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IMMUNIZATION WITH *Paracoccidioides brasiliensis* RADIOATTENUATED YEAST CELLS INDUCES TH1 IMMUNE RESPONSE IN BALB/C MICE

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ABSTRACT

Paracoccidioides brasiliensis is the agent of paracoccidioidomycosis, the most prevalent mycosis in Latin America. To date, there is no effective vaccine. In our laboratory yeast cells of *P. brasiliensis* were attenuated by gamma irradiation. We defined an absorbed dose in which the pathogen loses the reproductive ability, while retaining the morphology, the synthesis and secretion of proteins and the oxidative metabolism. The immunization with these cells was able to confer protection in BALB/c mice. The aim of the present work was evaluate the immune response pathway activated in mice immunized with *P. brasiliensis* radioattenuated yeast cells. The protector effect was evaluated in BALB/c mice groups immunized once or twice, respectively. Each group was divided in three sub groups that were challenge 30, 45 or 60 days after the immunization. These groups were called G1A, G1B and G1C in the group immunized once and G2A, G2B and G2C in the group immunized twice. Recovery of CFUs and cytokines determination (IFN - γ, IL - 10 and IL – 4) were performed three months post challenge. Quantitative RT-PCR was the method of choice used to quantify the expression of cytokines. The sera were collected weekly to evaluate the IgG antibody titers and the IgG1 and IgG2a pattern in the course of infection. A significant reduction in CFUs recovery was verified 90 days post challenge in mice submitted to one immunization: 73.0%, 96.0% and 76.3% for sub-groups G1A, G1B and G1C, respectively. In the group submitted to two immunizations, a remarkable increase in the protection was obtained. No CFUs was recovered from sub-groups G2B and G2C and very few CFUs (reduction of 98.6%) were recovered from the lungs of sub group G2A. In mice submitted to one immunization, Th1 and Th2 cytokines were simultaneously produced. In the group submitted to two immunizations, levels of IL-10 and IL-4 were very low, while IFN-γ production was maintained indicating that a Th1 pattern was dominant. For all sub-groups in group submitted to

one immunization significant difference between the levels of isotypes IgG1 and IgG2a was not observed. In contrast, IgG2a predominance in relation to IgG1 was verified in all sub-groups of the animals submitted to two immunizations. Altogether, the results of CFU recovery associated with those obtained from the cytokines and the IgG1 and IgG2a analysis suggested that mice submitted to one immunization developed a mixed Th1/Th2 response, which was less efficient in the infection control while a trend to a Th1 pattern was obtained with the use of two immunizations, promoting optimal elimination of *P. brasiliensis* yeast cells from mice tissues
Key Words: *Paracoccidioides brasiliensis*, vaccine, gamma irradiation, immunization

1. INTRODUCTION

Paracoccidioidomycosis (PCM) is an infectious granulomatous disease caused by the thermally dimorphic fungus *Paracoccidioides brasiliensis*. PCM constitutes the most prevalent deep mycosis in Latin America with extensive endemic areas in Brazil, Colombia, Venezuela and Argentina [1]. It is estimated that approximately 10 million people are infected, although most of them do not show clinical symptoms [2]. The disease is supposed to be acquired via the respiratory tract, primarily involving the lungs and then disseminating to other organs and systems. In the absence of drug therapy, the disease is usually fatal [3]. Up to the moment, no vaccine has been reported for *P. brasiliensis* or to any important pathogenic fungi infection in humans [4].
The potential of irradiation as a tool for creating highly effective attenuated vaccines has been recognized since the 50s. Irradiated pathogens frequently lost their virulence, but retained metabolic activity and morphology, and consequently are able to induce a high level of immunity. Radioattenuated live vaccines can expose their antigens sequentially to the host, as in a natural infection allowing the immune system to recognize them as viable agents without the risk of a progressive infection. Effective immunization of laboratory animals has been achieved with various irradiated protozoan parasites, helminths or nematodes [5].

