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200 Gy sterilised *Toxoplasma gondii* tachyzoites maintain metabolic functions and mammalian cell invasion, eliciting cellular immunity and cytokine response similar to natural infection in mice

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Abstract

200 Gy γ-irradiated *Toxoplasma gondii* RH tachyzoites failed to reproduce in vitro and in vivo. In short-term cultures, these parasites maintained a respiratory response, the ability to invade cells and preserved protein and nucleic acid synthesis. ELISA and Western blotting techniques demonstrated the similarity in humoral response between mice infected with γ-irradiated tachyzoites and animals infected with naive parasites and treated with sulfadoxine, higher than mice immunised with formaldehyde-killed tachyzoites. Splenocyte stimulation by *T. gondii* antigen produced lymphoproliferative response and cytokine profile (IL-10, IL-12, IFN-γ and TNF-α) similar to those produced by chronic natural infection. Mice immunised with irradiated tachyzoites had extended survival times after subsequent tachyzoite challenge, and displayed minimal cerebral pathology after cyst challenge. Irradiated tachyzoites lose their reproductive ability whilst maintaining metabolic function and may provide a novel tool for the study of toxoplasmosis and vaccine development. © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: Toxoplasmosis; γ-Radiation; Immunity

1. Introduction

Toxoplasmosis is a significant, life-threatening disease, infecting a large fraction of the population [1] as well as posing an economic problem with the loss of livestock, mainly sheep and goats [2]. This intracellular parasite, *Toxoplasma gondii*, has a complex life-cycle involving cats, both wild and domestic, as the definitive host, and other warm-blooded species, such as birds and mammals, including man, as intermediate hosts [3]. Transmission occurs via ingestion of raw or undercooked meat of the intermediate host, which contains cysts, or via ingestion of water contaminated with oocysts from cat faeces [4]. In humans, infection is generally benign with few symptoms, occasionally causing eye disease [5]. However, an acute infection can cause devastating disease in the foetus [1], AIDS patients [6], recipients of organ transplants [7] and patients receiving cancer therapy [8]. To date, there is no commercial vaccine for use in humans. One developed for veterinary use has low efficiency [2]. Several murine models were developed, using different antigens and routes, looking at specific antibody production and mouse challenge with conflicting results [9,10]. The immune response against *T. gondii* is complex, with quick and effective antibody production and cellular immunity. The latter is responsible for the elimination of infected cells via cytokine production and action on CD8 lymphocytes [11] or natural killer (NK) cells [12]. Specific responses of these cells, especially the production of IFN-γ, appears to be a crucial step in mounting an effective immune response [13]. A disrupted induced immunity could result in absent response or exacerbation of disease at challenge, and it has been suggested that the interaction of multiple antigens is necessary for an effective immune response [14].

Ionising radiation was used to sterilise *T. gondii* cysts in meat and oocysts on fruit and vegetables [15]. ^{60}Co irradiation on living cells is associated with mitotic death, by double DNA breakage and chromosomal disruption in daughter cells, and also to induction of apoptosis in lymphocytes,

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through ceramide production. Higher doses of radiation can have a direct effect on cell membranes by the production of free radicals [16]. Previous studies examined the immunity induced in experimental animals by irradiated forms of *T. gondii* but did not look at parasite viability, metabolism or host cell invasion [17,18]. As purified isolated tachyzoites are not involved in cell division, the main target of radiation, namely chromosomal structures, may be affected without interfering with other biological or metabolic processes. Allowing that irradiated parasites offer the same immunological challenge as intact organisms, this study presents data on the morphology, metabolism and cell invasion of ^{60}Co-irradiated tachyzoites, and their ability to induce an immune response in mice, comparing antibody production, cell proliferation and cytokine production with native parasites, and examining subsequent challenge of immunised mice with virulent tachyzoites or oral administration of cysts.

2. Materials and methods

All reagents used were of analytical grade, from commercial sources. All solutions were prepared with double distilled (MilliQ) water.

2.1. Parasites

T. gondii RH strain was maintained as frozen stabilates or by successive intraperitoneal (i.p.) passage in outbreed mice (Protozoology Lab., Instituto de Medicina Tropical de São Paulo, Brazil). *T. gondii* ME-49 strain was kindly donated by R. T. Gazzinelli (UFMG) and was also kept as stabilates or by serial passage in C57Bl/6j mice-oral gavage.

2.2. Mice

Isogenic C57Bl/6j, BALB/c or outbreed Swiss mice were obtained from a colony at the Faculdade de Medicina da Universidade de São Paulo, and maintained in sterilised cages with absorbent media and food and water ad libitum. All procedures were conducted according to the "Guide of Laboratory Animal Care", [19] and "The Principles of Ethics in Animal Experimentation" (COBEA—Colégio Brasiliero de Experimentação Animal).

