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MEASUREMENT OF IL-10 SERUM LEVELS IN BALB/C MICE TREATED WITH BETA-1,3 POLYGLUCOSE OR SULFADIAZINE AND ACUTELY INFECTED BY *Toxoplasma gondii*

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ABSTRACT: Acute infection by *Toxoplasma gondii* leads to suppression of cell-mediated immunity, facilitating chronic infection. One of the causes of immunosuppression is Interleukin-10 (IL-10) production. Glucan is used to stimulate phagocytosis. Our objective was to study IL-10 induction in male BALB/c mice with acute *T. gondii* BTU-2 strain infection, glucan immunostimulation, and sulfadiazine treatment. Animals were distributed into 7 groups: G1: infected with *T. gondii*; G2: infected with *T. gondii* and treated with sulfadiazine; G3: infected with *T. gondii* and immunostimulated with glucan; G4: infected with *T. gondii*, immunostimulated with glucan, and treated with sulfadiazine; G5: imunostimulated with glucan; G6: treated with saline; and G7: treated with sulfadiazine. IL-10 levels were determined by ELISA; the highest levels were found in G2, G3 and G4, and the lowest in G1 ($p<0.001$). Groups G1 to G5 and G7 had substantially higher levels than G6 ($p<0.001$). In this study, the highest IL-10 levels were found in groups treated with glucan.

KEY WORDS: *Toxoplasma gondii,* interleukin-10, glucan, immune response, sulfadiazine.

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INTRODUCTION

Toxoplasma gondii is an obligate intracellular parasite with a wide geographical distribution. Under normal conditions it is asymptomatic, but in immunocompromised individuals, such as AIDS patients, the parasite can rapidly disseminate causing severe disease (35).

The possibility that the parasite genotype influences the severity of the disease in humans is supported by differences in virulence observed in animal experimental models (30). *Toxoplasma gondii* presents a highly clonal populational structure made up of three strains: types I, II and III (30). *T. gondii* type I strain is extremely virulent, producing high levels of parasitemia, with increased risk of transplacentary transmission and increased severity of infection in developing fetuses, whereas types II and III strains lead to chronic infection and production of tissue cysts in mice (30). Da Silva *et al.* (9) described the BTU-2 strain originally isolated from a dog as a type III strain, although it shows high virulence to mice.

Sufadiazine and pyrimethamine are the two main drugs for treating toxoplasmosis (24). They have a beneficial action when given in the acute phase of the disease, when there is active parasite multiplication; however they usually do not indicate infection. These drugs are believed to have little effect on subclinical infection, although the growth of tissue cysts in mice has been restrained with sulphonamides (27). Other drugs such as diaminodiphenylsulphone, atovaquone, spiramycin, and clindamycin have also been used to treat difficult cases of toxoplasmosis (27). In a recent work, Montoya *et al*. (43) reported that the most effective treatment is the combination of sulfadiazine, pyrimethamine, and folic acid. This controls disease in acutely infected patients, children with congenital infection, and immunocompromised individuals with ocular disease (43).

Because immunity plays an important role in controlling acute toxoplasmosis, experimental models have often been used to analyse protective immune response against *T. gondii* (56). In both man and mice, acute *T. gondii* infection can suppress the immunological function (35).

The interaction between natural killer (NK) cells, macrophages (26, 32), Th1-type $CD4^+$ T cells (20), and antibodies (16) is believed to help resistance against toxoplasmosis. Many of these cells exert their protective effects by producing pro-inflammatory cytokines such as IL-1 (7, 13), IL-2 (54), IL-12 (36), IFN-γ (55), and

TNF-α (2, 33). However, Th2 cells and the substances they secrete suppress Th1 cells, antagonising their products and effects (17, 46).

