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ScienceDirect
Vaccine 25 (2007) 7893–7899
Vaccine
www.elsevier.com/locate/vaccine

Immunization with radioattenuated yeast cells of *Paracoccidioides brasiliensis* induces a long lasting protection in BALB/c mice

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Received 4 July 2007; received in revised form 27 August 2007; accepted 3 September 2007
Available online 21 September 2007

Abstract

Paracoccidioides brasiliensis is the fungus agent of paracoccidioidomycosis, a chronic systemic disease prevalent in Latin America. The aim of the present work was to evaluate the protection elicited by the immunization of BALB/c mice with radioattenuated yeast cells of *P. brasiliensis*. The immunization promoted a long lasting protection against highly infective yeast forms of *P. brasiliensis*. A 99.5% decrease in CFUs recovery was verified 90 days post challenge. At the same time the levels of IgG2a and IFN-γ were high while a very low production of IL-10 and IL-5 was verified, suggesting that a Th1 pattern was dominant. This work shows the potential of radioattenuated yeast cells for the development of vaccines against fungi infections.

Keywords: *Paracoccidioides brasiliensis*; Vaccine; Gamma irradiation

1. Introduction

Paracoccidioides brasiliensis is a thermally dimorphic fungus which causes the paracoccidioidomycosis (PCM), the prevalent deep mycosis in Latin America. The disease is widespread from Central America to Argentina [1] and is estimated that approximately 10 million people are infected, although the most of them do not show clinical symptoms [2]. Infection starts by inhalation of fungal propagules which reach the pulmonary alveolar epithelium, where they transform to the pathogenic yeast form at 37 °C. The infection can give rise to either an asymptomatic condition or to active disease. It initially causes lesions in the lungs but subsequently can disseminate to other organs and tissues [3].

Experimental and clinical evidences indicate that cellular rather than humoral immunity is the effective host defense mechanism that controls pathogenesis and evolution of PCM. The clinical manifestations depend upon the extent of cell immunity suppression [4]. The severe forms present strong T-cell disjunction, impaired delayed-type hypersensitivity, alterations in T-cell sub population's ratios and unbalanced level of cytokines production [5]. Furthermore, severe forms of PCM are associated with high levels of specific antibodies and hypergammaglobulinemia, which fails to protect against the disease [6].

The potential of irradiation as a tool for creating highly effective attenuated vaccines has been recognized since the 1950s. Gamma and X irradiation have consistently proved successful as attenuating agents for a remarkably wide range of helminthes, nematodes and protozoa's species. Irradiated pathogens frequently lose their virulence, but retain the

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doi:10.1016/j.vaccine.2007.09.011

metabolic activities and morphology, and consequently are able to induce a high level of immunity during its abbreviated lifespan in the host. However, they die before reaching the stages associated with pathogenicity. In some cases, the radioattenuated pathogens are more immunogenic than the normal counterparts [7].

Currently, there are no approved vaccines for the prevention or treatment of medically important fungi infections in humans [8]. Only one prophylactic fungal vaccine has been tested in humans and its immunogen consisted of formalin-inactivated spherules of *Coccidioides immitis*. However, the vaccine was unable to confer protection in a randomized trial [9]. There are only few reports describing the role of antigens on induction of immune responses for *P. brasiliensis*. The major diagnostic antigen gp43, or peptides derived from it, have shown to be a promise as potential protective antigens [10–12], and *P. brasiliensis* antigens fractionated by anion exchange chromatography, termed F0 and FII, have also been demonstrated to confer protection in mice [13].

The absence of fungal vaccines determines the need to search for new alternatives and the use of radioattenuated fungi for vaccine development was never explored. We obtained radioattenuated yeast cells of *P. brasiliensis*, which lose the reproductive ability, while retaining the morphology, the synthesis and secretion of proteins, the oxidative metabolism and the expression of the antigens present in the native yeast [14,15]. In the present study, the protective effect, cytokines profile, IgG dynamics and IgG isotypes production, elicited by the immunization with the radioattenuated yeast cells of *P. brasiliensis*, were evaluated in experimental PCM.