2.3. Purification of tachyzoites

Parasites were harvested from passage animals by sterile peritoneal lavage with phosphate buffered saline (PBS), pH 7.2. The parasite/cell suspension was centrifuged at $2000 \times g$ for 10 min, 4 °C, and the pellet re-suspended in PBS. The suspension was passed through a 0.1 mm gauge needle prior to being loaded on to a Sephadex G 50–80 (1 cm × 10 cm) column. The column was previously washed with sterile PBS. Unbound parasites were collected and recovered by centrifugation. To ascertain cell numbers and parasite viability and aliquot was diluted with one volume of Trypan Blue (0.4% in HBSS) and the total cell and stained cell (dead) count was performed using phase-contrast microscopy. Preparations with more than 1% mammalian cell contamination or viability less than 95% were discarded. The final concentration of parasites was adjusted to 10^8 tachyzoites/ml.

2.4. Irradiation

Tachyzoite suspensions, maintained in ice-cold baths, were irradiated at 20, 50, 100 and 200 Gy, in a uniform source of ^{60}Co γ-rays in a GammaCell™ (Atomic Energy of Canada Ltd.) at a dose rate of 370 Gy/h, in the presence of oxygen. Adequate controls were maintained outside the source. After irradiation viability of all samples was determined via Trypan Blue staining.

2.5. Electron microscopy

A suspension of irradiated and untreated control parasites were fixed in 1.5% glutaraldehyde solution buffered with 0.08 M cacodylate buffer (pH 7.4). After 1 h on ice the suspension was centrifuged ($1000 \times g$, 5 min) and the pellet re-suspended in 1% osmium tetroxide. After 1 h on ice, with occasional mixing, the suspension was centrifuged ($10,000 \times g$, 1 min) and the pellet processed for embedding in Araldite®. Ultra-thin sections were contrasted stained with uranyl acetate, observed and micrographed in a Zeiss™ EM109 electron microscope.

2.6. Cell invasion assays

LLC-MK cells were cultured in RPMI 1640 medium (Sigma) with 10% foetal bovine serum (FBS) and 50 μg/ml gentamicin. Trypsin-separated cells were seeded in Lab-tek™ chamber slides. On reaching semi-confluence, 10^5 tachyzoites, either 200 Gy-irradiated or wild-type (WT), were added. After 4 h the monolayers were washed carefully with PBS (4×), fixed with 100% methanol and Giemsa-stained. Representative field were photographed in an Axiophot planapochromatic microscope.

2.7. Mitochondrial respiratory assay

A purified parasite suspension of 10^7/ml in RPMI 1640 + 5% FBS + gentamicin + 1 mg/ml MTT (Sigma), was dispensed into 24-well plates and kept at 37 °C, 5% CO_2. Samples were taken at 1, 2, 4 and 18 h. Samples were fixed with an equal volume of 4% formaldehyde/sodium phosphate 0.02 M, pH 7.2 and centrifuged at $800 \times g$, 10 min. After discarding the supernatant, mitochondrial oxidative blue formazan was extracted from pellet with methanol (100%) and the absorbance read at 570 nm.

2.8. Protein and nucleic acid synthesis assays

Purified parasite suspensions (1×10^7), both irradiated and non-irradiated, were dispensed in 24-well plates in Dulbecco's MEM medium (Sigma) + 5% FBS + gentamicin and maintained at protein synthesis was determined by adding ^{3}H-proline (2 μCi per well) 37 °C, 5% CO_2. After 1, 2, 4 and 18 h aliquots were dispensed on to 2 cm^2 pieces of filter paper which were then dried and placed in 1% trichloroacetic acid (TCA) for 1 h. After two washes in ice-cold TCA and one wash in ethanol, the filters were dried and placed in scintillation vials with 10 ml scintillation fluid. Counts were determined in a Wallach^{TM} 1209 counter, at 66% efficiency, and expressed in counts per minute (cpm). For evaluation of nucleic acid synthesis, both RNA and DNA, a similar method was used ^{3}H-hypoxanthine, 1 μCi per well.

2.9. Reproductive ability of irradiated parasites

The reproductive ability of the parasites, irradiated at different doses, was assessed by two approaches: (a) Tachyzoites were seeded in a limiting dilution, on LLC-MK2 monolayers in 96-well plates. The plates were incubated for 7 days, with examination for cytopathic effects every 24 h. Giemsa-stained smears of culture overlay were also examined to determine the presence of free tachyzoites. (b) Groups of five mice were infected with different inoculums of irradiated parasites and survival time observed daily. When mice became clinically sick they were anaesthetised and peritoneal lavage, with PBS, was performed. Smears were Giemsa-stained and examined. The effective radiation dose was determined when the plates had maintained their integrity for 1 week or when all mice survived without sign of infection.

