Original article

Paracoccidioides brasiliensis: attenuation of yeast cells by gamma irradiation

Marina Cortez Demicheli,[1] Bernardo Sgarbi Reis,[2] Alfredo Miranda Goes[2] and Antero Silva Ribeiro de Andrade[1]

[1]*Laboratório de Radiobiologia, Centro de Desenvolvimento da Tecnologia Nuclear (CDTN), Belo Horizonte, MG and* [2]*Departamento de Bioquímica e Imunologia, Universidade Federal de Minas Gerais (UFMG), Belo Horizonte, MG, Brazil*

Summary

Paracoccidioides brasiliensis is the agent of paracoccidioidomycosis, the most prevalent mycosis in Latin America, and currently there is no effective vaccine. The aim of this study was to attenuate the yeast form of *P. brasiliensis* by gamma irradiation for further studies on vaccine research. *Paracoccidioides brasiliensis* (strain Pb 18) cultures were irradiated at doses between 0.5 and 8.0 kGy. After each dose the viability, reproductive ability and protein metabolism were evaluated. The comparison between the antigenic profile of irradiated and control yeast was made by Western blot and the virulence evaluated by the inoculation in C_{57}Bl/J6 mice. At 6.5 kGy the yeast lost its reproductive capacity. The viability and the incorporation of [L-^{35}S]-methionine were the same in control and up to 6.5 kGy irradiated cells, but 6.5 kGy-irradiated yeast secreted 40% less proteins. The Western blot profile was clearly similar in control and 6.5 kGy-irradiated yeast. No colony-forming unit (CFU) could be recovered from the tissues of the mice infected with the radioattenuated yeast. We concluded that for *P. brasiliensis* yeast it is possible to find a dose in which the pathogen loses its reproductive ability and virulence, while retaining its viability, metabolic activity and the antigenic profile.

Key words: *Paracoccidioides brasiliensis*, gamma irradiation, attenuation, vaccine.

Introduction

Paracoccidioides brasiliensis is a thermally dimorphic fungal agent of paracoccidioidomycosis, a deep-seated systemic infection of humans. The disease has a high incidence in Latin American countries, especially Brazil, Venezuela, Colombia and Argentina. It is estimated that approximately 10 million people are infected, although the most of them do not show clinical symptoms.[1] Airborne fungal propagules are thought to initiate the infection in the lungs after conversion to the yeast phase. The infection can proceed either as a mild, self-limited process, or be severe, eventually fatal, spreading to other organs and tissues.[2]

As other fungal pathogens, protective immunity against *P. brasiliensis* has been correlated with an exuberant cell-mediated immune response. Neither vaccine nor protective immunogen has still been reported, although the major diagnostic antigen gp43 has shown to be a promise as potential protective antigen.[3] *Paracoccidioides brasiliensis* antigens fractionated by anion-exchange chromatography, termed F0 and FII, have also been demonstrated to confer protection in mice.[4]

Ionising radiation has been successfully used to attenuate parasites and micro-organisms for vaccine development and research.[5–11] Irradiated pathogens frequently lost their reproductive ability or virulence, but retain the metabolic activities and morphology, and are able to stimulate a specific immune response. In

Correspondence: Dr. Antero Silva Ribeiro de Andrade, Laboratório de Radiobiologia, Centro de Desenvolvimento da Tecnologia Nuclear (CDTN), Rua Prof. Mario Werneck, s/n, Pampulha, Campus de UFMG, Caixa postal 941, CEP 30123-970, Belo Horizonte, MG, Brazil.
Tel: +55-31-3499 3182. Fax: +55-31-3499 3380.
E-mail: antero@cdtn.br

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some cases, the radioattenuated pathogens are more immunogenic than the normal counterparts.[12] An instructive example is the *Schistosoma mansoni* radiation-attenuated cercariae vaccine, which stimulates a level of immunity and protection that surpasses any generated by purified or recombinant molecules.[5] Although this schistosome vaccine has not been tested in humans, primarily for safety reasons, it has allowed that the mechanisms of induction of the protective immunity and the protective antigens were known.[13] The analogous sporozoite radiation-attenuated malaria vaccine was tested in human volunteers and elicited protective immunity.[14] The paradigm for a successful radiation-attenuated vaccine is that employed for *Dictyocaulus viviparous*, which causes parasitic bronchitis in cattle. This vaccine has been used with widespread success since its introduction in 1958.[15] Efforts were also accomplished to develop irradiation-attenuated vaccines against *Toxoplasma gondii*,[6] *Leishmania major*,[8] *Eimeria maxima*,[10] *Fasciola hepatica*,[11] among others.

