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In vivo inhibition of inducible nitric oxide synthase by aminoguanidine influences free radicals production and macrophage activity in *Trichinella spiralis* infected low responders (C57BL/6) and high responders (BALB/c) mice

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Summary

The influence of aminoguanidine (AG) – inhibitor of inducible nitric oxide synthase (iNOS), on macrophage activity and free radicals level was examined during *Trichinella spiralis* infection in two strains of mice: C57BL/6 and BALB/c. AG was administered either between 1 – 5 days post infection (dpi) for intestinal phase examinations or between 16 – 29 dpi for muscle phase examinations. The number of peritoneal macrophages and level of nitric oxide (NO) and hydrogen peroxide (H_2O_2) in biological fluids were determined in both strains after infection or infection together with AG treatment as well as in control uninfected mice. The performed studies have proved, that free radicals play role in host immune response during intestinal and muscle phase of *T.spiralis* infection in mice. Inflammatory response in peritoneal cavity was delayed during infection in low responders C57BL/6 mice in comparison with high responders BALB/c mice. C57BL/6 mice are Th-1 like strain and react stronger to AG in contrary to BALB/c being Th-2 like strain. It was manifested as changes and fluctuations of free radicals levels and in the number of peritoneal macrophages after AG treatment in C57BL/6 mice. A weak or no reaction on AG injection in BALB/c mice is responsible for more stable and more effective defense response of the host to *T. spiralis* infection.

Keywords: *Trichinella spiralis*; aminoguanidine; nitric oxide; hydrogen peroxide; macrophages; strain of mice

Introduction

The aim of our studies was examination of the influence of free radicals: nitric oxide (NO) and hydrogen peroxide (H_2O_2) on *T. spiralis* infection in mice. We used aminoguanidine (AG) – inhibitor of inducible nitric oxide synthase (iNOS) for treatment of mice. The population of peritoneal macrophages was examined for the production of free radicals; the presence of NO was searched also in blood plasma and urine. Aminoguanidine (AG) selectively inhibits inducible nitric oxide synthase (Griffiths *et al.,* 1993; Misko *et al.,* 1993). Overall, AG has also antioxidant properties and exhibited a significant dose-dependent effect against oxygen free radicals damage (Couderot-Masuyer *et al.*, 1999). Modulation of free radicals production by AG treatment is a good tool for studying the role of these substances during trichinellosis, because only iNOS-derived NO can play role in infected host organisms besides potential side-effects of AG administration are negligible (Misko *et al.*, 1993). It is known that iNOS activity is changing during *T. spiralis* infection (Bian *et al.*, 2001; Bian *et al*., 2005), so the inhibition of this enzyme by AG affects cytotoxic activity of macrophages, which could play role during infection. Our previous study showed that also macrophages play important role during intestinal phase of infection as well as revealed some new information about the role of free radicals during *T. spiralis* infection (Kołodziej-Sobocińska *et al*., 2006a,b) and reinfection (Kołodziej-Sobocińska *et al*., 2007).

NO is one of the free radicals, which plays crucial role in host defence, tissue pathology and in some autoimmune diseases (Singh *et al*., 2000; Bogdan, 2001). High levels of NO generated by iNOS have anti-microbial effects being the important factor of immune protection (Bogdan, 2001). Activated macrophages are able to produce high levels of iNOS derived NO as well as reactive oxygen species (ROS) such as: superoxide anion (O_2^-), hydrogen peroxide (H_2O_2). NO can react with O_2^- to form peroxynitrate anions ($ONOO^-$), which rapidly decay to release highly reactive hydroxyl radicals (Beckman *et al*., 1990; Zhu *et al*., 1992). These reactive substances are very effective in killing infectious agents: viruses, bacteria, protozoa and fungi (Pacelli *et al*., 1995). The protective role of NO in Th-1-inducing parasitic infections has been widely described (Liew *et al*., 1990; Liew, 1993; Rajan *et al*., 1996; Bhattacharjee *et al*., 2009; Cabrales *et al*., 2011). It has

been also proved that NO can cause immunosuppression (Eisenstein *et al*., 1994; Rockett *et al*., 1994; Dai & Gottstein, 1999; Ren *et al*., 2008) and tissue pathology (Garside *et al*., 1992; Kolb & Kolb-Bachofen, 1992; Pacher *et al.*, 2007). However, the role of NO in defence against *T. spiralis* and the other Th-2 inducing infections such as helminthoses have not been yet completely explained (James, 1995; Liew *et al*., 1997; Alonso-Trujillo *et al*., 2007; Gruden-Movsesijan & Sofronic-Milosavljevic, 2010). It is known that Th-2 immune response plays the main role during *T. spiralis* intestinal infection (Helmby & Grencis, 2003; Reiterová *et al*., 1999; Dvorožňáková *et al*., 2010). Hogaboam *et al*. (1996) proved that during the intestinal phase of the infection Th-1 response is suppressed.

Differences in *in vitro* NO production caused by antigens from encapsulated and non-encapsulated *Trichinella* species have been previously studied (Andrade *et al*., 2007). Otherwise, our previously published data (Kołodziej-Sobocińska *et al*., 2006b) have revealed that *in vivo* inhibition of NO production by AG influenced the number of *T. spiralis* parasites in BALB/c and C57BL/6 mice. It has occurred that AG treatment during intestinal phase of the infection caused opposite effects in two strains of mice. In BALB/c mice the diminution of adult worms was observed but in C57BL/6 mice the number of adult worms was even higher. The treatment of mice with AG at the beginning of muscle phase of infection caused that fewer larvae settled in mice muscles (Kołodziej-Sobocińska *et al*., 2006b).

In presented here examinations, similarly as in the quoted above experiment, two genetically and immunologically different strains of mice were used. The strain-specific differences in immunological response during nematode infection are known (Brown *et al.*, 2003). BALB/c mice (high responders) belonging to "Th2-like" strains, possessing M-2 macrophages, which can influence mainly Th-2 response and having small ability to produce NO (Mills *et al*., 2000). The mice of BALB/c strain respond rapidly to *T. spiralis* infection. The mice of strain C57BL/6 (low responders), belonging to "Th1-like" strains, having M-1 macrophages, more susceptible to produce NO and promoting mainly Th-1 response (Mills *et al*., 2000). These mice respond slowly to infection with *T. spiralis*. The strain differences can be helpful in explanation of mice responsiveness to free radicals production after *T. spiralis* infection as well as AG administration.

