Toxoplasma gondii: EFFECTS OF ^{60}CO IONIZING RADIATION IN THE VIABILITY AND INFECTIVITY, DETECTED *IN VITRO* IN LLC-MK2 CELLS AND *IN VIVO* IN C57BL/6J MICE

Roberto M.Hiramoto*#, Beatriz S.V.Almeida*, Roselaine P.A. Cardoso* & Heitor F.Andrade Jr.*

*Lab.Protozoologia do Instituto de Medicina Tropical de São Paulo - FMUSP - Av.Dr.E.C.Aguiar, 470, 05403-000, S. Paulo, SP, BRAZIL

Supervisão de Radiobiologia do Instituto de Pesquisas Energéticas e Nucleares(IPEN/CNEN-SP) Cx.Postal 11040, 05422-970, São Paulo, SP, BRAZIL

ABSTRACT

Toxoplasmosis, caused by *Toxoplasma gondii*, promotes devastating disease in fetus and AIDS patients. The longlife immunity of natural infection is inefficient in eliminate tissue infective cysts. Few immunization programs were tested, mostly with attenuated strains. Ionizing radiation were used with successful in vaccine production, without reproductive ability with a relatively normal physiology until reproduction. Here, we tested several schedules of ^{60}Co irradiation of tachyzoites from RH strain of *T.gondii*, from peritoneal exudate or suspensions of LLC-MK2 infected cells, to optimize the viability and sterility of the irradiated agents. The tachyzoites were exposed to 50, 100 and 200 Gy in a GammaCell 220 at 366 Gy/h. The viability was tested by motility, integrity and Trypan Blue dye exclusion. All irradiation schedules maintained a high(>90%) viability of the parasites. Dilutions were injected in C57Bl/6j mice with induction of specific antibodies, no clinical disease but uncertain sterility. Infection of LLC-MK2 cells showed that viable and reproductive parasites were often found in 50 Gy irradiated cells, rarely found in 100Gy irradiated cells, with no growth occurring with 200 Gy irradiated tachyzoites. Our data show that 200 Gy ^{60}Co irradiation blocks the reproductive capacity without affecting the short term viability of tachyzoites of *T.gondii*.

I. INTRODUCTION

Toxoplasmosis, a worldwide protozoan disease caused by *Toxoplasma gondii*, was usually benign or asymptomatic in most of the infected people[1], despite some minor behavioural alterations[2], affecting more than 60% of adult population in our region[3]. Transmission occurs by ingestion of latent cysts in undercooked meat or oocysts of cat feces in contaminated fresh food or water[4]. This infection presented a devastating course with bad prognosis when affects immunosupressed, like AIDS or transplant patients, or with immune immaturity, as the fetus of acutely infected pregnant women[5]. After the infection, an established immunity had a long life duration, despite its inefficiency in the eradication of tissue cysts, but enough to promotes the prompt elimination of released agents. Due to its apparently benign course, few efforts have been made in immunization schedules, but the HIV pandemic, the organ transplant receptors complications and possible behavioural interactions suggest that some sort of immunization to avoid this infection will benefit society. Those early attempts deals with attenuated strains, that result in infection with cysts persistence, without knowledge above behaviour effects or cysts reactivation induced be immunologic deficiency[6].

Ionizing radiation affects both nucleic acids and proteins of exposed cells, directly or due to action of free radicals from water radiolysis. Standardized protocols of ionizing radiation affect cells by abolishing its reproductive

ability without interfering in its short term physiology. This approach was used for the production of immunogens for parasitic diseases and other agents, resulting in better results as compared to chemically treated antigens[7].

Several studies were conducted in order to sterilize meat and other products that could be contaminated with *T.gondii* cysts by ionizing radiation, in order to improve meat quality for trading[8]. The immunity induced by irradiated forms of *T.gondii* was studied in experimental animals, usually without a careful analysis of viability and reproductivity capacity of treated agents. Most studies measure the effect by initial and after immunization challenge with infective forms, without any consideration to persistence of tissue cysts.

Here, we tested several schedules of ^{60}Co ionizing radiation of tachyzoites of RH strain of *T.gondii,* in order to detect viability, reproduction capacity *in vivo* in C57Bl/6j mice and *in vitro* in LLC-MK2 susceptible cells.

II. MATERIAL AND METHODS

Materials and reagents: All reagents were pro-analysis, with solutions made with MilliQ purified water. Reagents for electron microscopy were obtained from commercial sources, cited along the specific techniques when necessary. RH strain was maintained as cryo estabilates in liquid nitrogen, and expanded in Swiss mice before experiments. Mice were maintained according Animal Care definitions of Lab. Animal Science Assoc., in sterilized cages and absorbent media, with commercial food and water *ad libitum.*

Parasite purification: Mice were injected ip with 10^4 tachyzoites of RH strain obtained of the peritoneal exudate of a previous infected mouse. After 5 days, when death beginning to occurs, mice were killed by ether anesthesia and its peritoneal cavity washed with 2 batches of 5 ml of phosphate buffered saline(PBS). Recovered peritoneal washing were pooled, passed in a 28 gauge sterile needle, and immediately overlaid in a nylon wool glass column previously stabilized with room temperature PBS, with washing. The eluate was collected and centrifuged at 4°C, a 200g 5 min. The supernatant was recentrifuged a 1500g 10 min. The pellet tachyzoites were ressuspended in Dulbecco's modified MEM containing 10% FCS, with determination of cell viability by Trypan Blue and the number of reaminig contaminant host cells. Only batches with less than 1% of mice cell contamination were used.