In our laboratory, this approach was used for attenuation of yeast cells of *P. brasiliensis*. Yeast cells attenuated by gamma irradiation lose its reproductive ability, while retaining the oxidative metabolism, the synthesis and secretion of proteins, and the expression of antigens present in non-irradiated yeasts [6]. The gamma irradiation treatment did not disrupt the yeast morphology, although alterations in the nucleus and an extensive DNA fragmentation were observed [7]. The immunization of BALB/c mice with the radioattenuated yeast cells was able to confer a potent and durable protection against challenge with highly infective yeasts forms of *P. brasiliensis* [8], showing the potential of this approach for the development of live attenuated vaccines against fungi infections. The aim of the present work was evaluate the immune response pathway activated in mice immunized with *P. brasiliensis* radioattenuated yeast cells.

2. MATERIAL AND METHODS

2.1. Animals and *P. brasiliensis* strain

Male BALB/c mice 4-6 weeks old were purchased from Centro de Bioterismo, ICB-UFMG (Belo Horizonte, MG, Brazil) and maintained under standard laboratory care as previously described [9]. *P. brasiliensis,* strain *Pb* 18, was maintained in yeast-form, at 35^{o}C, in brain infusion agar medium (BHIA) supplemented with 1% glucose. The yeast cells were subcultured every 10 days. This work was approved by the Ethics Committee in Animal

Experimentation from Universidade Federal de Minas Gerais (CETEA/UFMG), under protocol number 132/2006.

2.2. Gamma irradiation.

Cultures of *P. brasiliensis*, in solid medium, were irradiated in the presence of oxygen and at room temperature. The irradiation was performed in a uniform source of ^{60}Co gamma rays at dose rate of 950 Gy h^{-1}. A dose of 6.5 kGy was used to achieve attenuation [6].

2.3. Mice immunization and challenge.

BALB/c mice were immunized through the ocular plexus with the injection of 10^5 radioattenuated yeast cells in PBS, without adjuvant. The challenges were performed by the same route of immunization using 10^5 viable yeast forms of virulent *P. brasiliensis* (strain Pb 18).

2.4. Protection assay.

Animals were divided into two groups: in group 1, BALB/c mice were immunized once and mice in group 2 were immunized twice with an interval of two weeks. For each group, mice were divided in three sub-groups (n = 3) that were challenged 30, 45 or 60 days after the second immunization. These sub-groups were denominated G1A, G1B, and G1C in group 1, and G2A, G2B and G2C in group 2, respectively. Organ colony-forming units (CFUs) were determined 90 days post challenge for lung, spleen and liver. The organs were removed, weighed, homogenized and washed three times in PBS by centrifugation. The final suspensions in PBS were plated on brain infusion agar medium (BHIA) supplemented with 4% fetal calf serum and 5% spent culture medium of *P. brasiliensis* as growth factor. Gentamicin and cycloheximide were added at 40 and 500 mg/L, respectively. The plates were incubated at 35^{o}C and counted after 20 days. The results were expressed as the number of *P. brasiliensis* CFUs per gram of tissue per mouse in each experimental group. Parts of the removed organs were also used for histopathological analysis. Non immunized controls were inoculated with viable virulent cells as used for the challenge of immunized animals.

2.5. Cytokine detection by real time PCR

Real-time PCR assays were performed to specifically quantify mouse IL-4, IL-10 and IFN - γ, transcripts. Briefly, lungs were excised from infected or immunized mice, mixed with Trizol LS Reagent (Invitrogen Life Technologies) and frozen in liquid nitrogen. Total RNA was extracted according to the manufacturer's instructions. Isolated RNA was incubated with 10 U of DNAse (RNAse free) (Promega) for 30 min at 37°C. The samples were then heat inactivated at 70°C for 10 min, chilled and reverse transcribed with SuperScript II (Invitrogen Life Techologies). Real-time quantitative PCR was carried out with 10 µl of SYBR green PCR master mix (Applied Biosystems), 4.0 µl of cDNA, and primers at a final concentration of 5 pmol, in a final volume of 20 µl. Cytokines and beta-actin specific primers were as in Giulietti et al [10]. Samples were first submitted to the temperatures of 50^{o}C for 2 min and 60^{o}C for 1 min and then subjected to 45 cycles of amplification (95°C for 15 s followed by 60°C for 1.0 min) using an ABI PRISM 7900 apparatus (Applied Biosystems). Dissociation curves occurred at 95^{o}C for 15 s and 60^{o}C for 15 s. All quantifications were normalized to the

housekeeping gene beta-actin. A non template control with no genetic material was included to eliminate contamination or non-specific reactions.