IL-10 was originally characterized as generated by Th2-type $CD4^+$ T cells and inhibiting cytokine synthesis by Th1-type CD4+ T cells. However, several other cell types were later identified as producing these cytokines, including $CD8^+$ T lymphocytes, monocytes, macrophages, B cells, mastocytes, and others (45). Today, we know that IL-10 can also block Th1 cell synthesis of cytokines, such as IFN-γ, regulating monocyte, macrophage, and neutrophil functions, and can exert important suppressive action on the effector activity of these cells. Thus, it can decrease macrophage microbicide activity by inhibiting the NO synthase enzyme, O_2 free radicals, and prostaglandin production (21). IL-10 is therefore considered a suppressive cytokine and potent anti-inflammatory agent (1). Studies on *T. gondii* RH strain, which causes acute lethal infection in mice, show that despite the high IL-12 and IFN-γ levels produced during infection, they were not able to exert a protective effect. The possible mechanism by which the RH strain may survive despite the presence of IFN-γ is by inducing cytokines that antagonise IFN-γ antimicrobicidal effects (31, 53). Studies have demonstrated that IL-10 production during *T. gondii* infection inhibits the capacity of IFN-γ to activate macrophages (61). In this way, IL-10 may inhibit IFN-γ and IL-12 protective activity when there is infection by *T. gondii* RH strain (61). However, Gazzinelli *et al*. (22) have reported that during acute infection by ME49, the avirulent cystogenic strain of *T. gondii*, IL-10+/+ mice were resistant, while IL-10-/- animals did not survive. The authors concluded that lack of IL-10 causes immune hyperactivity characterized by high IL-2 levels, IFN-γ, and mouse death. Treatment of IL-10-/- animals infected with anti-IL-12 or anti-IFN-γ antibodies led to increased survival, suggesting that IL-10 is necessary to control the high pro-inflammatory cytokine production during infection. Similarly, Suzuki *et al*. (56), using C57BL/6 mice infected with ME49 strain, reported serious intestinal infection followed by death in IL-10 deficient animals; however, normal and IL-10 deficient mice treated with anti-IFN-γ antibody survived after infection without developing intestinal lesions. These results also suggest that IL-10 has a role in regulating elevated IFN-γ production levels, which can lead to animal death.

The β-glucans are polysaccharides found in the cell wall of different microorganisms, including bacteria and fungi, and also some cereals, such as oats and barley. Glucan

is extracted from several sources for therapeutic use; one is the cell wall of *Saccharomyces cerevisae* (*S. cerevisae*) (8, 58, 62), where it is very abundant. The cell wall has a central linear structure of D-glucose molecules (linked β-1,3) with side chains of β-1,6-glucose of varying sizes that occur at different intervals through the central structure. Extraction provides a suspension of β-1,3 polyglucose particles (12).

The glucans stimulate the host system to exert a beneficial effect against bacterial, viral, fungal, and protozoal infections (42, 52, 59). They modulate both humoral and cellular immunity; their efficiency has also been confirmed against tumors (39) and as hematopoiesis stimulator (28).

There are reports on macrophage (57), neutrophil (51), and NK cell (14) activation by glucan and this substance is considered a promising candidate as a stimulating agent in immunocompromised individuals. Liang *et al*. (37) used *S. cerevisae*-derived glucan in rats to treat resistant *Stafilococcus aureus* (*S. aureus*) infection, and observed bacteria elimination followed by increased monocytes and neutrophils, and potentialized microbicidal activity. Kaiser *et al*. (34) also tested glucan with cephalosporin in *S. aureus* infected animals. The association of glucan with the antibiotic was 8 to 20 times more efficient than isolated antibiotic administration.

Other studies have reported that intravenous administration of β-1,3 and β-1,6 polysaccharides, before infection with *T. gondii* RH strain in mice, increased animal survival. The authors suggest that the higher resistance to *T. gondii* may be due to the fact that glucans increase macrophage activation (47). Similarly, a glucan protective effect has been demonstrated in experimental candidiasis (11) and human paracoccidioidomycosis (42), and an adjuvant action was observed in experimental immunization against *Leshmania donovani* (29).

The objective of this paper was to study IL-10 induction in BALB/c mice acutely infected by *T. gondii*, and treated with β-1,3 polyglucose and sulfadiazine.

MATERIALS AND METHODS

Mice

We used 150 male isogenic BALB/c mice, 8 to 12 week-old, weighing about 30 g each. They were supplied by the animal facility of the Tropical Diseases Research

Laboratory, Botucatu School of Medicine, and maintained at 21-23°C, with 12/12h day/night cycle with food and water *ad libitum*. They were divided into 7 groups:

G1: (n=30) infected with *T. gondii*.

G2: (n=20) infected with *T. gondii* and treated with sulfadiazine.

G3: (n=20) infected with *T. gondii* and immunostimulated with glucan.

G4: (n=20) infected with *T. gondii*, immunostimulated with glucan, and treated with sulfadiazine.

G5: (n=20) not infected with *T. gondii* and immunostimulated with glucan.

G6: (n=20) not infected with *T. gondii* and treated with saline.

G7: (n=20) not infected with *T. gondii* and treated with sulfadiazine.