2.10. Murine immunisation

After 200 Gy irradiation, groups of 5 mice were inoculated with 1×10^7 tachyzoites per mouse i.p., at the week intervals. The induced immune response of the challenges was compared to groups of mice inoculated either with formaldehyde-fixed tachyzoites or with naive parasites, and immediately treated with sulfadoxine and pyrimethamine [20]. Antibody response was determined by specific anti-*T. gondii* IgG detection ELISA using *T. gondii* extract as antigen [21]. Briefly, 96-well, high affinity ELISA plates (polystyrene multiwell plate, Sigma) were coated with 50 μl of a solution of 10 μg protein/ml antigen extract, diluted in 0.9 M carbonate buffer, pH 9.0, and incubated overnight at 4 °C. After washing with PBS/0.05% Tween 20 (Sigma) (PBS-T) residual binding sites were blocked for 1 h at 37 °C with PBS/3% fat-free lyophilised milk (PBSTM) in humid conditions. 100 μl aliquots of appropriate dilutions of serum samples from immunised mice were dispensed into the wells and incubated for 1 h at 37 °C. After washing with PBS-T, bound antibody was detected by peroxidase-conjugated anti-mouse IgG (Sigma) diluted in PBSTM, 1 h, 37 °C. After washing the reaction was developed by adding 30 μl per well of OPD solution (1 mg/ml *o*-phenylenediamine, 0.03% H_2O_2 in 0.2 M phosphate citrate buffer, pH 5). The reaction was terminated by adding 50 μl 4 N HCl, and the absorbance read at 492 nm.

2.11. Western blotting

Purified parasites (10^8/ml, PBS/H_2O 1:9 (v/v)) were submitted to ultrasound cycles until total cell rupture was achieved, and then centrifuged at 10,000 × *g*, 3 min. The collected supernatant was run on a 12.5% SDS-PAGE gel, with subsequent transfer to nitro-cellulose membranes [22]. The membranes were blocked with 5% PBSTM + 0.05% Tween 20, cut into 3 mm strips, dried and stored at −20 °C until use. Appropriate dilutions of sera from challenged animals were reacted with re-hydrated strips for 18 h, 4 °C. After careful washing with PBS-T bound antibody was detected by sequential incubation with peroxidase-conjugated anti-mouse IgG (Sigma) for 60 min, washed, and developed by adding PBS + 0.6 mg/ml 4-chloro-1-naphtol + 0.03% H_2O_2. The stained strips were dried, orientated according to molecular weight markers and digitally recorded.

2.12. Challenge of immunised mice

Groups of 5 mice were immunised with 1×10^7 200 Gy-irradiated tachyzoites per mouse (three doses), at the biweekly intervals and challenged with 1×10^3 RH tachyzoites i.p. with naive mice as controls. Symptoms and survival time were recorded weekly for 4 weeks, and data compared by Fisher's exact test. Similarly immunised mice with 255 Gy-irradiated tachyzoites per mouse were inoculated with 1, 5, 10, 20 and 25 cysts of ME-49 strain by oral gavage. Cysts were harvested from the brains of chronically infected C57Bl/6j mice. Briefly, after 4-week post-infection mice were euthanased and brain, heart, lung, liver and spleen were removed, fixed (10% formaldehyde/PBS, pH 7.2) and embedded in paraffin. Pieces of fresh brain were weighed and homogenised in a Dounce loose fitting homogeniser in five volumes of PBS (mg/ml). *T. gondii* cysts were expressed as (cyst/ml) homogenate, by counting under phase-contrast in volume fixed chambers (Neubauer). Brain sections (7 μm), after HE staining, were observed and photographed for *Toxoplasma* cysts and inflammatory lesions in an Axiophot photomicroscope.

2.13. Cell immunity assays

Mice were immunised with three biweekly 1×10^7 255 Gy-irradiated tachyzoites i.p. Spleens were collected 60 days after the first immunisation. Spleens were dissociated in RPMI 1640 + 5% FBS, penicillin 100 U/ml, streptomicin 100 μg/ml, amphotericin B 0.25 μg/ml. The cells were counted, their viability determined and washed in complete

medium by centrifugation. Lymphocytes were purified by Ficoll–Hypaque gradient and diluted to 2×10^6 cells/ml in RPMI 1640 + 10% FBS and antibiotics. Lymphocytes, at 2×10^5 cells per well, were stimulated by 48 h exposure to sonicated extract of tachyzoites at 5 μg/ml. ^{3}H-thymidine, 1 μCi per well, was added to the wells and incubated for a further 18 h. Incorporated label was harvested and determined. Concanavalin A (Con A), 5 μg/ml, was used as a non-specific internal control. For cytokine production, spleen cells from immunised and control mice were challenged with antigen as above, and the supernatant collected and stored at −20 °C after 48 h incubation and subsequent centrifugation. Cytokines IL-10, IL-12, IFN-γ and TNF-α were quantified in supernatants by specific OptEIA (Pharmingen) ELISA. Splenic lymphocytes from ME-49 infected mice were collected after 25-day post-infection and used for comparison, using ANOVA after variance check.

3. Results

3.1. Viability, structure and cell invasion

Purified parasites maintained their normal structure and morphology after irradiation as observed by phase-contrast microscopy. Viability was also not affected with 95% of cells viable as detected by Trypan Blue, regardless of radiation dose, time or process. By electron microscopy, 200 Gy-irradiated tachyzoites had the same morphology as untreated cells (Fig. 1A), with no evidence of nuclear or cytoplasmic damage. Conoid, rhoptries, micronemes and apicoplast maintained their integrity. There was no evidence of clumped chromatin or other evidence of radiation-induced apoptosis or mitochondrial swelling. Unirradiated parasites invaded LLC-MK2 cells using the apical complex structures, showing adhesion, orientation and penetration (Fig. 1B). 200 Gy-irradiated parasites displayed the same efficacy and step as untreated parasites, but degenerated after invasion (Fig. 1C).