2.6. IgG1 and IgG2a analysis

ELISA was performed using Maxisorp^{TM} surface plates coated with 0.5 μg per well of Mexo antigens [18] and blocked with PBS containing 1.6% casein. Sera from immunized or infected mice were tested by serial dilution starting at 1:100 and quantified with a IgG1 and IgG2a specific sub-class IgG-peroxidase conjugate (Sigma). The samples were analyzed 90 days post challenge. The results were recorded as OD_{490} readings taken in an automated ELISA reader.

2.7. Statistics

Data were analyzed statistically by one way ANOVA followed by the Bonferroni test or Student t-test associated when necessary to the non-parametric Mann-Whitney test with the level of significance set at $p< 0.05$.

3. RESULTS

A significant reduction in CFUs recovery was verified 90 days post challenge in mice submitted to one immunization (Group 1): 73.0%, 96.0% and 76.3% for sub-groups G1A, G1B and G1C, respectively (Figure 1A).
In the group submitted to two immunizations (Group 2), a remarkable increase in the protection was obtained. No CFUs was recovered from sub-groups G2B and G2C and very few CFUs (reduction of 98.6%) were recovered from the lungs of sub group G2A (Figure 1B).

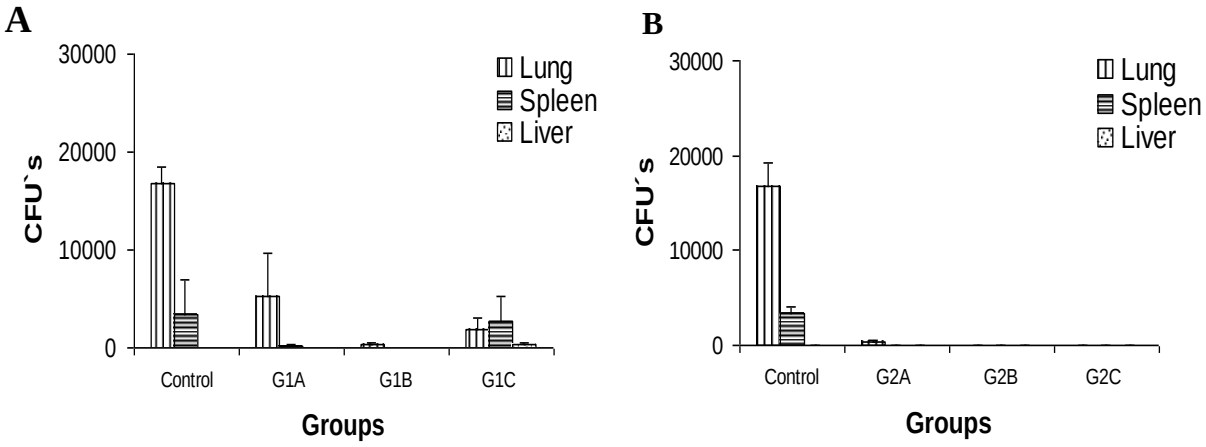

Figure 1 – CFU recovery from immunized mice organs 90 days post challenge. (A) Group immunized one time. Control – infected with the virulent yeast; G1A- sub-group challenged 30 days post immunization; G1B- sub-group challenged 45 days post immunization; G1C – sub-group challenged 60 days post immunization. (B) Group immunized two times. Control – infected with the virulent yeast; G2A- sub-group challenged 30 days post immunization; G2B- sub-group challenged 45 days post immunization; G2C – sub-group challenged 60 days post immunization. Data are reported as mean $\pm$ S.D. of CFU in each experimental group (n=3). The decrease in CFU recovery was significant for all sub-groups ($p<0.05$), except for the spleen in the sub group GIC.

