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Immunogenicity of Gamma-Irradiated *Toxoplasma gondii* Tachyzoites in Mice

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ABSTRACT

The Immunogenicity of γ-irradiated tachyzoites of a highly virulent strain of *Toxoplasma gondii* (RH strain) was investigated. Immunized mice survived challenge infection and displayed increased interferon gamma (INF-γ) production. On the other hand, nonimmunized controls died after 10 days and displayed a significantly decreased lymphoproliferative response to Concanavalin A (Con A). No tachyzoites were detected in peritoneal exudates of the immunized mice 10 days after challenge. These results indicate that the vaccination with γ-irradiated tachyzoites not only protects the mice against challenge infection, but may circumvent the danger of the vaccine being life-threatening because of remaining tachyzoites inside macrophages posing as a latent infection. *Iran. Biomed. J. 3 (3 & 4): 93-97, 1999*

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INTRODUCTION

Toxoplasma gondii is an intracellular parasite eliciting a protective immune response but persisting during latent infection in multiple tissues and organs [1]. Data from studies performed *in vitro* and *in vivo* in both laboratory animals and humans suggest that the major mechanism of resistance against *T. gondii* is cell- mediated immunity (CMI) [2, 3]. The effector arms of CMI against *T. gondii* appear to be monocytes, macrophages and CD8 T cells. IFN-γ which is released by CD8 T cells, macrophages and natural killer (NK) cells has the capacity to activate mouse macrophages *in vitro* [4, 5] and *in vivo* [6], and human macrophages *in vitro* to inhibit or kill *T. gondii* [5, 7]. These observations suggest that the IFN-γ is of primary importance in host resistance against *T. gondii* [8]. Thus, the efficacy of a *T. gondii* vaccine could be determined by the ability of immunized animals to survive challenge infection and to produce IFN-γ. Further, it was reported by Haque *et al.* [9] that spleen cells from infected mice failed to proliferate in response to *T. gondii* antigen (TgAg) and exhibited a significant reduction in the concanavalin A (Con A) induced lymphoproliferative response during the first week of infection. Our data show that the unlike controls, the mice immunized with γ-irradiated tachyzoites of *T. gondii* survive lethal challenge infection and reveal high *in vitro* lymphoproliferative responses to Con A and TgAg during the first week of infection. Besides, the production of IFN-γ following challenge infection is known to be significantly greater in immunized animals than in controls.

MATERIALS AND METHODS

Gamma-irradiated parasites and antigen preparation. The tachyzoites of a highly virulent strain of *T. gondii* (RH) were obtained from peritoneal cavity of BALB/c mice injected with 1´ 10^6 organisms 3 days earlier. The peritoneal cells were then disrupted by repeated passage through a 23-gauge needle and tachyzoites were separated from host cells by low speed centrifugation at 70 g for 5 min. They were then pelleted at 590 g for 10 min and adjusted to 1×10^6 organisms/ml in saline before being exposed to g -irradiation or prior to antigen preparation. A gamma beam 150 with a source of Co 60 that produces 445 rad/min was used. Preliminary experiments revealed that a dose of 25 Krad prevented the intracellular multiplication of the parasite, but tachyzoites were still capable of

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penetrating host cells. For TgAg preparation, tachyzoites were pelleted by centrifugation at 590 ×g for 10 min and repeatedly sonicated until no intact parasite remained. The supernatant was collected following centrifugation at 10,000 ×g for 20 min, dialyzed against PBS, its protein concentration determined by Bradford method with BSA as standard, and stored at –20°C.

Vaccination. Mice were immunized by i. p. inoculation with 2×10^4 γ-irradiated tachyzoites of RH strain and boosted 14 and 28 days later with 2×10^5 γ-irradiated tachyzoites.

Experimental design. Two hundred BALB/c mice were vaccinated with γ-irradiated tachyzoites 2 h after irradiation and a group of 200 control mice were injected with PBS only. To assess protective immunity, vaccinated mice, as well as controls, were challenged i.p. with 100 ml of 2×10^4 RH strain tachyzoites/ml two weeks after thelast inoculation. A third group of negative controls (200 mice), which were neither immunized nor infected were also included and survival monitored. Peritoneal cells were collected from animals 10 days post challenge and the total number of tachyzoites counted by light microscopy using trypan blue exclusion method. The lymphoproliferative responses of spleen cells from immunized and nonimmunized controls to Con A or TgAg were determined 7 days post infection, and culture supernatants were collected 72 h after stimulation with Con A or TgAg to measure IFN-γ production by sandwich ELISA.

