Original Article

Chronic infection with *Toxoplasma gondii* does not prevent acute disease after virulent strain reinfection in experimental mice

Eman M Gaballah, Aida A Abdel-Magied, Nora I Aboulfotouh, Goman A Elganainy

Department of Medical Parasitology, Faculty of Medicine, Mansoura University, 35516, Egypt

ABSTRACT

Background: Toxoplasmosis is a wide spread protozoan disease. It was generally believed that primary infection by *T. gondii* protects from reinfection, however, multiple cases of reinfection have been detected in immune mothers. This work reports results of re-infection of Swiss Webster (SW) mice with different strains of *T. gondii*.

Objective: To simulate the impact of reinfection in experimentally infected mice with lethal strain of *T. gondii* after prime infection with non-virulent genotype.

Material and Methods: The study was conducted on 36 female SW mice which were divided into four groups: 6 mice were infected with ME49 only (GI); 18 mice were infected with ME49 and re-challenged with RH on day 65, i.e. 8 weeks post ME49 infection (GII); 6 mice were infected with RH only (GIII); and 6 non-infected control mice (GIV). *Toxoplasma* RH strain re-challenged mice (GII) were monitored over two weeks observation period for mortality, clinical signs of acute illness (scored grades I-II), presence of intraperitoneal tachyzoites, brain cyst burden, and compared to chronically infected non-challenged mice (GI) and mice infected with RH only (GIII).

Results: Prolonged survival rate of re-challenged group of mice than in RH only infected group was the only significant result. Cyst number and diameter were higher in re-challenged group than in mice infected only with ME49. Tachyzoites were recovered from peritoneal lavage of all mice that received RH whether primarily infected with ME49 or not. Clinical grading (I-II) was the same for both groups and both reached grade II.

Conclusion: These results highlighted that mice with chronic toxoplasmosis developed acute disease when re-challenged with another virulent strain. Therefore, chronic infection with *T. gondii* apparently neither prevents acute disease nor impairs colonization of the brain with tissue cysts after virulent strain superinfection. The present work supports and explains the possibility of congenital toxoplasmosis in immune pregnant mothers when re-infected by a virulent strain of *T. gondii*.

Keywords: ME49 strain, toxoplasmosis, virulent strain superinfection.

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Corresponding Author: Aida A Abdel-Magied, **Tel.:** 01008370680, **E-mail:** aida.awad.new@gmail.com

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INTRODUCTION

T. gondii infects about one third of the whole population[1]. Human acquire infection *via* the infective stages; sporulated oocysts present in contaminated environment by felines stools, bradyzoites in tissue cysts during consumption of undercooked animal tissues, and finally tachyzoites which are mainly responsible for congenital infection[2]. The parasite persists in its host by conversion from the proliferative tachyzoite stage to the quiescent encysted bradyzoite stage. *T. gondii* strains belong to three clonal lineages: I, II and III[3], among which the genetic difference at the genome sequence levels are generally few. Several phenotypes such as growth, migration and transmigration were reported[4], however the best described of these is virulence to laboratory animals[5]. Type I strains (e.g. RH) cause lethal infection to all strains of laboratory mice even with low inoculum. In contrast, type III strains (e.g. NED) are typically non-virulent. On the other hand, type II strains (e.g. ME49) are characterized by moderate virulence for laboratory mice; however, they are associated with human infections creating chronic persistent tissue cysts, i.e. they are a cytogenic strain[6].

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[7]. In the chronic phase, TH1 $CD4^+$ cells and $CD8^+$ cytolytic lymphocytes produce INF-γ due to activity against tachyzoites or infected cells, preventing re-infection[8]. However, cases of congenital toxoplasmosis in immunocompetent women in the chronic phase have been reported, revealing the possibility of re-infection specially in pregnant women[9]. Hence, congenital transmission is considered as one of the most important complications of toxoplasmosis during pregnancy[10]. In congenital toxoplasmosis, transmission to the fetus occurs predominantly in women who acquire primary infection during pregnancy[11,12]. It was believed that primary infection with *T. gondii* can give lifelong immunity and protection against reinfection with another strain[2]. However, multiple cases of congenital infection

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of already immune mothers were recorded[13-15]. In immunocompetent mothers who were infected before conception, the immune mechanisms prevent transmission of the infection to their fetuses[16]. Still as reported, acquired immunity in toxoplasmosis does not fully protect against severe consequences to the child; caused either by reactivation of a latent infection in pregnant women with immunocompromised status or by re-infection[9,17]. In Egypt, multiple exposures to *T. gondii* likely occur with great frequency, yet little is known about the interaction between a chronically infected host and other *T. gondii* strains. A murine model was used to explore secondary infection of chronically infected mice with the highly virulent type I RH strain. Our aim is to demonstrate if previous infection by a certain avirulent strain of *T. gondii* can protect against reinfection by another strain or decrease sequel of re-infection.

