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Kinetics of specific humoral immune response of mice infected with low doses of *Trichinella spiralis, T. britovi,* and *T. pseudospiralis* larvae

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Summary

The specific humoral immune response of the host (mouse) to the infection with low doses of larvae of encapsulating (*Trichinella spiralis, T. britovi*) and non-encapsulating (*T. pseudospiralis*) species was studied. Mice were experimentally infected with 10 muscle larvae of the parasite to simulate natural conditions of the infection in rodents, important reservoirs of trichinellosis. The low infective dose of *T. spiralis* and *T. britovi* did not evoke an increased specific IgM response, which is typical for the acute infection. Only *T. pseudospiralis* induced a higher specific IgM level in the intestinal phase of the infection, till day 30 p.i. The low infective dose of *T. spiralis* larvae stimulated a specific IgG_1 production from day 20 p.i. with a strong increase on day 45 p.i., but *T. britovi* infection on day 60 p.i. Specific IgG_1 antibodies were not detected in *T. pseudospiralis* infection. The production of IgG_{2a} and IgG_{2b} antibodies was again earlier and more expressive in *T. spiralis* infection from day 45 p.i., in contrast to *T. britovi*, where these antibodies were increased on day 60 p.i. Only IgG_{2b} isotype was detected in *T. pseudospiralis* infection on days 45 and 60 p.i., however in very low values in comparison with encapsulating species.
Results conclude the low infective dose of *T. spiralis*, *T. britovi*, and *T. pseudospiralis* induced a late seroconversion in infected mice. *T. spiralis* caused earlier and more intensive specific antibody response, from day 45 p.i, when antigens from newborn and muscle larvae were accumulated, on the contrary to *T. britovi* and *T. pseudospiralis*, which induced specific antibody response from day 60 p.i.

Keywords: low infection, antibodies; *Trichinella spiralis*; *T. britovi*; *T. pseudospiralis*

Introduction

Trichinella spp. is the intestinal nematode parasite with worldwide distribution and which causes trichinellosis - a serious zoonosis. (Miterpáková *et al*., 2009; Paraličová *et al*., 2009). At present, the genus *Trichinella* comprises five encapsulating (*T. spiralis*, *T. nativa*, *T. britovi*, *T. nelsoni* and *T. murrelli*) and three non-encapsulating species (*T. pseudospiralis*, *T. papuae* and *T. zimbabwensis*)(Morgan, 2000). The characteristics of these species are based on biological, biochemical and genetic criteria. Species variation in infectivity and immunogenicity is very important (Bolas-Fernández, 2003). Experimental studies demonstrated significant differences in infectivity of well characterised domestic and sylvatic *Trichinella* genotypes and the dependence of infective dose on the effectiveness of infection (Kapel & Gamble, 2000; Cui *et al*., 2006; Reiterová *et al*., 2009). Infectivity of *Trichinella* species is also determined by the immune status of the host.
Larval cuticular fragments (Bruschi *et al*., 1992) and metabolites stimulate both specific and non-specific defense reactions during trichinellosis. *Trichinella* spp. infection induces T cell dependent inflammatory response of the host in which Th2 immune response is activated during the intestinal infection (Wakelin & Goyal, 1996) and Th1 response is suppressed (Hogaboam *et al*., 1996). The muscle infection with *T. spiralis* elicits a focal cellular immune response. Parasites survive in nurse cells in close association with macrophages, CD8+ and CD4+ T lymphocytes, and B lymphocytes (Beiting *et al*., 2004). B lymphocytes secreting antibodies, particularly IgG and IgE, may lead to an effective antibody-dependent cell mediated cytotoxic reaction against *T. spiralis* newborn larvae (Moloney & Denham, 1979; Wang & Bell, 1988). The earlier specific antibodies are bound to *Trichinella* antigens and form immune complexes, which are present in infected host at the beginning of the infection (Dziemian & Machnicka, 2000; Feldmeier *et al*., 1987). The protective isotypes IgG_1 and IgG_2 are involved in the inflammatory response. An elevation of IgG_1 accompanies the muscle phase of infection (Doligalska, 2000) and newborn larve are more sensitive *in vitro* to