Material and methods

Experimental animals

The experiments were carried out on 84 C57BL/6 and 84 BALB/c mice; 6 week old males orally infected with 400 *T. spiralis* larvae. Control groups consisted of uninfected mice, of the same age, untreated or treated with AG. Animals matched for age, weight and gender were housed under standard conditions (20 – 21 ºC, 50 – 60 % relative humidity, and 12 h light regime). They were fed ad libitum with AIN-76 diet (ICN Biomedicals) with the constant nitrite and nitrate contents to exclude fluctuations of NO levels in biological material connected with animals feeding. The larvae used in the infection were recovered from muscles of rats infected 2 – 3 months earlier by digestion with 1 % pepsin / HCl solution for 4 h at 37 ºC. The strain determined in Instituto Superiore di Sanita, Rome, Italy as *T. spiralis sensu stricto* T1 was maintained in W. Stefański Institute of Parasitology of the Polish Academy of Sciences since 1962 by serial passages in rats and was isolated originally from swine.

All procedures were performed in compliance with relevant Polish low and institutional guidelines. All procedures were approved by the appropriate institutional committee.

Experimental schedule

In the first experiment there were 4 experimental groups. The first group ("infected+AG") consisted of 12 C57BL/6 and 12 BALB/c infected mice, which were injected intraperitoneally with 50 mg / kg body weight (bw) of AG (Sigma-Aldrich, Germany) in 0.1 ml phosphate buffer saline (PBS) (Biomed, Poland) / 1 mouse. These mice were injected once a day at the beginning of the *T. spiralis* infection, from the 1st to the 5th days post infection (dpi). The second group ("infected") consisted of 12 C57BL/6 and 12 BALB/c infected mice which were injected with 0.1 ml / 1 mouse of PBS (Biomed, Poland) only. Two control uninfected groups ("uninfected+AG" and "uninfected") consisted of 12 C57BL/6 and 12 BALB/c mice each; treated with AG or PBS respectively with the same dose and volume as infected mice. Examinations were done at 6, 9, 15 and 20 dpi and the biological material was collected.

The second experiment consisted of the same four experimental groups: "infected+AG", "infected", "uninfected+ AG", "uninfected". Muscle phase of the infection was examined in 36 mice from each strain, which were injected once a day with AG or PBS at the beginning of muscle phase of *T. spiralis* infection from the 16th to the 29th dpi. Doses of AG and the volume of intraperitoneal injections were the same as in the first experiment. These mice were examined at 30, 35 and 41 dpi and the biological material was collected.

Blood and urine sample collection

Blood samples taken from the hearts of anaesthetized mice and collected in heparinized tubes were centrifuged at 1.500 x g for 10 min. Plasma samples were stored frozen until further examination. Urine was taken from live animals and kept frozen for detection of NO.

Peritoneal macrophages

Macrophages were purchased from the peritoneal cavity by washing with 5 ml of phenol red-free Hanks' Balanced Salt Solution (HBSS) (Sigma-Aldrich, Germany). Cells were washed three times in phenol red-free HBSS, centrifuged at 170 x g for 10 min at 4 ºC and the cell pellet was resuspended in RPMI 1640 medium (Sigma-Aldrich, Germany). The total number of cells and the number of macrophages isolated from peritoneal cavity were determined using a Bürker counting chamber. The viability of the cells was

assessed using the Trypan Blue (Sigma-Aldrich, Germany) exclusion assay.

Cultivation of peritoneal macrophages
Cells obtained from peritoneal cavity in 3 ml of sterile RPMI 1640 medium (Sigma-Aldrich, Germany) containing 10 % Fetal Calf Serum (FCS) (Sigma-Aldrich, Germany) were placed onto sterile plastic Petri dishes (Medlab Products, Poland) and incubated for 1.5 h at 37 ºC in 5 % CO_2. Next, the nonadherent cells were removed, peritoneal macrophages were washed twice with RPMI 1640 medium containing 10 % FCS and suspended in the cultivation medium: RPMI 1640 + 10 % FCS + penicillin G (100 U/ml) + streptomycin (100 µg/ml). The cultivation of macrophages was carried out for 48 h at 37 ºC in 5 % CO_2. Then, the culture supernatants were collected and frozen for the determination of NO level.

Detection of NO level in supernatants after peritoneal macrophages cultivation
Nitrite concentration in the supernatants was measured by a microplate assay method described by Ding *et al*. (1988). Briefly, to 100 µl of supernatant obtained from peritoneal macrophages cultivation, 100 µl of Griess reagent (1 % sulphanilamide in 5 % H_3PO_4; 0.1 % N-(1-naphthyl) ethylenediamine dihydrochloride in 5 % H_3PO_4; 1 vol/1 vol) (Sigma-Aldrich, Germany) was added. Probes were incubated at room temperature for 10 minutes. The absorbance of triplicate samples at 540 nm was determined in an Organon Teknika microplate reader (Netherlands). NO concentration in the samples was calculated with reference to a sodium nitrite standard curve of range 0 – 120 µM and expressed in µM of nitrite.

Detection of NO level in plasma and urine
The amount of NO was assayed using the method of Rockett *et al*. (1994).
Briefly, to 30 µl of sample, 15 µl of Nicotinamide Adenine Dinucleotide Phosphate (NADPH) (1.25 mg/ml) (Sigma-Aldrich, Germany) and 5 µl of nitrate reductase (5 U/ml) (Sigma-Aldrich, Germany) were added. Samples were incubated for 20 minutes at room temperature. Then, 100 µl of Griess reagent and 100 µl of 10 % trichloroacetic acid were added to each sample. Samples were centrifuged at 1400 x g for 15 minutes at room temperature. 200 µl of supernatants were transferred to 96 well microplates (Nunc, Denmark). The absorbance of duplicate samples was measured at 540 nm using an automatic reader (Organon Teknika, Netherlands). The amount of NO was defined from the standard curve of reduced $NaNO_3$. The results were expressed in µM of nitrite.

Microassay of hydrogen peroxide production
Hydrogen peroxide production by peritoneal macrophages was measured by a microplate assay method described by Pick, (1986). This assay is based on the horseradish peroxidase-dependent oxidation of phenol red by H_2O_2 leading to the formation of a compound that, at an alkaline pH, exhibits increased absorbance at 600 nm.
Briefly, cells suspension obtained from peritoneal cavity in 200 µl of sterile RPMI 1640 medium (Sigma-Aldrich, Germany) containing 10 % FCS (Sigma-Aldrich, Germany) were placed onto 96-well flat bottom microplates in duplicate (Nunc, Denmark). Cells were incubated for 1.5 h at 37 ºC in 5 % CO_2. After the incubation, the nonadherent cells were removed, peritoneal macrophages were washed twice in phenol red-free HBSS and the adherent cells were covered with 200 µl / well solution of 0.56 mM phenol red (Sigma Aldrich, Germany) and 20 U / 1ml horseradish peroxidase type II (HRPO) (Sigma Aldrich, Germany) in HBSS. The plates were covered with lids and incubated for 1 h at 37 ºC in 5 % CO_2. Then, the reaction was interrupted by adding 20 µl / well of 1 N NaOH. This step induced immediate cell death with consequent interruption of H_2O_2 production. The absorbance at 600 nm was read and H_2O_2 concentration in the samples was calculated with the reference to H_2O_2 standard curve of range 0-100 µM. Results were finally expressed as nanomoles H_2O_2 per 10^6 cells per time interval using the formula:
nanomoles H_2O_2 per well = Abs at 600 nm x 16.7.

