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255Gy IRRADIATED TACHYZOITES OF *Toxoplasma gondii* INDUCE INTESTINAL IMUNE RESPONSE IN C57BL/6J MICE IMMUNIZED BY ORAL ROUTE

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ABSTRACT

Toxoplasmosis, a prevalent widespread infection in man and animals, occurs mainly through ingestion of water and food contaminated with oocyst from cat feces, causing usually benign disease in humans, except in intrauterine fetal infection or in immunodeficient patients. We study the oral route for the development of a vaccine for toxoplasmosis, using parasites irradiated with 60 Cobalt, as an alternative for vaccine development to this worldwide parasitic infection. We evaluated the development of immunity at serum or mucosal levels, and their efficiency in protect the mice against challenge with oral cysts of the ME-49 strain. C57Bl/6j isogenic mice were immunized by oral route with 10^7 255 Gy irradiated tachyzoites from RH strain, at several protocols using milk as anti-peptic adjuvant and alum hydroxide as antacid. The preparations of irradiated tachyzoites induced production of serum IgG and IgA in immunized mice, as determined by ELISA, with IgG2a as the dominant subclass, similar to chronic infection. Their use with adjuvant allowed the excretion of significant amounts of IgA in stools also IgG, despite a lesser extent. All oral preparations induced some quantitative protection against challenge, which was similar to the parenteral route only isolated alum hydroxide was used as adjuvant. All these data support the possibility of the development of an oral vaccine against toxoplasmosis, using irradiated tachyzoites, which would be possible tool in near future for use in field baits, for immunizing either domestic or wild felids.

1. INTRODUCTION

Toxoplasma gondii is an apicomplexan protozoan, which can cause abortion or congenital birth defects in humans. Infection is usually asymptomatic in man with normal immune function, with occasional eye involvement [1]. Toxoplasmosis can cause severe disease in fetus of pregnant acutely infected woman, immunocompromized (AIDS) and therapeutically immune suppressed patients, as cancer or transplant recipients [2]. The infection is acquired by ingestion of water and food contaminated with oocysts of feline feces or raw meat contaminated with tissue cysts [3]. Once ingested, the cysts wall is digested within the lumen of the small intestine, and then the parasite infects the epithelial cells from which it is disseminated to other organs throughout the host, in particular, muscle and the central

nervous system [4]. To date no vaccine has been commercialized in the field of human parasitology, with some vaccines developed for veterinary use, but with low efficiency [5]. Several models were developed in mice, using different antigens and routes, all of then with conflicting results [6].
T. gondii irradiated tachyzoites inoculated by intra peritoneal route induced protection, with immune response similar to the chronically infected mice, with resistance to challenge [7]. In this work, we used irradiated parasites in the study of the intestinal immunity, which is the main infection way of infection by *T. gondii*, either for the felines as definitive hosts, or the intermediate hosts, the mammalians and birds, including the man, analyzing both the immune response and protection, key steps for the vaccine production.

2. MATERIALS AND METHODS

All reagents and conjugates were purchased from Sigma Co (St. Louis) and solutions were prepared with high quality water (Milli-Q®).

2.1. Parasites and animals

Two strains of *T. gondii*, RH and ME-49, cryopreserved and maintained by successive passage in mice (Protozoology Lab., Tropical Medicine Institute of São Paulo) were used in this study. RH strain was maintained routinely by intraperitoneal (i.p.) passage in outbreed mice. ME-49 strain was kindly donated by Prof. Dr. Fausto Araújo, UCLA, and was also kept serial passage in C57Bl/6j mice-oral gavage. Isogenic C57Bl/6J mice, were obtained from our colony (Centro de Bioterismo/FMUSP), and maintained in sterilized cages and absorbent media, with commercial food (Nuvital®) and water "*ad libitum*". The management of these animals before or during the experiments was according of "Principles of Laboratory Animal Care" (NIH Publication n^{o} 86-23, revised 1996) and the "Principles of Ethics in Animal Experimentation" (COBEA-Colégio Brasileiro de Experimentação Animal).

