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METACYCLIC PROMASTIGOTES OF *Leishmania amazonensis* SELECTION USING GAMMA IRRADIATION.

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ABSTRACT

Leishmania spp. causes a spectrum of human diseases, ranging from self-healing skin lesions to severe and lethal visceral disease. In previous work we demonstrated that the protein and nucleic acid metabolism and oxidative respiration were severely affected by irradiation, in a dose response way, but a small but representative fractions are relatively radio resistant, surviving after 800 Gy of ^{60}Co irradiation. The best explanation could be a selection of metacyclic promastigotes. In these forms, the G0 state allows the adequate correction of DNA repair after the irradiation insult. In this work, we are looking for the ideal radiation dose to select the higher proportion of metacyclic forms of *L.. (L.) amazonensis* in culture. Parasites were grown in RPMI 1640 medium, plus 20% fetal calf serum, than they were irradiated with differents doses rangin between 25 and 400 Gy. Parasites irradiated at 400 Gy infected, proportionally, more cells than parasites irradiated at other doses. To confirm this metacyclogenesis, a complement lysis assay was performed with 5, 10 and 20% of male guinea pig blood serum at 20ºC for 3 hours, and parasites counted. Guinea pig serum a 10% promotes more lysis, with 200 Gy irradiated parasites being less affected, probably due to metacyclic selection. These preliminary results suggests that the ionizing radiation, specially between 200 and 400 Gy, could be a alternative tool for the selection of metacyclic forms of *Leishmania amazonensis* in culture. Supported by LIMHCFMUSP, CAPES and IPEN/CNEN/SP

1. INTRODUCTION

The protozoan *Leishmania spp*. is an agent that causes a spectrum of human diseases, ranging from self-healing skin lesions to severe and lethal visceral disease[1]. The parasites are transmitted by a bite of a sand fly, and have two distinct phases on its life cycle: promastigotes, a flagellated form that occurs in the insect gut and axenic culture, and amastigote form, a rounded obligatory intracellular parasite without free [2,3].

The promastigotes form can be divided in two distinct stages: procyclic promastigotes, that multiply and lives in the light of the midgut attached to the epithelium by the flagella, and metacyclic promastigotes, the infective form for mammalians, which don't divides and are entrapped in a peritrophic matrix localized in the esophagus of the vector sand flies. Metacyclogenesis, the process of procyclic/metacyclic transformation, induces morphological and structural changes on parasite [4], as lipophosphoglycan (LPG) alteration, a surface molecule described in all species of the genus [5].

Ionizing radiation has direct and indirect effects on the cell molecules. The indirect effect shown a higher sensibility on the water diluted solutions on the oxygen presence, and a

protector effect on the others cells molecules, demonstrating that the final effect of radiation differ depending de irradiation conditions [6].
Studies using ionizing radiation to kill or sterilize protozoan parasites have been described as an efficient method [7].
Cists of T. Gondii were irradiated with doses of 550 Gy, and shown that they loose completely their infection capacity [8]. Other group shown that 200Gy gamma-irradiated Toxoplasma gondii RH tachyzoites failed to reproduce in vitro and in vivo, maintaining respiratory response, the ability to invade cells, and the protein and acid nuclei synthesis [9].
In this work, we demonstrated that the ionizing radiation could be used to select the metacyclic form in axenic cultures, based on the Bergonie & Tribondeau law, which say that cells in division are most radio sensible than non-divisible cells [10].

2. MATERIAL AND METHODS

1. Parasites: Promastigotes of *Leishmania amazonensis* (MPRO/BR/72/LV79) isolated from lesions of Balb/c mice were cultured in RPMI 1640 (SIGMA®), 20% of fetal calf serum plus.

2. Irradiation: The irradiation process was made at Instituto de Pesquisas Energéticas e Nucleares (IPEN/CNEN- SP) in ^{60}Co (GAMMACELL, Atomic Energy of Canadá Ltd). The doses of radiation used was 50, 100 e 200Gy, without attenuation; e 400, 800, 1000, 1100, 1200, 1300, 1400, 1500, 1600 e 3200Gy, with 90% of attenuation, for less direct effects on parasites.

3.Viable parasites growth: Non irradiated *Leishmania amazonensis* were cultured in RPMI 1640 medium, plus 20% fetal calf serum, at 24º C, for 7 days. Daily, the number of viable *Leishmania* was counted in Newbauer chamber. A curve for growth parasites determination was done using a GraphPad Prisma ® software.

4.Viable irradiated parasites count: The promastigote forms of *Leishmania* were recovered by centrifugation, suspended in fresh TC-100 medium without serum, adjusted to 10^7/ml promastigotes and irradiated with doses ranging between 25 and 400 Gy of ^{60}Co gamma-radiation. After irradiation, the number of surviving intact parasites was counted in hematocytometer chamber.