Statistical analysis
Data are presented as means ± SEM. The aim of the statistical analysis was evaluation of the differences among mean values of investigated biological parameters concerning: (1) two strains of mice; (2) infection *T. spiralis* versus control uninfected; (3) treatment of AG versus no treatment; (4) days after infection.
Main effects of 4 analysed factors and their all interactions were evaluated by 4-way analysis of variance (ANOVA) method. Hypotheses on main effects and interaction were tested by F-Fisher-Snedecor test. Multiple comparisons of significant effects were performed by least significant difference (LSD) based on t-Student test. The differences among the groups were considered significant at the value $p < 0.05$.

Results

Number of mice peritoneal macrophages during intestinal phase of infection
Infection with *T. spiralis* caused significantly elevated number of peritoneal macrophages in mice of both strains (***$p < 0.001$), which continuously increased from 6^{th} till 20^{th} dpi (***$p < 0.001$). There were more peritoneal macrophages in C57BL/6 mice infected with *T. spiralis* than in BALB/c mice (***$p < 0.001$). AG injections from 1^{st} to 5^{th} dpi caused diminution of peritoneal macrophage number in infected C57BL/6 mice at 6 and 9 dpi, just after AG treatment, and elevation of number of these cells at 15 and 20 dpi (*$p < 0.05$) (Fig. 1b). AG injections had no influence on macrophage number in peritoneal cavity of infected BALB/c mice (Fig. 1a) and uninfected mice of both strains during intestinal phase of *T. spiralis* infection.

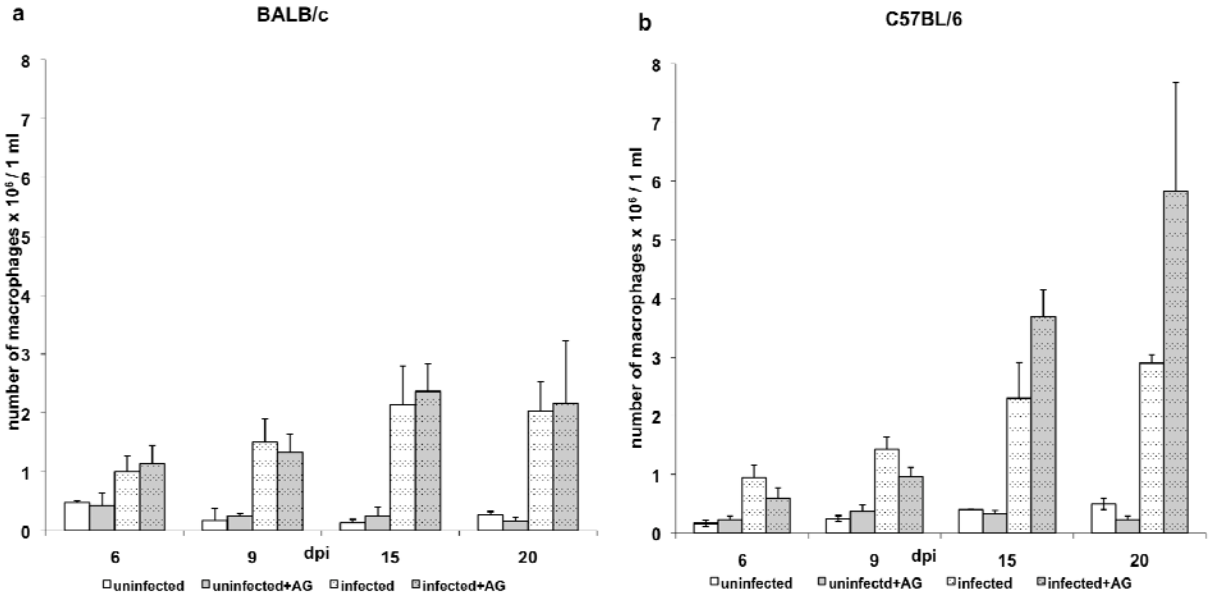

Fig. 1. Number of macrophages in peritoneal cavity of mice during intestinal phase of *T. spiralis* infection. Comparison of results from two strains of mice: BALB/c mice (a) and C57BL/6 mice (b) after infection with *T. spiralis* and with or without AG treatment between 1 and 5 dpi. n=4

Number of mice peritoneal macrophages during muscle phase of infection

Infection with *T. spiralis* caused significant elevation of peritoneal macrophage number in mice of both strains (***$p < 0.001$) during early muscle phase of the infection. In C57BL/6 mice infected with *T. spiralis* there were more peritoneal macrophages than in BALB/c mice (***$p < 0.001$). AG injections from 16th to 29th dpi caused diminution of peritoneal macrophages number in infected C57BL/6 mice (***$p < 0.001$) (Fig. 2b). AG injections had no influence on macrophages number in peritoneum of infected BALB/c mice (Fig. 2a) and uninfected mice of both strains during muscle phase of *T. spiralis* infection.

The nitric oxide production by mice peritoneal macrophages during intestinal phase of infection

Peritoneal macrophages isolated from C57BL/6 mice produced more NO than from BALB/c mice (***$p < 0.001$). Infection of C57BL/6 mice with *T. spiralis* caused higher NO production in comparison to uninfected, untreated with AG mice (*$p < 0.05$) (Fig. 3b). AG injections caused diminution of NO production in infected mice of both strains as well as in uninfected BALB/c mice. However, higher NO production after AG treatment in uninfected C57BL/6 mice was observed (***$p < 0.001$) (Fig. 3b). The level of NO production continuously decreased and at 20 dpi NO production was very low in animals from all experimental groups (*$p < 0.05$). (Fig. 3a-b).