2.2. Irradiation and immunization

The tachyzoites suspensions of *T. gondii* (RH strain), maintained in ice-cold baths, were irradiated at 255Gy (TX), in uniform source of ^{60}Co γ-rays (GAMMACELL™, Atomic Energy of Canada, Ltd.), in the presence of oxygen and dose rate of 6,41 kGy/h. The group control was maintained outside the source and after irradiation, the viability of samples was determined by Trypan Blue exclusion, being used only those with >95% viable parasites [7]. Groups of 5 mice were immunized with 1, 2 and 3 doses of 10^7 irradiated tachyzoites, suspended in pure defatted bovine milk, as anti-peptic solution or in aluminum hydroxide 3% suspension as antacid or a mix 1:1 of both vehicles, and immediately administered by oral gavage for each individual mouse. Blood samples was collected from tail of the mice in standardized filter papers that absorbs 5ul of blood, dried at room temperature and stored -20º C until use. Antibodies were recovered by extraction with 100µl PBS for 18h a 4^{o}C. The soluble extract was recovered by centrifugation and considered a 1/20 dilution of blood. Fecal sample were collected from groups mice, at the tree days intervals. The suspension, extracted with PBS, was vortexed and centrifuged for 15min. at 13000*g* [8]. All samples collected were stored at -20^{o}C until use.

2.3. *Toxoplasma gondii* antigen preparation and ELISA for antibody detection in serum and intestinal secretions

Tachyzoites of the RH strain were harvested from mice peritoneal cavity of previously infected mice in phosphate buffered saline (PBS), recovered by centrifugation, washed, counted and submitted to sonication (Sonic Dismembrator, Quigley-Rochester Inc., USA) for several periods of 4 cycles/30 seconds in an ice bath, until all parasites were destroyed, by phase contrast microscopy. The solution was isotonized by 0,3M NaCl addition (v/v) and cleared by centrifugation at 10000*g* for 3min. The protein content of the supernatant was determined [9] and aliquots were maintained at -70^{o}C [10]. Enzyme immunoassays were performed in 96 well microtration plates. The wells were coated overnight at 4°C with 100µl of carbonate buffer pH 9.6-0,1M containing 10µg of antigen of *T. gondii*. After three washes with PBS-0,05% Tween 20, remaining binding sites were blocked for 1h at 37°C with 200µl of PBS + BSA 2%. After washing, 100µl of the serum or feces extracts of mice were added and plates were incubated for 1h at 37°C. After washing above, 100µl per well of anti-mouse IgA and IgG peroxidase conjugate (Sigma®). After washing, the reaction was revealed by the addition of 100µl OPD (o-phenylenediamine 1mg/ml) for 30 min. at room temperature. The enzymatic reaction was stopped by 200µl per well of 4N HCl solution. The reading was done spectrophotometrically (Labsystems Multiskan MS®) at 492nm [11]. For IgG subclasses, serum dilutions, 100µl, were added to each well and the plates were incubated for 1h at 37°C. After additional washing with PBS-T, bound IgG were detected by incubation for 1h with peroxidase-conjugated anti-mouse anti-IgG1, anti-IgG2a and anti-IgG2b (Southern Biotechnology Associates®) at 37^{o}C, followed by 5 washings with PBS-Tween. The bound conjugate were developed with 100µl per well of OPD (o-phenylenediamine 1mg/ml) by 30 min., stopped with 100µl per well of citric acid 0,2M, and the absorbance read at 450nm in an ELISA reader (Dynatech MR4000®).

2.4. Challenge and histopathology of immunized mice

Tissue cysts of *T. gondii ME49 strain* were obtained from the brains of chronically infected C57Bl/6j. Brains were homogenized in 30% dextran in Hanks's balanced salt solution (HBSS). This mixture was centrifuged at 3000*g* for 10min. at 4ºC and the pellet was re-suspended in HBSS [12]. Tissue cysts were counted at optical microscope. The immunized mice were challenged after 15 days from the last dose with 10 cysts by oral route. The control with normal C57Bl/6j mice were challenged with the same quantity of cyst. The mortality of the animals was followed daily. After 30 days, all mice were killed and the brains were separated for histology and cyst counting. Half brain was homogenized in ten volumes of saline solution and was observed for cysts in at least 0.1 ml under phase contrast microscopy, allowing the estimation of number of cysts/ brain. Remaining intact brain tissue were fixed in several changes of phosphate buffered (pH 7,2) 4% formaldehyde. Those organs were included in paraffin, and 5µm thick section were stained with hematoxylin-eosin. When necessary, representatives filed were photographed in a Zeiss Axiophot microscope, with planapochromatic optics.