IgG_1 in antibody-dependent cellular cytotoxicity (Moskwa, 1999). Antibodies significantly participate in *Trichinella* entrapment and rapid expulsion of infective larvae L1, reduce adult worm fecundity and kill newborn larvae (Appleton & Usack, 1993). The consistent release of circulating antigens by the larvae plays a major role in sustaining the host immune response until the calcification of the parasites (Li *et al*., 1999). Circulating antigens are present in plasma and urine of infected organisms about 30 days after the infection (Machnicka *et al*., 2001; Kolodziej-Sobocińska *et al*., 2006). Specific antibodies remain detectable for a very long time after the infection. Polyclonal lymphocyte activation of T-cells, but particularly B-cells, is responsible for the high levels of immunoglobulines IgG, IgM, and IgE observed in infected animals and humans (Murrel & Bruschi, 1994).
Specific anti-*Trichinella* IgM antibodies are first found, during second week of the infection (Li & Ko, 2001; Kolodziej-Sobocińska *et al*., 2006). Specific IgG antibodies were found also 6 or 8 months after infection, even after 3 years (Dziemian & Machnicka, 2000; Kolodziej-Sobocińska *et al*., 2006; Morales *et al*., 2002). Stimulation of ES antigens production by larvae and their penetration through the capsules (Pritchard, 1985) as well as degradation of larvae by inflammatory cells (Candolfi *et al*., 1989) explains the long-lasting presence of IgM, IgG antibodies. Immunoglobulines IgG_1 represent Th2-cell activation and IgG_2 antibodies reflect Th1 response (Else & Finkelman, 1998). A significant elevation of IgG_1 is often observed in trichinellosis (Li & Ko, 2001; Kolodziej-Sobocińska *et al*., 2006). Both IgG_1 and IgG_2 are responsible for antilarval activity of peritoneal eosinophils that are involved in the inflammatory response (Doligalska, 2000).
Potential differences in a development of the host immune response to different *Trichinella* species and host-parasite interactions are rarely studied under *in vivo* conditions. Kapel and Gamble (2000) observed variances in the infectivity and antibody responses of pigs to domestic and sylvatic *Trichinella* spp. after a high infective dose. Andrade *et al*. (2007) described interspecies results from *in vitro* study, where the differences in NO production of macrophages after stimulation with L1 antigens from encapsulated and non-encapsulated trichinellas were demonstrated. Immunochemical variety of larval *T. spiralis* and *T. pseudospiralis* antigens had been confirmed by Turčeková *et al*. (1997).
The aim of this study was to observe the dynamics of specific antibody response in mice after the infection with low doses of larvae of encapsulating (*Trichinella spiralis*, *T. britovi*) and non-encapsulating (*T. pseudospiralis*) species.

Materials and methods

The experiment was carried out on male BALB/c mice (n = 144) weighting 20 – 25 g. Mice were kept under a 12-h light/dark regime at room temperature (22 – 24°C) and 56 % humidity on a commercial diet and water. The experimental protocol was approved by the Parasitological Institute Animal Care Commitee. Animals were divided randomly into four groups as folows: Group 1 (n = 24) was uninfected and untreated (control), mice in Group 2 (n=40) were infected *per os* with 10 *T. spiralis* larvae per mouse on day 0 of the experiment. Mice in Groups 3 (n = 40) and 4 (n = 40) were infected *per os* with 10 *T. britovi* and *T. pseudospiralis* larvae per mouse, respectively.
Samples of blood were obtained on days: 0 (prior infection), 5, 10, 15, 20, 30, 45 and 60 post infection (p.i.) from all groups, from 5 infected and 3 uninfected mice. The blood was centrifuged at 3000 *g* for 10 minutes and serum samples were stored at -20 ^{o}C until the examination.

The infective larvae Trichinella spp.:
The reference isolates of *Trichinella spiralis* (ISS 004), *T. britovi* (ISS 1088) and *T. pseudospiralis* (ISS 013) (obtained and assigned codes from the Trichinella Reference Centre in Rome), maintained by serial passage in ICR mice at the Parasitological Institute SAS, were used for the infection. Larvae were released by artificial digestion (1 % pepsin, 1 % HCl for 4 h at 37 °C) of tissue following the standard protocol and kept saline solution until inoculation of experimental mice.