In the present study, the effects of gamma irradiation on the reproductive ability, metabolic activity, antigenic profile and virulence of the yeast form of *P. brasiliensis* were analysed. The purpose was to attenuate the pathogen for further studies on immunity in the paracoccidioidomycosis and vaccine research.

Material and methods

Culture conditions

Paracoccidioides 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.

Yeast cell counting

The cells were collected and diluted in a counting solution (0.9% NaCl, 4% formaldehyde and 4% Tween-20). The mixture was vigorously mixed up in order to disperse the aggregated cells. The counting was performed in a Neubauer chamber (BOECO, Hamburg, Germany).

Gamma irradiation

Cultures of *P. brasiliensis*, in solid medium, were irradiated at doses ranging from 0.5 to 8.0 kGy, 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}. Adequate controls were maintained outside the source. After each dose the growth, viability and metabolic activity were estimated.

Yeast growth analysis

Control and irradiated cells were transferred to phosphate-buffered saline (PBS) solution, vortexed and counted. The suspensions, containing 10^2 to 10^7 colony-forming units (CFU), were spread on Petri dishes with a high plating efficiency medium. The plates were incubated at 35 °C for 7 days and colonies counted under a magnifying glass. The mean colony counting was obtained from triplicate determinations.

Agar plates with high plating efficiency were prepared adding 20 g of agar to 910 ml of distilled water. The solution was autoclaved for 15 min at 121 °C, and cooled to 56 °C. Then, 40 ml of fetal bovine serum and 50 ml of water extract of *P. brasiliensis* were added. The water extract was obtained by culturing the strain Pb 18, at 35 °C for 5 days, on BHIA supplemented with 1% glucose. About 5 ml of the pelleted yeast cells were suspended in 45 ml of distilled water and autoclaved. The suspension was left to rest for 24 h at room temperature and centrifuged at 1000 *g* for 15 min. The supernatant was used as the extract.[16]

Yeast viability analysis

The viability was determined by using the modified vital dye Janus green method[16] and the methylene blue method.[17] In brief, for Janus green, 20 μl of 0.05% solution of the dye was added to an equal volume of the fungal cell suspension. The methylene blue test was performed by adding 20 μl of methylene blue solution (0.05 mmol l^{-1} in PBS, pH 7.2) to the same volume of fungal cells suspension. The suspensions were allowed to interact with the dye for 10 min. The counting was performed in a Neubauer chamber. Viable cells remained unstained and dead cells stained blue for both methods.

Protein synthesis assay

Controls and irradiated yeast cells were transferred to 10 ml of liquid medium (brain heart infusion, BHI), at an initial inoculum density of 1×10^6 cells ml^{-1}. To each sample were added 10 μCi of [L-^{35}S]-methionine and cells were incubated for 12 h at 35 °C. The cells were harvested, washed in PBS and disrupted using glass beads. After the centrifugation at 10 000 *g*, the protein concentration in the supernatant was determined by the Lowry method. Then, a volume

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containing 0.3 mg of protein was placed in scintillation vials and 4.5 ml of scintillation fluid was added. Counts were determined in a liquid scintillation analyzer (Insta-Gel Plus, Perkin Elmer, Wellesley, MA, USA) and expressed in counts per minute (cpm).

