

Susceptibility to re-infection in C57BL/6 mice with recombinant strains of *Toxoplasma gondii*

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ABSTRACT

This work reports results of re-infection of BALB/c and C57BL/6 mice with different recombinant strains of *Toxoplasma gondii*. Mice were prime-infected with the non-virulent D8 strain and challenged with virulent strains. PCR-RFLP of *cS10-A6* genetic marker of *T. gondii* demonstrated that BALB/c mice were re-infected with the EGS strain, while C57BL/6 mice were re-infected with the EGS and CH3 strains. Levels of IFN- γ and IL-10 after D8 prime-infection were lower in C57BL/6 than in BALB/c mice. Brain inflammation after D8 prime-infection was more intense in C57BL/6 than in BALB/c mice. It was shown that re-infection depends on mice lineage and genotype of the strain used in the challenge.

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1. Introduction

Primary infection with *Toxoplasma gondii* gives life-long immunity, preventing re-infection (Filisetti and Candolfi, 2004). In the early phase of toxoplasmosis, resistance in mice is related to the initial production of IL-12 and INF- γ by natural killer cells (Buzoni-Gatel et al., 2006). In the chronic phase, TH1 CD4⁺ cells and CD8⁺ cytolytic lymphocytes produce INF- γ (Pfaff et al., 2007) through activity against tachyzoites or cells infected with *T. gondii* (Subauste et al., 1991), preventing re-infection. However, cases of congenital toxoplasmosis in immunocompetent women in the chronic phase have been reported, showing the possibility of re-infection at least in pregnant women (Elbez-Rubinstein et al., 2009). Resistance and susceptibility in mice experimentally infected with *T. gondii* varies depending on the route of infection, type of inoculum, parasite strain, and the host immune response (Dao et al., 2001; Brandão et al., 2009).

The genes of major histocompatibility complex (MHC) exert considerable influence on the outcome of experimental toxoplasmosis in mice (Blackwell et al., 1993; McLeod et al., 1996). The class I MHC genes (H-2 haplotype) perform critical functions in resistance to encephalitis and control of cerebral cyst loads (Brown

et al., 1995). BALB/c mice display the allele d, which confers resistance against encephalitis caused by *T. gondii* infection, while C57BL/6 mice have the allele b, which is associated with susceptibility (Suzuki et al., 1994). The genotype of *T. gondii* strain also plays a role in determining resistance to encephalitis in mice (Fux et al., 2003). However, little is known about the influence of genetics on the host co-infection with different recombinant strains of *T. gondii*, such as those commonly occurring in Brazil (Ferreira et al., 2006; Dubey and Su, 2009).

This study reports results of re-infection of BALB/c and C57BL/6 mice after prime infection with a non-virulent recombinant strain of *T. gondii* isolated in Brazil as well as of the influence of the host on resistance to re-infection with *T. gondii*.

2. Material and methods

2.1. *T. gondii* strains

Three different recombinant *T. gondii* strains isolated in Brazil were used in this study. The differences in their recombinant genotypes after analysis of eight genetic markers (Fux et al., 2003) were described in details elsewhere (Ferreira et al., 2006). The D8 (non-virulent) and CH3 (virulent) strains, both cystogenic, were isolated from a dog and a chicken, respectively. The EGS strain (virulent, cystogenic) was isolated from a human with congenital toxoplasmosis.

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2.2. Mice

Female Swiss, BALB/c and C57BL/6 mice, 8 weeks of age, were obtained from the animal breeding facility (Centro de Bioterismo – CEBIO) of the Universidade Federal de Minas Gerais (UFMG). Swiss mice were used for parasite maintenance and to obtain tachyzoites for DNA extraction. BALB/c and C57BL/6 mice were used for re-infection experiments and bioassays. BALB/c and C57BL/6 mice were kept in the animal house facility of the Biological Sciences Institute, Federal University of Minas Gerais, Brazil. Food and water were available to the animals throughout the experiment. All experimentation with animals was performed in accordance to the guidelines of the Institutional Ethics Committee (CETEA-UFMG protocol No. 038/05).

