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## Influence of *Echinococcus multilocularis* infection on immune response of mice and their offspring

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### Summary

Parasitic infection during pregnancy represents a serious stress factor and affects the course of pregnancy and the foeto-maternal relationship. The infection may not clinically manifest itself, however it can modulate the immune response of the offspring for a long-time. The influence of secondary *Echinococcus multilocularis* infection on the proportion of CD4+ and CD8+ T cells and the level of anti-*Echinococcus* antibodies were studied in Balb/c mice. The female mice were infected with homogenised metacestode material containing 2000 *E. multilocularis* protoscoleces (Group 1, 2). Group 1 was fertilised on day 60 post infection, while Group 2 remained unfertilised. Group 3 was uninfected and fertilised on the same day as Group 1. The numbers of both T cell subpopulations were higher in non-pregnant than in pregnant mice. In late pregnancy, the decline of CD4+, however, the increase of CD8+ T-cell subtypes were observed in both, infected and uninfected mothers, respectively. The strong humoral response with the high production of IgM and IgG2b antibodies in infected mice was detected. In infected mothers, IgG2b level was higher than in infected nonpregnant mice during almost whole monitored period. In Group 1, delivery caused suppression of Th2 immune response, represented by IgG1, under the level observed in uninfected mothers. The findings show the changes in helper regulatory and cytotoxic immunity mechanisms of infected mothers. In offspring of infected mothers all IgG subclasses were detected, however specific IgM were not transmitted neither transplacental, nor transmammary.

Keywords: *Echinococcus multilocularis*; mouse; pregnancy; immune response; offspring

### Introduction

*Echinococcus multilocularis*, an intestinal cestode parasite of red foxes (*Vulpes vulpes*) and other carnivores, is the

causative agent of human alveolar echinococcosis which is rare but mostly lethal infection in high percentage of untreated patients (Ammann & Eckert, 1996; Gottstein & Hemphill, 1997). The first confirmed finding of *E. multilocularis* in red foxes in Slovakia was recorded in 1999 at the border area with Poland (Dubinský *et al.*, 1999). At present, this serious zoonosis occurs in animals and humans nearly on the whole territory of Slovakia (Kinčeková *et al.*, 2005; 2008; Miterpáková *et al.*, 2009).

The pregnancy and a simultaneous infection are important risk factors for mothers and their embryos and can modulate the subsequent immune response of the offspring for a long-time. Alveolar echinococcosis is a serious zoonosis caused by the larval stage of *E. multilocularis*. Larvocysts develop in different organs of intermediate hosts, as well as in humans (Eckert & Deplazes, 1999; Vuitton, 2002). Secondary infection model, where mice are injected intraperitoneally with isolated protoscoleces from metacestode material, is characterised by switch from Th1 immune response in the early infection phase to Th2 response during chronic infection (Dvorožnáková *et al.*, 2004). This type of alveolar echinococcosis infection lacks the “early antigen phase”, when the oncosphere and the early developing metacestode are presented to the immune system, and has an atypical localisation of the metacestode (peritoneal cavity). Changes in immune response caused by both, parasitic infection and pregnancy, do not influence reproduction potential of infected mice (Antolová & Reiterová, 2010).

The proportion of CD4+ and CD8+ T lymphocytes and the balance between Th1 and Th2 cytokine profile appear to be crucial for metacestode growth (Bauder *et al.*, 1999; Vuitton 2002; Borošková *et al.*, 2003). Beside the significant cellular immune response, the humoral response has no direct restrictive role on the parasite growth but participates in immunopathological processes with a long term production of specific antibodies (Manfras *et al.*, 2004). The aim of the study was to follow up the influence of

secondary *Echinococcus multilocularis* infection on cellular and humoral immune responses of mice, especially on the course of gravidity.

## Materials and methods

### Biological material

*E. multilocularis* metacestode (T1/85 strain, kindly provided by prof. Dr. Herbert Auer, Department of Medical Parasitology, Clinical Institute of Hygiene and Medical Microbiology, Medical University of Vienna, Austria) was maintained in Institute of Parasitology by intraperitoneal passage through Mongolian gerbils (*Meriones unguiculatus*). Protoscoleces were isolated from vesicular cysts 4 months post infection (p.i.). Parasite material was cut into small pieces in sterile RPMI-1640 medium (Sigma-Aldrich, Germany), supplemented with antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin (Sigma-Aldrich, Germany) and passed through a cell dissociation sieve tissue grinder kit using apertures ranging from 380 to 45.7 µm (Sigma-Aldrich). Protoscoleces collected after the last filtration were counted and maintained in RPMI until use.