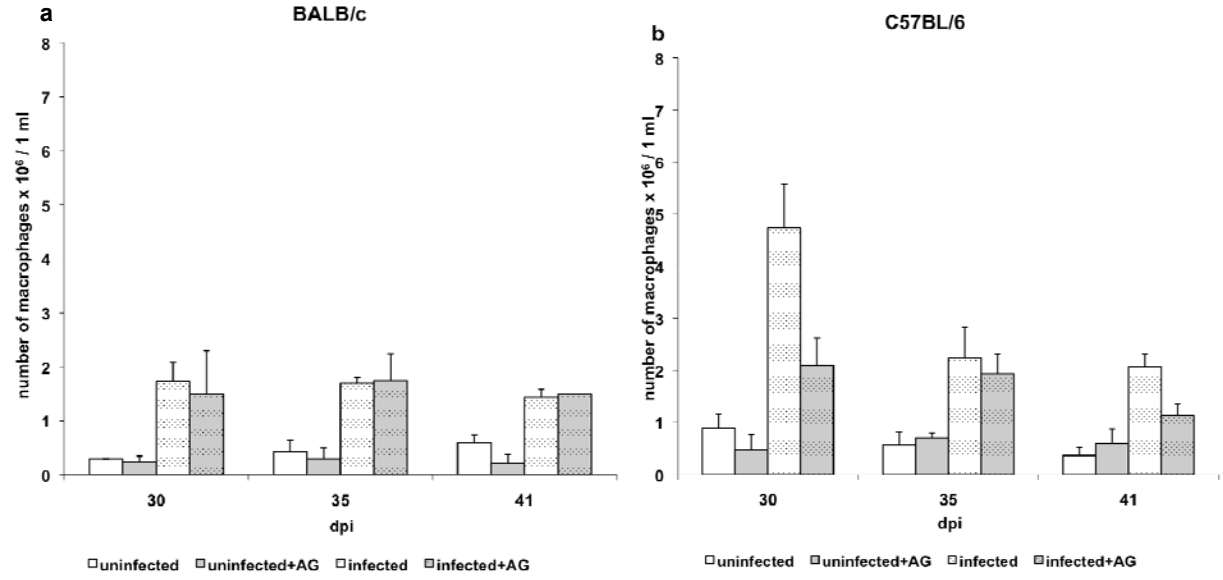

Fig. 2. Number of macrophages in peritoneal cavity of mice during muscle phase of *T. spiralis* infection. Comparison of results from two strains of mice: BALB/c mice (a) and C57BL/6 mice (b) after infection with *T. spiralis* and with or without AG treatment between 16 and 29 dpi. n=4

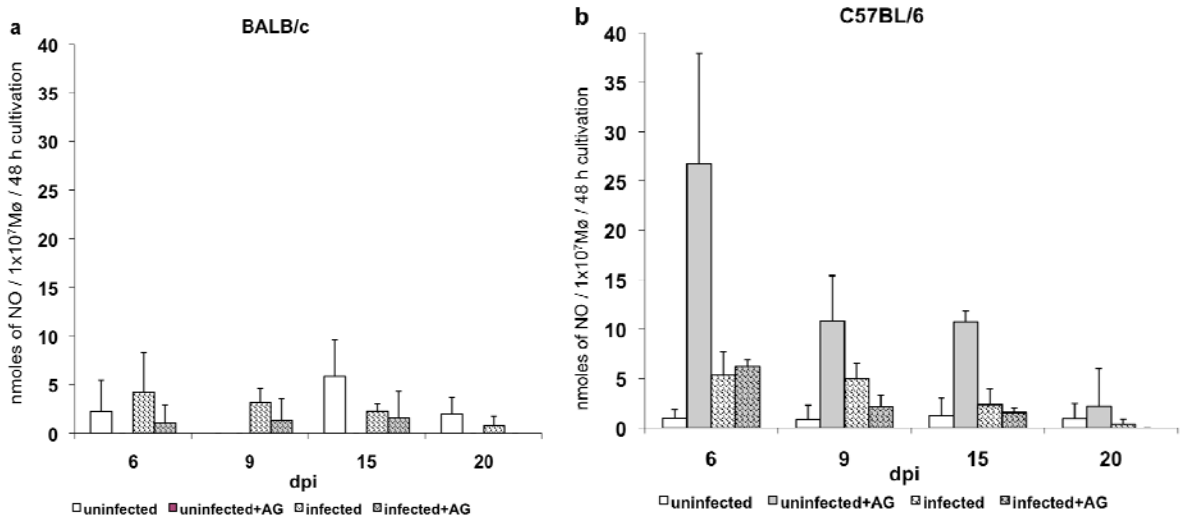

Fig. 3. The production of nitric oxide by peritoneal macrophages during intestinal phase of *T. spiralis* infection. Comparison of results from two strains of mice: BALB/c mice (a) and C57BL/6 mice (b) after infection with *T. spiralis* and with or without AG treatment between 1 and 5 dpi. n=4

The nitric oxide production by mice peritoneal macrophages during muscle phase of infection

None of studied factors had statistical significant influence on NO production by peritoneal macrophages during muscle phase of *T. spiralis* infection. Only the tendency of lower NO production by macrophages isolated from infected mice of both strains was observed ($p = 0.058$) (Fig.4a-b).

The hydrogen peroxide production by mice peritoneal macrophages during intestinal phase of infection

Peritoneal macrophages isolated from infected mice of both strains produced less H_2O_2 than from uninfected mice during the whole intestinal phase of infection (***$p < 0.001$). Additionally, AG treatment of infected mice caused inhibition of H_2O_2 production but in uninfected mice the stimulation of H_2O_2 production was observed (*$p < 0.05$) (Fig. 5a-b).

The hydrogen peroxide production by mice peritoneal macrophages during muscle phase of infection

The strong inhibition of H_2O_2 production by peritoneal macrophages of infected mice of both strains was observed during muscle phase of infection (*$p < 0.05$). AG treatment caused elevated H_2O_2 production in macrophages of both uninfected and infected mice (*$p < 0.05$) (Fig. 6a-b).

Level of NO in plasma during intestinal phase of infection

AG treatment of C57BL/6 mice caused lower NO level in plasma at the beginning of the experiment in comparison to BALB/c mice treated with AG at the same time (*$p < 0.05$). Later, during intestinal phase of *T. spiralis* infection, NO

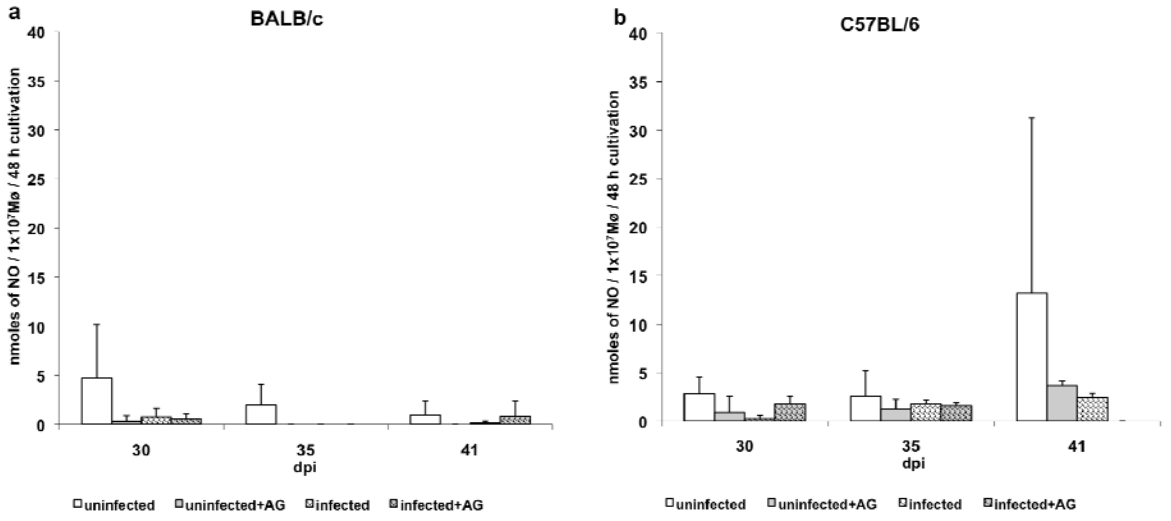

Fig. 4. The production of nitric oxide by peritoneal macrophages during muscle phase of *T. spiralis* infection. Comparison of results from two strains of mice: BALB/c mice (a) and C57BL/6 mice (b) after infection with *T. spiralis* and with or without AG treatment between 16 and 29 dpi. n=4