2. Material and methods

2.1. Animals

This work was approved by the Ethics Committee in Animal Experimentation from Universidade Federal de Minas Gerais (CETEA/UFMG), protocol number 132/2006. Adult male BALB/c mice 6–8-weeks-old were purchase from Centro de Bioterismo, ICB-UFMG (Belo Horizonte, MG, Brazil) and maintained under standard laboratory care as previously described [16].

2.2. P. brasiliensis strain

P. brasiliensis, strain Pb 18, was maintained in the yeast form, at 35 °C, in brain infusion agar medium (BHIA) supplemented with 1% glucose. The yeast cells were subcultured every 10 days.

2.3. Gamma irradiation of P. brasiliensis

Cultures of *P. brasiliensis*, in solid medium, were irradiated in the presence of oxygen and at room temperature. The irradiation was performed with a uniform source of ^{60}Co gamma rays. A dose of 6.5 kGy at a dose rate of 950 Gy h^{-1} was used [14].

2.4. Mice immunization and challenge

BALB/c mice were immunized twice, at 2 weeks intervals, by the ocular plexus with the injection of 10^5 radioattenuated yeast cells in PBS, without adjuvant. The challenges were performed by the same rout of immunization using 10^5 viable yeast forms of virulent *P. brasiliensis* (strain Pb 18).

2.5. Fungal recovery in lung, spleen and liver

Animals were divided in three groups: Group 1 were BALB/c mice infected with the virulent strain, Group 2 were inoculated with the radioattenuated yeast cells, Group 3 were BALB/c mice immunized with the radioattenuated yeast and challenged with highly infective yeast forms of *P. brasiliensis* (the same used in the Group 1). The mice of Group 3 were divided in three sub groups that were challenged 30, 45 and 60 days after the last immunization. These groups were called 3A, 3B, and 3C, respectively. For all groups, organ colony-forming units (CFUs) were determined in the lung, spleen, and liver, 30 and 90 days after the challenge or infection. The organs were removed, weighed, homogenized and washed three times in PBS by centrifugation. The final suspensions in PBS were plated on brain heart infusion (BHI) agar supplemented with 4% fetal calf serum and 5% spent culture medium of *P. brasiliensis* as a growth factor [17]. Gentamicin was added at 40 mg/l. The plates were incubated at 35 °C and read after 20 days. The results were expressed as the number of *P. brasiliensis* CFUs per gram of tissue per mouse in each experimental group ($n = 3$). Parts of the organs were kept for histopathological analysis.

2.6. Antibody response to the radioattenuated yeast infection or immunization

Antibody responses were determined weekly after inoculation by the ocular plexus. ELISA was performed using Maxisorp™ surface plates coated with 0.5 μg per well of Mexo antigens [18] and blocked with 0.15 M PBS containing 1.6% casein. Sera from immunized or infected mice were tested by serial dilution starting at 1:100 and quantified with an anti-mouse IgG-peroxidase conjugate. For IgG1 and IgG2a isotype determination a specific sub class IgG-peroxidase conjugate (Sigma) was used. The samples were analyzed 30 and 90 days post challenge. The results were recorded as OD_{492} readings taken in an automated ELISA reader.

2.7. Cytokine detection by real-time PCR

Real-time PCR assays were performed to specifically quantify mouse IL-10, IL-5, TNF-α, and IFN-γ transcripts.

Briefly, lungs were excised from infected or immunized mice at specific times, mixed with Trizol LS Reagent (Invitrogen Life Technologies) and frozen in liquid nitrogen. Total RNA was extracted according to the manufacturer's directions. 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. [19]. Samples were first submitted to the temperatures of 50 °C for 2 min and 60 °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 PRIM 7900 apparatus (Applied Biosystems). Dissociation curves occurred at 95 °C for 15 s and 60 °C for 15 s. All quantification's were normalized to the housekeeping gene beta-actin. A nontemplate control with no genetic material was included to eliminate contamination or nonspecific reactions.

2.8. Histopathology of lung, spleen, and liver of experimental groups

BALB/c mice were sacrificed 1 and 3 months after the challenge or infection. The lung, spleen, and liver were excised, fixed in 10% buffered formalin, and embedded in paraffin for sectioning. The sections were stained with hematoxylin–eosin and examined microscopically.

