

HUMORAL IMMUNE RESPONSE OF C57Bl/6j AND BALB/c MICE IMMUNIZED WITH IRRADIATED TACHYZOITES OF *Toxoplasma gondii* RH STRAIN AND ORAL CHALLENGE WITH ME-49 STRAIN

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ABSTRACT

Toxoplasmosis, a prevalent widespread infection in man and animals, is mainly transmitted by oral route, through ingestion of oocysts from water and food contaminated with cat feces or infected animal tissue cysts in undercooked meat. Vaccine development implies in effective intestinal immunity, the first site of parasite entry. Radiation (255Gy/⁶⁰Co) sterilized *T. gondii* RH strain tachyzoites (RST) induced significant protection when parentally administered, similar to chronically infected and acute disease protected animal. We study the humoral immune response in C57Bl/6j and BALB/c mice immunized with 10⁷ RST, by oral (with aluminium hydroxide 3%) or parenteral 3 biweekly administrations. *T. gondii* antigens specific ELISA for IgG, IgA, IgG1, IgG2a and IgG2b detection was performed in weekly blood samples during immunization. Also we evaluate of the intestinal epithelial of immunized mice the integrity of the radiated parasites by electronic microscopy. After 2 weeks, immunized and control animals were challenged with 10 cysts of ME-49 strain p.o. Protection was determined at the 30th day by brain cyst counting. As it was possible to observe in the intestinal mucosal, the aluminium hydroxide seems to maintain unchanged the parasite morphology and its mechanisms of invasion, probably due to keeping it safe from extreme pH condition of stomach. All immunized groups presented significant protection when challenged with ME-49; however, BALB/c mice showed better protection levels, with only one positive animal on brain microscopic analysis. IgG production in the serum of the animals was higher in groups immunized by i.p route, however, IgA and IgG1 levels were higher in BALB/c mice immunized by oral route. This higher protection found in BALB/c group could probably also be related to the Th2 response, demonstrated by higher IgG1 levels. All these data provide insights in oral immunization schedules for toxoplasmosis prevention, suggesting that oral vaccines could be an alternative in the prevention of toxoplasmosis and the block of chain transmission.

1. INTRODUCTION

Toxoplasma gondii is an apicomplexan protozoan, which can cause abortion or congenital birth defects in humans. Toxoplasmosis can cause severe disease in fetus of pregnant acutely infected woman, immunocompromized (AIDS) and therapeutically immune suppressed patients, as cancer or transplant recipients. The infection is acquired by ingestion of water and

food contaminated with oocysts of feline feces or raw meat contaminated with tissue cysts [1]. 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 [2]. To date no human vaccine has been development, with some vaccines developed for veterinary use, but with low efficiency [3]. Several models of vaccines were developed in mice, using different antigens and routes, with conflicting results. *T. gondii* irradiated tachyzoites (RH*) inoculated by intra peritoneal route induced protection, with immune response similar to the chronically infected mice, with resistance to challenge [4]. 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 the immune response and protection, key steps for the vaccine production.

2. MATERIALS AND METHODS

All reagents and conjugates were purchased from commercial sources, mainly of 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). 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 or Swiss mice-oral gavage. C57Bl/6J and BALB/c 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 no 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 (RH*), in uniform source of ⁶⁰Co γ -rays (GAMMACELL™, Atomic Energy of Canada, Ltd.), in the presence of oxygen and dose rate of 6,41 kGy/h [5]. Groups of 5 mice were immunized with 1, 2 and 3 doses of 10⁷ irradiated tachyzoites (i.p or v.o), suspended 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 -20°C until use. Antibodies were recovered by extraction with 100µl PBS for 18h a 4°C. All samples collected were stored at -20°C until use [5].

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

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 10000g for 3min. The protein content of the supernatant was determined and aliquots were maintained at -70°C. 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 + Milk 3%. After washing, 100µl of the serum 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 50µl per well of 4N HCl solution. The reading was done spectrophotometrically (Labsystems Multiskan MS®) at 492nm [6]. 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°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 of immunized mice:

Tissue cysts of *T. gondii* ME-49 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 3000g for 10min. at 4°C and the pellet was re-suspended in HBSS. 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 controls with normal 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 estimation of number of cysts/brain [7].

2.5. Eletron microscopy:

The intestinal sections of immunized mice were fixed in 1.5% glutaraldehyde solution buffered with 0.08M cacodylate buffer (pH 7,4). After 1h on ice the organ was centrifuged (1000g, 5min) and re-suspended in 1% osmium tetroxide. After 1h on ice, with occasional mixing, the samples had been processed for embedding in Araldite® [8]. Ultra-thin sections were contrasted stained with uranyl acetate, observed and micrographed in a Zeiss™ EM109 electron microscope.