3.2. Metabolism

Irradiation (200 Gy) did not affect major metabolic functions of *T. gondii* tachyzoites. Oxidative function was maintained (Fig. 2) with levels mirroring non-irradiated controls. A slight increase in function was observed at later time points (Fig. 2A) suggesting radiation-induced metabolic activity. Amino acid incorporation, measured by ^{3}H-proline, was maintained (Fig. 2B). All samples displayed a clear increase

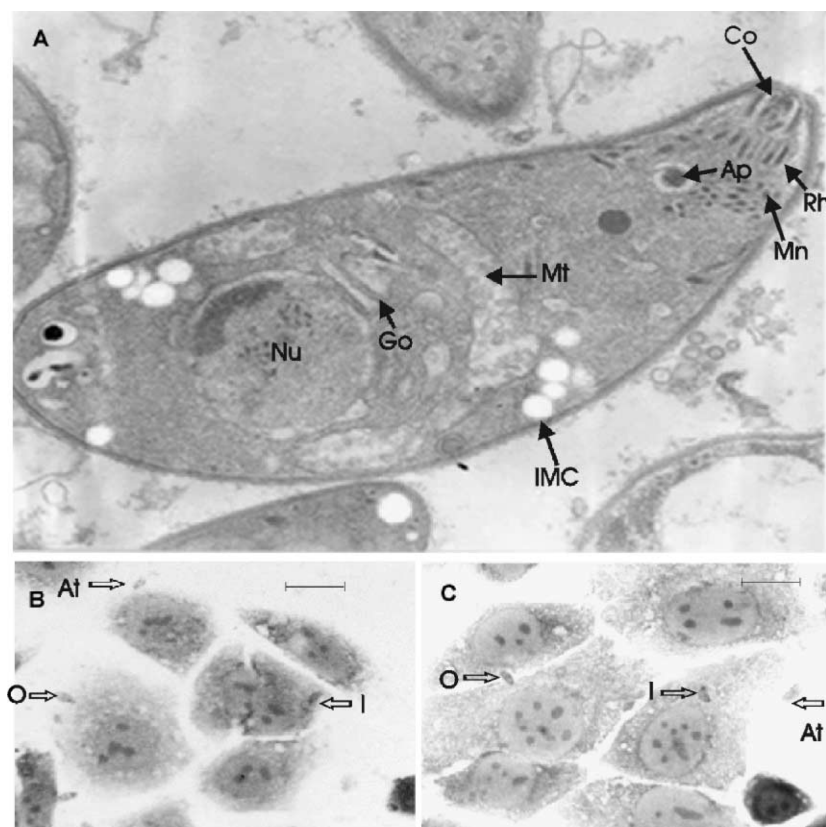

Fig. 1. Morphology and cell invasion characteristics of irradiated tachyzoites. (A) Electron microscopy of 200 Gy-irradiated tachyzoites showing preservation of structures, (20,000×). Nu, nucleus; Go, Golgi complex; Mt, mitochondrion; Ap, Apicoplast; IMC, inner membrane complex; Rh, rhoptry; Mn, micronemes; Co, conoid. (B) and (C) cell invasion in vitro on LLCMK2 cells, Giemsa-stained. Bar represents 16 μm. At, attached tachyzoite; O, oriented tachyzoite; I, internalised tachyzoite. (B) Controls tachyzoites; (C) 200 Gy-irradiated tachyzoites.