### Experimental design

The experiment was carried out on 160 inbred female Balb/c mice, 2 months old, weighing 25 – 30 g, kept under a 12-hour light/dark regime at room temperature ( $21 \pm 3^\circ\text{C}$ ) and 50 – 60 % humidity on commercial diet and water.

Animals were randomly divided into 3 groups. Groups 1 and 2 (60 animals per Group) were intraperitoneally infected with homogenised metacestode material containing 2000 protoscoleces of *E. multilocularis* per animal. The control group (Group 3/40 animals) composed of healthy female mice exposed to a sham procedure by the administration of saline solution on the same day as experimental groups. Mice in Group 1 were fertilised on day 60 post infection, while Group 2 remained unfertilised and Group 3 was uninfected and fertilised on the same day as mice in Group 1. Offspring of infected and uninfected dams created Group 4 and 5, respectively.

In the course of 170 days post infection the production of different antibody classes and subclasses (IgM, IgG1, IgG2a, IgG2b) were monitored in the both age categories, mothers and their offspring, respectively. The blood samples were taken from mothers on days 28, 42, 56, 63, 70, 77, 84, 98, 112, 126, 140 and 170 post infection and from their offspring on days 1, 3, 5, 7, 21, 35 and 42 after birth. On the stated days five mice from each group and all their offspring were euthanized and the blood samples were examined separately.

Experiments were performed in accordance with national guidelines of the Slovak Republic. The experimental protocol was approved by the Institute of Parasitology Animal Care Committee.

### Detection of CD4+ and CD8+ T lymphocytes

Lymphocytes isolated from mice spleens were resuspended in PBS (pH 7.2) at final concentration  $1 \times 10^6$  cells/ml. Cells were then incubated with directly-labelled rat anti-mouse CD4+ fluorescein isothiocyanate-conjugated and rat anti-mouse CD8+ phycoerythrin-conjugated monoclonal antibodies (Becton Dickinson Biosciences, PharMingen, Belgium) at the concentration  $0.4 \mu\text{g}/10^6$  cells at  $4^\circ\text{C}$  for 30 min. After washing of cells three times with PBS containing 0.1 % sodium azide, their number was analysed by Flow cytometry method (Dvorožnáková *et al.*, 2000). Data for  $10^4$  cells per sample falling within forward scatter (FSC) and side scatter (SSC) set for lymphocytes were collected with a FACScan flow cytometer (Becton Dickinson Biosciences, Germany). All data were analysed with CellQuest software.

### Enzyme-linked immunosorbent assay for determination of specific antibody production

The production of various specific antibody classes and subclasses in mouse sera was determined by an indirect enzyme-linked immunosorbent assay (ELISA) (Havasiiová-Reiterová *et al.*, 1995) using partially purified antigen prepared from *E. multilocularis* metacestodes (Auer *et al.*, 1988). Anti-mouse IgM, (Sigma, Germany) and IgG1,

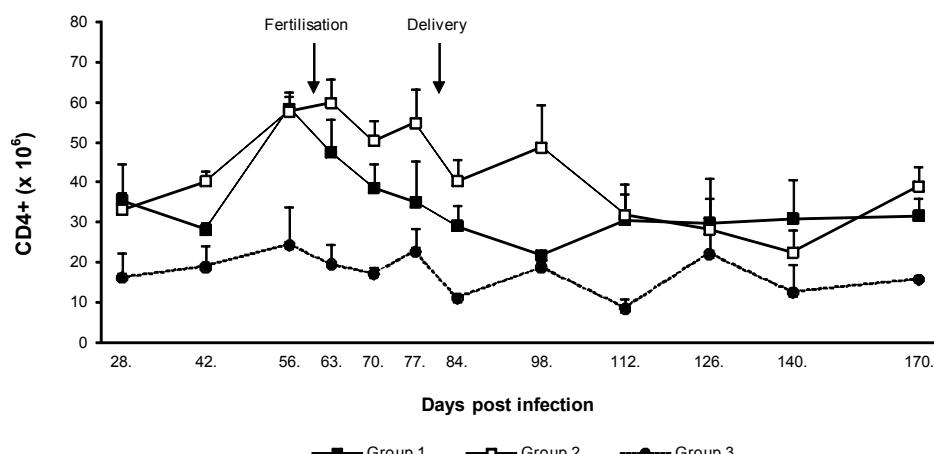


Fig. 1. Number of CD4+ T lymphocytes in fertilised and unfertilised mice infected with *Echinococcus multilocularis* and in uninfected fertilised control

