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# CD8$^+$ T-cell Mediated Protection against Acute Toxoplasmosis in Mice Induced with X-ray Irradiated *Toxoplasma* Tachyzoites

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## Abstract

Mice vaccinated with X-ray irradiated tachyzoites of *Toxoplasma gondii* have developed complete resistance to a lethal challenge with a highly virulent *Toxoplasma* strain (RH). To determine the roles for T cell subpopulations responsible for this protection, CD4$^+$ or CD8$^+$ T cells were depleted in primed BALB/c mice or were transferred into naive mice, which were then subjected to challenge infection. We found that primed mice depleted of CD4$^+$ T cells were protected while mice depleted of CD8$^+$ T cells became susceptible. As expected, CD8$^+$ T cells from primed mice could transfer protection to naive mice, even in the absence of host CD4$^+$ T cells. Contrary to our expectation, CD4$^+$ T cells were also competent in conferring protection so long as host CD8$^+$ T cells existed. These results suggest that primed CD8$^+$ T cells mediated protection as essential effector cells; primed CD4$^+$ T cells may work to help unprimed CD8$^+$ T cells to differentiate into effector cells upon challenge infection. X-ray-irradiated *Toxoplasma gondii* may serve as a useful tool for elucidating the mechanisms of the protective immunity for controlling toxoplasmosis.

**Key words:** *T. gondii*; mice; protective immunity; CD8$^+$ T cell; CD4$^+$ T cell.

## Introduction

*Toxoplasma gondii* (*T. gondii*) is an obligate intracellular protozoan parasite that infects all warm-blooded animals including man. Although generally asymptomatic for healthy individuals, a primary infection acquired during early pregnancy can lead to serious damage to the fetus such as stillbirth, blindness, mental retardation and occasionally death (Frenkel, 1988). In addition, in people suffering from acquired immunodeficiency syndrome (AIDS), toxoplasmosis is often lethal through reactivation of encysted parasites that persist in the host tissues following a primary oral infection (Levy *et al.*, 1985). In farm animal breeding, abortion and neonatal loss due to the parasite are important economic problems world-wide (Dubey, 1993). Therefore, understanding the mechanisms of protective immunity and prophylactic measures, such as a vaccine for controlling the infection, would be highly valuable.

Experimental and farm animals have been vaccinated successfully against fatal toxoplasmosis with live *Toxoplasma* mutants (Buxton *et al.*, 1993; McLeod *et al.*, 1988; Suzuki and Remington, 1988; Waldeland and Frenkel, 1983). Mice immunized with a live, temperature-sensitive mutant (ts-4) of *T. gondii* develop resistance mediated predominantly by γ-IFN-producing CD8$^+$ cytotoxic T cells (Hakim *et al.*, 1991; Suzuki and Remington, 1990; Waldeland and Frenkel, 1983). The parasite has been shown to lack the ability to form tissue cysts and to infect the host persistently (Waldeland *et al.*, 1983). However, vaccination with these live mutants has a potential risk because they may persist or even cause severe disease when the host is immunocompromised (Waldeland *et al.*, 1983).

The usefulness of tachyzoites attenuated by gamma irradiation as a candidate vaccine has been assessed previously (Bakal and Veld, 1979; Chhabra *et al.*, 1979; Seah and Hucal, 1975). For example, tachyzoites of the RH strain of *T. gondii* irradiated with 150 Gy remained infective to host cells *in vitro*

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but were unable to multiply intracellularly (Lund *et al.*, 1961). In addition, substantial protection was induced against a subsequent infectious challenge in mice primed with gamma-irradiated RH tachyzoites (Bakal and Veld, 1979; Chhabra *et al.*, 1979; Seah and Hucal, 1975). However, the effector mechanisms of this protective immunity have not been analyzed. Therefore, we characterized the effector T cell subsets participating in this protection in mice primed with irradiated RH strain tachyzoites of *T. gondii*.

## Materials and Methods

*Mice*

Female 8- to 10-week-old BALB/c and C57BL/6 were purchased from Charles River Japan (Kanagawa, Japan) and C.B-17/Icr-*scid* were from CLEA JAPAN, Inc. (Tokyo, Japan). Mice were housed in Laboratory Animal Center for Biomedical Research in Nagasaki University School of Medicine (Nagasaki, Japan).