3. RESULTS

The IgA and IgG production in the serum was clearly seen after the first oral dose, increasing thereafter, more intense in the IgG response, but immunized mice produced smaller levels of serum antibodies as compared to chronically infected mice (Fig. 1).

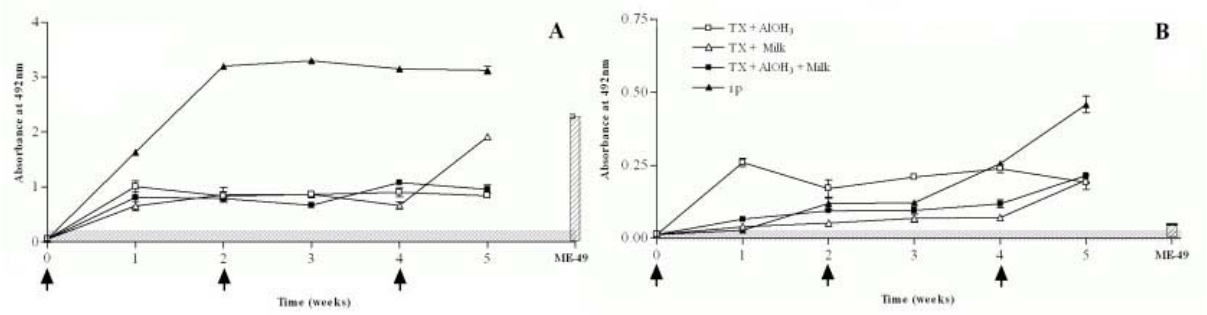

Figure 1 – Specific anti-*Toxoplasma gondii* IgG(A) and IgA(B) antibody in C57Bl/6j mice serum immunized with 1, 2 and 3 doses with 10^7 tachyzoites RH strain, 255 Gy irradiated (oral route) with aluminum hydroxide ($TX+AlOH_3$) and/or suspended in bovine milk (TX+Milk), detected by ELISA. Days of immunization procedures are indicated by black arrows.

The levels of IgG subclasses were analyzed by ELISA to determine the proportion produced in the immunized groups with irradiated tachyzoites, with different adjuvants. The group immunized i.p. and the group with ME-49 presented the higher level of 3 classes analyzed. The groups immunized presented a increase in the level of IgG2b subclasses, as observed in the control groups (Fig. 2).

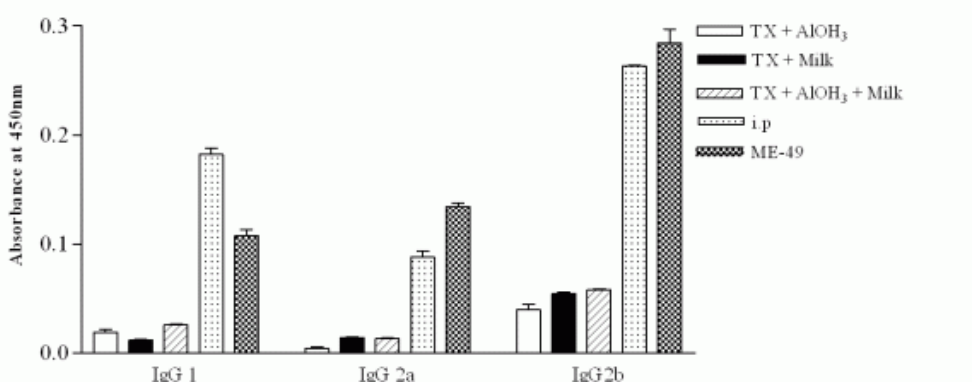

Figure 2 – Evaluation of IgG subclasses produced in C57Bl/6j in response to oral immunization with irradiated tachyzoites of *T. gondii*, in different adjuvants or i.p. (3doses).