2.3. Prime infection and re-infection of mice with *T. gondii*

The strains D8, EGS and CH3 were maintained *in vivo* as cysts in Swiss mice carriers. Maintenance was performed by successive per oral (p.o.) inoculation of cysts isolated from brains of previously infected animals. In the case of EGS and CH3, the infected Swiss reservoirs were treated with sulfadiazine for 10 days after infection to obtain cysts. For infection experiments, BALB/c and C57BL/6 mice were divided in groups of 7–10 animals (Table 1) and inoculated with a p.o. dose of 20 cysts of the D8 strain. Forty-five days after primary D8 infection (recent chronic toxoplasmosis), animals were challenged with 20 cysts p.o. of either EGS or CH3 strains. As controls, animals were inoculated with the D8 strain and kept without challenge. Groups of animals inoculated with EGS or CH3 strains, without the D8 primary infection, were also used as controls. A detailed description of all groups is shown in Table 1. After challenge, mortality was followed over 30 days. The mice that survived were sacrificed by cervical dislocation; the brains were removed and homogenized in 1 ml of phosphate buffered saline, pH 7.2 and examined for DNA analyses by PCR–RFLP (Brandão et al., 2009) and bioassay. For bioassay, only one normal BALB/c or C57BL/6 mice was infected i.p. with 20 brain cysts from each mouse that survived challenge. Bioassay was considered positive when tachyzoites in the peritoneal fluid or cysts in the brain were found. Bioassay was used as an auxiliary criterion to PCR–RFLP, demonstrating re-infection by mortality rate.

2.4. Polymerase chain reaction – restriction fragment length polymorphisms (PCR–RFLP)

The main criteria to confirm re-infection with *T. gondii* was PCR–RFLP. We performed genotyping of *T. gondii* samples from infected animals using the *c510-A6* genetic marker (Su et al., 2002), which was shown to distinguish D8 from EGS and CH3 strains (Ferreira et al., 2006). First, we recovered brain cysts from each BALB/c or C57BL/6 that survived challenge infection and inoculated them by i.p. injection of 200–300 cysts in Swiss mice. After 5–7 days of infection, Swiss mice were sacrificed and peritoneal washes were performed under aseptic conditions to obtain tachyzoites. Then, DNA samples were extracted from tachyzoites with phenol–chloroform and submitted to PCR–RFLP, as described elsewhere (Ferreira et al., 2006; Sambrook et al., 1989). Amplifications were performed with primers 5'-CTGGTTACATTTTCGCCTATCA-3' and 3'-CCTAGTCCAACTAGGGCTTGA-5', producing a 341 bp fragment. PCR products were digested with restriction enzyme *RsaI*. The DNA banding pattern was resolved in 5% polyacrylamide gels and silver stained. The RH88 (type I), ME49 (type II) and VEG (type III) strains were used as references.

2.5. Splenocyte cultures and cytokine measurement

Spleens were removed from D8 infected and non-infected control animals at 45 days after primary infection and macerated to obtain single cell suspensions. Red blood cells were lysed using a buffer containing 0.16 M NH₄Cl and 0.017 M Tris–HCl pH = 7.5. Splenocyte suspensions were washed three times and adjusted to 10⁷ cells/ml in RPMI-1640 supplemented with 10% fetal calf serum, 25 mM HEPES and 40 mg/l gentamicin. The cell suspension was distributed (500 µl/well) in 24-well tissue culture plates and cultured with 10 µg/ml STAG, 5 µg/ml Con A or RPMI medium alone for 72 h at 37 °C in 5% CO₂. The cytokines IL-10 and IFN-γ were quantified in supernatants of the spleen cells using the BD OptEIA® kits (BD Biosciences), according to the manufacturer instructions.

2.6. Histopathology

A quarter of the brain of the surviving mice was collected in 10% formalin in PBS and subjected to histopathology evaluation.

Table 1

Survival, PCR and bioassay of BALB/c and C57BL/6 mice prime infected with D8 strain and challenged after 45 days with EGS or CH3 strains of *Toxoplasma gondii*.

Group ^a	Strains	Re-infection		Bioassay of survivors	
		Survival n/N ^b (%)	PCR–RFLP n/N ^c (%)	Survival n/N ^d (%)	Cysts n/N ^e
BALB/c					
A	D8 + EGS	7/7 (100)	5/7 (71)	6/7 (86)	6/6
B	D8 + CH3	8/10 (80) [*]	0/8 (0)	8/8 (100)	8/8
C	D8	10/10 (100)	ND ^f	10/10 (100)	10/10
D	EGS	0/10 (0)	NS ^g	–	–
E	CH3	0/10 (0)	NS	–	–
C57BL/6					
F	D8 + EGS	6/7 (85.7) ^{**}	6/6 (100)	1/6 (16.7)	1/1
G	D8 + CH3	6/7 (85.7) ^{**}	6/6 (100)	1/6 (16.7)	1/1
H	D8	6/7 (85.7) ^{**}	ND	3/6 (50)	3/3
I	EGS	0/10 (0)	NS	–	–
J	CH3	0/10 (0)	NS	–	–

^a Groups A and B: BALB/c mice challenged with EGS or CH3 strains after primary D8 infection. Groups C, D and E: BALB/c mice prime infected with D8, EGS or CH3 strains (BALB/c controls). Groups F and G: C57BL/6 mice challenged with EGS or CH3 strains after primary D8 infection. Groups H, I and J: C57BL/6 mice prime-infected with D8, EGS or CH3 strains (C57BL/6 controls). After challenge, mortality was observed during 30 days. The experiment was repeated twice and provided similar results.