Table 1. Proportion of CD4+/CD8+ T lymphocytes

Days p.i.	28	42	56	63	70	77	84	98	112	126	140	170
<b>Group 1</b>	2.4	1.8	2.1	1.9	1.9	1.8	2.3	1.9	1.9	1.8	1.9	1.2
<b>Group 2</b>	2.5	2.1	2.2	2.4	2.1	1.8	1.9	1.9	1.5	1.4	1.9	1.8
<b>Group 3</b>	2.1	2.3	2.3	2.0	2.1	1.9	2.0	2.1	2.0	2.0	1.9	1.5

IgG2a, IgG2b (Serotec Ltd, Oxford, UK) peroxidase-labelled conjugates were used at a dilution of 1:5000 and 1:1000, respectively. Chromogen o-phenylenediamine (Sigma-Aldrich, Steinheim, Germany) with H<sub>2</sub>O<sub>2</sub> was applied as substrate. The reaction was stopped with 2 M H<sub>2</sub>SO<sub>4</sub>. Optical densities (OD) were recorded spectrophotometrically at 492 nm (Revelation Quicklink, Opsys MR, Dynex Technologies, USA). Cut off values were determined individually for each antibody type by taking the average OD of uninfected control mice plus 3 standard deviations (S.D.).

#### Statistical evaluation

Statistical differences were assessed using Kruskal-Wallis analysis of variance (a value of P < 0.05 was considered to be significant), which allowed a comparison between the two groups. The analyses were performed using the Statistica 6.0 (Stat Soft, Tulsa, USA).

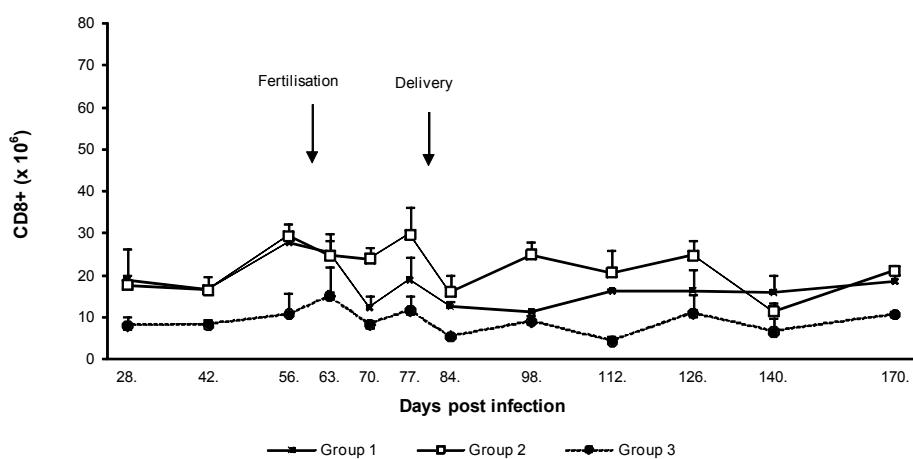


Fig. 2. Number of CD8+ T lymphocytes in fertilised and unfertilised mice infected with *Echinococcus multilocularis* and in uninfected fertilised control

tometrically at 492 nm (Revelation Quicklink, Opsys MR, Dynex Technologies, USA). Cut off values were determined individually for each antibody type by taking the average OD of uninfected control mice plus 3 standard deviations (S.D.).

#### Results

##### Number of CD4+ and CD8+ T lymphocytes

The number of both CD4+ and CD8+ T lymphocytes was higher in non-pregnant infected mice (Group 2) than in the both groups of pregnant animals, infected and uninfected ones (Groups 1 and 3).

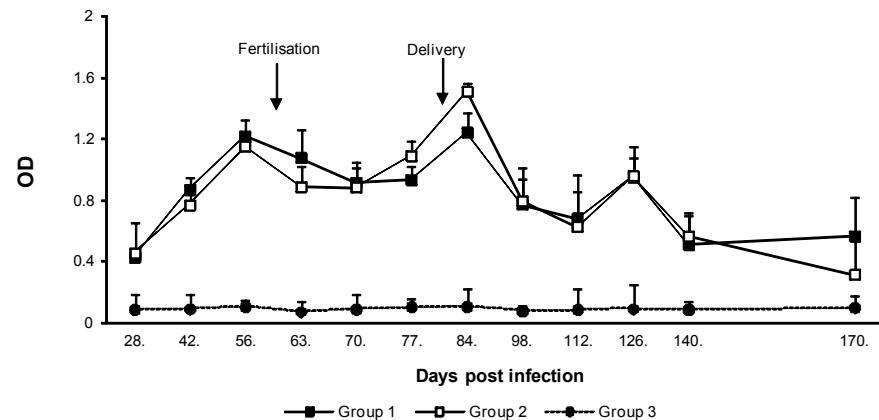


Fig. 3 Course of IgM antibody response in Balb/c mice infected with *Echinococcus multilocularis*