2.9. 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

The protection assay showed that a significant reduction in the CFUs recovery took place in the immunized mice group (Fig. 1). Thirty days after the challenge (Fig. 1A), a reduction of 100% was verified for the sub group 3A, 92% for the sub group 3B and 51% for the sub group 3C. A remarkable decrease in the organs CFUs was verified 90 days after the challenge (Fig. 1B) since no CFU could be recovered from sub groups 3B and 3C and very few CFUs (98% reduction) were recovered from the lungs of sub group 3A. After 90 days the CFU recovery reduction was significant for all sub groups ($p < 0.05$) and the overall decrease was of 99.5%, considering the three sub groups. The histopathological analysis confirmed these results showing the tissues free from yeast, granulomas or inflammation in three sub groups (Fig. 2).

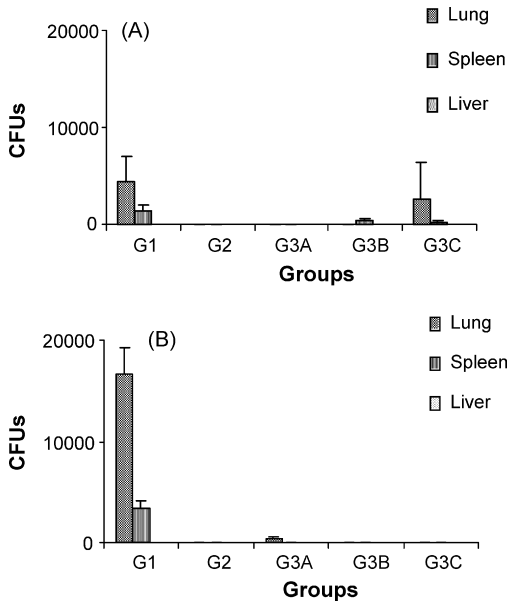

Fig. 1. Fungal recovery in lung, spleen and liver. The CFUs were estimated 30 days (A) and 90 days (B) post infections or challenge. G1, group infected with the virulent yeast; G2, group inoculated with the radioattenuated yeast; G3A, group challenged 30 days post immunization; G3B, group challenged 45 days post immunization; G3C, group challenged 60 days post immunization. Data are reported as mean ± 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 in the lungs of the sub group G3C, 30 days after the challenge.

This high level of protection verified after a prolonged time of exposition to the fungi (90 days), is an important aspect of the protection against PCM, due to the *P. brasiliensis* ability to stay dormant for long periods before initiating an infection. It is not uncommon for patients to fall ill more than a decade after leaving an endemic region [20]. These results pointed out that a long lasting protection able to eliminate the fungi of the organism was elicited.

The results also confirmed that the radioattenuated yeast lost the virulence. In the group infected with the virulent strain (Group 1), CFUs were recovered from all organs but no CFU could be recovered (30 or 90 days after the infection) from mice infected with the radioattenuated yeast (Group 2) (Fig. 1). Tissue destruction and exudative epithelioid granulomas with numerous multiplying fungi were visualized in the tissues of Group 1, but the histopathological analysis did not detect fungal cells, granulomas or inflammatory focus in the tissues from mice infected with the radioattenuated yeast (Fig. 2), demonstrating that the yeast cells were cleared from the analyzed mice organs.

The antibody responses in the animals of Groups 1, 2 and 3 were shown in Fig. 3. The virulent yeast stimulates the production of antibodies able to recognize the antigen Mexo while the radioattenuated yeast did not elicit a significant antibody production. This is an interesting behavior for a vaccine candidate in view of high antibody level is a bad prognostic in PCM. The severe forms of PCM are associated with high