^b Number of survivors (n) of the total number of challenged mice (N).

^c Number (n) of mice presenting the two strains (positive PCR–RFLP at *c510-A6* locus) of the total number of survivors after challenge (N).

^d Number of survivors (n) of the total number of bio-assayed animals (N).

^e Number of mice with brain cysts (n) of the total number of bio-assayed mice (N).

^f ND – Not done.

^g NS – No survival.

^{*} Death occurred 17–19 days after challenge.

^{**} Death occurred 9–12 days after challenge.

Following processing, the samples were set in paraffin, sectioned into 3–5 μm slices stained with hematoxylin–eosin and examined under a light microscope. Morphometrical studies of glial nodules and perivascular inflammatory infiltrates were performed by analyzing images of 20 randomly selected fields (total area $1.5 \times 10^6 \mu\text{m}^2$) of tissue fragment sections on a single slide per animal. All analyses were performed using a 40 \times objective and images were analyzed using Leica QWin software (Leica Microsystems®).

2.7. Statistical analysis

The intensity of inflammation in the brain as well as cytokine levels in the supernatants from the splenocyte cultures obtained from the different mice groups were analyzed by the Student's *t* test. The differences were considered statistically significant when $p < 0.05$.

3. Results

3.1. Mortality

The results of challenge infections are shown in Table 1. We observed that the primary infection of BALB/c mice with the D8 strain prevented mortality caused by EGS strain (group A) and significantly reduced the mortality caused by CH3 strain (group B). The cause of death of two BALB/c mice challenged with CH3 strain was not determined. In the case of C57BL/6 mice, priming with D8 strain lead to a significant reduction of mortality caused the EGS and CH3, from 100% to around 14% mortality (groups F and G, respectively). Both BALB/c and C57BL/6 survivors had brain cysts, independent of the strain of challenge. The cysts loads were similar in all groups and comparable to number of brain cysts in animals that received only D8 infection (data not shown).

3.2. Genotyping of *T. gondii* by PCR–RFLP

To evaluate if the challenge inoculation could induce re-infection and co-existence of two different strains of *T. gondii* in the brain of the same animal, we performed analysis of the *T. gondii* marker *cS10-A6* in DNA samples obtained from parasites recovered from infected animals. Table 1 shows that the EGS strain successfully re-infects animals that were previously inoculated with D8 strain, since 71% of specimens from BALB/c group A and 100% of specimens from C57BL/6 group F showed positive PCR results for both strains.

On the other hand, successful re-infection with CH3 strain appears to depend on the strain of mice. BALB/c are resistant to

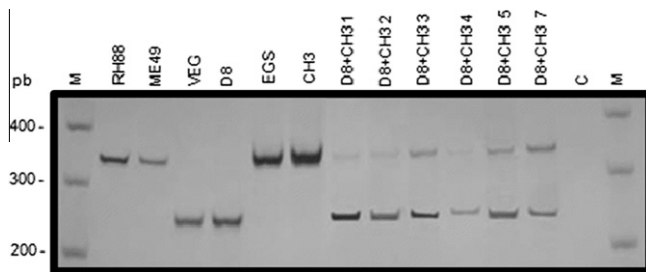


Fig. 1. Polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) of *Toxoplasma gondii* at *cS10-A6* locus. Products were digested with restriction endonuclease *RsaI* and revealed in 5% polyacrylamide gel to verify re-infection of C57BL/6 mice challenged with the CH3 strain, 45 days after primary D8 strain infection. RH88 (Type I), ME49 (Type II) and VEG (Type III) strains were used as reference. D8 + CH3 = Six mice challenged with the CH3 after primary D8 infection. M = molecular weight marker (Promega 100pb), C = negative control, without DNA. The experiment was repeated twice and provided similar results.

re-infection by CH3 since it was observed that none of the survivors from group B, analyzed by PCR–RFLP, showed concomitant presence of D8 and CH3 strains. C57BL/6 mice seem to be more susceptible to re-infection than BALB/c, taking in account that all specimens from group G showed co-existence of D8 and CH3 strains. Representative results of PCR–RFLP analysis of *T. gondii* DNA from C57BL/6 mice challenged with CH3 strain are shown in Fig. 1.