*Parasites*

RH stain tachyzoites of *T. gondii* (Sabin, 1941) were passaged serially in human B lymphoma cells (ARH) in RPMI 1640 (Gibco, Grand Island, NY, USA) with 1% fetal bovine serum (FBS), penicillin (100 μg/ml), streptomycin (50 μg/ml), and 0.1% Nutridoma NS (Boehringer Mannheim, Mannheim, Germany) as previously described (Yano *et al.*, 1989). The parasites were partially separated from host cells by a low speed centrifugation (70×g, for 10 min) for further use.

*Attenuation of parasites*

RH strain tachyzoites were attenuated by 120 Gy irradiation for 35 min with an X-ray generator (EXS-300, Toshiba, Tokyo, Japan: filter 0.5 Al + 0.5 Cu, 200 KV, 15 mA). The irradiated tachyzoites remained infective to host cells *in vitro* but lost their virulency for mice *in vivo*, as previously reported (Kobayashi and Jacob, 1963; Lund *et al.*, 1961). In preliminary experiments, no morbidity was detected in BALB/c mice inoculated with $1\times10^6$ of 120 Gy-irradiated RH tachyzoites. Also, C.B-17-*scid* mice, which are deficient in both competent T and B cells, showed no morbidity after inoculation with the irradiated parasites (data not shown), indicating that this loss of virulency was not dependent on the immunocompetency of the host mice. Tests on surviving mice revealed no cysts in their brains one month after inoculation (data not shown). Based on these observations, RH tachyzoites irradiated with 120 Gy were used to prime mice for the subsequent experiments.

*Immunization and challenge infection*

Mice were primed once intradermally in a hind footpad with $1\times10^6$ X-ray-irradiated RH strain tachyzoites in 10 μl of Hank's balanced salt solution (HBSS, Nissui, Tokyo, Japan). The primed mice as well as age-matched controls were challenged various days after priming by an i.p. inoculation with $1\times10^4$ RH strain tachyzoite (approximately $1{,}000\times LD_{50}$). Morbidity was monitored daily and survival was determined at 30 days post-infection (PI).

In vivo *T cell depletion experiments*

Subsets of T cells were depleted by an i.p. inoculation with anti-CD4 mAb (0.5 mg) (GK1.5) (Dialynas *et al.*, 1983) or anti-CD8 mAb (0.5 mg) (2–43) (Sarmiento *et al.*, 1980) (kindly supplied by Dr. N. Shinohara, Mitsubishikasei Life Science Institute, Tokyo) 4 and 2 days before the challenge infection and every third day thereafter. The specificity of the depletion was confirmed by flow cytometry analysis on both axillar lymph node cells and splenocytes from treated mice at the time of challenge infection, and in no case we detected >2% of unwanted cells.

*Adoptive cell transfer experiments*

Immune T cells, and subsets of T cells obtained from the draining lymph nodes 5 days after priming were prepared by the panning method. Briefly, tissue-culture grade 100-mm petri dishes (Corning Glass Works, Corning, NY, USA) were coated overnight at 4°C with 6 ml of either anti-I-E mAb (ISCR.3) (Watanabe *et al.*, 1983), anti-CD4 mAb (GK1.5) or anti-CD8 mAb (2-43) (10 μg/ml in 5 mM phosphate-buffered saline (PBS), pH 7.2). The plates were blocked with 6 ml of a 1% (w/v) bovine serum albumin (BSA, B oehringer Mannheim, Mannheim, Germany) solution in PBS for 4 hr at r.t..

Lymph node cells were distributed first in the anti-I-E-coated dishes in 6 ml of a 1% BSA/PBS solution ($5\times10^7$–$10\times10^7$ cells/dish) and kept for 1 hr at 4°C. Non-adherent cells were collected by decanting and used as immune T cells for the adoptive cell transfer experiments. To further enrich $CD4^+$ or $CD8^+$ T cells, T cells were subsequently incubated in either the anti-CD4 mAb- or anti-CD8 mAb-coated dishes for 30 min at 4°C. After the removal of the non-adherent cells, the adherent cells were detached by vigorous pipeting, and used as purified T cell subset for the adoptive cell transfer experiments. This protocol routinely yielded >97% pure cell populations as determined by flow cytometric analysis (data not shown). Naive recipient mice were inoculated i.p. with $1\times10^7$ lymph node cells 24 hr before the challenge infection.