Irradiation: The purified tachyzoites were irradiated with 50, 100 and 200 Gy in Gammacell 220(Atomic Energy of Canada) at a doses rate of 366 Gy/h, with temperature monitored and in presence of atmospheric oxygen. After irradiation, parasites were maintained in 4°C until use. Samples were observed in phase contrast for motility, incubated with Trypan Blue for viability, stained by Giemsa, for morphology and, after glutaraldehyde fixation, processed by electron microscopy. Aliquots were stored in 4°C for a few days.

In vivo assay of virulence and infectivity: C57Bl/6j mice were inoculated ip with a Poisson distribution of irradiated or controls parasites, and its survival and parasites in peritoneal exudate monitored weekly. After four weeks, tail blood was collected for specific antibody detection.

In vitro assay of reproductive capacity: LLC-MK2 cells, a susceptible epithelial-like established cell culture, was grown in Dulbecco MEM at a monolayer in plasticware. For *in vitro* assays, the cells were grown in 96 wells plates, or in 75 cm^2 plastic bottles for long term culture. Poisson adjusted parasite concentrations were then seed over the monolayers. The characteristic cytopathic effect of the agent, a destruction of the monolayer, was monitored each day. For long term culture, the cells were seed at 1/10 initial concentration and observed for 21 days. Supernatant media was applied to glass slides, stained with Giemsa, and carefully observed three times a week.

III. RESULTS

The parasites maintained its usual morphology and movement after the three irradiation schedules. No significative changes occurred as related to irradiation strength. The integrity of the agents was also shown in electron microscopy, as shown in fig. 1, where intact parasites could be seen, presenting the usual conoid structure and without any evidence of nuclear or cellular damage. The nucleus of the cell presented also no clumped chromatin or other evidence of apoptosis.

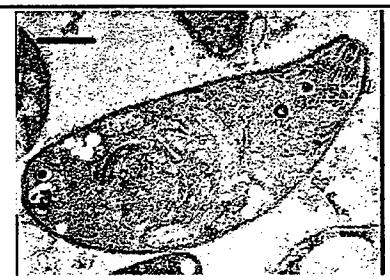

Fig.1: Electron micrography of a tachyzoite irradiated with 200 Gy, maintaining its organelles and internal structures. Bar represents 1μm.

The *in vivo* assays were performed using groups of five mice injected with 10^5, 10^4, 10^3, 10^2 and 10^1 irradiated or control parasites. All mice injected with irradiated parasites or with 10^2 and 10^1 intact parasites survived without clinical evidence of disease, contrasting with all deaths that occurred in higher inoculate($>10^2$). Based in these data, we defined 10^3 as a lethal dose of the agent. We performed an indirect immunofluorescence assay in blood sampled 28 days after the inocula, showing that most irradiated injected animals presented few if any specific antibodies in its sera.

The *in vitro* assay was performed in monolayers of LLC-MK2 cells, showing that control parasites killed all the cells in one week, with characteristic cytopathic effect, despite of lower concentrations including 10 parasites/well.

When irradiated samples were tested, in the multiwell assay, a progressive direct effect could be seen, with few parasites surviving in the 50 Gy irradiated parasites, rare parasites surviving at 100 Gy. Quantification was difficult to perform, but a rough estimate showed that 50 Gy furnishes a surviving reproductive population around 0.1% and 100 Gy around 0.001%, but the confidence intervals were quite higher, avoiding further analysis. Those data were also confirmed in a whole bottle assay.

The 200 Gy irradiated parasite did not present reproductive capacity tested in both assays, allowing an estimation of less than 0.00001% surviving population, practically achieving sterility.

IV. DISCUSSION

Our data shown that tachyzoites of *T.gondii* presented a relatively high resistance to gamma irradiation, with 200 Gy requirement for complete sterility. This fact was similar in magnitude with those found by others authors, but without adequate testing, using only experimental animals as a probe[9,10,11]. The short-term structure and physiology of the parasite appears intact, as shown by viability testing and ultrastructural findings. Interesting, others authors found a lower resistance of those forms, ascribing this to this intense reproductive activity[6], but our data are quite similar to those found for cysts, a quiescent form of the agent. The morphology of the parasite after irradiation suggests that an indirect phenomenum, like radio induced apoptosis, are not the main damage inducing the late cell death.

Our data suggest that *in vivo* assays using mice in order to evaluate adequate changes on viability of *T.gondii* is a bad approach, as elsewhere suggested[6] due to possible low virulence of the strain or better host immune response[6]. The LLC-MK2 cell monolayers are better form to detect this fact, both for viability or reproduction. Moreover, we can analyze the reproductive status of the parasite both in terms on destruction of monolayer or its detection in supernatant media.

Gamma radiation (^{60}Co) induces changes on *T.gondii*, leading to decreased or abolished reproduction, but maintaining viability, and probably physiology, of these parasites. Other attempts, using inactivated or chemically treated antigens, resulted in lower or no response (our data not shown). These data suggest that efficient immune response to *T.gondii* depends of its viability, maintaining its physiology until complete recognition by the host immune system.

Thus, ^{60}Co gamma rays seems a better tool to produce a toxoplasmosis vaccine with special focus to maintenance the viability, lack of infectivity and immunogenicity.

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