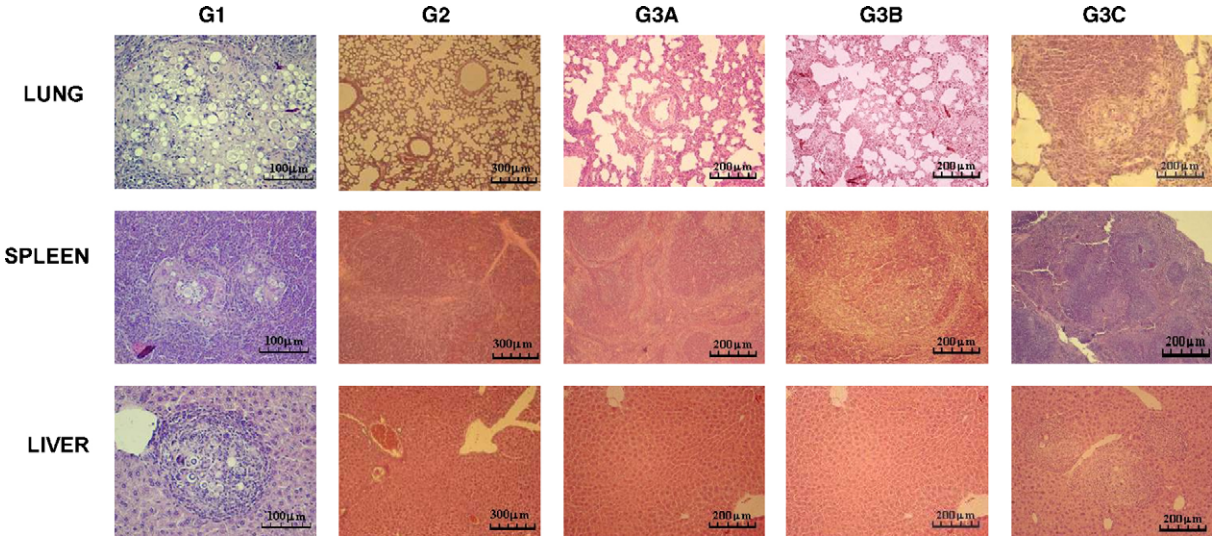

Fig. 2. Representative organs histopathology of G1, G2 and G3 mice groups analyzed 90 days after infection or challenge. G1, group infected with the virulent yeast; G2, group inoculated with the radioattenuated yeast; G3A, group challenged 30 days post immunization; G3B, group challenged 45 days post immunization; G3C, group challenged 60 days post immunization. Tissue destruction and exudative epithelioid granulomas with numerous multiplying fungi were visualized in the tissues of Group 1. Histopathological changes, granulomas or yeast cells were not detected in the other groups.

levels of specific antibodies and hypergammaglobulinemia, which fails to protect against the disease while mild forms of the disease leading to healing are parallel to production of low levels of specific antibodies and maintained cellular immune responses [6]. In the immunized mice, the antibody level increased in all sub groups after the challenge, indicating that the production of some IgG isotypes play an important role in the establishment of the protection.

The levels of the immunoglobulin isotypes IgG1 (Th2 regulated) and IgG2a (Th1 regulated) and their correlation with the establishment of a pattern related to the disease control observed in the protection assay was evaluated. The results were shown in Fig. 4. IgG1 predominance in relation to the IgG2a was verified in the sera collected 30 days after the challenge. In contrast, in the sera collected 90 days after the challenge was verified a higher production of IgG2a in relation to IgG1 for all sub groups (G3A, G3B and G3C) and the levels of IgG2a where significantly higher than verified in the Group 1 ($p < 0.05$). This profile suggested that the mice had not developed a polarized pattern of Th1/Th2 response up to 30 days but a trend to a Th1 pattern was evident 90 days post challenge.

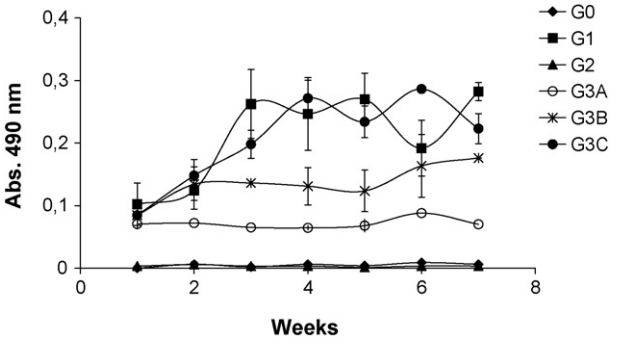

Fig. 3. IgG production in the sera of G1, G2 and G3 mice groups. G0, group not infected or immunized; G1, group infected with the virulent yeast; G2, group inoculated with the radioattenuated yeast; G3A, group challenged 30 days post immunization; G3B, group challenged 45 days post immunization; G3C, group challenged 60 days post immunization. Antibody responses were determined weekly for each group ($n = 3$) after the first week post inoculation or challenge.