*Flowcytometory*

Cells ($5\times10^5$/sample) were simultaneously stained with FITC-anti-CD8 (83-12-5, (Leo *et al.*, 1987)) and biotin-anti-CD4 (RL172.4, (Lowenthal and MacDonald 1987)), both of which are non-crossreactive with the corresponding mAbs used for *in vivo* depletion experiments, using a phycoerythrin (PE)-streptavidin conjugate (Serotec, Oxford, U.K.) as a second-stage reagent. After washing, cells were analyzed by FACScan (Becton Dickinson & Co. Mountain View, CA, U.S.A.) excluding dead cells by propidium iodide gating.

*Statistical analysis*

Comparisons between groups of mice were evaluated statistically using the log-rank sum method, and $p$ value less than 0.05 was considered statistically significant.

## Results

BALB/c and C57BL/6 mice were primed intradermally in the hind footpad with $1\times10^6$ X-ray-irradiated RH tachyzoites. These mice were challenged i.p. with $1\times10^4$ tachyzoites 2 weeks after priming to assess their ability to resist a parasite. Unprimed control mice died within 14 days of the challenge and showed intense signs of disease, such as ruffled fur, hyperpnea and weight loss. In contrast, all of the primed mice survived longer than 30

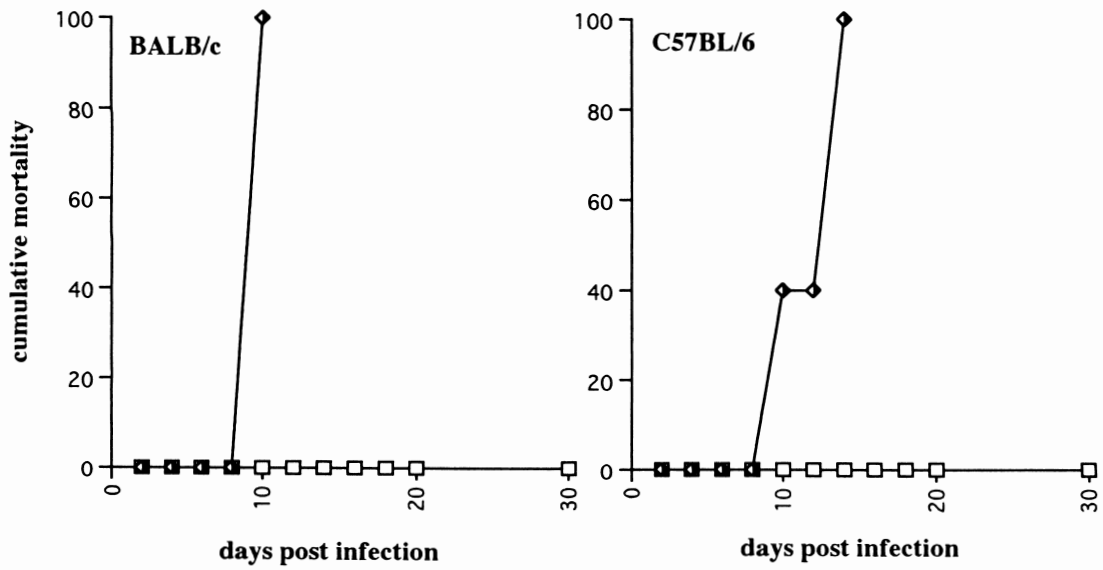


Fig. 1 Effect of priming mice with X-ray-irradiated tachyzoites on the resistance against acute toxoplasmosis. Groups of mice (n=5) were primed intradermally with $1\times10^6$ X-ray-irradiated RH tachyzoites (-□-) and were challenged intraperitoneally with $1\times10^4$ RH tachyzoites 14 days post-priming. Control mice (-◆-) were challenged with RH tachyzoites without priming. The representative data shown here are from one of three experiments performed.

days after the challenge. In addition, the priming was effective in two mouse strains that differ in their genetic background, including H-2 (Fig. 1).

To examine the subsets of T cells that are responsible for this resistance against the parasite, primed mice were treated with either anti-CD4 or anti-CD8 mAb *in vivo*, and were subjected to a challenge infection. The treatments resulted in a depletion of more than 98% of the corresponding T cell population and did not affect the unrelated T cells (Table 1). Treatment with anti-CD8 mAb significantly reduce resistance to *T. gondii* ($p<0.05$), with 80% of the treated mice dying between 20 and 24 days after a challenge. This result suggests that $CD8^+$ T cells play an essential role in protective immunity against *T. gondii* (Fig. 2). In contrast, treatment with anti-CD4 mAb had minimal effect; only one out of five mice treated with anti-CD4 mAb died 40 days PI (Fig. 2).