Lymphocyte preparation and cell culture. Cell culture studies were performed in RPMI 1640 supplemented with 10mM L-glutamine, penicillin-streptomycin (10,000 units/ml penicillin & 100 m/ml streptomycin), 39.4 m M2-mercaotoethanol, 10% FCS, and adjusted to pH 7.0 by the addition of 10mM HEPES. Following infection, the mice were sacrificed, the spleen removed aseptically, transferred into 15 ml ice-cooled complete medium containing 5% foetal calf serum (FCS), trimmed free of any excess fat, cut into small pieces, and then squeezed through sterile mesh. Cell suspension was passed repeatedly through a 21-gauge needle using 20 ml plastic syringe to produce a single cell suspension. Spleen cells were pelleted by centrifugation at 400 ×g for 10 min, erythrocytes were lysed by Tris ammonium chloride and cell suspension was washed twice at 4ºC in medium supplemented with 5% FCS by centrifugation at 400 ×g for 10 min. The viability of cells, determined by trypan blue exclusion method, exceeded 85%. For proliferative assays, 100 ml of the cells were cultured in triplicate wells at a concentration of 2×10^6 cells/ml of complete medium supplemented with 10% FCS in 96-well flat-bottom culture plates. 100m l of Con A or TgAg was then added to each well at the concentrations of 5 mg/ml. The cells were cultured for 4 days in a humified chamber at 37°C containing 5% CO_2 and were plused with 1 uCi of [^{3}H] thymidine (Amersham, UK) 18 h before harvesting on glass filter strips. The radioactivity was determined by liquid scintillation and data presented as c.p.m. For culture supernatant preparations, cells were cultured in the same conditions as for proliferation assay but in a 24-well plate for three days. Culture supernatants were collected and stored at -70°C until IFN-γ to be measured using sandwich ELISA.

ELISA for IFN-γ. A sandwich ELISA was employed to assay the levels of IFN-γ in only 10 samples each as previously described [3, 10]. IFN-γ was measured in the supernatant of cell proliferation assays of each group by ELISA method [11]. The reaction was performed using protein G rat anti mouse IFN-γ mAb R4-6A2 [12], biotin-labelled rat anti-mouse IFN-γ mAb An18.17.24 [13] and horseradish peroxidase-conjugated biotin-sterept-avidine (BRL-Gaitherburg, MD). All cell lines kindly were provided by DNAX Research Institute, Palo Alto, Calif. The plates were analyzed on an ELISA reader at 490 nm. The specific IFN-γ concentration was calculated on the basis of the standard curve of recombinant IFN-γ. Concentration from the supernatants higher than minimal values obtained from the standards was considered positive (minimal value is 30 pg/ml).

Statistics. Statistical analysis was performed with minitab release 10 software using student's *t*-test. The p value of < 0.05 was considered significant.

RESULTS

Animal survival and presence of tachyzoites in the peritoneal exudates. 78% of the mice immunized with γ-irradiated tachyzoites of *T. gondii* survived primary infection with RH strain, whereas all controls died within 10 days post challenge. Peritoneal exudates from immunized mice revealed no tachyzoites, but those from controls increased to

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Table 1. The number of Tachyzoites in peritoneal exudates of immunized (n=18) and non-immunized (n=18) mice 10 days after the challenge infection with 100m l of 2´ 10^4 RH strain of tachyzoites

Animals	Days post-infection	
	0	10
Immunized	2×10^4 tachyzoites	0
Non-immunized	2×10^4 tachyzoites	5×10^6 tachyzoites

5×10^7 organism/ml 10 days post challenge. Data isshown in Table 1.

Splenocyte proliferative response to Con A and TgAg. As shown in Fig. 1, splenocytes from control mice had reduced proliferative responses to Con A 7 days post-infection (P<0.05). Further both negative (naive) and positive controls failed to response to TgAg. The mice immunized with γ-irradiated tachyzoites displayed a marked proliferative response to Con A or TgAg (P<0.05).

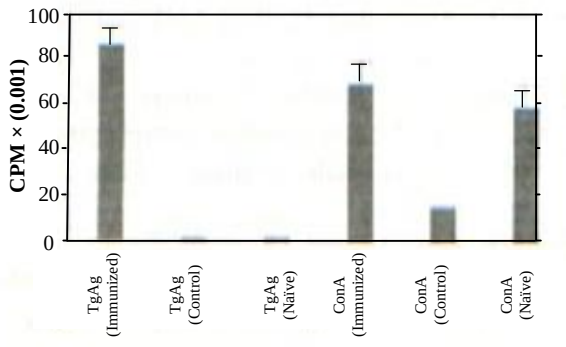

Fig. 1. Lymphoproliferative responses of spienocytes from naive, control and immunized mice to Con A and to *T. gondii* antigen (TgAg) seven days after challenge infection with 2,000 *T. gondii* tachyzoites.

Production of IFN-γ. IFN-γ production by splenocytes from both controlled and immunized mice cultured with Con A or TgAg seven days after challenge infection are depicted in Fig. 2. The immunized cells revealed marked IFN-γ production in cultivation with both Con A and TgAg. There were also significant differences between controlled and immunized cells to produce IFN-γ when cultured with Con A (P=0.008) or TgAG (P<0.001).

