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Gamma irradiation of *Toxoplasma gondii* protein extract improve immune response and protection in mice models

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ABSTRACT

Gamma radiation induces protein changes that enhance immunogenicity for venoms, used in antivenin production. *Coccidian* parasites exposed to gamma radiation elicit immune response with protection in mice and man, but without studies on the effect of gamma radiation in soluble acellular extracts or isolated proteins. Toxoplasmosis is a highly prevalent coccidian disease with only one vaccine for veterinary use but with remaining tissue cysts. Total parasite extracts or recombinant proteins used as immunogen induce usually low protection. Here, we study gamma radiation effect on *T. gondii* extracts proteins (STAG) and its induced immunity in experimental mice models. By SDS-PAGE, protein degradation is seen at high radiation doses, but at ideal dose (1500 Gy), there are preservation of the antigenicity and immunogenicity, detected by specific antibody recognition or production after mice immunization. Immunization with STAG irradiated at 1500 Gy induced significant protection in mice immunized and challenged with distinct *T. gondii* strains. In their blood, higher levels of specific $CD19^+$, $CD3^+CD4^+$ and $CD3^+CD8^+$ activated cells were found when compared to mice immunized with STAG. Irradiated *T. gondii* tachyzoites extracts induce immune response and protection in mice in addition, could be a feasible alternative for Toxoplasma vaccine.

1. Introduction

Biological effects of gamma radiation are usually related to lethal genotoxic aspects, as in nuclear accidents or sterilization [1]. Nucleic acids effects are prominent and occur in lower doses than in other biological compounds as proteins. Vaccines involving gamma radiation of intact organisms were designed for malaria caused by Plasmodium, a coccidian parasite. X-rays irradiated mosquitos previously infected with *P.berghei* induced significant protection in mice [2] and irradiated sporozoites, the infective form of the agent, has been proposed for human use [3]. *Toxoplasma* tachyzoites gamma irradiated produce a protection in mouse models [4,5].

Subcomponent proteins and not intact organisms compose vaccines for several diseases but they need adjuvants for promoting adequate immunity and protection. Ionizing gamma radiation on isolated or mixed proteins solutions were used to improve the production of antisera against snake venom [6] and isolated recombinant proteins immunity [7]. These effects were attributed to radicals produced in the radiolysis of water by gamma radiation and not a direct effect on the structure of the protein [8]. Ionizing radiation promote the formation of poorly soluble protein aggregates [6], which can be compared to some Alum-type adjuvants that act as slow delivery systems [9]. Effective subcomponent protein vaccines require the combination of strategies such as delivery systems and adjuvants for protective, adequate and efficient immune response. The adaptive immune response to protein antigens are initiated by their uptake by antigen-presenting cells which are unable to bind most soluble proteins, which explains why isolated proteins induce a weak immune response [10].

Adjuvants are compounds that promote low solubility of the antigen and inflammation at the injection site, with influx of neutrophils and APCs, thus allowing the contact of proteins with immune cells [11]. To activate the immunogenicity of the proteins present in a vaccine, adjuvants mostly induce an acute inflammation with influx of activated neutrophils [12]. Neutrophils are potent sources of oxidants such as

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hypochlorous acid (HOCl) and induce the oxidation of proteins present at the site of inflammation [13].

Toxoplasmosis is a widely distributed infection caused by the intracellular parasite *Toxoplasma gondii* [14]. One-third of the world's human population has specific antibodies, which makes this parasitic infection successful, usually asymptomatic but with infrequent eye disease [15]. Acute maternal infection can cause damage or even death of the fetus and patients with defective immune response, as HIV or transplanted patients, reactivation of a latent infection may induce severe encephalitis [16]. To date, the only available vaccine for the control of toxoplasmosis is based on the attenuated live strain S48, restricted to ovine vaccination [17].

However, as a short-lived live vaccine, it causes protection but also remaining cysts in lamb of vaccinated animals, unfit for human consumption [18]. Irradiated *T. gondii* tachyzoites induces a good protection in mice with efficient humoral and cellular immune response [4,19], but there are no studies on irradiation of protein extracts of those agents for inducing protective immunity. Protein extracts are much easier to handle than irradiated intact parasites that require more sophisticated forms of preservation and short half-life [4]. Untreated tachyzoites extracts demands adjuvants for inducing partial protection as reported [20] and isolated or associated recombinant proteins induced conflicting results, also demanding adjuvants for any protection [21]. In order to study if gamma irradiation of soluble protein extracts could have the same immunity inducing effect seen in snake venoms and recombinant proteins, we study the protection and immune response of mice immunized without adjuvants with gamma irradiated *T. gondii* tachyzoites soluble protein extracts.

2. Material and methods

2.1. Parasites and SPF isogenic mice

Parasites were *T. gondii* RH and ME-49 strains maintained as elsewhere described [5] and experimental SPF mice were bought from our colony of the Faculty of Medicine of the University of São Paulo and kept in adequate conditions according to Experimental Animal Use Committee of Tropical Medicine of the University of São Paulo (CPE-IMT 2012/115).