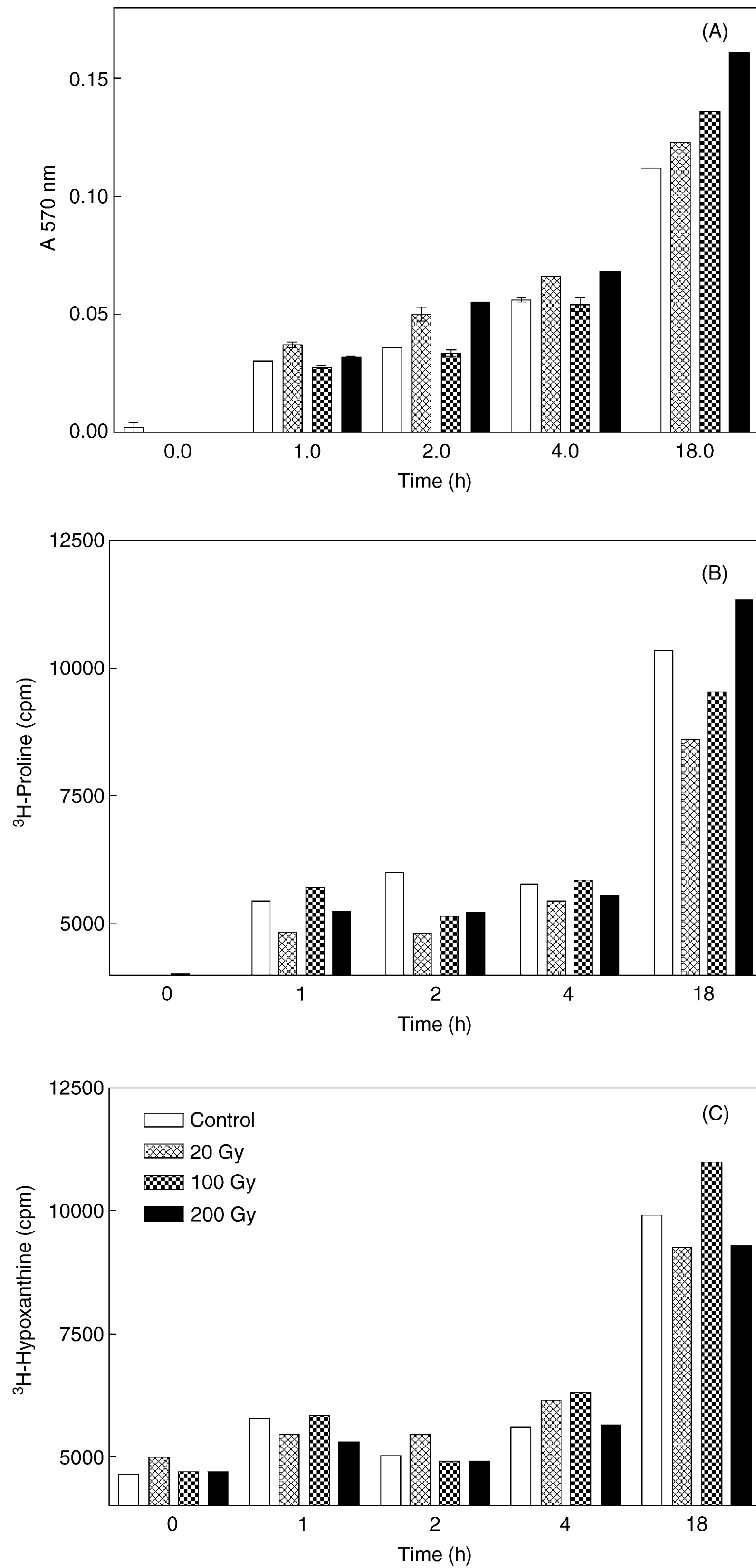

Fig. 2. Metabolic activities in *T. gondii* irradiated tachyzoites: (A) oxidative metabolism; (B) protein synthesis; (C) nucleic acid synthesis. Errors bars: S.E.M.

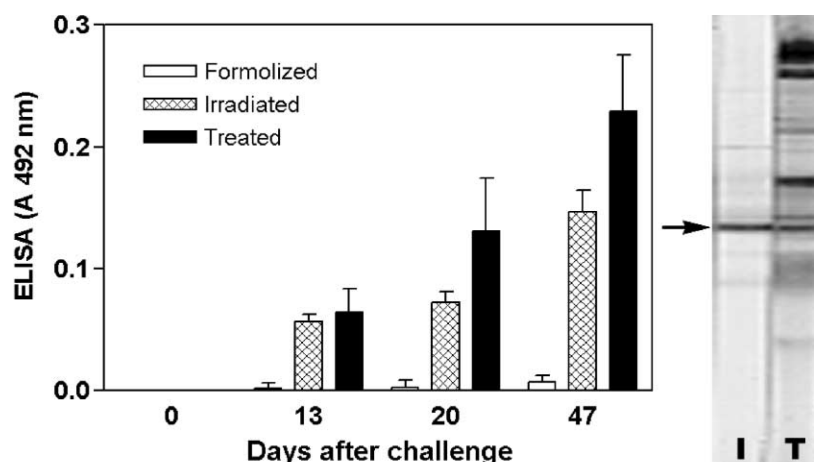

Fig. 3. Follow-up of specific IgG antibody production by ELISA in serum from groups of mice challenged with 10^7 tachyzoites i.p., formaldehyde treated, 200 Gy-irradiated tachyzoites or viable tachyzoites, with immediate treatment with pyrimethamine and sulfametoxazol. Errors bars: S.E.M. Insert: immunoblot analysis of the specific IgG antibody response in C57Bl/6j mice. I, mice immunised with three i.p. biweekly shots of 10^7 irradiated tachyzoites; T, mice infected with *T. gondii* RH strain and treated with sulfadoxine and pyrimethamine. Arrow indicates *T. gondii* SAG1.

in TCA-precipitated radioactivity, with irradiated parasites showing a slight increase at later time points, probably due to repair of radiation effects. Nucleic acid incorporation, measured by ^{3}H-hypoxanthine, appeared to be time-dependent, with irradiated tachyzoites showing similar values as controls (Fig. 2C). ^{3}H-thymidine incorporation was also assayed but, as expected for tachyzoites, no significant incorporation occurred in any sample.