Detection of specific antibody production by iELISA:
Specific *Trichinella* spp. antibodies in serum were detected by indirect ELISA according to Reiterová *et al*. (1999). Somatic antigens *(T. spiralis, T. britovi, T. pseudospiralis)* diluted at 2 μg/ml carbonate buffer (pH 9.6) were bound to the microtitrate plates (Nunc, Denmark) overnight at 4 °C. After triple washing of wells with phosphate buffered saline (PBS, pH = 7.2) with 0.5 % Tween 20 (PBS-T) non-specific bonds were blocked with by 0.5 % skimmed milk PBS after 1 hour incubation at room temperature. After triple washing with PBS-T the serum samples and conjugates were added step by step for 1-hour incubation at 37 ^{o}C. Sera were diluted 1:100 in PBS-T. Anti-mouse horseradish peroxidase conjugates (all Sigma-Aldrich, Germany) were diluted: IgM (1:2000), IgG_1 (1:2000), IgG_{2a} (1:500) and IgG_{2b} (1:500). The substrate o-phenylene diamine (Sigma-Aldrich, Germany) at 0.05 mol/l in citrate buffer (pH 4.7) with 0.005 % H_2O_2 was used for a visual reaction. The reaction was stopped by 1M H_2SO_4 after 20 minutes incubation at room temperature in the dark. Plates were measured for the optical density at 490 nm (Revelation Quicklink, Opsys MR, Dynex Technologies, USA).

Intestinal worm burdens:
The intestinal phase of infection was investigated on days 5, 10, 15 and 20 p.i. Small intestine was cut into 5-10 cm long pieces, placed into a sieve and incubated in conical pilsner glasses in 37 °C NaCl (0.9 % saline) overnight. After incubation, gut pieces were discarded and the sediment was counted under stereomicroscope at 60 x magnification.

Isolation of muscle larvae:
The muscle phase of infection was examined on days 20, 30, 45 and 60 p.i. Whole eviscerated carcasses were

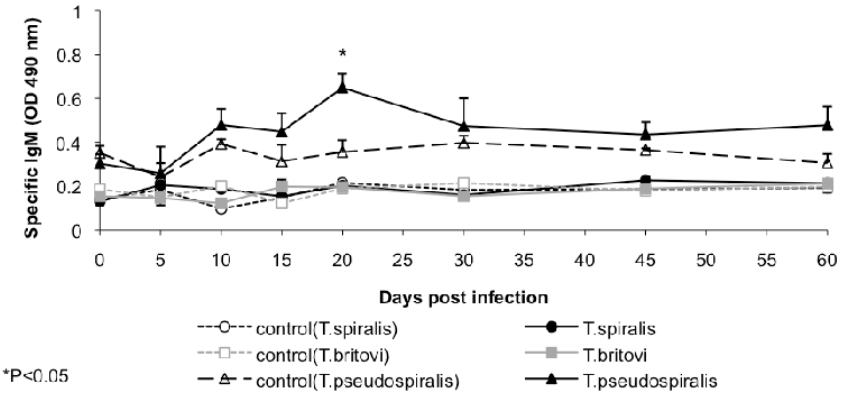

Fig. 1. Specific antibodies IgM in mice infected with 10 larvae of *Trichinella spiralis*, *T. britovi* and *T. pseudospiralis*. *(P<0.05) statistically significant from control uninfected mice.

minced and artificially digested (1 % pepsin, 1 % HCl for 4 h at 37 °C; both Sigma-Aldrich, Germany) according to Kapel and Gamble (2000). Samples were allowed to settle for 20 min before the supernatant was discard and the sediment was poured through a 180 μm sieve into a conical glass and washed with tap water. The sediment was finally transferred to a gridded Petri dished and counted using a stereomicroscope at 40 x magnification. Depending on the density of larvae either a sub-sample or the whole sample was counted.

Statistical evaluation:
Statistical differences were assessed using one-way ANOVA, followed by post hoc Tukey's test (a value of $P < 0.05$ was considered significant), which allowed comparison between each two groups at each time point. The analyses were performed using the Statistica 6.O (Stat Soft, Tulsa, USA) statistical package.