Fig. 5 shows the cytokines analyses accomplished by real-time PCR in the lungs of Groups 1, 2, and 3. The Th1 related cytokines IFN-γ and TNF-α, and the Th2 related IL-10 and IL-5 were analyzed. Fig. 5 shows that the mice infected with the radioattenuated yeast (Group 2) presented a higher production of IFN-γ ($p < 0.05$) in relation to the animals infected with the virulent strain (Group 1). The immunized group (3A, 3B and 3C) maintained an elevated and consistent production level of IFN-γ after the challenge, significantly higher than the observed in the Group 1 ($p < 0.05$). These results clearly showed that the immunization induced the IFN-γ production and suggested that this cytokine plays an important role in the establishment of the protection. The tumor necrosis factor (TNF)-α was strongly induced by the radioattenuated yeast inoculation (Group 2) but was moderately produced by the immunized groups after the challenge. The IL-10 is a cytokine able to down-regulating a broad spectrum of pro-inflammatory cytokines in mycosis [21]. The mice infected with the virulent strain produced high levels of IL-10 but, interestingly, it was not induced by the infection with the radioattenuated yeast and the immunization abolished the secretion of IL-10 after the challenge. The eosinophil differentiation factor IL-5 was ephemerally secreted by the immunized group. Mice of sub groups 3B and 3C presented a significant production of IL-5 30 days after the challenge ($p < 0.05$), but the cytokine was not detected 90 days post challenge.