3.3. Reproduction of irradiated tachyzoites

Reproductive ability in infected monolayers was examined. In 10 replicates of 200 Gy-irradiated parasites no reproduction, and no cytopathic effects, were observed in the cell layers, 100 Gy-irradiated parasites a few foci (five per flask) of cytopathic effects were seen after 7-day incubation. Lower doses of irradiation or non-irradiated parasites promoted destruction of the monolayer in less than 5 days, with many parasites free in the medium. C57Bl/6j mice infected with native tachyzoites died after 7-day post-infection with tachyzoites in the peritoneal exudate. All mice, inoculated with 50, 100 or 200 Gy-irradiated parasites, survived with no symptoms of infection. Peritoneal exudate washings were clear of parasites up to 2-week post-infection. Despite this data presence of benign infection in some mice cannot be excluded, especially in groups which received tachyzoites irradiated at a lower dose, as the RH strain is characteristically non-cystogenic in mice [10].

3.4. Immunogenicity and protection assays

The immunogenicity of 200 Gy-irradiated, formaldehyde treated tachyzoites and a natural infection treated early then challenged, was compared. ELISA quantitatively detected IgG, the specificity being detected by Western blot analysis, as shown in Fig. 3. Serial blood sampling of mice infected with either irradiated or native parasites, showed similar antibody profiles with titers of the former being lower. Formaldehyde-treated parasites failed to promote a significant antibody response. Antibody specificity was comparable in both models. The protection afforded by 200 Gy-irradiated parasites was investigated. Immunised mice survived longer than naturally infected control mice (RH strain) (Fig. 4, $P < 0.05$) and demonstrated less cerebral pathology when challenged with the cystogenic ME-49 strain (Fig. 5). Brain histology showed a paucity of cysts and inflammatory foci. Antigen nucleic acid was detected by PCR (B1 gene primers—kindly carried out by T.S. Okay, Instituto da Criança, FMUSP) confirming that irradiated tachyzoites only provided partial protection against both challenges.

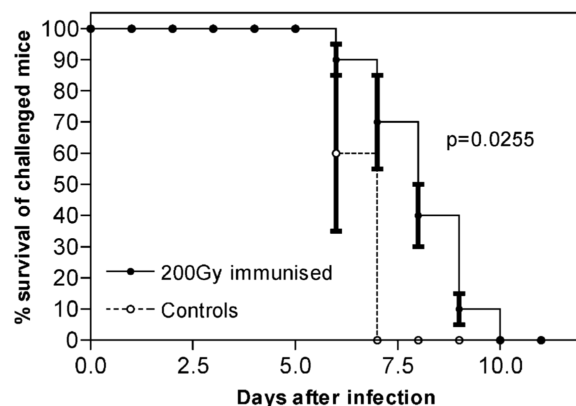

Fig. 4. Mortality of mice immunised with 10^7 200 Gy-irradiated tachyzoites, challenged on day 30 with 10^3 tachyzoites of RH strain. Solid line, immunised mice; dashed line, controls mice. Bars represent 95% confidence interval of proportions. Significance was determined by Fisher's exact test.

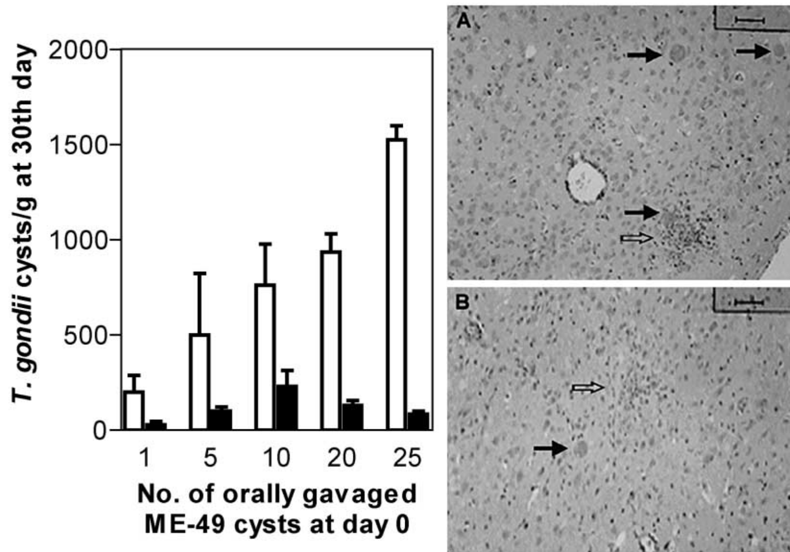

Fig. 5. Resistance of immunised mice to oral challenge with *T. gondii* Me49 strain cysts as detected by number of brain cysts 30 days after challenge. Solid bars, immunised mice with three i.p. biweekly shots of 10^7 irradiated tachyzoites; open bars, naive mice. Error Bar: S.E.M. Inserts of representative histology of brain of C57Bl/6j mice challenged with 25 cysts of *T. gondii* Me49 strain: (A) naive mice; (B) immunised with three biweekly i.p. 10^7 irradiated tachyzoites on day 60. Black arrows indicate *Toxoplasma* cysts and white arrow inflammatory foci in brains. Bars represents 50 μm.