The immunoglobulin isotypes IgG1 and IgG2a were determined 90 days post challenge (Figure 4). For all sub-groups in Group1 a significant difference between the levels of both isotypes was not observed. In contrast, IgG2a predominance in relation to IgG1 was verified in all sub-groups of Group 2.

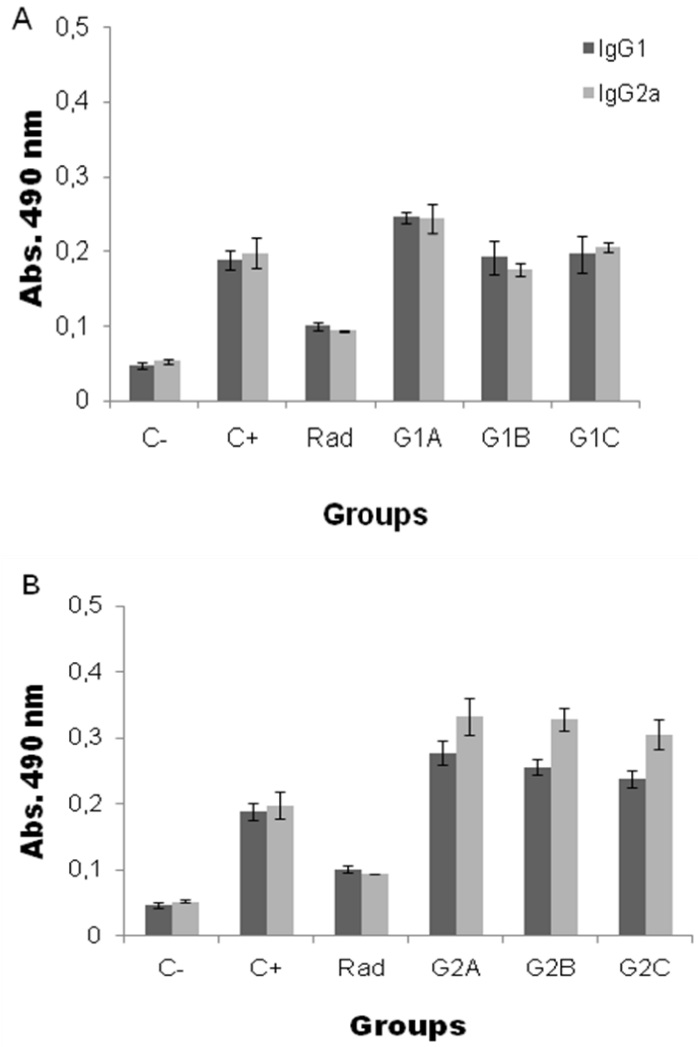

Figure 4 - Determination of immunoglobulin isotypes IgG1 and IgG2a. A) Group submitted to one immunization. C^- - not infected; C^+ - infected with the virulent yeast; Rad – inoculated with the radioattenuated yeast; G1A- sub-group challenged 30 days post immunization, G1B- sub-group challenged 45 days post immunization; G1C – sub-group challenged 60 days post immunization. B) Group submitted to two immunizations. C^- - not infected; C^+ - infected with the virulent yeast; Rad – inoculated with the radioattenuated yeast; G2A- sub-group challenged 30 days post immunization; G2B- sub-group challenged 45 days post immunization; G2C – sub-group challenged 60 days post immunization.

Cytokine analysis was accomplished by real time PCR 90 days post challenge in the lungs of mice of Groups 1 and 2 (Figure 5). The Th1 related cytokine IFN - γ, and the Th2 related cytokines IL-10 and IL-4 were analyzed. In mice submitted to one immunization, Th1 and Th2 cytokines were simultaneously produced. In the group submitted to two immunizations, levels of IL-10 and IL-4 were very low, while IFN - γ, production was maintained indicating that a Th1 pattern was dominant.