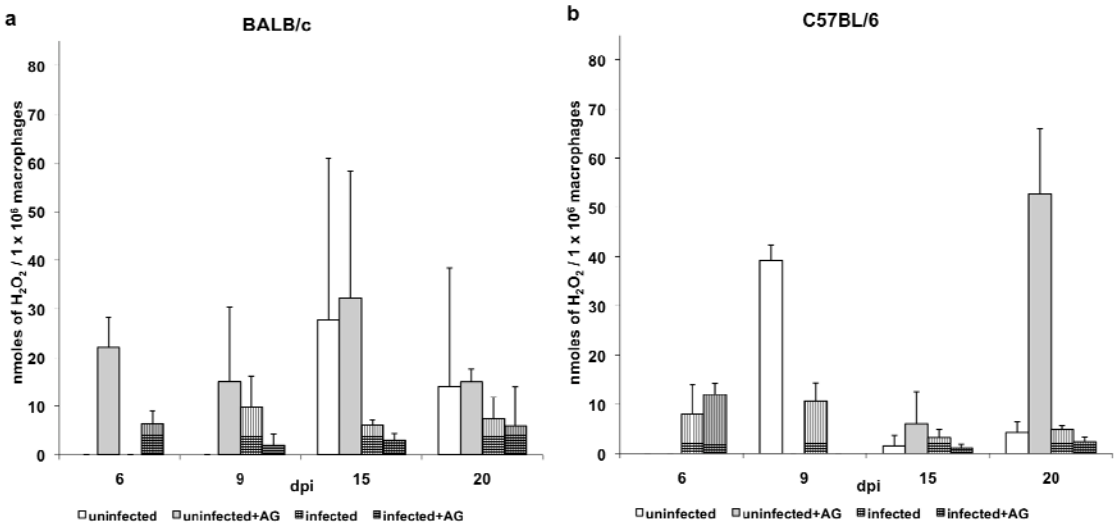

Fig. 5. The production of hydrogen peroxide by peritoneal macrophages during intestinal phase of *T. spiralis* infection. Comparison of results from two strains of mice: BALB/c mice (a) and C57BL/6 mice (b) after infection with *T. spiralis* and with or without AG treatment between 1 and 5 dpi. n=4

level was rising in plasma of C57BL/6 mice and was decreasing in plasma of BALB/c mice (***$p < 0.001$) (Fig. 7a-b).

Level of NO in plasma during muscle phase of infection

Elevated NO level in plasma of infected mice in comparison to uninfected mice, both untreated with AG, was observed at 30 and 41 dpi in BALB/c mice (Fig. 8a) and at 35 and 41 dpi in C57BL/6 mice (Fig. 8b). AG treatment caused higher NO level only in plasma of uninfected mice of both strains (*$p < 0.05$).

Level of NO in urine during intestinal phase of infection

In both strains of mice the level of NO in urine of infected animals was lower in comparison to uninfected controls during intestinal phase of the infection. Additionally, analysis of this parameter has shown strong interdependence of time and this free radical level. The strongest decreasing of NO level in urine during the experiment was observed in infected BALB/c mice (***$p < 0.001$) (Fig. 9a). On the contrary, in infected C57BL/6 mice treated with AG the level of NO was increasing and it was the highest at the end of intestinal phase of the infection (***$p < 0.001$) (Fig. 9b). Treatment of infected mice with AG causes higher NO level in urine.

Level of NO in urine during muscle phase of infection

Analysis of NO level in mice urine during muscle phase of the infection has shown strong interdependence of time and this free radical level in mice infected with *T. spiralis*. In infected BALB/c mice untreated with AG the level of NO was increasing significantly (*$p < 0.05$) but was lower

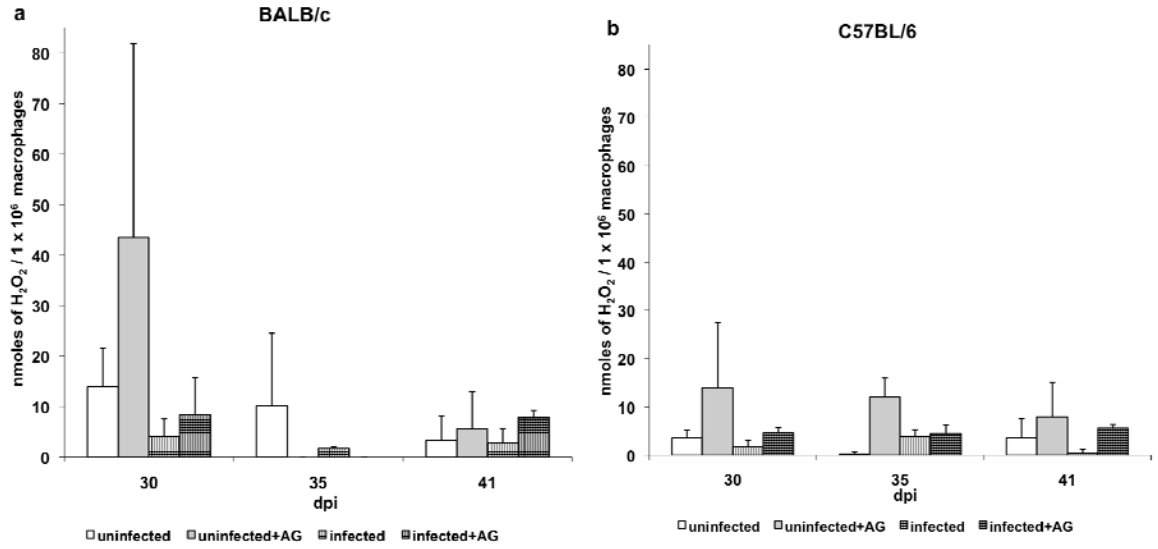

Fig. 6. The production of hydrogen peroxide by peritoneal macrophages during muscle phase of *T. spiralis* infection. Comparison of results from two strains of mice: BALB/c mice (a) and C57BL/6 mice (b) after infection with *T. spiralis* and with or without AG treatment between 16 and 29 dpi. n=4