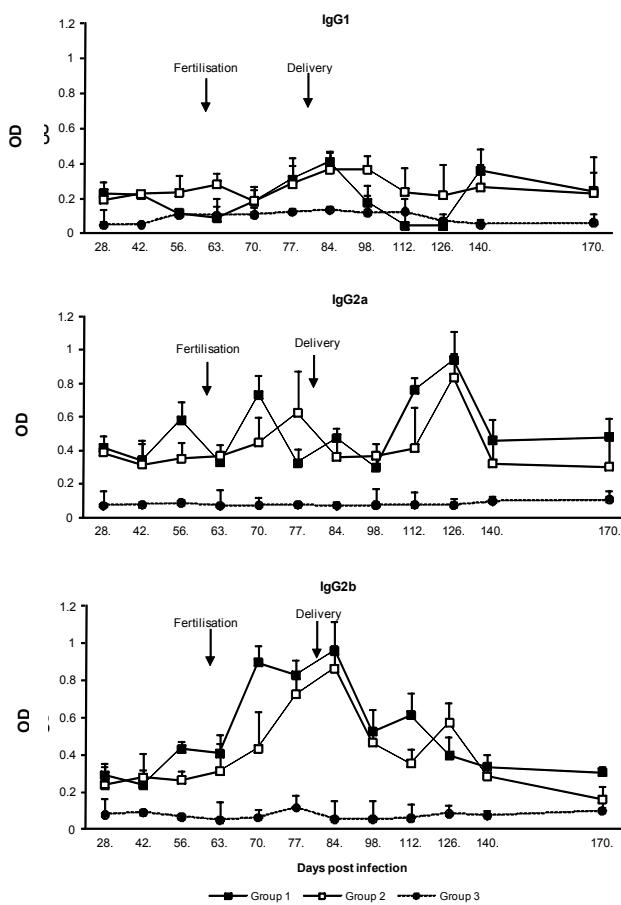


Fig. 4. Kinetics of specific IgG1, IgG2a and IgG2b antibodies in Balb/c mice infected with *Echinococcus multilocularis*

In infected mothers the decrease in CD4+ T lymphocyte population was observed between days 56 and 98 post infection (p.i.). Their number elevated on day 112 p.i. and remained stable till the end of observation. Significant differences in the proportion of CD4+ subpopulation were detected between infected fertilised and unfertilised mice (Groups 1, 2) only on day 98 p.i. ( $P < 0.05$ ) (Fig. 1). The

number of CD8+ T lymphocytes was also higher in both infected groups then in uninfected mothers. In infected mothers (Group 1) its decrease was observed after the first trimester of pregnancy. Then, before the delivery, the increase of CD8+ T cell number was recorded, following by stabilisation of its level until the end of experiment. Significant differences in the proportion of CD8+ subtype were detected between infected mice (Group 1, 2) on day 70 and 98 p.i. ( $P < 0.05$  and  $P < 0.01$ , respectively) (Fig. 2). The proportion of CD4+/CD8+ T lymphocytes between examined Groups of mice varied between rates 1.2 and 2.5, however without any significant differences on particular days of experiment (Table 1).

#### Specific antibody production

Strong humoral IgM response was detected in both infected groups, without any significant differences during pregnancy and in *post partum* period (Group 1 versus Group 2) (Fig. 3).

The production of IgG1 subclass was slightly depressed in the first trimester of pregnancy of infected mice (Group 1). Then, its production was similar than in infected unfertilised mice (Group 2). After delivery antibody level decreased and reached lower values than in uninfected mothers (Group 3) on days 112 and 126 p.i.. After 140 dpi the level was comparable to infected unfertilised mice (Group 2) (Fig. 4).

IgG2a antibody response was similar in both infected groups (Group 1 and 2) during the whole monitored period.