Results

Dynamics of specific immunoglobulines IgM, IgG_1, IgG_{2a}, IgG_{2b} (Figs. 1 – 4)

The low infective dose of 10 larvae of *Trichinella spiralis* and *T. britovi* did not induce an increase in specific IgM antibody response, no seroconversion was observed in comparison with the control. However, *T. pseudospiralis* infection evoked the increase IgM immunoglobuline level in murine serum from days 5 to 20 p.i., i.e. during the intestinal phase of the infection (Fig. 1).
The low infective dose of *Trichinella spiralis* stimulated the production of specific IgG_1 antibodies from day 20 p.i., with a sharp increase from day 45 p.i., but *T. britovi* infection induced the generation of specific IgG_1 antibodies only on day 60 p.i. *T. pseudospiralis* infection did not affect the IgG_1 production (Fig. 2).
The productions of IgG_{2a} and IgG_{2b} were again earlier and more expressive after *T. spiralis* infection from day 45 p.i., on the contrary to *T. britovi*, where the antibody concentrations in serum were increased only on day 60 p.i. The low infective dose of *T. pseudospiralis* did not influence IgG_{2a} antibody response, but IgG_{2b} immunoglobuline level started increasing from day 45 p.i., although with a lower intensity as in infections with encapsulating species (Figs. 3, 4).

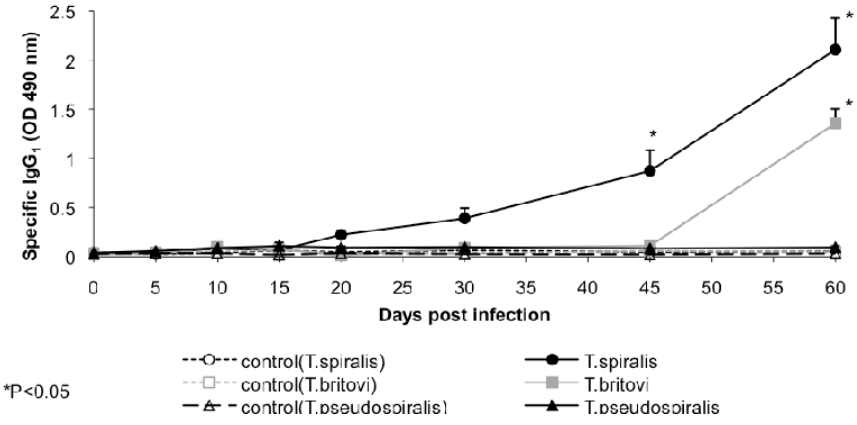

Fig. 2. Specific antibodies IgG1 in mice infected with 10 larvae of *Trichinella spiralis*, *T. britovi* and *T. pseudospiralis*. *(P<0.05) statistically significant from control uninfected mice.

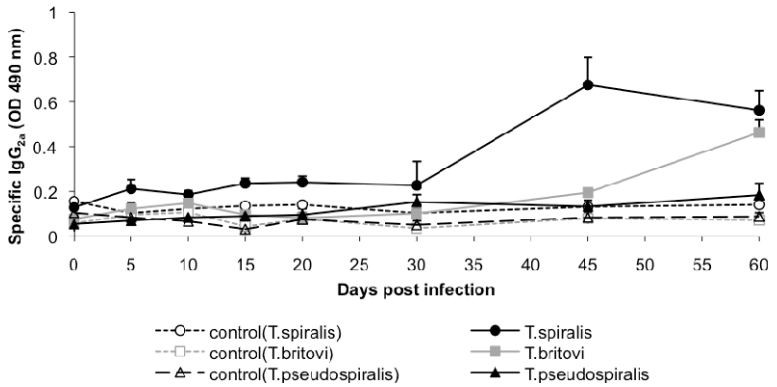

Fig. 3. Specific antibodies IgG2a in mice infected with 10 larvae of *Trichinella spiralis*, *T. britovi* and *T. pseudospiralis*.