MATERIAL AND METHODS

This experimental case control study was carried out at Medical Parasitology Department, Faculty of Medicine, Mansoura University during the period from February to December 2017.

Experimental animals: Fifty-one female SW mice, weighing 20 gm in average and six weeks old were purchased from Mansoura Experimental Research Center. Mice were bedded on autoclaved wood shavings. Water and standard pelleted diet were offered ad libitum. A temperature of 25 ± 20°C, humidity of 60% ± 10 and a 12 h day/night schedule was maintained.

Parasites: Two *Toxoplasma* strains were used; ME49 avirulent strain, belonging to lineage II (kindly provided from Department of Epidemiology and Zoonotic Diseases, National Research Center, Doki, Giza), and RH virulent strain belonging to lineage I (kindly provided from Parasitology Department, Faculty of Medicine, Alexandria University).

Study design: Thirty-six mice were divided into four groups: 6 mice infected with ME49 only (GI); 18 mice infected with ME49, then re-challenged with RH on day 65, i.e. 8 weeks post ME49 infection (GII); 6 mice infected with RH only (GIII); and 6 control mice (non-infected GIV) (Table 1). For maintenance of strains, 10 mice were used to maintain and propagate ME49, and 5 mice for RH every 3rd day. Mice were observed for two weeks to estimate the mean percentage of survival rate; to record and grade the clinical signs of acute illness and search for tachyzoites in the peritoneal aspirate in groups II and III. In addition, estimation of the mean brain cyst burden and diameter was conducted only in GI and GII because ME49 is cystogenic while RH strain is not[6].

Table 1. Experimental schedule

Day	No. of mice	Action
0	6*	Non-infected controls (GIV).
	34*	Infected with ME49 strain; 6 mice were used for GI, 18 mice for GII and 10 mice to confirm infection.
60	10	ME49-infected mice were sacrificed to confirm infection.
	5*	Maintenance and propagation of RH strain every three days.
	6*	Infected with RH strain only (GIII).
	18	ME49-infected mice were challenged with RH strain (GII).
65-78	36	Observation period.
79		End of the experiment.

NB: * Total number of mice included in the study.

Parasite maintenance, propagation and mice infection: The avirulent ME49 strain was maintained through passage into ten mice. For animal infection, mice were orally inoculated by gavages with brain suspension containing *T. gondii* cysts (10 cysts/mouse). For preparation of brain suspension, eight weeks after infection, mice were sacrificed, brains were removed and homogenized in a tissue homogenizer (Wheaton, USA) with 1 ml sterile 0.9% NaCl solution each[18]. The virulent RH strain was maintained by continuous intraperitoneal passages into five mice every three days[19]. For animal infection, tachyzoites were harvested from peritoneal exudates of infected mice on the fourth day of infection, debris and host cells were removed by filtration through a sheet of glass wool fibers. The filtrate was washed three times and diluted with phosphate buffer saline (PBS), pH 7.4. About 2500 viable tachyzoites/mouse were injected intra-peritoneally for induction of acute infection model[20].

Clinical grading: Mice were closely observed for behavior changes, recording and grading clinical signs of acute illness. Clinical grading was described as grade 0: glossy coat, bright and active mice; grade I: hunched and stiff coat; grade II: reluctant to move[21].

Count of tachyzoites in the peritoneal lavage: After euthanasia in groups II and III, mice were submitted to peritoneal lavage with 10 ml sterile 0.9% NaCl solution. Samples were centrifuged at low speed (500 g for 30

sec). The supernatant was filtered and centrifuged two more times at 500 g for 5 min. The pellets were re-suspended in a 0.9% NaCl solution and standardized by counting tachyzoites in a hemocytometer. Tachyzoites were detected in the peritoneal lavage by direct fresh smear.

Total count of *Toxoplasma* cysts in the brain: At the end of the experiment after sacrifice, the brain of each mouse infected with ME49 strain (GI) was homogenized in one ml PBS and sieved. The total number of cysts in each brain was determined by microscopy (40x). The mean number of successive 10 µl aliquots was multiplied by 100[22]. Brain cysts diameter in homogenate were measured using calibrated slide.

Statistical analysis: Data were analyzed with SPSS version 21. The normality of data was first tested with Shapiro-Wilk test. Qualitative data were described using number and percent. Association between categorical variables was tested using Chi-square test. Continuous variables were presented as mean ± SD. The two groups were compared with Student *t* test. Analysis of variance (ANOVA test) was used for comparison of means of more than two groups. For all statistical tests done, the threshold of significance is fixed at 5% level; the results were considered significant when *P* value ≤ 0.05.