2.2. Soluble antigen of Toxoplasma gondii (STAG)

The soluble extract was prepared as elsewhere described [4]. Briefly, tachyzoites were filtered through in 5 µm polycarbonate filter (Millipore, Massachusetts, EUA), recovered by centrifugation, adjusted to 10^8 tachyzoites/mL in water and sonicated for complete cell lysis. After addition of one volume of 0.3 M NaCl to the lysate, the suspension was cleared by centrifugation at 10.000 *g* for 15 min at 4 °C, and supernatant defined as soluble tachyzoites antigen (STAG). The protein concentration was determined by the Bradford method and adjusted to 0.1 mg/ml and stored frozen (−70 °C) until the moment of use. STAG were irradiated at 250, 1000, 1500, 2000 and 4000 Gy from Cobalt-60, using a GammaCell™ (Atomic Energy, Canada Ltd.) at a dose rate of 1.03 Gy/h, in the presence of oxygen. For molecular weight profiles and immunogenicity, STAG proteins were separated in 12.5% SDS-PAGE and stained or transferred to nitrocellulose membranes as elsewhere reported [22,23]. Transferred proteins were reacted with sera from ME-49 strain infected animals for 18 h, 4 °C and bound antibody was detected by commercial peroxidase-conjugated anti-mouse IgG (Sigma-Aldrich, St. Louis, Missouri, USA) for 60 min, with detection by adding 3,3',5,5'-Tetramethylbenzidine (TMB) for membrane (Sigma-Aldrich, St. Louis).

2.3. Immunization and challenge with viable parasites

Groups of 05 BALB/c mice were immunized with 3 biweekly s.c doses of 20 µg of each STAG type in PBS. No mice were irradiated only the antigen were irradiated but non-radioactive. Blood and Serum was collected before the immunization and 15 days after the last immunization for immunological studies. The blood samples were obtained by sectioning of the tail end of the immunized and control BALB/c mice, collected on filter paper dried and, stored at −20 °C. Elution was performed by the addition of 300 µL of buffer containing 10 mM Tris/HCl pH 7.5, 150 mM NH4Cl and 10 mM NaN3 for erythrocytes lysis and conversion of hemoglobin to stabilize cyanohemoglobin [24]. After elution, sample absorbance 540 nm were determined and blood content adjusted for 1:100 dilution. For challenge, we used both models of acute or cystogenic oral infection as elsewhere reported [5]. For acute infection, 10^3 RH tachyzoites were injected intraperitoneally in mice, and symptoms and survival were observed daily until 21 days after infection, with data compared by Log-Rank test. Cystogenic ME49 *T. gondii* challenge was performed orally with 10 cysts from the brains of chronically infected mice. After 30 days of infection, the mice were euthanized, brains removed and homogenized in 10 mL of sterile saline. Cyst counts were determined in phase contrast microscopy. DNA from homogenate was extracted and the absolute number of parasites determined by real-time PCR using sense B1JW63 and antisense B1JW62 primers for the B1 gene [25] and Power SYBR Green® PCR Master Mix (Applied Biosystems, Foster City, California, EUA) according to the manufacturer's instructions in a real-time PCR System®7500 (Applied Biosystems California, EUA)

2.4. Immune response determination in immunized mice

Total antigenic extract of *T. gondii* tachyzoites (1 µg protein/ml) was adsorbed in 96-well, high affinity ELISA plates (Costar, Washington, D.C., EUA), incubated with dilutions of serum samples from immunized mice; bound antibody detected by peroxidase-conjugated anti-mouse IgG (Sigma-Aldrich, St. Louis), developed with commercial TMB solution (Sigma-Aldrich, St. Louis). After the 30 min, the reaction was stopped by addition of 4 N HCL. The absorbance readings were performed on a Multimode microplate reader Microplate Reader Filter Max FS5 (Molecular Devices®, Sunnyvale, California) in the absorbance filter 450 nm. The reactivity index of the samples was determined as the ratio of the absorbance from each samples by the mean absorbance of the negative control samples, used for intratest quality control for conjugate variation. (IR = Abs of positive samples/mean of Abs. of negative samples). IgG avidity was determined by using a 6 M Urea washing after IgG binding and before conjugate incubation. Avidity was expressed as percent of Urea resistant IgG.

Cell phenotyping was determined in spleen and blood cells (2×10^6 cells/well) stimulated by total extract antigen of *T. gondii* tachyzoites (20 µg/mL), cultured for 6 days in 24-well plates, as elsewhere reported [26]. Splenic and blood cells were purified by Ficoll-Paque™ Premium 1084 (GE Healthcare, Little Chalfont, UK) and labeled with Carboxyfluorescein succinimidyl ester (CFSE, BD Biosciences, Franklin Lakes, New Jersey, EUA), according to the manufacturer's instructions. Phenotypes were determined by incubation with adequate anti-mouse monoclonal antibodies: anti-CD3-Pacific Blue (BD Biosciences, New Jersey, EUA), anti-CD4 V500 Horizon (BD Biosciences, New Jersey, EUA), anti-CD8 APC-H7 (BD Biosciences, New Jersey, EUA), and anti-CD19 PE-Cy7 (BD Biosciences, New Jersey, EUA), according to the manufacturer's instructions, with adequate controls and submitted to a Fortessa Flow cytometer. The data were collected (50.000 events) and analyzed both by the BD FACSDIVA® software for acquisition and by the FlowJoX® software for analysis. All samples were compensated using cells from non-immunized animals. After selecting the lymphocyte population, we defined the singlet population to exclude debris and doublets. From the singlet population we separated the proliferated $CD19^+$ and $CD19^-$ populations (B cells). Of the population $CD19^-$, we determined the populations of T cells ($CD3CD4^+$ and $CD3CD8^-$).