Infection with T. gondii

Mice were infected with tachyzoites of BTU-2 *T. gondii*, a type III strain that presents high virulence to mice (9), from a naturally infected dog, kindly provided by the Zoonosis Service, Department of Veterinary Hygiene and Public Health, Botucatu School of Veterinary Medicine and Animal Husbandry. *Toxoplasma gondii* strain was maintained in the laboratory by continuous intraperitoneal passage in BALB/c mice. Tachyzoites were counted in a Newbauer chamber, $1x10^5$ tachyzoites/ml saline. All mice in groups G1 to G4 were intraperitoneally infected with 0.1 ml inoculum on day zero.

Immunostimulation with glucan

Glucan was obtained from the University Hospital Hemocenter, Botucatu School of Medicine. It was manufactured from 3000 mg of bread yeast (*S. cerevisae*), divided into two lots, according to Di Luzio *et al.* (11). To each lot, 2 L of 3% NaOH was added and boiled for 4h in water bath. The mixture was allowed to rest overnight; supernatant was then aspirated. This procedure was performed three to four times to obtain alkaline hydrolysis. Later, acid hydrolysis was performed by adding 2 L of 3% HCL and boiling for 4h in water bath. The mixture was allowed to rest overnight and supernatant was then aspirated. This procedure was performed three to four times. The precipitate was washed twice in distilled water and centrifuged. The resulting residues were washed in boiling water and then centrifuged. The residues in both recipients were resuspended in 1 L ethanol and kept at room temperature for a few

days. After filtration, glucan was constantly homogenized at 37ºC until dry. Ethyl ether was added and the procedure repeated. The resulting particulate powder was placed in flasks (50 mg glucan/flask), autoclaved and kept at 4ºC. Suspensions were made by adding 5 ml isotonic solution to each flask of glucan for animal administration. G3, G4, and G5 mice received 0.1 ml suspension (1.0 mg/mouse) intraperitoneally on days -7, -4, -1, +2, and +4.

Treatment with sulfadiazine

Two 500-mg sulphadiazine tablets were each dissolved in 45 ml of distilled water to obtain a concentration of 375 mg/kg/day. G2, G4, and G7 received a single dose by gavage of 0.5 ml/day of a suspension containing approximately 11 mg sulfadiazine on days +1, +2, +3, and +4.

Treatment with saline

G6 mice received 0.1 ml saline solution intraperitoneally on days -7, -4, -1, +2, and +4.

Determination of Interleukin-10

Blood samples were obtained by cardiac puncture on days -6, -4, -3, -1, and zero, from G5 and G6, and on days zero, +1, +2, +3, +4, and +5, from all groups. Sera were obtained after centrifugation (15 min at 1600 x g), aliquoted into 2-ml vials and kept at -70°C until analysis. IL-10 serum levels were determined using ELISA kit (Catalog M1000, R&D Systems, Minneapolis, M.N.), according to the manufacturer's instructions. Detection limit was 4 pg/ml.

Statistical Analysis

The experiment was analysed in two stages, before and after infection with *T. gondii*.

Stage 1: before infection - Two groups, G5 (glucan) and G6 (saline) were evaluated at five different times: T1=-6; T2=-4; T3=-3; T4=-1; and T5=0 (day zero, infection day).

Stage 2: after infection - All seven groups, G1 (*T. gondii*); G2 (*T. gondii* + sulpha); G3 (*T. gondii* + glucan); G4 (*T. gondii* + glucan + sulpha); G5 (glucan); G6 (saline), and

G7 (sulpha), were evaluated at five different times after infection day (day zero) T6=+1; T7=+2; T8=+3; T9=+4; and T10=+5.

Analysis of Repeated Measurements was used to compare groups at each time and compare times within each group (44). F and p statistics were calculated for both analyses. Differences were statistically significant when $p<0.05$ (44).

RESULTS

There was no alteration in G6 pre-infection IL-10 serum levels at all five times (Stage 1: T1 to T5). However, there was a significant increase in G5 IL-10 serum levels starting at Stage 1, T4 to T5 ($p<0.001$) (Table 1).

In Stage 2, G5 IL-10 serum levels at T6 and T7 were significantly higher ($p<0.001$) (Table 2) than in Stage 1 (T4 and T5). Mean IL-10 values decreased ($p<0.001$) at T8, T9, and T10, but with mean T10 value four times higher than Stage 1 ($p<0.001$).

Tables 1 and 2 show that IL-10 levels were higher in G1, G2, G3, G4, G5 and G7 ($p<0.001$) than in G6. Groups G3, G4 and G5 showed the highest levels ($p<0.001$) at T6 and T7, but G5 IL-10 dropped significantly at T8 ($p<0.001$). The only infected groups that maintained high IL-10 levels were G3 and G4 ($p<0.001$). G1, G2 and G7 also showed significantly higher levels of IL-10 than G6. However, in G2, the levels were higher than in G1, starting at T8 ($p<0.001$). The lowest IL-10 means throughout the experiment were seen in G6, G1 and G7.