3.3. Bioassay

Bioassay was conducted as an auxiliary criterion for re-infection. Twenty cysts obtained from surviving BALB/c mice were used to infect naïve BALB/c, and mortality in recipients groups was followed for 30 days (Table 1). A similar bioassay was performed with C57BL/6 mice: Twenty brain cysts of each surviving mouse were used to infect one naïve C57BL/6 mouse. Fourteen percent mortality (one of seven animals) was observed in naïve BALB/c mice that received cysts obtained from survivors of group A (primed with D8 and challenged with EGS). Bioassay performed with cyst samples from survivors of group B (challenged with CH3) did not induce mortality. Bioassay of *T. gondii* cysts obtained from C57BL/6 mice survivors (groups F and G) induced 83.3% mortality. On the other hand cysts from group H animals caused 50% of mortality in the naïve recipients.

3.4. Cytokine measurement

Synthesis of IFN- γ and IL-10 by splenocytes of BALB/c and C57BL/6 mice was evaluated 45 days after prime-infection with

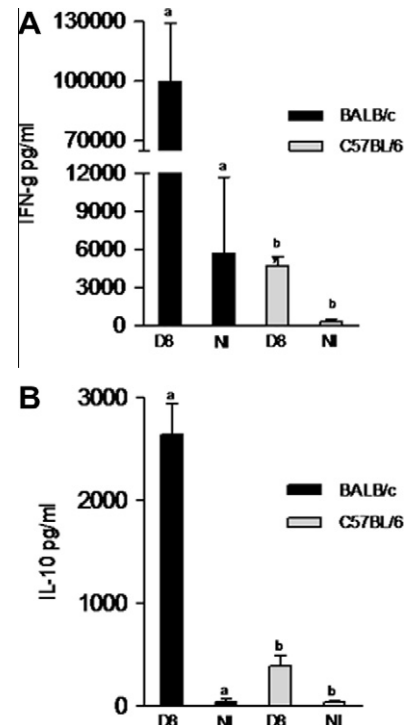


Fig. 2. Levels of IFN- γ (A) and IL-10 (B) in BALB/c and C57BL/6 mice orally infected with 20 cysts of the D8 *Toxoplasma gondii* strain (D8) and non infected (NI). IFN- γ and IL-10 were measured in the supernatant of splenocytes cultured with STAG. Results obtained in the supernatant of splenocytes cultured with Con A or RPMI medium alone are not shown. The values shown are the means of eight animals. The experiment was repeated twice and provided similar results. Significant differences in the production of IFN- γ or IL-10 in BALB/c (a) and C57BL/6 (b) mice are shown with lower case letters.

Table 2
Pathological findings in the brain of mice infected with D8 and challenged with EGS or CH3 strains of *Toxoplasma gondii*.

Strains of <i>T. gondii</i>	BALB/c			C57BL/6		
	Group	GN ^a	PVI ^b	Group	GN ^a	PVI ^b
D8 + EGS	A	1.37 ± 1.50 ^c	1.12 ± 1.12	F	2.16 ± 0.4	2.16 ± 2.2
D8 + CH3	B	1.42 ± 1.61	1.14 ± 1.2 ^d	G	2.5 ± 2.34	2 ± 1.67
D8	C	0 ± 0	0.25 ± 0.70	H	1.5 ± 1.37 ^e	1.83 ± 1.47 ^e

^a Mean number of glial nodules in a total area of $1.5 \times 10^6 \mu\text{m}^2$.

^b Mean number of perivascular inflammatory infiltrates in a total area of $1.5 \times 10^6 \mu\text{m}^2$.

^c Significant increase ($p < 0.05$) of glial nodules in relation to the non-challenged control – group C.

^d Significant increase ($p < 0.05$) of perivascular inflammatory infiltrates in relation to the non-challenged control – group C.

^e Significant increase ($p < 0.05$) of perivascular inflammatory infiltrates and glial nodules in relation to non-challenged BALB/c mice – group C.

D8 strain of *T. gondii*. In BALB/c mice, there was a significant increase in the production of IFN- γ and IL-10 after stimulation with STAg, compared with the non-infected animals submitted to the same stimulus (Fig. 2A and B). In C57BL/6 mice, there was a significant increase in the production of IFN- γ after stimulus with STAg, compared to non-infected animals submitted to the same stimulus (Fig. 2A). Production of IL-10 in C57BL/6 mice did not reach significant levels compared to BALB/c mice (Fig. 2B).