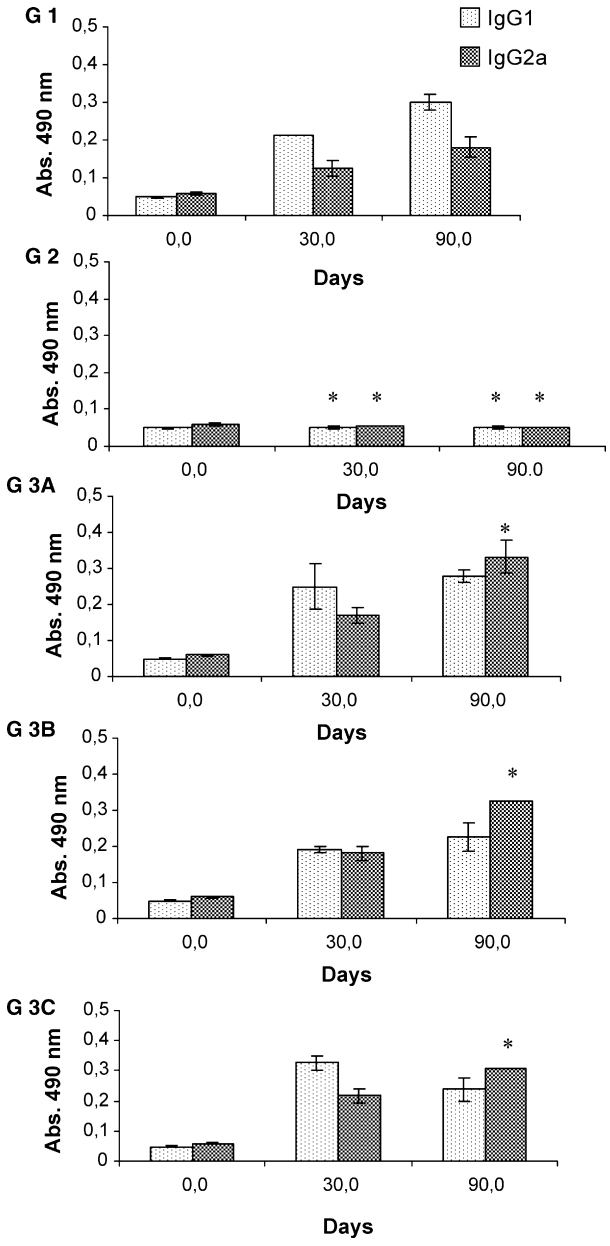

Fig. 4. IgG1 and IgG2a in the sera of G1, G2 and G3 mice groups. The antibody responses were determined 30 and 90 days post infection or challenge by ELISA. Data were reported as mean ± S.D. of optical density at 490 nm in each experimental group ($n = 3$). G1, group infected with the virulent yeast; G2, group inoculated with the radioattenuated yeast; G3A, group challenged 30 days post immunization; G3B, group challenged 45 days post immunization; G3C, group challenged 60 days post immunization. $^{*}p < 0.05$ in relation to G1 group.

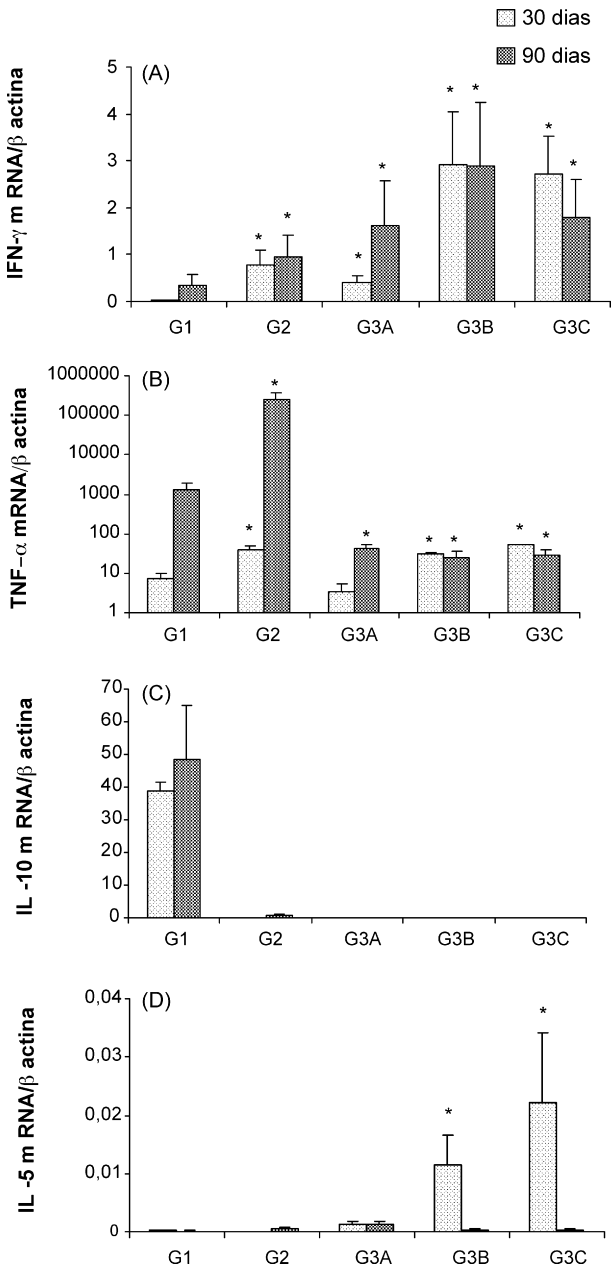

Fig. 5. Cytokine production in the G1, G2 and G3 mice groups. The relative expression of the cytokines of IFN-γ (A), TNF-α (B), IL-10 (C) and IL-5 (D) by lung cells were determined by real-time PCR, 30 and 90 days post infection or challenge. G1, group infected with the virulent yeast; G2, group inoculated with the radioattenuated yeast; G3A, group challenged 30 days post immunization; G3B, group challenged 45 days post immunization; G3C, group challenged 60 days post immunization. Bars represent the mean ± S.D. in each experimental group ($n = 3$). $^{*}p < 0.05$ in relation to G1 group.