The course of IgG2b production was also similar in both, fertilised and unfertilised infected mice groups, except for the pregnancy period, when IgG2b antibody level in Group 1 was slightly enhanced (Fig. 4).

The IgM antibodies were transmitted neither transplacental nor transmammary from mothers to their offspring (Fig. 5). Specific antibodies of IgG2a and IgG2b subclasses in offspring of infected mothers (Group 4) were detected already on the first day *post partum*, while IgG1 appeared on day 3 *post partum*. The temporary increase in IgG1 and IgG2a subclasses, on 21. and 35. dpi respectively, suggests their transmammary transmission (Fig. 6).

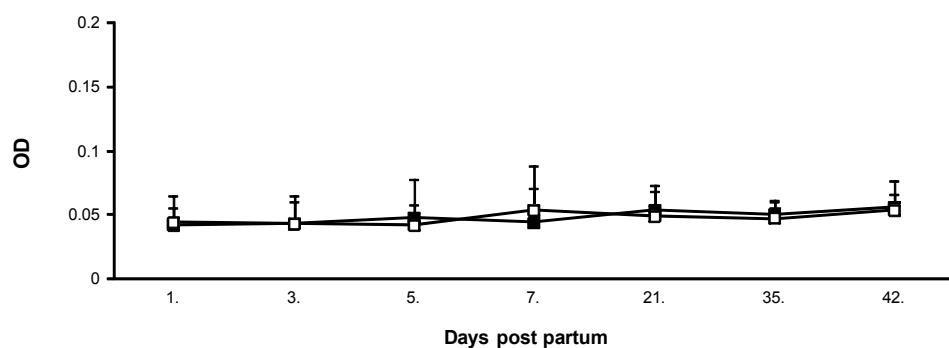


Fig. 5. IgM antibody level in offspring of Balb/c mice infected with *Echinococcus multilocularis*

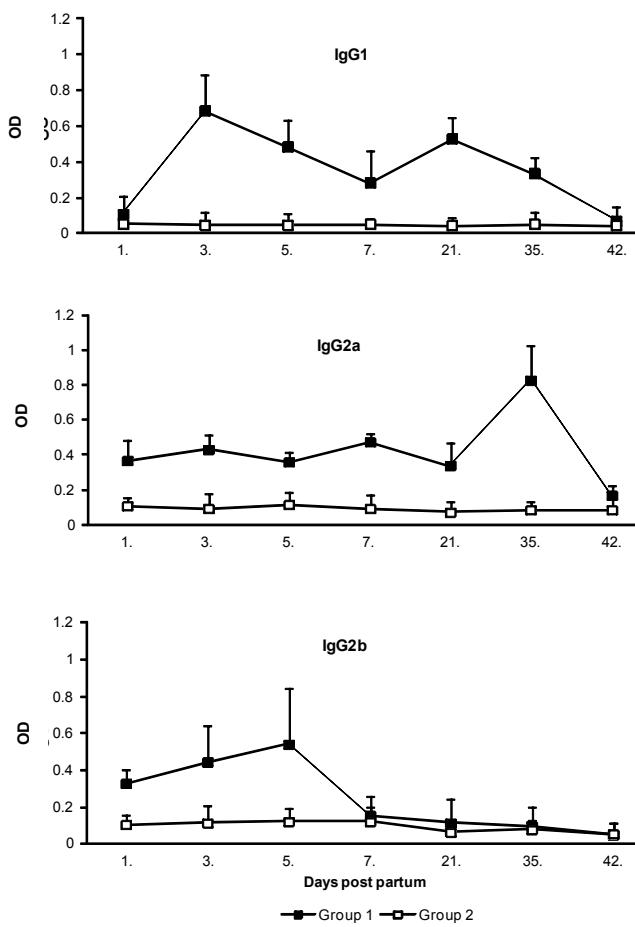


Fig. 6. Kinetics of specific IgG1, IgG2a and IgG2b antibodies in offspring originating from Balb/c mice infected with *Echinococcus multilocularis*