Table 1: Means (x), standard deviation (sd) of IL-10 serum levels (pg/ml) in BALB/c mice treated with glucan (G5) and saline (G6), measured in five different moments before *Toxoplasma gondii* infection. (Stage 1).

Time Group	T1 x±sd	T2 x±sd	T3 x±sd	T4 x±sd	T5 x±sd
G5	19.17 ± 0.58 (A;c)	18.17 ± 2.02 (A;c)	18.17 ± 2.02 (A;c)	30.50 ± 0.50 (A;b)	38.83 ± 0.58 (A;a)
G6	21.33 ± 3.33 (A;a)	17.33 ± 1.53 (A;a)	17.67 ± 0.29 (A;a)	18.33 ± 1.26 (B;a)	19.33 ± 1.26 (B;a)

T1, T2, T3, T4, and T5: 6th, 4th, 3rd, 1st day before *T. gondii* infection, and infection day. Different uppercase letters indicate significant differences between groups ($p<0.05$). Different lowercase letters indicate significant differences among times after infection ($p<0.05$).

Comparison between groups at each time:

	T1	T2	T3	T4	T5
Hypothesis	G5=G6	G5=G6	G5=G6	G5=G6	G5=G6
Statistical Analysis	F=1.23; p>0.10	F=0.32; p>0.50	F=0.18; p>0.50	F=241.50; p<0.001	F=592.43; p<0.001
Comments	G5=G6	G5=G6	G5=G6	G5>G6	G5>G6

Table 2: Means (x), standard deviation (sd) of IL-10 serum levels (pg/ml) in BALB/c mice treated or not with glucan and sulfadiazine, measured in five different moments after *Toxoplasma gondii* infection. (Stage 2).

Time Group	T6 x±sd	T7 x±sd	T8 x±sd	T9 x±sd	T10 x±sd
G1	54.33±6.17 (D,c)	100.83±4.16 (D,a)	70.00±3.61 (D,b)	72.00±6.56 (C,b)	77.33±3.06 (D,b)
G2	56.50±6.54 (D,b)	117.00±3.90 (C,a)	119.00±2.00 (C,a)	110.33±5.86 (B,a)	115.00±3.46 (B,a)
G3	98.17±3.88 (C,b)	154.83±6.25 (B,a)	151.33±3.51 (B,a)	150.67±1.53 (A,a)	150.0±4.58 (A,a)
G4	123.00±2.29 (B,c)	166.17±4.65 (A,ab)	175.00±8.72 (A,a)	152.00±7.94 (A,b)	156.00±4.00 (A,b)
G5	137.17±5.01 (A,b)	177.83±4.04 (A,a)	104.67±11.59 (C,c)	84.33±9.07 (C,d)	78.33±2.08 (D,d)
G6	21.67±1.89 (E,a)	18.83±1.53 (E,a)	20.33±1.53 (E,a)	19.33±3.21 (D,a)	19.67±2.31 (E,a)
G7	62.83±6.33 (D,d)	103.00±4.58 (D,a)	81.33±12.79 (D,c)	80.17±15.27 (C,c)	92.83±7.01 (C,b)

T6, T7, T8, T9, and T10: 1st, 2nd, 3rd, 4th, and 5th day after *T. gondii* infection. Different uppercase letters indicate significant differences between groups ($p<0.05$). Different lowercase letters indicate significant differences among times after infection ($p<0.05$). G1 - *T. gondii* infected; G2 - *T. gondii* infected treated with sulfadiazine; G3 - *T. gondii* infected immunostimulated with glucan; G4 - *T. gondii* infected, immunostimulated with glucan, and treated with sulfadiazine; G5 - Glucan; G6 - Saline; and G7 - Sulfadiazine, post infection (Stage 2)

Comparison between groups at each time:

	T6	T7	T8	T9	T10
Hypothesis	G5=G6	G5=G6	G5=G6	G5=G6	G5=G6
Statistical Analysis	F=35.54; p<0.001	F=0.32; P>0.50	F=0.18; p>0.50	F=241.50; p<0.001	F=592.43; p<0.001
Comments	G5>G4>G3>(G1=G2=G7)>G6	(G5=G4)>G3>G2>(G1=G7)>G6	G4>G3>(G2=G5)>(G1=G7)>G6	(G4=G3)>G2>(G1=G5=G7)>G6	(G4=G3)>G2>G7>(G1=G5)>G6