Parasite burden – numbers of adults and muscle larvae (Figs. 5, 6)

Mice infected with the low dose of larvae absolutely eliminated parasite adults from the small intestine till day 20 p.i. in both encapsulating species. On day 15 p.i. the occurrence of *T. spiralis* adults in the intestine was sporadic (0.13 ± 0.35) in contrast to *T. britovi*, that were found in higher numbers of worms (2.43 ± 2.51). *T. pseudospiralis* adults were isolated from the small intestine also on day 20 p.i. (0.62 ± 0.74) (Fig. 5).

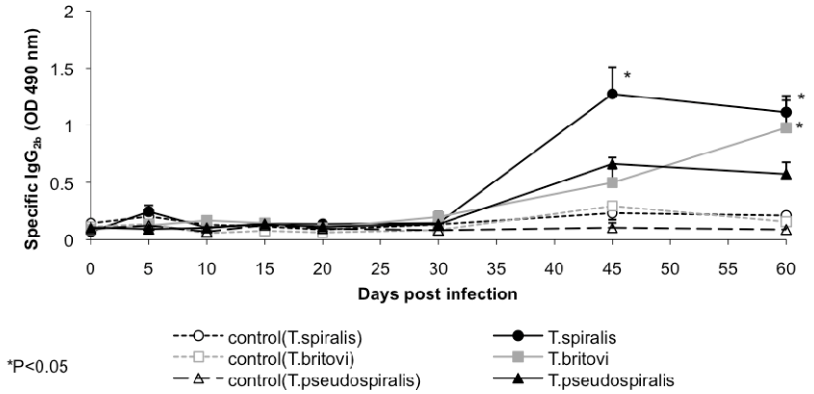

Fig. 4. Specific antibodies IgG2b in mice infected with 10 larvae of *Trichinella spiralis*, *T. britovi* and *T. pseudospiralis*.
*(P<0.05) statistically significant from control uninfected mice.

Numbers of muscle larvae obtained after *T. spiralis* infection were the highest of all infections, what is related to the high reproductive capacity of this species. The maximum numbers of *T. spiralis* larvae were found on day 45 p.i. (3060 ± 1859.1 larvae/mice), numbers of *T. britovi* and *T. pseudospiralis* peaked on day 30 p.i. (847.2 ± 609.6 and 645.5 ± 269.1 larvae/mice, respectively) (Fig. 6).

Discussion

The presence of immune complexes in the vascular system is connected with the symptoms of trichinellosis at the beginning of the infection (Feldmeier *et al*., 1987). Later, when the parasite has been settled in muscles, neither *Trichinella* antigens nor immune complexes are detectable in blood, but there are detectable specific antibodies. This phenomenon is caused by excretory-secretory antigens from larvae settled in muscles (Pritchard, 1985; Li *et al*., 1999). Humoral immune response is important in the host defence against migrating newborn larvae. The absence of antigen on the cuticular surface of adult worms is in a sharp contrast to the findings described in muscle larvae, whose surface antigenicity is identical to that of stichocyte a-granules (Appleton *et al*., 1991). Therefore, no direct immune attack is likely to be exerted against adults. It has been suggested that the effects of serum antibodies on worms may be indirect and that worm expulsion is the result of inflammatory processes in the intestines evoked by the infection (Wakelin & Wilson, 1979).

An increased specific IgM antibody response, which is typical for acute infection, was not found in our experiment after the low infective dose of 10 larvae of *T. spiralis* and *T. britovi.* In comparison with control, neither seroconversion was detected. On the contrary, Reiterová *et al.* (2009) infected mice with 50 larvae of *T. spiralis* and recorded the seroconversion on day 30 p.i., but IgM antibody production overdrew a cut-off only a little. The low number of antigens might be bound into immune complexes. Contrary, *T. pseudospiralis* induced a higher level of specific IgM immunoglobulines during the intestinal phase till

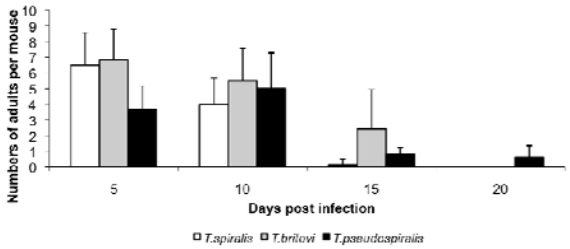

Fig. 5. Numbers of adults isolated from the small intestine of mice infected with 10 larvae of *Trichinella spiralis*, *T. britovi* and *T. pseudospiralis*.