Adoptive cell transfer experiments also supported the idea that $CD8^+$ T cells serve as essential effectors of vaccine-induced immunity (Table 2). Mice transferred with $1\times10^7$ immune $CD8^+$ T cells survived after a challenge infection, even in the absence of host-derived $CD4^+$ T cells (Table 2). Unexpectedly, immune $CD4^+$ T cells could also transfer protection, so long as host $CD8^+$ T cells were not depleted

Table 1 Effect of *in vivo* administration of anti-CD4 or anti-CD8 mAb on axillar lymph node and spleen T-cell populations of mice primed with X-ray-irradiated RH tachyzoites

| treatment* | T cell subpopulations (%)† | | | |
|---|---|---|---|---|
| | axillar lymph node | | spleen | |
| | $CD4^+$ | $CD8^+$ | $CD4^+$ | $CD8^+$ |
| untreated | 56.9 | 22.4 | 29.8 | 17.4 |
| anti-CD4 | 0.3 | 50.3 | 0.7 | 19.4 |
| anti-CD8 | 74.7 | 0.4 | 30.3 | 1.1 |

* Mice received mAbs 4 and 2 days before challenge infection.

† Mean percent of cells in each category at the time of challenge infection. Each group consisted of two primed mice.

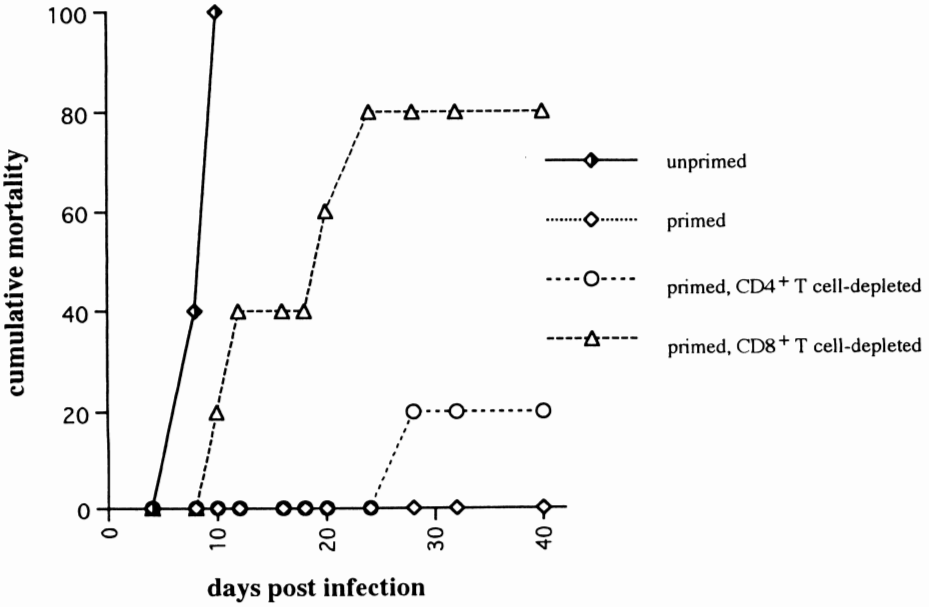


Fig. 2 Requirement of T cell subset for the maintenance of the protective immunity against acute toxoplasmosis. Primed BALB/c mice were treated starting 4 days before challenge and periodically thereafter with anti-CD4 (-O-) or anti-CD8 mAb (-△-). All animals (n=5) (including primed (-◇-) and control non-primed mice (-◆-) were challenged with $1\times10^4$ RH tachyzoites.

Table 2 Effect of adoptive transfer of immune T cell subsets on resistance to *Toxoplasma* infection in BALB/c mice

| Immune lymph node cells* | mAb | Cell number | Survival† |
|---|---|---|---|
| none | none | 0 | 0/7 |
| T cells | none | $1\times10^7$ | 7/7 |
| $CD4^+$ T cells | none | $1\times10^7$ | 7/7 |
| $CD4^+$ T cells | anti-CD8‡ | $1\times10^7$ | 0/7 |
| $CD8^+$ T cells | none | $1\times10^7$ | 6/7 |
| $CD8^+$ T cells | anti-CD4‡ | $1\times10^7$ | 4/4 |

Groups of seven syngeneic naive BALB/c mice were either injected or not injected with immune lymph node cells. These mice were challenged with RH tachyzoites 24 hr after transfer. Survival was determined 30 days PI.