3.5. Cellular immune responses after immunisation with irradiated tachyzoites

Spleen cells from mice immunised with irradiated tachyzoites showed distinct proliferate responses when stimulated, in vitro, with *Toxoplasma* antigen (Fig. 6). Stimulation with Con A induced higher non-specific responses, showing the usual response of murine lymphoid cells. Cytokine production of these spleen cells was examined in vitro (Fig. 7). IFN-γ production was higher than controls but lower than levels produced by cells from naturally-infected, ME-49 strain, mice (Fig. 7A). TNF-α production was similar to chronically infected mice, being smaller than the acute stages of a natural infection (Fig. 7B). The production of IL-10 by spleen cells from all groups of mice presented no significant differences (Fig. 7C). IL-12 production by spleen cells from immunised mice was lower than those from naturally infected mice and higher than control groups (Fig. 7D). The data suggests that acute infection promotes higher levels of the cytokines tested, especially IFN-γ, which was found at a lower level in immunised mice, indicating a cellular immune response to irradiated tachyzoites mirroring that of a natural infection.

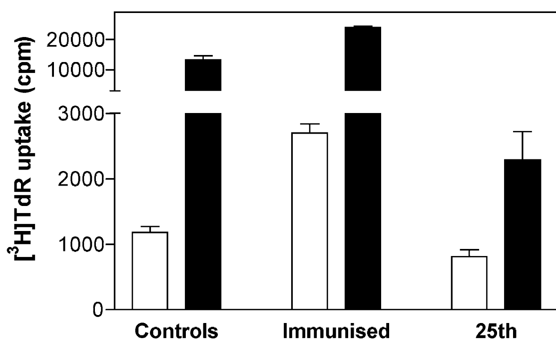

Fig. 6. Proliferative response of spleen cells in C57Bl/6j mice, stimulated by *T. gondii* antigens (open bars) or Con A (solid bars). Controls, spleens from normal mice; immunised, from mice immunised with three i.p. biweekly shots of 10^7 irradiated tachyzoites on day 60; 25th, mice infected with cysts of *T. gondii* Me49 strain by 25 days after infection. Spleen cells were cultured for 48 h, followed by addition of 1 μCi [^{3}H]-TdR and then harvested 24 h later. Error bar: S.E.M.

4. Discussion

The results show that the primary effect of exposing *T. gondii* tachyzoites to increasing doses of radiation is to abrogate reproductive ability without significant interference with other cellular functions such as metabolic functions, protein and nucleic acid synthesis. Morphologically, no clearly defined features could be attributed to radiation effects. This is not common in most instances where cell growth is disrupted, with evidence of cellular destruction, mitochondrial swelling and loss of membrane integrity for example [23].

Previously, the effect of irradiating *T. gondii* tachyzoites at a dose of 100 Gy or 10 krad had been evaluated by their ability to reproduce and cause lethality [24]. These studies failed to detect non-lethal infection and did not utilise more sensitive in vitro assays and did not use a virulent strain of parasites. The in vitro data in this study that a few tachyzoites can survive the lower dose of radiation, enabling a non-lethal infection. Complete block of reproductive ability