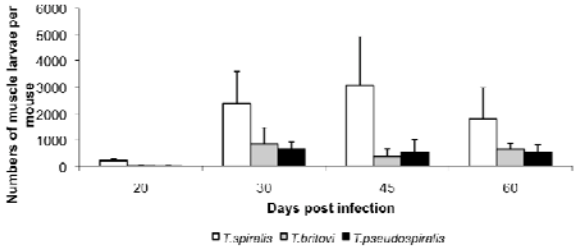

Fig. 6. Numbers of muscle larvae isolated from mice infected with 10 larvae of *Trichinella spiralis*, *T. britovi* and *T. pseudospiralis*.

day 30 p.i., what reflects the acute stage of the infection.

An elevation of IgG_1 accompanies the muscle phase of infection (Doligalska, 2000) and newborn larve are more sensitive *in vitro* to IgG_1 in antibody-dependent cellular cytotoxicity (Moskwa, 1999). The low infective dose of 10 larvae of *T. spiralis* in our study stimulated IgG_1 antibody production from day 20 p.i., with a significant elevation from day 45 p.i., but *T. britovi* infection induced the generation of IgG_1 antibodies not until day 60 p.i. It correlates to IgG antibody response in outbred ICR mice after *T. spiralis* infection with low dose of 5 larvae (Reiterová *et al*., 2009). *T. pseudospiralis* infection did not show a positive specific IgG_1 antibody response. The production of specific IgG_{2a} and IgG_{2b} was again more expressive and earlier after *T. spiralis* infection from day 45 p.i. in contrast to *T. britovi* infection, where these antibodies increased their serum levels not until day 60 p.i. Only isotype IgG_{2b} was detected in *T. pseudospiralis* infection on days 45 and 60 p.i., however at very low values in comparison to encapsulating *Trichinella* species.

Study by Furze and Selkirk (2005) compared antibody response in mice after *T. spiralis* a *T. pseudospiralis* infection with 500 larvae, whereby all classes of parasite-specific antibody were present in serum, but there were differences in the timing. Infection with *T. spiralis* notably induced greater amounts of IgM, IgG_1, IgG_{2b} and IgG_3 in serum during the muscle phases of infection.

The low infective dose of *T. spiralis*, *T. britovi*, and *T. pseudospiralis* in our study induced a late seroconversion in infected mice. Interspecies differences were found in immunogenicity of *T. spiralis* and *T. britovi*, which showed the similar trend, but varied in the intensity of the host antibody response. The species *T. spiralis* appeared to be more immunogenic and evoked more intensive and earlier specific antibody response of the host from day 45 p.i., when the antigen material had been markedly accumulated from the newborn and muscle larvae. As the reproductive capacity of *T. britovi* and *T. pseudospiralis* is lower in contrast to *T. spiralis*, the start of specific antibody response in *T. britovi* and *T. pseudospiralis* infections was recorded from day 60 p.i. The immune response to *T. pseudospiralis* infection suggested the biggest deviations. *T. pseudospiralis* adults persisted in the host small intestine for a longer time, untill day 20 p.i. Only mice infected with *T. pseudospiralis* reacted by low IgM antibody production during the intestinal phase, which was not observed in infections with encapsulating *Trichinella* species.

All species *T. spiralis*, *T. britovi* and *T. pseudospiralis* are intracellular parasites of muscle cells, essentially with the same life cycle, which differ in only the fact, that complex "nurse cell" is or is not surrounded with a collagen capsule. The found differences in the host immune reactions to different *Trichinella* species in our study suggest that the immune response variations can be caused not only with distinctions of muscle larval L1 excretory-secretory antigens (Robinson *et al*., 2007; Milcheva *et al*., 2009), but also with parasite stages L2-4 and adults. Identification and characterization of species-specific proteins of development stages play an important role for understanding of mechanisms involved in the parasite-host interaction, which provide a longterm surviving for the parasite in the host organism.

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