5. RAW cells infection assay: Irradiated promastigotes were challenged to $2x10^5$/ml RAW cells in round cover slips, in RPMI 1640 medium, plus 10% fetal calf serum, at 37ºC 5% CO_2 in 24-well plates by 3 hs. The cover slips were fixed, stained with Giemsa, determining the number of infected cells counting one hundred cells per slips.

6. Complement interaction assay: Parasites were irradiated as described previously. After irradiation, the concentration of parasites was adjusted to 10^8 *Leishmanias*/ ml, and incubated for 90 minutes at 37ºC in HBSS plus 10% or 20% of male guinea pig. After incubation the same volume of cold PBS cation free was added, and the vials were maintained in ice. The number of intact promastigotes were counted at optical microscopy in a hemacytometer chamber. The results were expressedwith a percentage of intact cells *versus* the initial number of parasites.

3. RESULTS

1. Viable parasites growth: Our data shows the growth curve of an axenic culture of Leishmania amazonensis promastigotes in RPMI 1640 medium. The culture started with a concentration of $2X10^6$ /ml, increasing quickly on the first four days until 4,4 X 10^7 parasites /ml. After two days (day six) the parasites concentration increased slowly and stopped in $7X10^7$ parasites/ml. On day seven the number of parasites started to decrease as shown in figure 1:

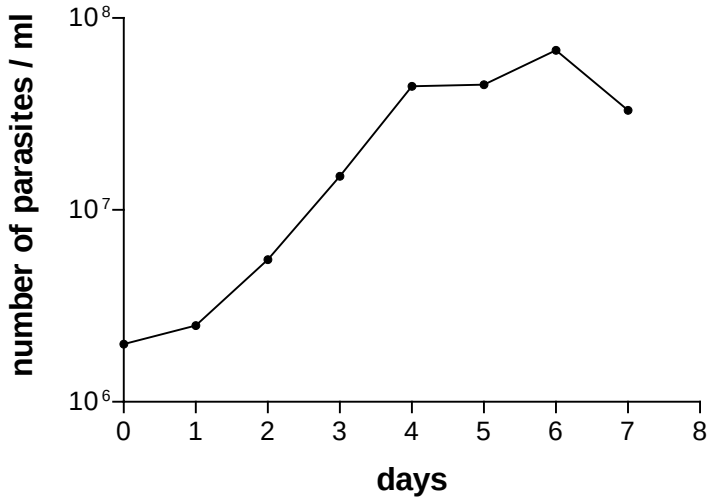

Fig1: Promastigotes of *Leishmania amazonensis* growth in axenic culture for 7 days. The graphic shows the log phase until day six, a stability for one day and the decrease of parasites concentration starting at day seven.

2. Viable irradiated parasites count: The promastigotes form of *Leishmania amazonensis* were irradiated with differents doses ranging beween 25 and 400 Gy of gamma radiation to stabilish the most effective dose capable to select only metacyclic form at the culture. The decrease on the number of the parasites at the culture is proportionally to de increase of the dose. Except on the dose 100 Gy wich is less agressive to the parasites, while the number of non irradiated parasites remains intact. The dose response curve is shown on the figure 2. This curve was obtained using a non linear regerssion sthatiscal analysis:

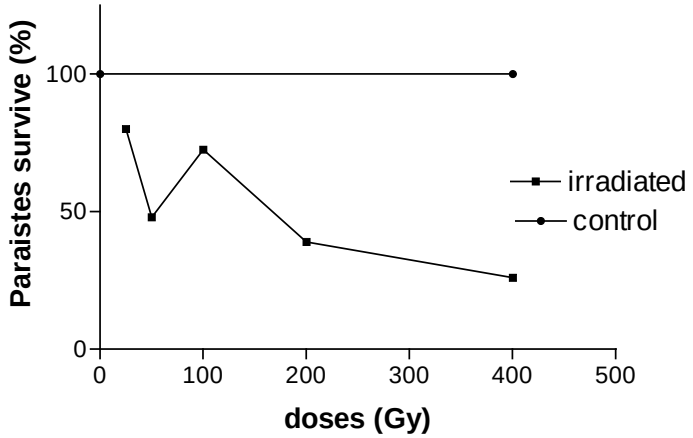

Fig 2: Number of survival promastigotes of *Leishmania amazonensis* irradiated or not (control) with different doses ranging between 25 and 400 Gy of ionizing radiation (^{60}Co).

3.RAW cells infection assay: Promastigotes forms of *Leishmania amazonensis* were irradiated with the same doses early discribed. After the irradiation the parasites were applied oneself to RAW cells and incubated for 3 hours 37ºC, 5% CO_2. This proceeding results an infection wich were measured counting the number of infected cells in one hundred of total cells. The increase of dose results in less infection when compared with that occured in the control (non irradiated) parasites, about 75% of infection. Although, the parasites who recieved 50 Gy and 400 Gy of ionizing radiation infected more RAW cells than the parasites irradiated with other doses.