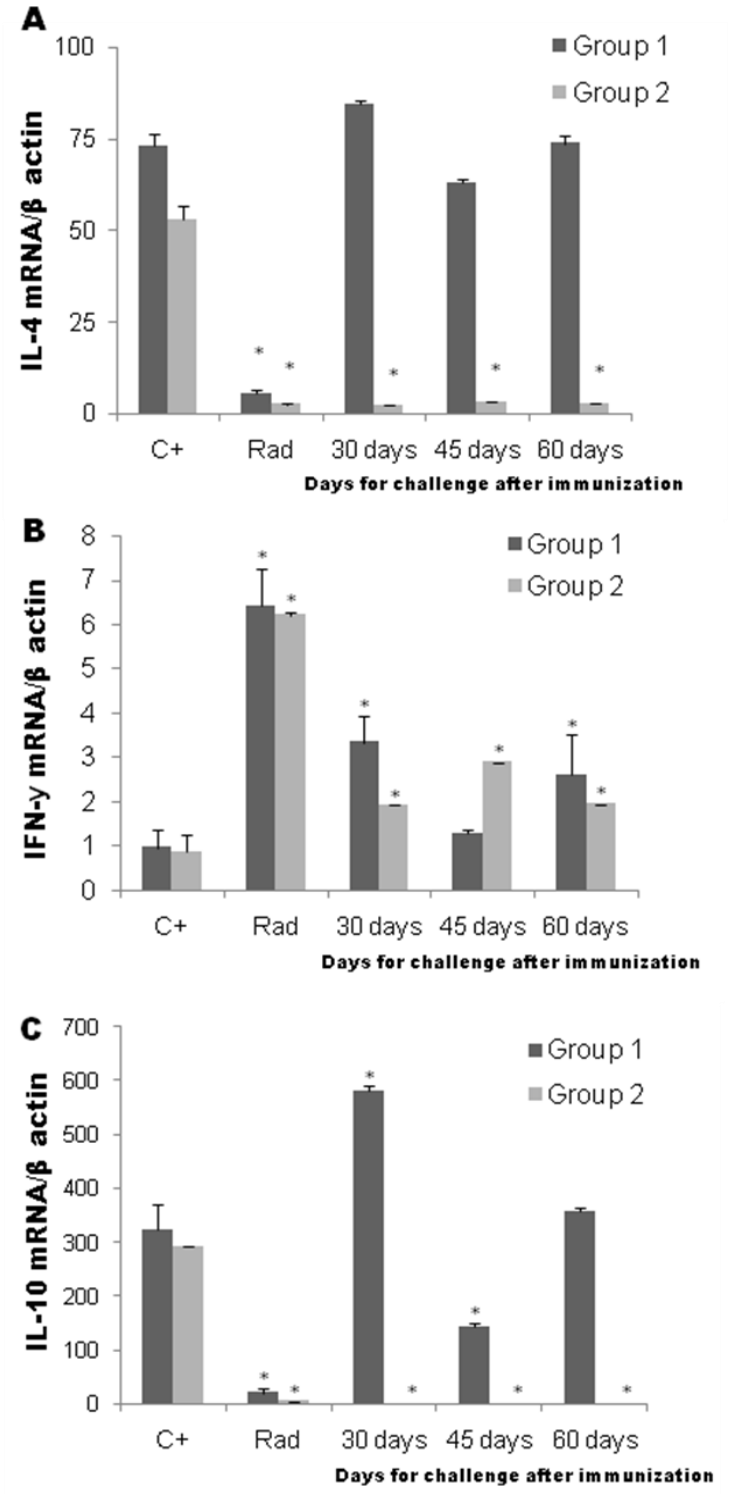

Figure 5- Cytokine profiles in mice submitted to one or two immunizations. The relative expression of the cytokines IL-4 (A), IFN - γ (B), IL-10 (C) was measured in the lung cells by real time PCR, 90 days post challenge. C^+ - infected with the virulent yeast, Rad – inoculated with the radioattenuated yeast. Group 1 – submitted to one immunization, Group 2 – submitted to two immunizations. (*) $p<0.05$ in relation to C+.

4. CONCLUSION

The results of CFU recovery associated with those obtained from the cytokines and the IgG1 and IgG2a analysis suggested that mice submitted to one immunization developed a mixed Th1/Th2 response, which was less efficient in the infection control while a trend to a Th1 pattern was obtained with the use of two immunizations, promoting a long lasting protection able to eliminate the fungal cells from tissues.

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