DISCUSSION

In primary infection, *T. gondii* can induce potent cellular immunity characterized by Th1 response with high levels of pro-inflammatory cytokines (19). IL-10 induction is also seen in this stage of the infection (18), and is produced by macrophages and Th2 cells, being a potent inhibitor of Th1-response pro-inflammatory cytokine production. IL-10 is an important modulator of macrophage effector functions against different pathogens including *T. gondii* (10). In Gazzinelli *et al*. (22) study, it is suggested that IL-10 has a role in host protection against harmful effects of an exacerbated cellular immune response during acute infection. IL-10-/- mice have produced elevated IFN-γ, TNF-α, and IL-12 levels, followed by alterations in the liver and lungs, and increased mortality in the initial acute stages of the infection. This suggests that IL-10-/- mice succumbed to *T. gondii* infection due to the production of elevated levels of inflammatory cytokines. Linke *et al*. (38) saw that IL-10-/- mice infected with *Plasmodium chabaudi* died before control animals. They suggest that animal death may have been due more to lethal inflammatory response than to sudden parasitemia. Therefore, IL-10 could prevent infection-induced immune hyperactivity; this may not be restricted to *T. gondii*, but also to other infections.

Results obtained in this study in animals that received only β-glucan [G5] showed that it was also capable of inducing IL-10 production. However, the consequent fall in IL-10 levels at T8 can be explained by all available cell receptors being occupied, which prevented the β-glucan linking to these receptors. Studies have demonstrated that β-glucans are recognized by receptors on the surface of cells such as macrophages, neutrophils, NK cells, dendritric cells, and fibroblasts (5, 6, 23). Therefore, the β-glucans can interact with these cells through specific receptors; these include CR3, lactosylceramide, scavenger receptors, and Dectin-1 (5). Other studies have also reported that vitronectin and fibronectin have been used as glucan ligand proteins increasing cytokine release by macrophages (48, 60).

In infected animals that received β-glucan [G3], IL-10 levels were elevated and did not decrease; this was probably due to constant stimulation of the cells by the parasite, which made them more active, increasing the number of receptors available for linking to the glucan administered during the experiment. In this case, in the opposite way to in animals with only *T. gondii* infection [G1], IL-10 could have reduced the inflammatory effects produced during infection. It has already been

suggested that glucan has the capacity to stimulate the immune response by macrophage activation, increasing their number, size and functions; and by cytokine secretion, such as TNF-α by the macrophage itself (42). Studies performed in this laboratory have demonstrated an increase in TNF-α levels in paracoccidioidomycosis patients (42) and in IFN-γ in leishmaniosis patients who received glucan weekly (41). In recent studies, Marcondes-Machado (40), using a similar experimental model, found that glucan immunostimulated animals infected by a virulent *T. gondii* strain showed elevated levels of cytokines such as IFN-γ, IL-12, and TNF-α. This author has also demonstrated that the highest TNF-α levels were detected at the end of infection; this increase was followed by the animal death.

In this study, sulfadiazine-only treatment [G7] was also capable of stimulating IL-10 production. There is evidence that T cells are responsible for initiating and regulating the specific immune response to drugs (4, 25). These drugs can interact with T cell receptors (TCR) after linking with MHC-peptide complex (15), generating a potent immunogenic signal for T cells and leading to cellular activation (49). In a recent paper, Engler *et al*. (15) confirms the capacity of inactive drugs, such as sulfamethoxazole, to stimulate T cells through TCR to produce cytokines. This can explain the elevated IL-10 levels in animals treated only with sulfadiazine.

The very high IL-10 levels obtained in mice with acute *T. gondii* infection and treated with sulfadiazine [G2] can be explained by the double stimulus they received: on one hand, sulfadiazine inducing IL-10 production as already suggested; and on the other, the protozoa, which despite sulpha parasiticide action, still persist. Piketty *et al.* (50) reported that sulfamethoxazole reduces *T. gondii* parasitic load in mouse tissue. Other authors, investigating the effects of sulfadiazine treatment in BALB/c mice infected with the avirulent strain, detected a decrease in the parasitic load of organs such as the spleen, liver, and lung. However, there was no parasite reduction in the heart, brain, and skin (3). This suggests that the anti-toxoplasmic effects of the drug cannot be simply attributed to its direct effect against the parasite, but to its cooperation with other host defense mechanisms as well as to INF-γ (3, 50).

In the same way, the triple stimulus of acute *T. gondii* infection, β-glucan immunostimulation, and sulfadiazine treatment [G4] can explain the permanently elevated IL-10 levels.

Although high levels of IL-10 were observed in animals treated with glucan, further studies are necessary to confirm its role as immunomodulator over Th1 cytokines production.

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