4. Discussion

P. brasiliensis has the ability to initiate an infection after prolonged periods of dormancy in the host, therefore is mandatory for a *P. brasiliensis* live vaccine the guarantee that the attenuated cells have irreversibly lost the virulence and have been cleared of the host tissues after the immunization. These assumptions were confirmed by the results obtained with Group 2, composed of mice inoculated with the radioattenuated yeast cells. No CFU were recovered 30 or 90 days after the infection, neither histological changes nor yeast cells were observed in the mice tissues. The virulence of the radioattenuated yeast cells was also evaluated in athimic nude (nu/nu) mice and the same results were obtained (data not shown). The stability of the avirulence

was a consequence of the extensive DNA fragmentation produced by the gamma irradiation treatment [15].

Fig. 3 shows that the infection with the virulent *P. brasiliensis* (Group 1) elicited a high level of IgG production while the inoculation with the radioattenuated yeast cells (Group 2) did not change the IgG level significantly. The mice inoculated with the radioattenuated yeast presented an increase in the IFN-γ and TNF-α production and a very low synthesis of IL-10, while the mice of Group 1 showed a high production of IL-10 and a smaller production of IFN-γ and TNF-α (Fig. 5). These results pointed out that the inoculation with the radioattenated yeast has elicited the cellular immunity, in opposition to the infection with the virulent strain that appeared to be stimulated a humoral response and the suppression of the cellular immunity. As stated before cellular rather than humoral immunity is the effective host defense against PCM.

In this work, the mice were submitted to two consecutive immunizations and a high level of protection was evident, nevertheless, the use of just one immunization was not enough to confer a long lasting protection (data not shown). The mice immunized once presented a significant reduction in the CFUs recovery when examined 30 days after the challenge. However, 90 days post challenge the number of CFUs recovered from all organs increased, indicating that some fungi focus was not eliminated.

The mice immunized twice presented a significant degree of protection 30 days after the challenge, but the results were more expressive when analyzed 90 days after, showing that a long lasting protection able to eliminate the fungi cells of the tissues was elicited. The protection level evaluated after a long time of exposition to the fungi (90 days) is a stringent and appropriate criterion of protection since not only the initial infection control is considered but mainly the elimination of possible quiescent forms of the fungi.

These results together suggested that 30 days post challenge a mixed Th1/Th2 response occurred as evidenced by the synthesis of IFN-γ and TNF-α together with IL-5, and a higher production of IgG1 in relation the IgG2a. But in the sera collected 90 days after the challenge, the presence of TNF-α associated to high levels of IFN-γ without the presence of IL-5 and IL-10, parallel to a shift to IgG2a production in all sub groups, strongly indicated that a protective Th1 pattern was dominant. A similar behavior was verified in mice immunized with peptides derived from gp63 as adjuvants to chemotherapy [22].

Our findings pointed out that the protective effect could be attributed to IFN-γ production. The immunized mice did not produce IL-10, but significant levels of IFN-γ. This ratio in cytokine production could increase the microbicidal activity and antigen-presentation capacity of macrophages. The hole of IFN-γ on mechanisms of protection against *P. brasiliensis* infection playing a pivotal role through macrophage activation was documented [23].

In this work, it was shown for the first time that the immunization with radioattenuated yeast cells was able to confer a potent and durable protection against highly infective yeast forms of *P. brasiliensis*. Radioattenuated yeast cells are a powerful tool for understanding the factors that contribute to protective immunity in experimental PCM and open new perspectives in the research for a *P. brasiliensis* vaccine. Even the use of viable multicomponents imunogens as vaccine present problems, due to potential toxicity issues and compositional changes in different preparations, live attenuated vaccines for other pathogens, such the Bacille Calmette-Guérin (BCG) vaccine against tuberculosis, are widely used. Therefore, the use of radioattenated yeast cells for the development of vaccines against fungi of medical or veterinary relevance must be considered.

Acknowledgements

This research was supported by Fundação de Amparo a Pesquisa do Estado de Minas Gerais (FAPEMIG) (CBB580/06) and International Atomic Energy Agency (IAEA) (13239/R0).

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