3. RESULTS

Serum IgG and IgA production was detected by ELISA in different mice strains. Serum IgG production was higher in all i.p. immunized groups, showing an expressive increasing with the subsequent inoculations, as observed in Fig. 1A. When serum IgA response was evaluated in the same groups, was observed that the response was increased in oral route immunized group after the first inoculation, whereas in subsequent inoculations was noticed a low increased response in i.p. group, with similar response profiles in both groups (Fig. 1B). Considering C57Bl/6j mice, serum IgA response showed a higher humoral response development after the second oral route inoculation, what did not happen with BALB/c mice, which presented more efficient response when animals were oral route immunized, with a profile compared to i.p. group (Fig. 1D).

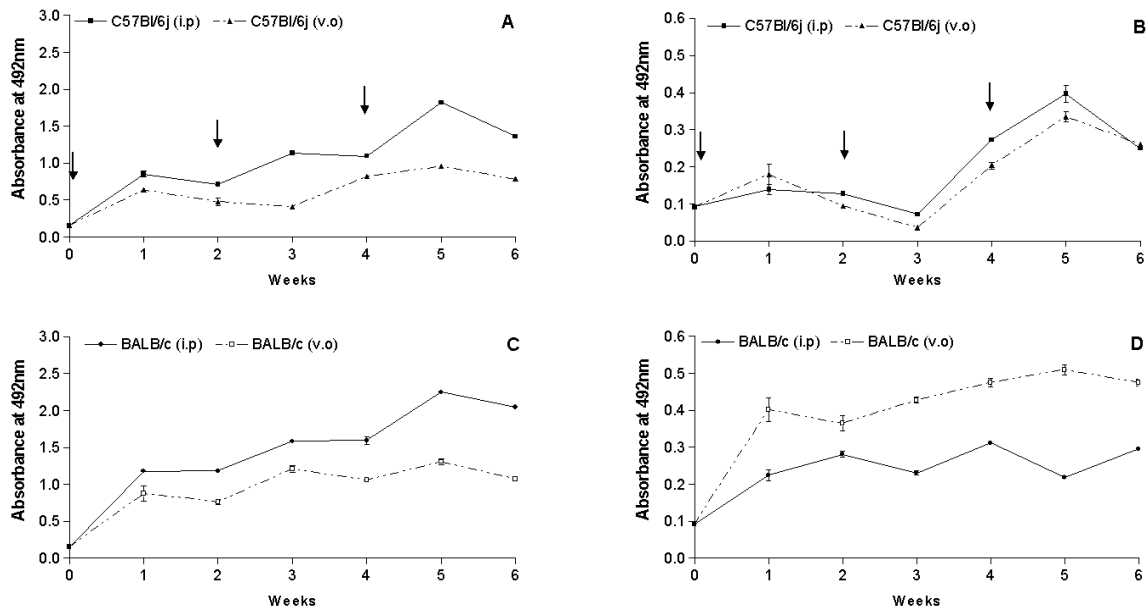


Figure 1. Specific anti-*Toxoplasma gondii* IgG(A/C) and IgA(B/D) antibody in C57Bl/6j and BALB/c mice serum immunized with 3 doses (i.p or v.o) with 10^7 tachyzoites RH strain, 255 Gy irradiated with aluminum hydroxide (oral route), detected by ELISA. Days of immunization procedures are indicated by black arrows.

The levels of IgG subclasses were analyzed by ELISA to determine the proportion produced in the immunized groups with irradiated tachyzoites, using different strains of mice. The BALB/c mice immunized i.p. presented the higher level both in IgG1 and IgG2a subclasses. The C57Bl/6j mice (i.p.) presented an increase in the level of IgG2b subclasses. In oral immunization the BALB/c mice showed an increase in the levels of IgG1 and IgG2b subclasses (Fig. 2).

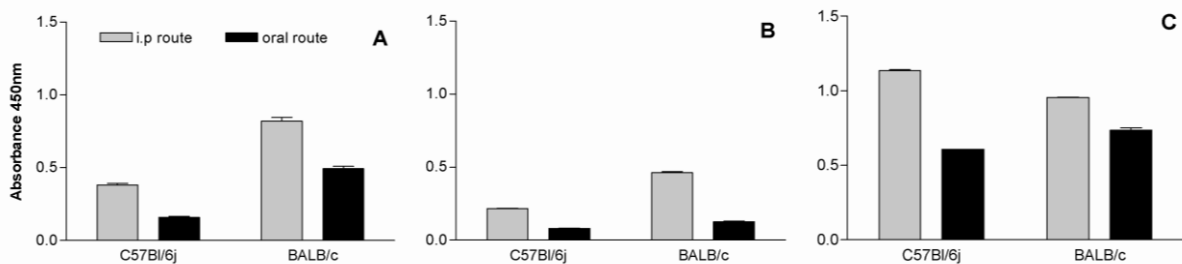


Figure 2. Evaluation of IgG subclasses, IgG1(A), IgG2a(B) and IgG2b(C), produced in C57Bl/6j and BALB/c mice in response to immunization (3 doses), with irradiated tachyzoites of *T. gondii*, in different routes.