3.5. Histopathology

Infection with D8 strain of *T. gondii* induced significant pathology in the brain of C57BL/6 mice but not in the brain of BALB/c mice (group C). BALB/c mice challenged with EGS strain (group A) presented a significant increase in the number of glial nodules compared to the animals infected only with D8 strain (Table 2). BALB/c mice challenged with CH3 strain (group B) presented an increase in the number of perivascular inflammatory infiltrates. No significant difference was observed in the number of perivascular infiltrates and glial nodules in C57BL/6 mice challenged with the strains EGS (group F) and CH3 (group G), compared to the animals infected only with D8 strain (group H).

4. Discussion

When BALB/c mice were prime infected with the non-virulent D8 strain of *T. gondii* and challenged after 45 days with the virulent EGS or CH3 strains, the mortality rate significantly decreased, compared to the mortality of mice infected only with the strains used in the challenge. This probably occurred as a consequence of the adaptive immune response conferred by the D8 strain. Such results are similar to that previously described (Dzitko et al., 2006). Mice challenged with a lethal dose of a virulent strain after a prime-infection were found to survive due to the development of a strong adaptive immune response capable of preventing the death of the host. Re-infection with CH3 strain cannot be ruled out as cause of death of two BALB/c mice. The PCR-RFLP of tissues of these animals could clarify this situation but unfortunately it was not possible to obtain their organs for analysis.

The C57BL/6 mice prime-infected with D8 strain and challenged with EGS or CH3 strains also had a lower mortality rate than that seen in prime-infected control animals. PCR-RFLP confirmed the co-existence of D8 and EGS strains in the brain of five (71%) surviving BALB/c mice challenged. DNA analysis showed that 100% of the surviving C57BL/6 mice were re-infected with EGS and CH3 strains. The presence of two clonal lines of *T. gondii* in the brain of mice after experimental re-infection had been previously demonstrated (Dao et al., 2001). These authors observed that primary infection with the Prufgal *T. gondii* strain did not impair tissue cyst formation upon re-infection with the Ned strain of *T. gondii*, which belongs to another *T. gondii* clonal genotype. Bioassay mortality

rates confirmed greater susceptibility of C57BL/6 to re-infection with recombinant strains of *T. gondii* compared to BALB/c mice. However RFLP-PCR has greater sensitivity than bioassay to detect low brain cysts load of the strain used in the challenge.

Splenocytes of C57BL/6 mice produced significant smaller amounts of IFN- γ than those of BALB/c mice and IL-10 was not observed at significant levels in C57BL/6 mice. The lower amount of IFN- γ and IL-10 in C57BL/6 mice probably favored re-infection and the greater mortality rate observed in these animals compared to BALB/c mice. IFN- γ is crucial as mediator of adaptive immunity (Pfaff et al., 2007), while IL-10 acts on the regulation of the pro-inflammatory response (Lang et al., 2007). Also, IFN- γ and IL-10 produced by T cells are crucial for the elimination of pathogens with minimum pathology or development of persistent infection (O'Garra and Vieira, 2007). However the production of IFN- γ and IL-10 by splenocytes may not reflect the presence of these cytokines in the brain, where pathology is observed.

The inflammation in the brain of C57BL/6 mice infected only with the D8 strain was more pronounced than in BALB/c mice. Inflammation observed in BALB/c mice challenged with CH3 strain probably occurred as a result of re-infection undetectable by PCR-RFLP due to the low amount of CH3 strain DNA compared to D8 strain DNA. The inflammation observed in C57BL/6 mice does not correspond to the smaller amount of IFN- γ produced by these animals, compared with BALB/c mice. This suggested that pathology in the brain of C57BL/6 mice can be attributed to a higher parasite burden, associated with inability of this mouse line to control parasite replication via pro-inflammatory response. Other factors associated with increased susceptibility of C57BL/6 mice to re-infection still remain to be studied. It was recently observed that increases IL-17 expression contributes to the inflammatory response and susceptibility in C57BL/6 mice after *T. gondii* infection (Guiton et al., 2010). Further studies are required in order to investigate cytokines levels in the brain and their involvement in the pathology after re-infection by *T. gondii*.

Previously, we have shown that the genotype of the *T. gondii* strain used in the challenge as well as the time between prime-infection and challenge influence the re-infection rate (Brandão et al., 2009). In this work, the occurrence of experimental re-infection by *T. gondii* varied as a function of the parasite strain and mouse line. These observations lead to the conclusion that the occurrence of re-infection is related not only to the genotype of the parasite but also to the immune response of the host, which is dependent on the mouse line used.

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