* The transferred cells were prepared from the draining lymph nodes of mice 5 days after priming with X-ray-irradiated RH tachyzoites.

† Number of survived mice/total number of mice.

‡ Recipient mice were treated with an i. p. injection with 0.5 mg of anti-CD8 (2-43) or anti-CD4 mAb (GK 1.5) 4 and 2 days before transfer, and additional doses every third day after transfer.

(Table 2). Mice transferred with immune $CD4^+$ T cells succumbed to challenge infection with a significant ($p<0.05$), but slight delay compared to control mice when host $CD8^+$ T cells were depleted in advance (Fig. 3).

The fact that the $CD8^+$ T cells were competent in transferring protection as early as 5 days after priming (table 2) prompted us to examine the kinetics of the induction of the protective immunity against *T. gondii*. Groups of primed mice were challenged with unirradiated tachyzoites on the same day, the following day, or 5 days after priming. Surprisingly, six of seven primed mice, when challenged on the same day with $1\times10^3$ live RH tachyzoites ($100\times LD_{50}$), survived, although only one out of seven primed mice was resistant to a simultaneous challenge with $1\times10^4$ RH tachyzoites (Table 3). These results indicate that the inoculation with irradiated tachyzoites induces immunity rapidly enough to overcome a concurrent lethal infectious challenge.

## Discussion

Our study demonstrates that a potent protective

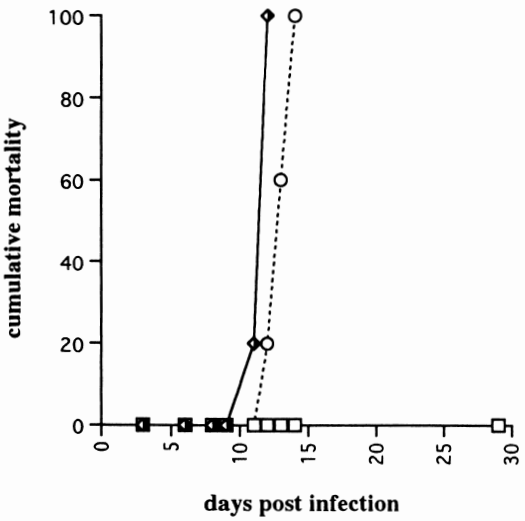


Fig. 3 Requirement of host $CD8^+$ T cells for the transfer of protection by immune $CD4^+$ T cells. One $\times 10^7$ immune $CD4^+$ T cells were transferred into either naive mice (–□–) or mice treated beforehand with anti-CD8 mAb (–○–). The mice (n=5) were subsequently challenged with $1\times10^4$ RH tachyzoites on the next day. Control mice (–◆–) were challenged without cell-transfer. The representative data shown here are from one of three experiments performed.

Table 3 Development of protective immunity to a challenge infection following priming with X-ray-irradiated RH tachyzoites

| Group | Challenge dose* | Challenge date† | Survival‡ |
|---|---|---|---|
| unprimed | $1\times10^3$ | d 0 | 0/5 |
| | $1\times10^4$ | d 0 | 0/5 |
| | $1\times10^4$ | d 1 | 0/5 |
| | $1\times10^4$ | d 5 | 0/5 |
| primed | $1\times10^3$ | d 0 | 6/7 |
| | $1\times10^4$ | d 0 | 1/7 |
| | $1\times10^4$ | d 1 | 4/5 |
| | $1\times10^4$ | d 5 | 5/5 |

Groups of five BALB/c mice were either unprimed or primed with X-ray-irradiated RH tachyzoites and subjected to a challenge infection various times after priming. Survival was determined at 30 days PI.

* Number of RH tachyzoites used in the challenge.

† Day(s) post-priming.