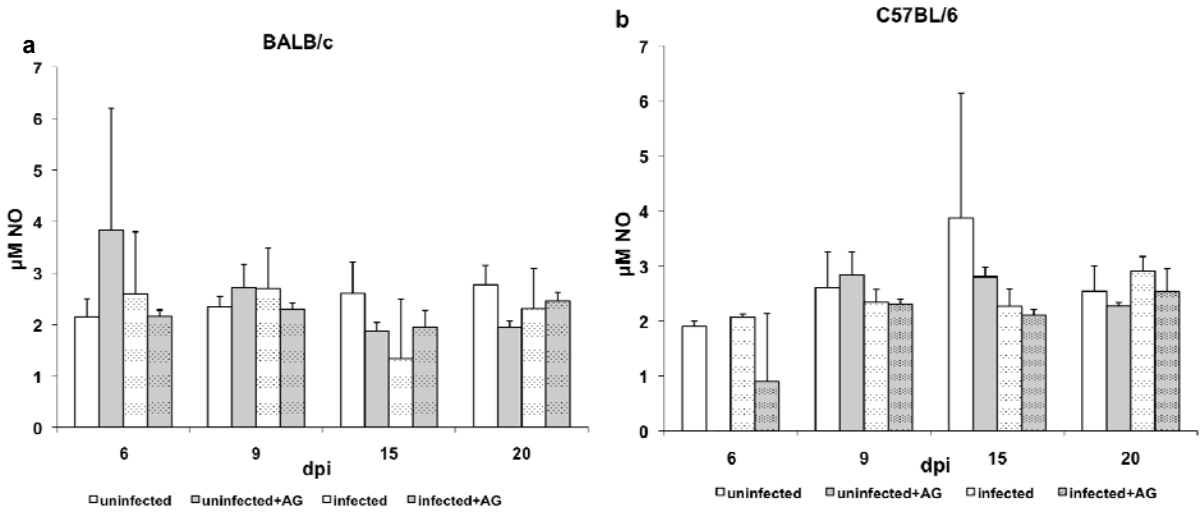

Fig. 7. Level of nitric oxide in plasma of mice during intestinal phase of *T. spiralis* infection. Comparison of results from two strains of mice: BALB/c mice (a) and C57BL/6 mice (b) after infection with *T. spiralis* with or without AG treatment between 1 and 5 dpi. n=4

in comparison to control uninfected mice (Fig. 10a). In C57BL/6 mice the level of NO in urine of infected animals was also increasing significantly but only in mice untreated with AG (***$p < 0.001$) (Fig. 10b). Treatment of infected mice with AG had no statistical significantly influence on NO level in mice urine. In uninfected mice of both strains the level of NO in urine was constant and no statistical significant differences were observed.

Discussion

The aim of the performed study was to examine the influence of free radicals: nitric oxide and hydrogen peroxide on *T. spiralis* infection in mice. Two strains of mice: C57BL/6 and BALB/c, which differ in immunological response, where used. Also the influence of aminoguanidine – inhibitor of inducible nitric oxide synthase on the cytotoxic immune response was studied.

It is known that activated macrophages produce among others, highly reactive inorganic compounds, which are toxic for pathogens. However, little is known about their role in defense against *T. spiralis* infection. The performed studies have proved that free radicals take part in the host immune response during both intestinal and muscle phase of *T. spiralis* infection in mice.

Infection of mice belonging to both strains resulted in elevation of the number of peritoneal macrophages, even during muscle phase of the infection (Figs. 1 and 2). It is adverse parallel to the number of adult parasites in mice of both strains but more pronounced in C57BL/6 mice (Kołodziej-Sobocińska *et al*., 2006b). The changes in macrophages number in time was observed only in C57BL/6

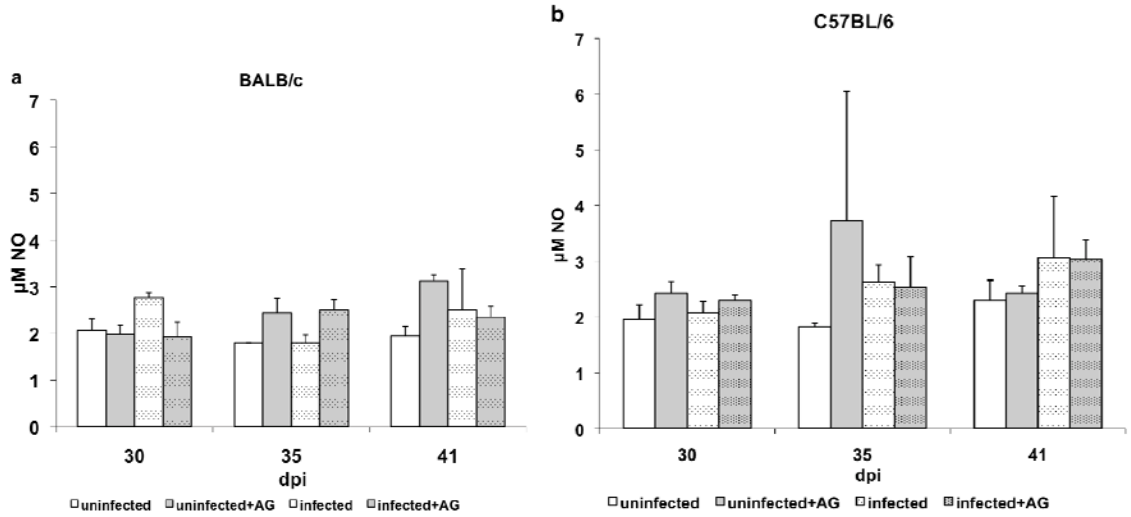

Fig. 8. Level of nitric oxide in plasma of mice during muscle phase of *T. spiralis* infection. Comparison of results from two strains of mice: BALB/c mice (a) and C57BL/6 mice (b) after infection with *T. spiralis* with or without AG treatment between 16 and 29 dpi. n=4