Protein secretion

The control and the irradiated yeast cells were transferred to 10 ml of 0.65% yeast nitrogen-based medium (Sigma, St Louis, MO, USA), supplemented with 2% glucose. The initial inoculum density was 1×10^6 cells ml^{-1}. After 4 days the cell densities were determined, the cultures pelleted and the supernatants harvested. The supernatants were lyophilised and the resulting powders dissolved in 0.5 ml of 0.01 mol l^{-1} borate buffer (pH 8.0). These solutions were dialysed against the same buffer using a 1000 Da cut-off membrane. After that, protein concentrations were estimated by the Lowry method. The total secreted protein was normalised by the growth factor of each individual culture.

Western blotting

The controls and the 6.5 kGy-irradiated yeast (16 h after irradiation) were disrupted using glass beads. After the centrifugation at 10 000 *g*, the supernatants were collected and 20 μg of each sample submitted to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) in a 12.5% running gel, with subsequent transfer to nitrocellulose membrane. The membrane was incubated in 0.3% Tween-20 in PBS, for 1 h, and then twice in 0.05% Tween-20 in PBS, for 30 min, and stored (dried) until being used. Appropriately dilute sera, from *P. brasiliensis*-infected patients, were incubated for 90 min at room temperature with the membrane. The antibodies were detected by sequential incubation with peroxidase-conjugated antibodies, for 1 h at room temperature, and incubated with the staining solution (0.6 mg ml^{-1} 4-cloro-1-naphtol and 0.03% hydrogen peroxide in PBS). After development the membrane was washed in distilled water and dried.

Virulence assay

C_{57}Bl/J6 mice were inoculated by ocular artery with 50 μl of PBS containing 10^5 radioattenuated yeast cells. Controls were inoculated with the same number of viable virulent cells. Organ CFUs were determined after 1 month of infection in the lung, spleen and liver. The organs were removed, weighed, homogenised and washed three times in PBS by centrifugation. The final suspensions in PBS were plated in the medium described in *Yeast growth analysis* section. The plates were incubated at 35 °C and examined after 20 days. The results were expressed as the number of *P. brasiliensis* CFUs per gram of tissue per mouse in each experimental group ($n = 3$).

Results

The reproductive ability of irradiated cells was monitored by their capacity to form colonies in a high plating efficiency solid medium. The survival curve was presented in Fig. 1. The number of surviving cells falls off with increasing doses of radiation. A typical tailoring effect, or a tail, was observed in the curve after 2.0 kGy. This behaviour has been well documented with radiation and other environmental stresses[18] and it represents a yeast population that exhibits higher radiation resistance. A reduction of 5 $\log_{10}$ cycles in the number of colonies was achieved at 6.0 kGy and at 6.5 kGy, no colonies could be recovered, even if large inoculum and incubation time (40 days) were used. The 6.5 kGy-irradiated yeast cells were also unable to grow in liquid BHI and PYG (Bactopeptone, yeast extract, glucose) media (data not shown).

The viability of irradiated cells was first evaluated using the Janus green and methylene blue dyes (Fig. 1). The vital dye exclusion test with Janus green is a very popular method for the determination of cell viability of fungi and useful for estimating cell viability of yeast form of *P. brasiliensis*.[16] The analysis by this method showed that up to 6.5 kGy the cells were viable, retaining the membrane permeability control. A similar result was obtained with methylene blue that evaluates the status of the oxidative metabolism. Although

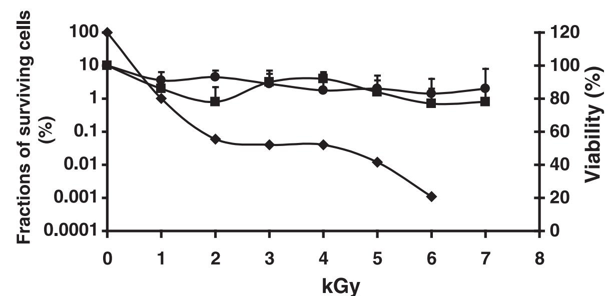

Figure 1 Effect of gamma irradiation on survival and viability of yeast cells of *Paraccocicioides brasileinsis*. Yeast cells were irradiated with increasing doses of external gamma radiation and the fraction of surviving cells determined (◆). The viability was measured by Janus green (■) and methylene blue (●). The bars represent the standard deviations of triplicate determinations.