‡ Number of survived mice/total number of mice.

immunity can be induced in mice with x-irradiated RH tachyzoites against a lethal infectious challenge with *Toxoplasma* parasites. In addition, $CD8^+$ T cells are the major effector cells in this immunity. These findings are in agreement with previous observations (Gazzinelli *et al.*, 1991; Khan *et al.*, 1994; Nagasawa *et al.*, 1991; Suzuki and Remington, 1990) that $CD8^+$ T cells are the major effectors of protective immunity against *T. gondii*. The adoptive transfer of $CD8^+$ T cells from ts-4-vaccinated mice or a $CD8^+$, SAG1-specific T cell clone could confer resistance against lethal toxoplasmic infections (Khan *et al.*, 1994; Suzuki and Remington, 1990). We and others (Aosai *et al.*, 1994; Hakim *et al.*, 1991; Subauste *et al.*, 1991; Yano *et al.*, 1989; Yano *et al.*, 1992) have detected MHC class I-restricted CTL activity in *Toxoplasma*-specific $CD8^+$ T cells against *Toxoplasma*-infected cells. The identification of $CD8^+$ T cells restricted to MHC class I molecules as decisive effectors against infection is consistent with the fact that *T. gondii* attacks a variety of nonphagocytic somatic cells, such as hepatocytes and myocardial cells (Frenkel 1988), that do not normally express MHC class II molecules.

The direct loading of pathogen-derived antigens into the cytoplasm of antigen presenting cells is assumed to be a prerequisite for those antigens to enter the MHC class I presentation pathway (Moore *et al.*, 1988; Yewdell *et al.*, 1988). We previously reported that, soon after *T. gondii* enters host cells, some fraction of the outer membrane of the parasite fuses with the parasitophorous membrane of the host cells forming channel-like structures, through which the parasite antigens may directly enter the cytoplasm of the host cells (Yano *et al.*, 1992). Although not investigated in this study, X-ray-irradiation might not affect the way the tachyzoites penetrate the host cells and the subsequent formation of parasitophorous membranes, as indicated by Endo *et al.* (1981). Our findings, together with the fact that most vaccines that utilize killed, non-viable organisms (i.e. heat-, formalin-treated, *etc*) have been unsuccessful in producing effective immunity, may illustrate the absolute requirement of the parasite's ability to actively penetrate into host cells for successful induction of the parasite-specific $CD8^+$ cytotoxic T cells *in vivo*.

Another finding in our study is that immune $CD4^+$ T cells play a substantial, but minor role in the protective immunity against *T. gondii*, which confirms previous observations (Araujo 1991; Gazzinelli *et al.*, 1991; Vollmer *et al.*, 1987) in which $CD4^+$ T cells contribute to protective immunity to *T. gondii*. Because immune $CD4^+$ T cells could transfer protection only if host $CD8^+$ T cells coexisted (Fig. 3), those $CD4^+$ T cells might not have functioned as effectors, but rather helped to generate $CD8^+$ effectors upon challenge infection in unprimed hosts. Alternatively, immune $CD4^+$ T cells might have played a role in resistance against a lethal toxoplasma infection (Fig. 2), provided that other effector arms, such as parasite-specific antibodies and/or NK cell populations (Hauser *et al.*, 1982), were adequately recruited together with those $CD4^+$ T cells.

In the light of the relatively rapid induction of the protective immunity (Table 2), an important question is why an infection with the RH strain is lethal to mice. One possible reason is that infection-induced disintegration within the antigen presenting cells may limit the prompt induction of protective immunity *in vivo*. In cultured cells, RH tachyzoites multiply rapidly, causing cell death within 2 days after infection. The organisms might kill the antigen presenting cells before they can induce immunity against the parasite. Alternatively, the RH tachyzoites might produce a putative "virulence-associated" molecule(s) during their division within the host cells, which might suppress antigen processing and/or the presentation function of the infected cells, or the differentiation and functional maturation of the effector cells. Evidence shown by Haque *et al.* suggested that *T. gondii* excretes or secretes a factor(s) that stimulates the production of the immune downregulatory cytokine, such as IL-10, during an *in vitro* experimental model of infection (Haque *et al.*, 1995). Irradiation of tachyzoites or some lymphokine(s) produced from immune $CD4^+$ T cells, such as interferon $\gamma$, might prevent tachyzoites from producing such a molecule(s), thereby allowing uninterrupted induction of protective immunity against the parasite.

In conclusion, $CD8^+$ T cells are the major effector cells in the protective immunity induced in mice with X-ray-irradiated RH strain tachyzoites of *T.*

*gondii*. Induction of the protection was rapid enough to resist a concurrent lethal infectious challenge. X-ray-irradiated *T. gondii* may serve as a useful alternative to the avirulent, temperature-sensitive mutant ts-4 strain for elucidating the mechanisms of protective immunity against a *Toxoplasma* infection.

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