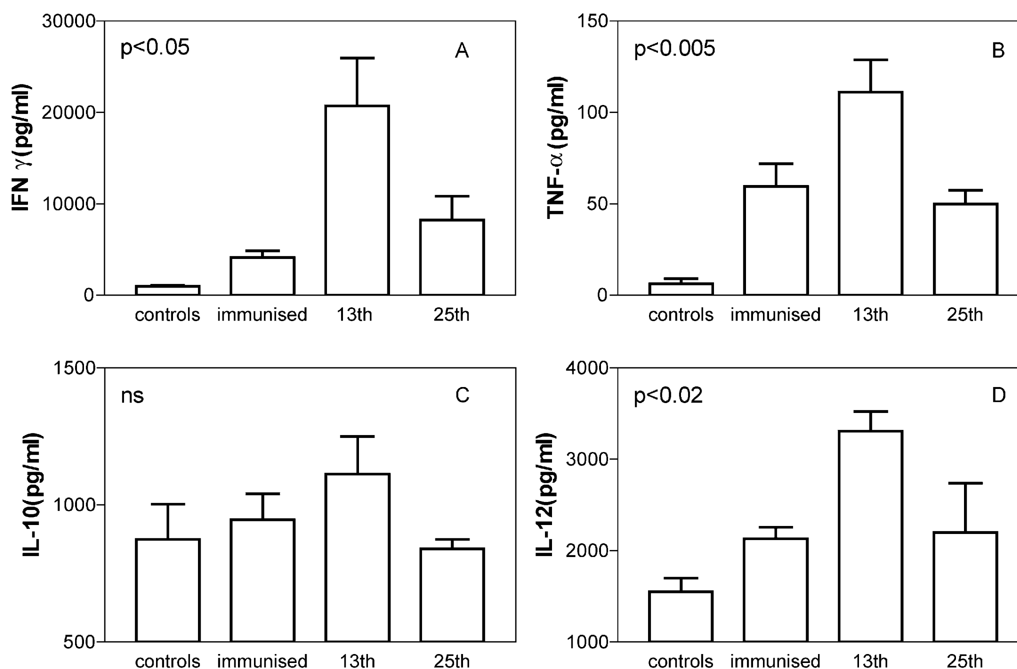

Fig. 7. Cytokine production by spleen cells in response to *T. gondii* antigen (TA) in C57Bl/6j mice. Controls, from normal mice; immunised, from mice immunised with irradiated tachyzoites; 13th, from mice on day 13 of infection with cysts of Me49 *T. gondii* strain; and 25th, from mice on day 25 of infection with cysts of Me49 *T. gondii* strain. (A) IFN-γ; (B) TNF-α; (C) IL-10; and (D) IL-12. Bars represent S.E.M. ANOVA significance described in each graph.

was only achieved by more the 200 Gy γ-radiation in this more sensitive assay. At this dose, the data shows that the sterilising effect is not associated with major effects on parasite viability as demonstrated by the dye exclusion test, or morphology, or interference with somatic cell function, namely protein and RNA synthesis. Maintenance of nucleic acid synthesis was unexpected, as other reports documenting ^{3}H-uracil uptake of irradiated parasites did not examine metabolic function [25]. The tachyzoite is in fact the non-dividing form of the parasite and does not incorporate labelled thymidine (data not shown), however, hypoxanthine can be incorporated into other nucleic acids such as mRNA or tRNA by the tachyzoite [26].

The irradiated parasite's ability to invade host cells was probably due to ready-formed organelles, and follows reported steps of cell invasion processes, partially observed previously [25], and is parasite driven [27].

Double DNA breaks in chromosomal strands of irradiated tachyzoites probably results in an inability to reproduce. This effect has also been observed in actively dividing cells, which were previously irradiated, resulting in an unbalanced chromatin exchange in daughter cells and loss of essential genetic material [28]. These events may explain how the irradiated tachyzoite maintains its metabolic function and other cellular activities. The complex genetic structure of the tachyzoites, 11 chromosomes [29], may favour this effect at low doses of radiation as compared to the effect on simpler genomic structures, e.g. in bacteria [30]. The few double DNA breaks maybe located outside operons, allowing adequate function of the genes until cell division, leading to mitotic death [31]. As we expected few random breaks along the chromatids, analysis by pulse field electrophoresis would have resulted in loss of definition of specific bands and so was not tried [32]. The triggering of apoptotic cell death by irradiation was examined by TUNEL [33]. No evidence of this was seen in 200 Gy-irradiated tachyzoites (data not shown).

The irradiated tachyzoites promoted similar antibody responses in immunised mice when compared to control treated mice, with the same bands appearing in Western blot analysis. Formaldehyde-treated parasites failed to elicit specific antibodies when administered without adjuvant. This unexpected result may be to the destruction of these tachyzoites by complement and/or neutrophils in acute response [27,34]. We have shown that irradiated tachyzoites have similar characteristics as native parasites, without their reproductive ability, but exposing their antigens sequentially to the host as in a natural infection.

Mice immunised with irradiated tachyzoites and subsequently challenged with viable parasites, survived longer (RH) and displayed less pathology (ME-49). This mirrors previous findings using other immunisation schemes; intact parasites [35], purified antigens [36] or attenuated parasites with non-sterile immunity (2). Use of recombinant protein produces results similar to those found with irradiated parasites [37,38].

The study of cytokine production is essential for the understanding of the induced immune response by *Toxoplasma*

vaccines. We found similar cytokine production, although at lower levels, when comparing the irradiated tachyzoite immunisation schedules with the usual cyst-forming experimental model, especially when comparing late infection levels. The induction of IFN-γ was notable as the elimination of infection in experimental models appears to be related to the production of this cytokine [12]. The concomitant antigen-induced production of TNF-α and IL-12 in those mice is also notable, due to the interaction with IFN-γ or NK cell activation in an effective immune response in natural infections [13]. Thus, immunisation with viable, functional parasites, minus their reproductive ability, appears to induce the same as a natural infection, avoiding problems associated with single antigen immunisation schedules [14]. Despite our promising data, the problem of vaccination against toxoplasmosis remains unsolved, as the immune response needs to be induced at the normal site of infection, the gastric–intestinal tract. Most studies utilising systemic immunisation are contentious, as the tools for studying gut immunology remain limited.

Immunity induced by *T. gondii* is complex, having both antibody and cellular components, and with tissue cysts remaining viable for the lifetime of the host. Doubts remain whether an acute infection promotes sterile immunity or if it only succeeds in abolishing clinical signs of new infection-specific opsonisation of tachyzoites leading to phagocytosis but circulating parasites remain [39].

Irradiated tachyzoites could be an alternative immunisation schedule for vaccine development, perhaps as a boosting antigen in patients with positive *T. gondii* serology and who are at risk of severe immunosuppression, e.g. undergoing cancer chemotherapy. Alternative, they could be used as a tool in the study of the re-infection phenomenon of toxoplasmosis.

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