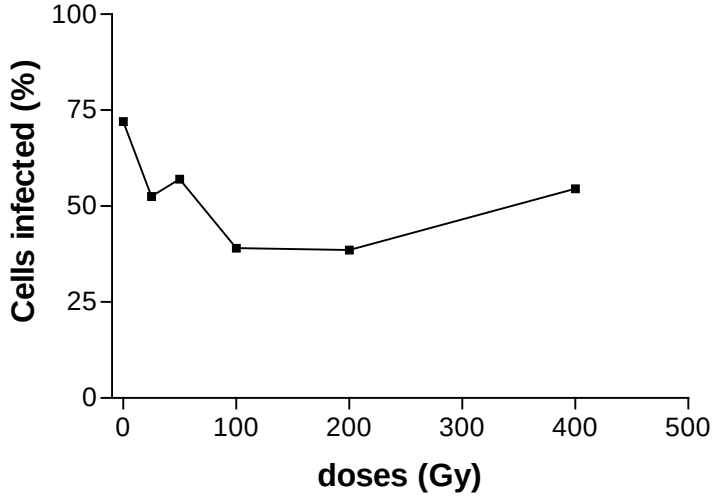

Fig 3: Percentage of infected RAW cells, by irradiated and naive promastigotes of *Leishmania amazonensis*. The graphic shows how many cells were infected at any 100 cells after 3 hours incubation aside irradiated promastigotes, with doses ranging since 25 Gy to 400 Gy .

4. Complement interaction assay: Irradiated and naive promastigotes of *Leishmania amazonensis* were incubated with 10% and 20% of male guinea pig serum in an axenic culture. The figure 4 shows the percentage values of the survive parasites after 3 hours incubation at 37º C, 5% CO_2. The parasites were counted before and after incubation in a hematocytometer chamber, and the values were analyzed in GraphPad ® software:

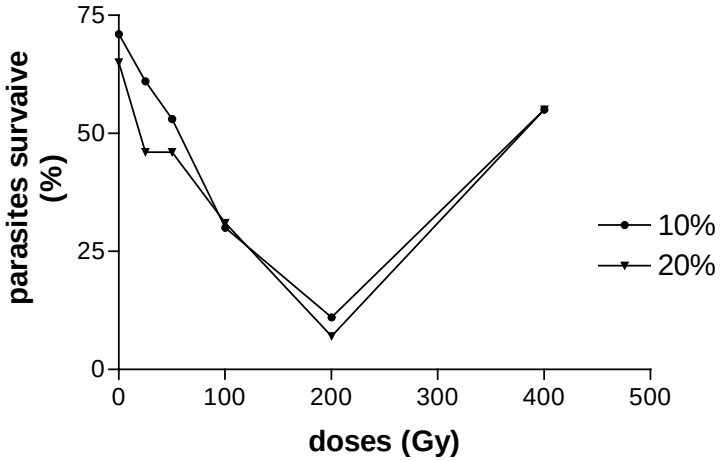

Fig 4: Percentage value of survival parasites after 3 hours of incubation with 10% or 20% of male guinea-pig. The values were obtained counting the parasites in a hematocymeter chamber before and fter incubation.

4. DISCUSSION

Metacyclogenesis is the process which the parasites stop the replication and switch their sugar and proteins of the cell surface being ready to infect mammalian cells. [11]. The ionizing radiation seems to be an efficient tool to select the metacyclic parasites in axenic culture [12].
Promastigotes forms of *Leishmania amazonensis* were cultured in RPMI 1640 and grow quickly through some days, decreasing this growth after five days, when the culture stabilized. Our data suggests that the metacyclogenesis process occur on the 6th day of culture. Ionizing radiation was used as a tool for selects this form on culture and compared with the selection occured using male guinea pig serum, wich has high concentration of Complement proteins, an important immune activator.
Different doses ranging between 25 and 400 Gy of ^{60}Co ionizing radiation were tested to access the better dose to select the metacyclic forms. The 400 Gy dose shown the lower concentration of survivor parasites (around 30%) suggesting the prior presence of metacyclic forms. When these parasites were incubated *in vitro* aside RAW cells, the parasites irradiated with the dose of 400 Gy infected proportionally more cells than the other irradiated parasites.
To confirm that those parasites were priory metacyclics form, they were incubated with male guinea-pig serum, to react with the complement system. Non-metacyclic cells are disrupted by the complement while metacyclic parasites resists to this interaction [13, 14]. The data shown that the survivor parasites irradiated with the 400 Gy dose have resistance to the complement, and that dose is the ideal dose to select metacyclic forms of *Leishmania amazonensis* in culture. The prior presence of metacyclic in culture can be used on the preparation of efficient specific immunogens on vaccines develops.

5. CONCLUSION

The preliminar results suggests that ionizing radiation can be used to select metacyclic forms of Leishmania amazonensis in axenic culture, and could be an efficient tool to produce specific immunogens for vaccines develops.

6. ACKNOWLEDGMENTS

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