Fecal IgG production was absent after immunization but IgA production was higher than controls during the first doses but decays after this, suggesting tolerance, with chronically infected mice presented higher levels of IgA in feces (Fig. 3).

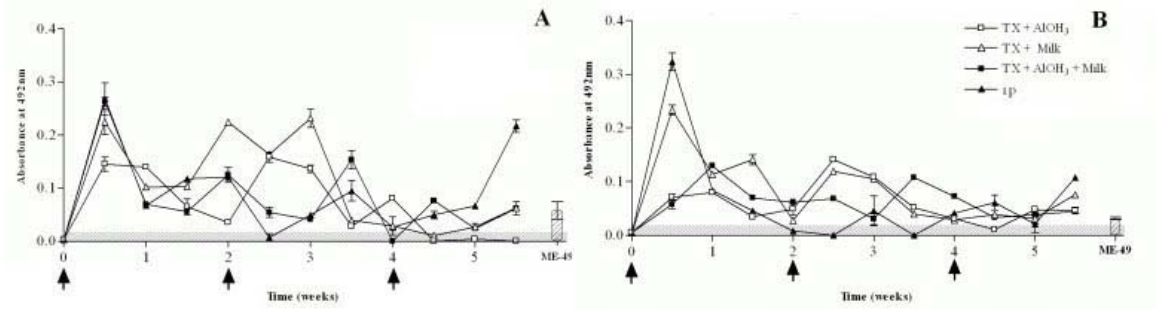

Figure 3 – Specific anti-*Toxoplasma gondii* IgG(A) and IgA(B) antibody in C57Bl/6j mice feces immunized with 1, 2 and 3 doses with 10^7 tachyzoites RH strain, 255 Gy irradiated (oral route) with aluminum hydroxide ($TX+AlOH_3$) and/or suspended in bovine milk (TX+Milk), detected by ELISA. Days of immunization procedures are indicated by black arrows.

To evaluate the vaccine efficiency, the immunized groups were challenged with 10 cysts of ME-49 strain by oral route. Only in the control group occurred the death of 1 animal after 13 days from challenge, which presented large numbers of cyst in brain, showed in squash preparation on phase contrast microscopy. The immunized groups did not show any death and all mice presented some protection after challenge, when they were compared with the non-immunized group ($P<0.001$), with the higher protection occurring in the group immunized by parenteral route and in the group immunized with aluminum hydroxide ($P<0.001$), the difference of the cyst numbers were higher and less cysts were found in the brain, usually less than 500 brain cysts/animal and similar to i.p. immunized mice (Fig. 4A). The brain histopathology of immunized animals with irradiated tachyzoites and challenged with 10 cysts of ME-49 strain, showed similar protection found in the quantitative studies of cyst brain load (Fig. 4).