DISCUSSION

A vaccine consisting of live attenuated tachyzoites that successfully prevents abortion in sheep is already commercially available in several countries [14], and a similar vaccine has been shown to limit experimental infections in pigs [15, 16]. However, vaccinating sheep fails to prevent the vertical disease transmission and the development of cysts in lambs [17].

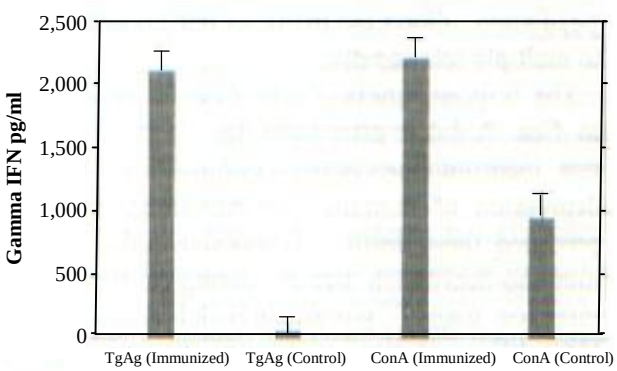

Fig. 2. INF-γ production by splenocytes of control and immunized mice cultured with Con A and *T. gondii* antigen (TgAg) seven days after infection with 2,000 *T. gondii* tachyzoites.

Preliminary experiments revealed that γ-irradiated tachyzoites must be used less than 6h post-irradiation, otherwise they will be reactivated. Storing these parasites either at room temperature or at 4°C for 24 h causes reactivation and development of acute fatal toxoplasmosis in infected animals. Our data indicate that vaccination of mice with γ-irradiated tachyzoites of *T. gondii* (RH strain) can protect up to 78% of animals against infection. The peritoneal cells of vaccinated mice were free of tachyzoites following challenge infection, whereas those of controls contained an increased number of tachyzoites. This shows that, apart from 78% efficacy of γ-irradiated tachyzoites, the clearance of tachyzoites from peritoneal cells was roughly 100%. Thus, vaccinated animals did not become carriers of *T. gondii* and there was no state of latent infection. This may circumvent the danger of vaccine being life-threatening. The RH strain is most frequently associated with congenital infection [15]. However, trypan blue exclusion is not a sensitive method to detect low levels of intracellular parasites and it has to be replaced by immunohistochemical staining using an anti-toxoplasma serum as well as by the subinoculation of peritoneal exudates into naive mice and the subsequent evaluation of seroconversion or by PCR technology. It is not clear that how long this

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immunity may last. Since tachyzoites disappeared in the immunized animals, immune system may not be exposed to *T. gondii* antigens over time. This may be a disadvantage and affect the duration of specific immunity against infection. This possibility needs to be explored in future studies. Preliminary experiments indicated that a dose of 25-30 Krad of γ-radiation allows the parasites remain infective but to multiply less rapidly.

The responsiveness of cells from immunized mice to Con A 7 days after challenge infection indicated that their immune system was functional, whereas a depression of immune responsiveness in controls preceded their death. It was shown that a state of immuno-depression occurs during the first week of infection with *T. gondii* in both humans and mice [16, 17]. Haque *et al.* [9] demonstrated that macrophages from infected mice displayed reduced lymphoproliferativeresponses to Con A. Immunodepression allows *T. gondii* to multiply and to kill infected animals.

Macrophages secrete a variety of factors with immunosuppressive activities, including IL 10, which inhibits T cell proliferation by Con A [18]. It was well documented that IL10 inhibits synthesis of IFN-γ by human and mouse NK cells [19]. In the murine models activation of NK cells is one of the first events to occur following infection with *T. gondii* [20]. These cells produce IFN-γ early in the course of infection, resulting in resistance of *T. gondii* [21]. This may account for our finding a significant difference between immunized and nonimmunized mice in their IFN-γ production when their spleen cells were stimulated with Con A following challenge infection. Data from studies performed *in vitro* and *in vivo* suggest that IFN-γ is essential for resistance to acute *T. gondii* infection [21]. IFN-γ can activate macrophages to inhibit or kill *T. gondii* without collaboration of any other lymphokine [4, 5, 7]. This cytokine increases the expression of MHC-I products on cells [22] and by doing so may enhance the recognition of antigen on infected cells by immunized animals.

The spleenocytes of vaccinated mice revealed significant proliferative responses to TgAg, whereas this was not the case for controls. These results suggest that *in vitro* lymphocyte transformation tests (LTT) could be used as a diagnostic method to detect patients with a latent toxoplasmosis, as their peripheral blood lymphocytes may reveal a proliferative response to TgAg *in vitro.*

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