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unreliable when used with *P. brasiliensis*, the methylene blue test can be used for crude viability estimation. A good agreement was obtained by the two methods. The irradiated yeast cells remain viable for 3 weeks (data not shown).

The protein synthesis was analysed by the incorporation of [L-^{35}S]-methionine (Fig. 2). The results showed that the yeast cells, irradiated up to 6.5 kGy, maintained a similar synthetic protein metabolism level than controls. The protein secretion was another metabolic parameter evaluated (Fig. 3), as proteins secreted by *P. brasiliensis* have important roles in the establishment of the infection, in the stimulation of the immune system and include some virulent factors.[19, 20] A decrease of 40% in the protein secretion occurred at 6.5 kGy.

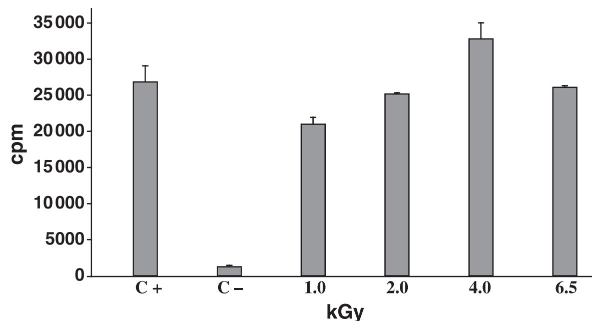

Figure 2 Evaluation of protein synthesis after gamma irradiation. Yeast cells were irradiated with increasing doses of external gamma radiation and the metabolism of protein synthesis monitored by the incorporation of [L-^{35}S]-methionine. The negative control (C−) was cells killed by heating. The positive control (C+) was no irradiated cells. The values represent the median and standard deviations of three independent experiments.

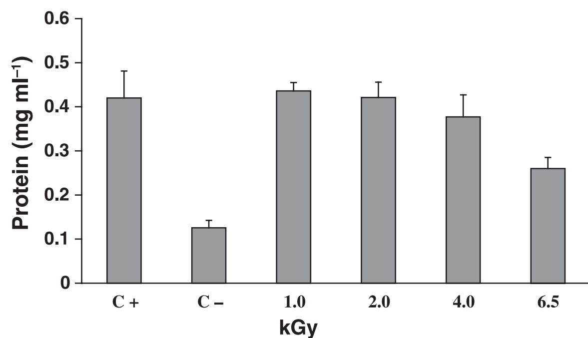

Figure 3 Gamma irradiation effect on protein secretion. The negative control (C−) was cells killed by heating. The positive control (C+) was no irradiated cells. The values represent the median and standard deviations of three independent experiments.

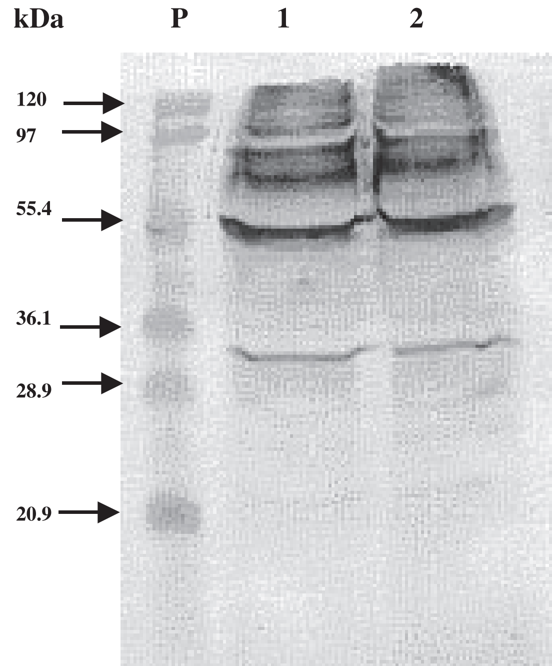

Figure 4 Comparison between antigenic profiles of irradiated and control yeast by Western blot. Sera from *Paracoccidioides brasiliensis*-infected patients were used. P, molecular weight standards; 1, control yeast; 2, 6.5 kGy-irradiated yeast. The same protein bands were recognised in control and 6.5 kGy-irradiated yeast. A 12.5% acrylamide gel was used for electrophoresis protein separation.