Different protection levels were determined among mice strains, which were immunized with 255Gy irradiated tachyzoites by oral and i.p. routes. In C57BL/6j group we observed death rates of 40% in oral route immunized animals one week after challenging. In both immunized groups, we could notice over 50% protection when compared to control group and similar protection levels between the groups, as observed in Fig. 3A. BALB/c mice showed higher protection levels, which were over 90%, cysts were observed in only one animal of each immunized group, as observed in Fig. 3B.

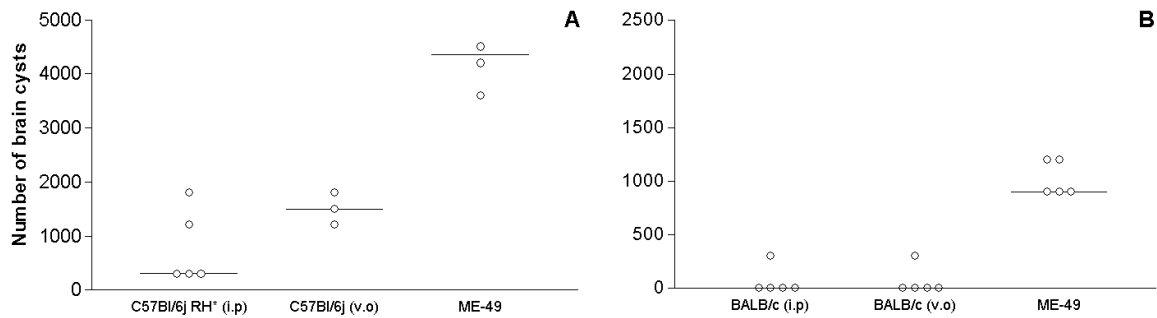


Figure 3. Number of brain cysts, after 30 days from oral challenge with 10 cysts of ME-49 strain, in mice immunized with different routes. C57Bl/6j mice (A) and BALB/c mice (B).

For study of the integrity of the vaccine preparation when passing through the gastrointestinal tract, evaluation by electronic microscopy was performed, as described in methods. In this assay we can notice that the administration of irradiated parasites, by oral route, didn't cause inflammation or necrosis of tissues the intestinal mucosa. The integrity of the microvilli and the enterocytes was observed during all evaluated period. As we can observe in the Fig. 4A, after 30 minutes of immunization with radiated tachyzoites, we notice that these already had penetrated in the intestinal epithelium and after 4h and 30 minutes the presence of early parasitophorous vacuoles was observed around the parasites, as can be observed in the Fig. 4B. These data suggest that the parasites were kept intact and viable during the transit for the digestive treatment, not suffering alteration for the action from any condition of digestive tract.