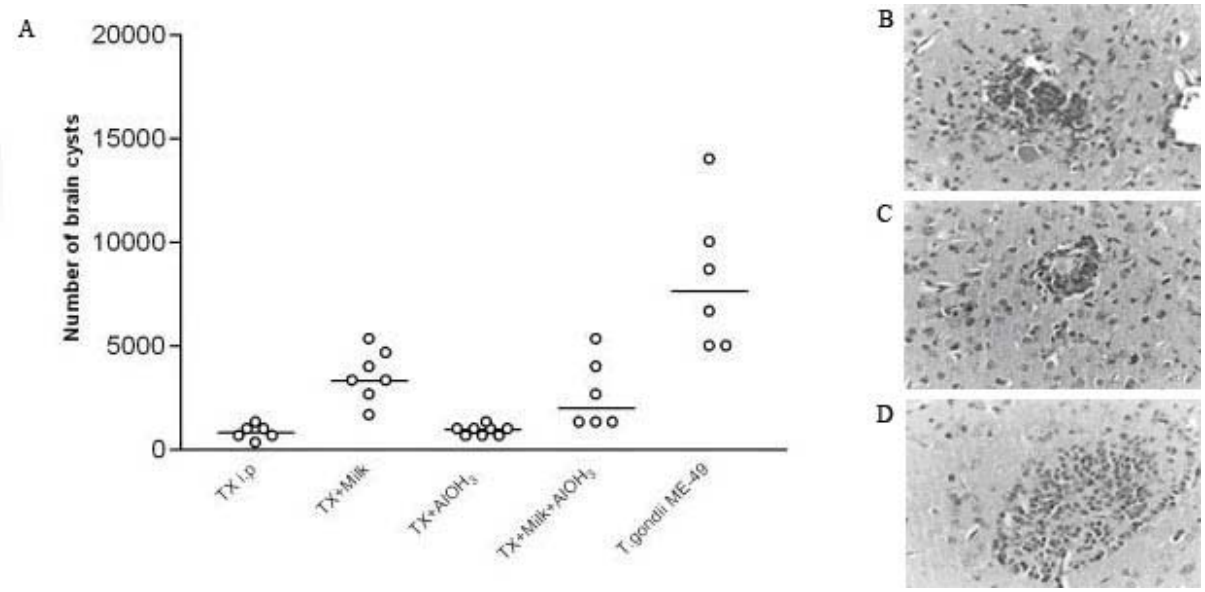

Figure 4 – (A) Number of brain cysts, after 30 days from oral challenge with 10 cysts of ME-49 strain, in mice immunized with differents doses and immune project. Brain histopathology of mice challenged with ME-49 strain, without immunization (A). Immunized by parenteral route (B) or immunized by oral route with aluminum hydroxide as adjuvant (C).

4. DISCUSSION AND CONCLUSIONS

The use of antacids or protein in order to enhance the survival of the agent at stomach level was demonstrated by the 100% killing of mice groups, with acute disease of few days duration, in experiments when aluminum hydroxide or protein competitor as bovine milk are used as vehicle for non-irradiated viable tachyzoites. This was expected by the increased virulence when mice was challenged with milk or home-made cheese contaminated with viable tachyzoites [13].

When those vehicles are used for irradiated tachyzoites, no infection or deaths was present as expected and a clear humoral response could be seen, showing that those tachyzoites became antigenic to those mice, despite an lower titer of antibody after the 3rd oral dose, that could be ascribed to an tolerance effect, as suggested by others in oral vaccines [14]. When stools are used as antibody source, low levels of IgG was found, similarly to previously reported data dealing with *T. gondii* antigen associated to cholera toxin [15]. When IgA was tested in

those samples, the production was observed only in the first two doses of oral challenge, mostly on preparations with antipeptics.
The IgG subclasses results showed similar levels of production of each subclass, both in parenteral vaccinated mice or natural infection, which showed similar immune response in this model as natural infection. When oral route of vaccination was analyzed, the production was IgG subclasses was much smaller, but present, especially when alum hydroxide is used as adjuvant, which could be attributed to a protection to the acid environment of the gastric juice.
When we submit those immunized animals to oral challenge with cysts of ME-49 strain, we note that all groups, both oral or parenteral vaccinated, presented some protection to the infection, as compared to the control group. Again, we also observed a higher efficiency of aluminum hydroxide as adjuvant, resulting in protection similar to parenteral vaccine. This data is not associated with higher levels of serum IgG subclasses, despite the higher levels found in this model, but much lower than the obtained in parenteral vaccination [7]. Thus the protection must be related in some order to antibody production but other factors could also be involved in this process. Interestingly, the best protection showed the higher levels of IgG2a, usually associated to a Th1 immune response, usually associated with a cytotoxic CD8 response [16].
All this data show the possibility of development of an oral vaccine to toxoplasmosis, which much more feasibility than parenteral ones. This vaccine could be useful as attractive baits in the environment to affect the free living cats, promoting a decrease in excretion of oocysts by cats and reducing the environment transmission of toxoplasmosis, in an approach ecologically correct, without the risks of direct intervention in the human populations.

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