação do complemento na blastomicose sul-americana, com antígenos polissacarídicos. **Arq. Cirurg. clin. exp., 18**: 197-254, 1955.

6. KAMEGASAWA, A.; VIERO, R. M.; REZKALLAH-IWASSO, M. T. & FRANCO, M. F. — Protective effect of prior immunization on ocular paracoccidioidomycosis in guinea -pigs. **Mycopathologia (Den Haag), 103**: 35-42, 1988.

7. KONG, Y. M.; SAVAGE, D. C. & KONG, L. N. L. — Delayed dermal hypersensitivity in mice to spherule and mycelial extracts of **Coccidioides immitis. J. Bact., 91**: 867-883, 1966.

8. LOWRY, O. H.; ROSEMBOURG, N. J.; FARR, A. L. & RANDALL, R. J. — Protein measurement with the Folin phenol reagent. **J. biol. Chem., 193**: 265-275, 1951.

9. MARQUES, S. A.; FRANCO, M. F.; MENDES, R. P.; SILVA, N. C. A.; BACCILI, C.; CURCELLI, G. D.; FERACINI, A. C. M.; OLIVEIRA, C. S.; TAGLIARINI, J. V. & DILLON, N. L — Aspectos epidemiológicos da paracoccidioidomicose na área endêmica de Botucatu (São Paulo — Brasil). **Rev. Inst. Med. trop. S. Paulo, 25**: 87-92, 1983.

10. MENDES, E. & RAPHAEL, A. — Impaired delayed hypersensitivity in patients with South American Blastomycosis. **J. Allergy, 47**: 17-22, 1971.

11. MOTA, N. G. S.; REZKALLAH-IWASSO, M. T.; PERAÇOLI, M. T. S.; AUDI, R. G.; MENDES, R. P.; MARCONDES, J.; MARQUES, S. A.; DILLON, N. L. & FRANCO, M. F. — Correlation between cell-mediated immunity and clinical forms of paracoccidioidomycosis. **Trans. roy. Soc. trop. Med. Hyg., 79**: 765-772, 1985.

12. MUSATTI, C. C.; REZKALLAH, M. T.; MENDES, E. & MENDES, N. F. — In vivo and in vitro evaluation of cell — mediated immunity in patients with paracoccidioidomycosis. **Cell. Immunol., 24**: 365-378, 1976.

13. PAPPAGIANIS, D.; HECTOR, R.; LEVINE, H. B. & COLLINS, M. S. — Immunization of mice against coccidioidomycosis with a subcellular vaccine. **Infect. Immun., 25**: 440-445, 1979.

14. PERAÇOLI, M. T. S.; MOTA, N. G. S. & MONTENEGRO, M. R. — Experimental paracoccidioidomycosis in the Syrian hamster: morphology and cell-mediated immunity. **Mycopathologia (Den Haag), 79**: 7-17, 1982.

15. PRESTES, F. R. C.; REZKALLAH-IWASSO, M. T. & FRANCO, M. F. — Contribuição ao estudo da imunidade celular ao **Paracoccidioides brasiliensis** no camundongo. **Rev. Ciênc. bioméd.** (S. Paulo), **4**: 77-84, 1983.

16. RESTREPO, A.; RESTREPO, M.; RESTREPO, F.; ARISTIZABAL, L. H.; MONCADA, L. H. & VELEZ, H. — Immune responses in paracoccidioidomycosis. A controlled study of 16 patients before and after treatment. **Sabouraudia, 16**: 151-163, 1978.

17. TUESTA, W. E. G. — Relaciones biológicas entre **Paracoccidioides, Blastomyces** e **Histoplasma. An. Fac. Med.** (Lima), **49**: 80-108, 1966.

Recebido para publicação em 8/11/1991.
Aceito para publicação em 22/4/1992.