Table 1 Recovery of CFUs from mice infected with the radioattenuated yeast of *Paracoccidioides brasiliensis*.

Organ	Control yeast (CFU[1])	Radioattenuated yeast (CFU[1])
Lung	4750 (±1041)	0
Spleen	417 (±158)	0
Liver	123 (±44)	0

[1]Colony-forming units (CFUs) per gram of tissue per mouse ($n = 3$).

The comparison between the antigens recognised by the sera from patients with paracoccidioidomycosis, in 6.5 kGy-irradiated yeast and control yeast, was realised by Western blot (Fig. 4). The profile was clearly similar in both cases, indicating that the irradiated yeast retained the expression of the same antigens that are present in the wild yeast.

The 6.5 kGy gamma-irradiated yeast of *P. brasiliensis* fails in producing infection in C_{57}Bl/J6 mice. Table 1 shows the number of CFUs recovered by each organ from mice infected with the irradiated yeast in relation to controls. No CFU could be recovered from mice

infected with the radioattenuated yeast, indicating that the pathogen was attenuated.

Discussion

Paracoccidioides brasiliensis yeast cells, like many fungi, exhibited high radioresistance relative to other micro-organisms and mammalian cells.[21] A dose of several thousand grays was required to kill, in the reproductive sense, the yeast cells whereas the lethal dose for mammalian cells is only a few grays. The mechanisms responsible for these differences are not well understood, but could involve more efficient mechanisms of DNA repair of the damage caused by gamma rays. The inability of 6.5 kGy-irradiated cells to divide results from unrepaired double DNA breaks, leading to a cell cycle arrest or unbalanced chromatin exchange in daughter cells and consequent mitotic death.[22, 23] However, these lesions do not impair the protein synthesis, likely because most of the breaks occur outside operons, usually a minor part of the genome, allowing adequate functions of the genes until cell division. The lost of the reproductive ability seems irreversible. No colonies were observed in the plating experiment, despite of long incubation times, and no CFU could be recovered from infected mice. However, *P. brasiliensis* has the ability to initiate the infection after prolonged period of dormancy. For example, it is not uncommon for patients to fall ill more than a decade after leaving an endemic region.[1] As a consequence, the hypothesis that the radioattenuated yeast may, after long times, recover the cell division capacity cannot be excluded. This possibility could limit the utilisation of a radioattenuated yeast vaccine in humans and confine its utilisation for experimental paracoccidioidomycosis on animal models.

Among the metabolic parameters evaluated, only protein secretion was changed with the gamma irradiation dose that eliminated cell division. Now, we are evaluating if the secretion of antigens and virulent factors was negatively affected.

The 6.5 kGy dose do not disrupt the expression of the known antigens of *P. brasiliensis*. This is a very favourable result that indicates that the radioattenuated yeast is able to stimulate the host immune system at least as the native yeast. So, the irradiated yeast could be a valuable tool for the evaluation if the interaction of multiple antigens is necessary for an effective immune response.

We concluded that for the yeast cells of *P. brasiliensis*, it is possible to find an absorbed dose (6.5 kGy) in which the pathogen loses its reproductive ability and virulence, while retaining its viability, metabolic activity and antigenic profile. This behaviour allows the immune system to recognise the irradiated yeast as a viable agent without risk of progressive infection. The radioattenuated yeast provides a novel tool for immunological studies in experimental paracoccidioidomycosis and vaccine research, because 'live' vaccines can expose their antigens sequentially to the host, as in a natural infection, avoiding problems associated with single immunisation schedules. Currently, there are no approved vaccines for the prevention or treatment of medically important fungi infection in humans. This failure determines the need to explore new alternatives and there are no studies using radioattenuated fungi for this purpose. Now, we are evaluating the capacity of the radioattenuated yeast to stimulate a protective immunity against a *P. brasiliensis* infection.

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