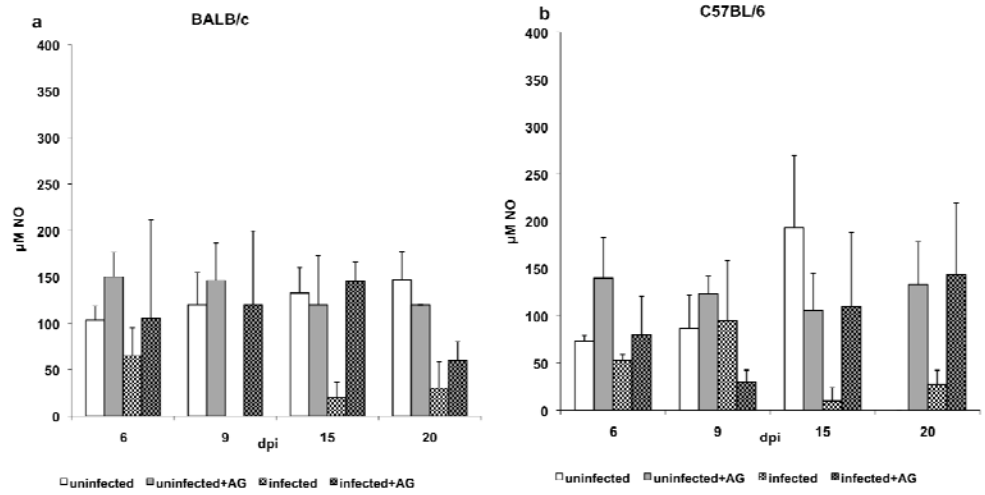

Fig. 9. Level of nitric oxide in urine of mice during intestinal phase of *T. spiralis* infection. Comparison of results from two strains of mice: BALB/c mice (a) and C57BL/6 mice (b) after infection with *T. spiralis* with or without AG treatment between 1 and 5 dpi. n=4

infected mice, where the highest numbers of these cells were observed from 15 till 30 dpi, and then their significant drop was observed (Figs. 1 and 2). In BALB/c infected mice the number of macrophages was also higher in comparison to uninfected controls, but the values were stable during the all experiment. Interestingly, the inflammatory response with high number of macrophages is strongly activated after early AG treatment of infected C57BL/6 mice (Fig.1b). In uninfected mice treated with AG such reaction is not observed, so it is unquestionable that both factors together: *T. spiralis* infection and AG treatment caused this reaction. Unexpectedly, in C57BL/6 mice treated with AG in the early muscle phase of the infection the number of macrophages was much lower (Fig. 2b), whether AG treatment of these mice at the beginning of *T. spiralis* infection caused higher macrophages number (Fig. 1b). The role of NO production modulators in therapy of other diseases is known (Morley & Flood, 1991; Cooke & Tsao, 1992; Cayatte *et al*., 1994; Olesen *et al*., 1994). So, the probe of the recognition of AG role in trichinellosis could be also important for probable therapy. AG treatment of infected mice of both strains (BALB/c, C57BL/6) caused that less muscle larvae settled in muscles. In C57BL/6 mice infected with *T. spiralis* but untreated with AG the highest muscle larvae number was observed at 30 dpi and, then strong reduction of muscle larvae was observed till 41 dpi (Kołodziej-Sobocińska *et al*., 2006b). It could be explained by stronger activation of cytotoxic mechanisms in this Th1-like strain. It is known that macrophages are present in the neighborhood of larvae in muscles for a long time (Dąbrowska *et al*., 2004) and that they play role in elimination of larvae from muscles of infected host. The role of Th-2 response and secretion of Th2-cytokines (IL-4, IL-5, IL-9, IL-10, IL-13) by activated

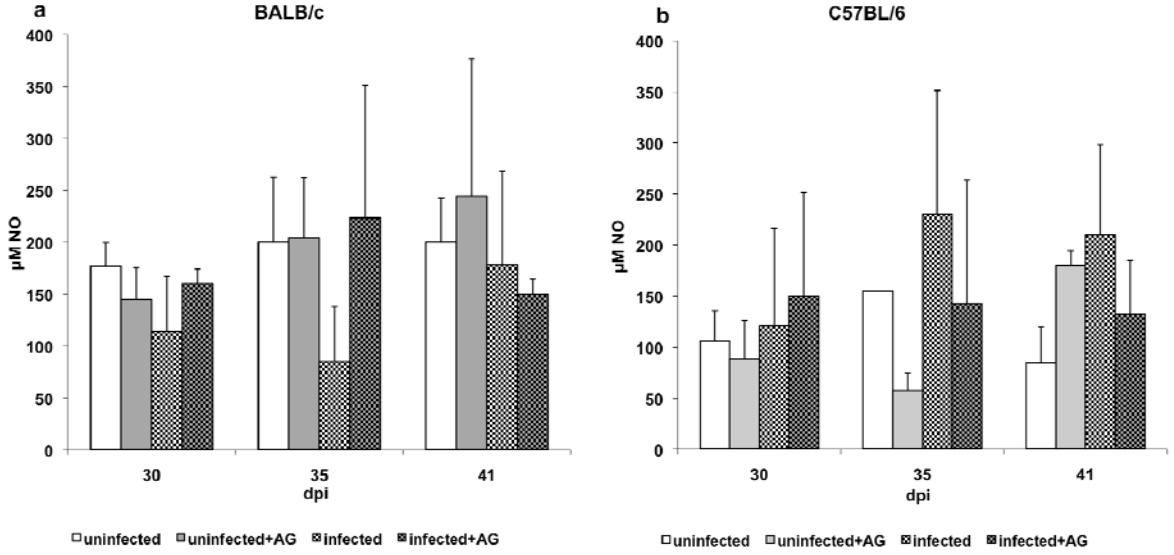

Fig. 10. Level of nitric oxide in urine of mice during muscle phase of *T. spiralis* infection. Comparison of results from two strains of mice: BALB/c mice (a) and C57BL/6 mice (b) after infection with *T. spiralis* with or without AG treatment between 16 and 29 dpi. n=4

lymphocytes, during intestinal phase of *T. spiralis* infection has been proved (Faulkner *et al*., 1997; Urban *et al*., 2000; Khan *et al*., 2001; Helmby & Grencis, 2003). Recently published by Dvorožňáková *et al*. (2011) results showed the increased Th-2 response at the intestinal phase (elevated level of IL-5 and IL-10) in mice infected with small doses of both: encapsulating (*T. spiralis, T.britovi*) and non-encapsulating (*T. pseudospiralis*) species of *Trichinella*, what simulates infections naturally occurred in the environment. However, it has also occurred that at the beginning of *T. spiralis* infection also cytotoxic immune response is activated. Karmańska *et al*. (1997) detected macrophages in gut mucosa at 7 dpi in infected with *T. spiralis* mice. The immunological reaction in peritoneal cavity could be caused by the newborn larvae migration through body cavities. Shanta and Meerovith, (1967) detected *T. spiralis* newborn larvae in peritoneal cavity between 6 and 25 dpi, when we observed very high number of peritoneal macrophages (Fig. 1). Inflammatory reaction in peritoneal cavity is strongly delayed in low responders mice (C57BL/6). It seems to be connected with worm expulsion, which is also delayed in this strain of mice in comparison to high responders mice (BALB/c) (Kołodziej-Sobocińska *et al*., 2006b). Similarly, from the 15th dpi the increasing of IFN-γ level (Th-1 response) was observed in experimentally infected with small doses of three *Trichinella* species (*T. spiralis, T. britovi, T. pseudospiralis*) mice, what is connected with persistence of migrating newborn larvae at this time of the infection (Dvorožňáková *et al*., 2011).