Ethical considerations: The study was approved by both the Ethics Review Committees and the Institutional Review Boards of the faculty of medicine, Mansoura University, Egypt (IRB No. 16.06.83).

RESULTS

Mice with confirmed chronic toxoplasmosis (ME49 strain) were re-challenged on day 65 with another virulent strain (RH) (GII), monitored over a period of 2 weeks and compared to the other two groups; one was already infected by ME49 strain only (GI), and the other was infected by RH strain only (GIII). Mice of GI survived throughout the whole experiment time (79 days). The survival rate of re-challenged GII was significantly (*P*=0.001) prolonged than that of mice infected with RH only (GIII) (12.5±2.22 versus 4.17±0.98 days, respectively), but insignificantly shorter than ME49 infected only (GI) (77.5 versus 79 days, respectively) (Figure 1).

It was observed that all females in GI remained healthy with clinical grading 0 (glossy coat, bright and active). Both re-challenged GII and RH infected only (GIII) recorded clinical changes ranging from grade I (hunched and stiff coat) on third day (day 68), then deteriorated to grade II (reluctant to move) and finally all died (Figure 2). Reinfection effect was so obvious on brain cyst burden which was significantly (*P*=0.001) higher in re-challenged GII than ME49 only group (G1) (Table 2, Figure 2). The same was noticed regarding cyst diameter in the same two compared groups (*P*=0.006) (Table 2). Concerning peritoneal aspirate examination, tachyzoites were found in all mice of both groups who received RH infection regardless whether previously infected with avirulent strain or not. The experiment was repeated twice and gave similar results.

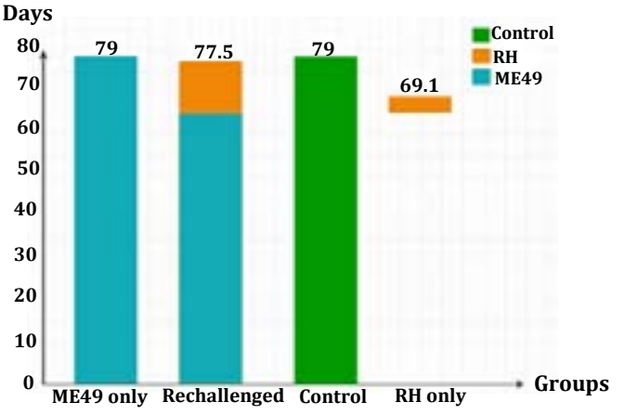

Fig. 1. Comparison between different groups in relation to survival.

Table 2. Brain cyst burden and diameter of GI and GII mice.

Brain cysts	ME49 only group GI (No. =6)		Re-challenged group GII (No. =18)		Statistical analysis
	Mean ± SD	Min-Max	Mean ± SD	Min-Max	
Number/Brain	4366.67 ± 445.72	3800 - 5000	5866.66 ± 657.98	5000-7000	*t*=5.16, *P*≤0.001
Diameter (µm)	19.90 ± 0.96	18.6 - 21.3	24.40 ± 3.52	18.6 - 30.0	*t*=3.05, *P* =0.006

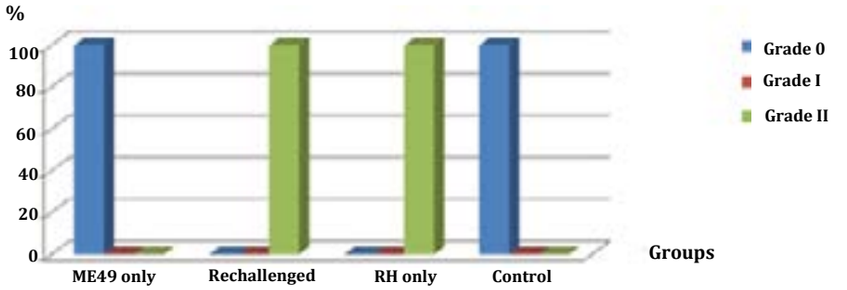

Fig. 2. Clinical grading of all studied mice groups (3 days after RH infection).