## Discussion

Balb/c mice strain has been found to be particularly susceptible to *E. multilocularis* infection with an intensive intraperitoneal metacestode proliferation and metastasis formation, in various inner organs (Gottstein & Felleisen, 1995). T-lymphocytes play a major role in the control of immune response in *E. multilocularis* infected intermediate host (Dvořáková *et al.*, 2008). The helper CD4+ T lymphocytes are essential for activation, differentiation, and isotype regulation of B lymphocytes and are of significant help in antigen presentation. After the antigen stimulation they release a whole range of cytokines, as regulatory mediators that determine the development of immune response to Th1 or Th2 type (Emery *et al.*, 1996; Dvořáková *et al.*, 2009). Cytotoxic CD8+ T lymphocytes are responsible for the destruction of structures carrying an antigen on their surface, participate in the inhibition of immune response and regulate eosinophilia (Owhashi *et al.*, 1998). Proportions of CD4+ and CD8+ T cell subsets appear to be important for the metacestode growth. Relatively high number of CD4+ T cells correlates with abortive or slow development of the larvae, while CD8+ T cells

are predominant in severe cases of alveolar echinococcosis (Vuitton, 2002; Vuitton *et al.*, 2006; Yang *et al.*, 2005). During the course of gravidity and *post partum* period the number of both subtypes of T lymphocytes was lower in pregnant animals comparing with non-pregnant mice suggesting the down-regulation of T cells subpopulations caused by pregnancy. The decline of CD4+ and rise of CD8+ T cell numbers observed in both, infected and uninfected mothers, in late pregnancy correlates with findings of other authors (Landers *et al.*, 1991), who observed the decrease of CD4+ and increase of CD8+ T cells at the terminal phase of physiological pregnancy.

Basically, the production of IgM and all IgG subclasses is stimulated by antigens from metacestode laminar layers, the source of host's antigen stimulation in chronic course of the disease. Th2 response is characterised by the activation of B cells and by a subsequent production of IgE, IgG1, IgG4 and IgA antibodies. On the other hand, Th1 response is characterised by IgG2a, IgG2b and IgG3 production (in hosts resistant to infection).

In our experimentally infected dams, strong humoral response with high production of IgM and IgG2b antibodies was detected. IgG1 level, related to Th2 immune response, was suppressed in infected mothers after the delivery, when, the antibody levels declined under the level of uninfected mothers. IgG2a and IgG2b antibody response was similar in both infected groups. Both antibody subclasses slightly declined in infected dams in the third trimester of gravidity. On the other hand, in different mouse strains (C57BL6J), susceptible to *E. multilocularis* infection, low production of IgG2a immunoglobulins have been observed (Bauder *et al.*, 1999).

A specific antibody production in progeny starts already in the early gestation period, but due to the sterile surroundings of the foetus its level is very low (Buc, 1997). In our experiment, IgM antibodies were transmitted neither transplacental nor transmammary. IgG1 subclass seems to be transmitted only transmammary, because it was detected on the 3<sup>rd</sup> day *post partum* and persisted for 5 weeks. IgG2a and IgG2b subclasses were detected in offspring of infected mothers already on the first day *post partum*. Later temporary increase of IgG2a subclass suggests also its transmammary transmission.

Alveolar echinococcosis is not transmitted to offspring; therefore no impulse for specific antibody production in young ones exists. After the birth, antibodies acquired from mothers progressively decline and usually persist in the blood circulation only for several weeks *post partum*. In our experiment, IgG2a persisted almost at the same level for 5 weeks, while IgG2b rapidly decreased after 5<sup>th</sup> day *post partum*.

A successful pregnancy results from a state of "tolerance" of the mother. A special type of cells called a regulatory T cells, is known to prevent immune system from attacking own tissues. These regulatory T cells now seem to play a key role in preventing mother from rejecting her embryo (Raghupathy, 1997). In our former studies, in the group of infected, fertilised mice increased Th2 cytokine (IL-10)

concentrations from the day 70 p.i. until the end of experiment was found, what correlates with observed more intensive metacestode growth (Antolová & Reiterová, 2010). We can hypothesise that changes in Th1 and Th2 response could influence both, the course of gravidity and infection. *E. multilocularis* infected mice were fertilised on day 60 p.i., in the period characterised by relatively rapid cyst growth. Although, not observed in this experiment, Th2 overactivity induced by pregnancy can be associated with the more rapid growth of metacestodes in fertilised mice observed by Antolová & Reiterová (2010). After all, these changes should influence also humoral immune response with the production of specific antibody subclasses, as well as their transmission into the progeny.

In the light of these data the changes in Th1/Th2 production did not significantly influence the course of gravidity. On the other hand, more intensive metacestode growth was observed after the delivery, which confirmed the ability of host organism to adapt to severe damage caused by *E. multilocularis*, not only in normal physiological, but also during pregnancy (Antolová & Reiterová, 2010). Understanding the immune system is crucial for both, medical and bioscience studies, with new research revealing yet more secrets year on year.

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