During *T. spiralis* infection macrophages isolated from peritoneal cavity of C57BL/6 mice produced more NO than from BALB/c mice (Figs. 3 and 4). C57BL/6 mice are Th1-like strain, where are present M-1 macrophages, which produce high levels of NO after activation (Mills *et al*., 2000). It could be suspected that AG also easily inhibits these cells and they do not produce NO. Therefore, the compensation of interrupted cytotoxic response is needed and more macrophages migrate into peritoneal cavity of infected animals. These suggestion has been proved by our studies, because indeed high number of macrophages after AG treatment of infected mice does not correlate with high level of NO as well as H_2O_2 at the beginning of infection (Figs. 3 and 5). The highest activation of NO production in peritoneal cavity of infected, untreated mice was observed at 6 and 9 dpi (Fig. 3). Bian *et al*. (2001) observed high iNOS expression in intestine of *T. spiralis* infected mice but only during first 3 dpi. The reaction in peritoneal cavity is delayed in comparison to reaction activated in intestine of mice infected with *T. spiralis*. That could be the reason of high iNOS expression till 3 dpi in intestine (Bian *et al*., 2001) and higher NO production by peritoneal macrophages from 6 dpi in our studies. Later, during muscle phase of *T. spiralis* infection, AG treatment of C57BL/6 infected mice caused diminution of macrophages number (Fig. 2b), these cells produced more NO at 30 dpi (Fig. 4b) and H_2O_2 at 30 and 41 dpi (Fig. 6b) in comparison to infected but untreated with AG mice. It could be explained by the compensation, when small number of cells under inflammatory conditions elicited by *T. spiralis* infection are forced to produce more free radicals to make the immune response as strong as it is necessary for the host defense. So, it is suggested that the differences in both: free radicals production and the number of macrophages observed after AG treatment in two strains of mice are related to the host immune response caused by parasite infection but not to AG mode of action. BALB/c mice infected with *T. spiralis* as well as control uninfected animals do not react to AG treatment (Fig. 2). During the early muscle phase of *T. spiralis* infection in the neighborhood of nurse-cell the presence of CD4+, CD8+ lymphocytes as well as macrophages was stated (Karmańska *et al*., 1997). In muscles the presence of iNOS was also stated (Boczoń *et al*., 2004) but it is known that expression of iNOS mRNA as well as iNOS presence in tissues do not testify to NO persistence (Luss *et al*., 1994; Luss *et al.*, 1997). The studies of the influence of AG on both NO and H_2O_2 level is reasonable because this selective NO inhibitor is known also as antioxidant factor in both *in vitro* (Courderot-Masuyer *et al*., 1999) and *in vivo* (Giardino *et al*., 1998) conditions. AG has several functions; apart of selective iNOS inhibition (Griffiths *et al*., 1993; Misko *et al*., 1993), AG inhibits also reactive oxygen species (ROS) formation, lipids peroxidation and induced by ROS apoptosis (Giardino *et al*., 1998).

The levels of NO in plasma at the beginning of *T. spiralis* infection was stable (Fig. 7) but later, during muscle phase of the infection higher NO level in plasma of infected mice was observed (Fig. 8). Wandurska-Nowak and Wiśniewska, (2002) also stated higher NO level between 5 and 8 weeks post infection (wpi) in BALB/c mice. Interestingly, the level of NO in urine of infected mice during intestinal phase of the infection was lower than in uninfected mice (Fig. 9). The level of NO in urine is known as the indicator of systemic cytotoxic response activity (Granger *et al*., 1991). This parameter confirms the main role of Th-2 response at the beginning of *T. spiralis* infection. The second reason of the lower NO level in urine of infected mice could be caused by the renal reabsorption of nitrate (Zeballos *et al*., 1995). The similar reaction was observed in horses after endotoxin treatment, where also lower NO level in urine was detected despite the stable level of NO in plasma (Bueno *et al*., 1999). It has occurred that reabsorbed nitrates are accumulated in intracellular niches, not in blood (Zeballos *et al*., 1995). During the muscle phase of *T. spiralis* infection, the increase of NO level in urine of infected mice of both strains was noticed, especially in C57BL/6 mice, where the level of NO in urine of infected animals was even higher than in uninfected controls (Fig. 10). It confirms that Th-1 response is important during muscle phase of *T. spiralis* infection. Other studies showed higher IFN-γ level and lower IL-5 level after 15 dpi in BALB/c mice infected with *T. spiralis*, what additionally confirms the role of Th-1 response (Dvorožňáková *et al*., 2005). Cytotoxic activation of macrophages was also suggested by Beiting *et al*. (2004) who demonstrated IL-10

participation in local inflammatory response suppression in the neighborhood of nurse-cell as well as studies where revealed strong growth of antioxidant enzymes (ex. superoxide dismutase (SOD)) level in blood and muscles (Derda & Hadaś, 2000; Derda *et al*., 2004). So, it has occurred that strong inflammatory reactions provoked by the parasite in muscles activate mechanisms, which protect the host from itself tissue destruction exposed to elevated cytotoxic activity of macrophages accumulated around *T. spiralis* muscle larvae.

In conclusion, this study has shown that free radicals play role in control of *T. spiralis* infection. In the intestinal phase of *T. spiralis* infection cytotoxic immune response is activated in mice peritoneal cavity and in the muscle phase, the local immune response activated in the neighborhood of larvae in muscles appeared as the higher level of free radicals in blood and urine. Additionally, it has occurred that administration of AG between 1-5 dpi causes opposite reactions in two different strains of mice. However, it must be stressed that there are no differences between two strains of mice after treatment with AG between 16 and 29 dpi. So, our studies confirmed that the differences in host background genotype are important in activation of free radicals production as well as the responsiveness to AG treatment. In low responders mice AG works as an inhibitor and in BALB/c mice AG is a stimulator just after finishing injections. Mills *et al*., (2000) revealed that M-1/M-2 macrophages stimulated with LPS activated different way of arginine metabolism what elicited opposite effects in, for example, inflammatory reactions. Our results confirm diversity in reactivity between M-1 and M-2 macrophages expressed in different reactions of two strains of mice. Additionally, it has occurred that the roles of selective iNOS inhibitor - AG depend on the genetic background of the infected host and the phase of *T. spiralis* infection. Overall, inflammatory response in peritoneal cavity is observed later during the infection in low responders (C57BL/6) mice in comparison with high responders (BALB/c) mice. Th-1 like mice (C57BL/6) react stronger to AG treatment than Th-2 like mice (BALB/c). It occurs as changes and fluctuations in free radicals levels and the number of peritoneal cells after AG treatment in C57BL/6 mice. Weak or no reaction on AG injections in BALB/c mice is responsible for more stable and more sufficient defense response of the host to *T. spiralis* infection.

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