DISCUSSION

When SW mice were prime infected with avirulent ME49 strain of *T. gondii* and challenged on day 65 with virulent RH strain, the survival rate of re-challenged group was significantly prolonged when compared with the survival of mice infected only with the strain used in the challenge. This likely occurred because of the adaptive immune response produced by ME49 strain and is in accordance with Brandã *et al.,*[23], who found that the survival rate of mice prime infected with the non-virulent D8 strain and superinfected with virulent one significantly increased when compared with the mortality of mice infected only with the virulent strains. It was documented from their study that when mice were prime infected with the non-virulent D8 strain and then challenged with a virulent one, they became susceptible to reinfection with a different recombinant *T. gondii* strain and this susceptibility correlated with increase of IL-10 production. The presence of two genotypes of *T. gondii* in the brain of mice after experimental reinfection was previously demonstrated[24]. The investigators observed that the primary infection with the Pru *beta* gal *T. gondii* strain did not impair tissue cyst formation upon re-challenge with the Ned strain, which belongs to another *T. gondii* clonal genotype.

In our study, both ME49 infected and control groups showed mean survival rate (79±0 days) as they lived throughout the experiment. Re-challenged group with RH showed a survival mean of 77.5±2.22 days, the difference wasn't significant. Our result matches with another study by Araujo *et al.,*[25] which was conducted on SW mice primarily infected with ME49 strain. The researchers detected zero mortality after 60 days of infection. Mice infected with RH only group showed mean survival 4.17±0.98 days, and started to record death from the 3rd to the 5th day. This is in accordance with El Temsahy *et al.,*[26] who recorded that RH infected mice did not remain alive beyond the seventh day post infection with a mean survival time of 5.9±0.88 days.

Clinical observation of the four studied groups of mice showed that both ME49 (GI) and control group (IV), remained unchanged through the whole experiment with clinical grading 0. On the other hand, both re-challenged GII and RH only GIII recorded clinical changes reaching grade II by the end of the experiment. These findings proved that chronicity in primarily infected mice with ME49 strain, maintained the survival of mice. However, the chronicity didn't offer protection against secondary infection with lethal RH that succeeded to invade tissues and replicate as proved by tachyzoites presence in mice peritoneal lavage. Furthermore, this lethal strain caused manipulation of immune system in mice and reactivated the dormant infection.

Brain cyst number and diameter were the basic parameters for evaluation used in our experiment. Cyst diameter was significantly higher in re-challenged GII than GI (P=0.006). Larger tissue cysts contain more bradyzoites presenting evidence for replication of bradyzoites[27]. Also, cyst number was significantly higher in re-challenged GII than GI (P<0.001). This can be explained by matching with the study done by Liesenfeld *et al.,*[28] who demonstrated that both RH and ME49 have different immunological, inflammatory and pathological pathways. RH parasites have the genetic ability to replicate and reach higher parasitic burden in tissues, much higher than ME49. The virulent strain acts by over production of INF-ɤ. This cytokine in a suitable dose masters control on parasite replication and enhanced tissue cyst formation. But in the case of RH strain, overproduction of INF-ɤ leads to uncontrollable pathological reactions and lethal effect. That may be explained by the fact that despite the high number of macrophages stimulated by IFN-ɤ, there are low levels of nitric oxide as well as low levels of RH inducible nitric oxide synthase gene induction. This may be due to stimulation of "alternatively activated" or "M-2" macrophages which secrete IL-10 and IL-1R antagonist known for down regulation of immune response[29]. The same authors added that RH parasites induce apoptosis of spleen cell population. All those factors can contribute to the high cyst number recorded in our work after RH challenge infection. Decreased T-lymphocytes number due to apoptosis induction renders the mice in a state similar to that of

HIV infected patients in whom dormant tissue cysts rupture and new cysts form.

On the other hand, Brandã *et al.,*[23] reported results of re-infection of BALB/c and C57BL/6 mice with virulent strains of *T. gondii* after prime-infection with the non-virulent D8 strain. The BALB/c mice were susceptible to re-infection with EGS but not with CH3 strain of *T. gondii*; while C57BL/6 mice were susceptible to re-infection with EGS and CH3 strains of *T. gondii*. The researchers concluded that re-infection depends on: route of infection, type of inoculum, host immune response, mice lineage and genotype of *T. gondii* used in the challenge

In conclusion, chronic infection with *T. gondii* neither prevented acute disease nor impaired colonization of the brain with tissue cysts after virulent strain superinfection. This result explains and supports the clinical observations that pregnant women previously exposed to *Toxoplasma* can develop congenital infection upon re-exposure. Taking this result in account, pregnant women should be advised to follow the hygienic and dietary preventive measures even though they had past toxoplasmosis. Our study enhances motivation towards further work on murine models to evaluate the efficiency of prophylaxis to prevent reinfection during pregnancy.

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Author contribution: AA Abdel-Magied, GA Elganainy and NI Aboulfotouh designed the study protocol, and shared EM Gaballah in performing the laboratory studies. All authors contributed equally in writing and reviewing the manuscript.

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