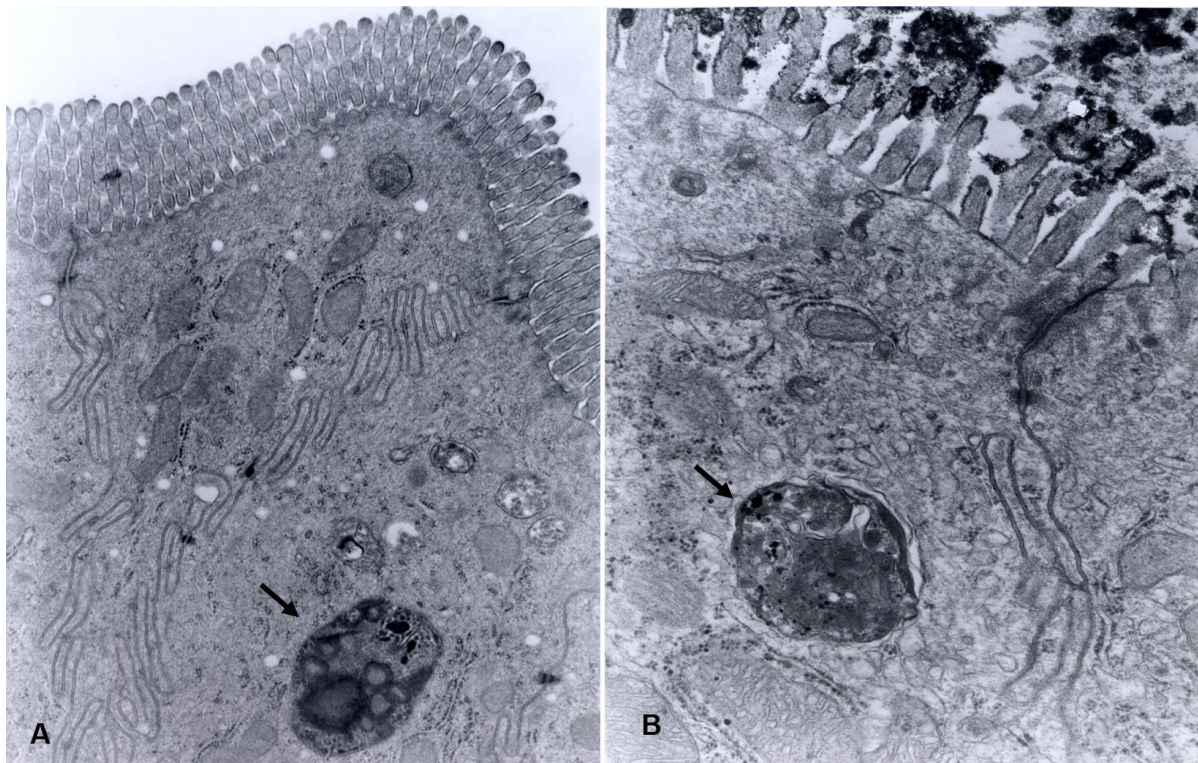


Figure 4. Electron microscopic of the intestinal section in C57Bl/6j mice, after oral immunization with irradiated tachyzoites (255Gy). (A) 30 minutes and (B) 4h and 30 minutes, after oral inoculation. Arrows indicates *T. gondii* parasites in intestinal epithelium.

4. DISCUSSION AND CONCLUSIONS

Serum IgG levels detected in different mice strains immunized with 255Gy-irradiated tachyzoites were present but lower in oral route immunized animals, as compared to parenteral challenged animals. These events had been already detected in C57Bl/6j mice when oral route immunized with irradiated tachyzoites [5]. Exposition to antigens on gut can induce humoral response, but with lower intensity if compared to parenteral route, as elsewhere described [9], specially related to IgG production. Serum IgA levels were produced by BALB/c mice challenged by oral route, with levels present since the first inoculation in oral route immunized animals. IgA was the main immunoglobulin for mucosal secretion and our data suggest that also serum IgA has an important role as one of the host defense lines against *T. gondii*. It is well known that serum IgA plays an important role in bacterial infections in blood, avoiding septicemia and disease [10]. In addition, immunization with *Giardia duodenalis* rCWP2 recombinant protein showed that serum IgG was very important on inhibition of cyst releasing, and then reducing disease transmission [11]. Serum levels of specific IgA could indirectly reflects the presence of this immunoglobulin in mucosal sites and luminal secretion, and could an alternative index for luminal protection.

The IgG subclasses results showed similar levels of production of each group of animals, but, as expected, parenteral-vaccinated mice presented the higher level of subclasses. When oral route of vaccination was analyzed, the production of IgG subclasses was present but smaller, but the differences were related only to the intensity of production, with the same subclass proportions.

When different immunized animal groups were challenged with cysts of ME-49 *T. gondii* strain, all groups presented partial protection if compared to control group. In all vaccination schemes, response was close to those we had already found by using C57Bl/6j mice, when i.p. or oral route immunized. However, BALB/c mice presented higher protection levels (over 95%) after challenged with cystogenic ME-49 strain. This higher protection in BALB/c is related to the higher levels of antibody induced by oral challenge, which could indirectly represents a shift to an Th2 immune response, under influence of higher IL-4 production than in C57Bl/6j mice, which similar response was more discrete, as well as lower serum antibody levels [12]. This fact must be carefully investigated, probably looking for cytokine in situ production or circulating levels, which have been reported as a specific role in of different immunization schedules groups, in order to evaluate the response and specific schedule which provides better efficiency against parasites when oral route immunization is applied.

Using electron microscopy, we could observe preservation of the parasites through the digestive tract probably due both to the use of the aluminum hydroxide used as vehicle for gastric juice protection but also the characteristic of parasitic membrane structure, which allows the detection of maintenance of viability signs as cell invasion and penetration, keeping all the conditions so that the tachyzoites are active and preserved, but without reproductive capacity, resulting in subsequent reproductive death [4]. This fact was also indirectly proven by the achieved serum and intestinal antibody produced for the mice oral immunized [5]. This data clearly show the arrival and cell invasion of irradiated tachyzoites by oral route, however, the success of the oral immunization imply in an effective presentation of antigens in for immune response induction in the intestinal mucosa (Peyer's patches and intestinal epithelium) [13], acting as mediator of communication between the lumen and the mucosal immune system.

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 in an attractive-bait form that could be spread in the environment to protect 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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