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Immunophenotyping of cells involved in early immune response to experimental *Trichinella spiralis* gut infection

Frane Božić[1]*, Albert Marinculić[2], and Relja Beck[2]

[1]*Department of Pharmacology and Toxicology, Faculty of Veterinary Medicine, University of Zagreb, Zagreb, Croatia*

[2]*Department of Parasitology, Faculty of Veterinary Medicine, University of Zagreb, Zagreb, Croatia*

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ABSTRACT

Peyer's patches (PP) are believed to be the principal sites for the induction of immune responses to invading mucosal pathogens, yet the phenotype of T cells recruited within the PP during the early phase of *Trichinella spiralis* gut infection is largely unexplored. In order to gain an insight into this issue, in the present study C57BL mice were orally infected with the parasite at day 0 and necropsied at 1, 4, 7 and 14 days post infection (p. i.). Uninfected mice served as control. Absolute number of mononuclear PP cells and cell surface marker expression of PP T cell subpopulations in the infected mice are compared with those from spleen. Our results showed that early during the intestinal phase of infection, total number of PP mononuclear cells gradually declined from p. i. day 1 onward, whereas the number of splenocytes dramatically increased at 4 and 14 days p. i. At the same time, no major changes in the phenotype of PP T cells were observed in infected versus uninfected mice. However, lymphocytes from spleen underwent significant kinetic changes related to the infection.

Key words: *Trichinella spiralis*, immunophenotyping, T cells, flow cytometry

Introduction

Because of its dramatic re-emergence in many areas around the world over the past 10-20 years, trichinellosis can be classified as emerging zoonosis (MURRELL and POZIO, 2000). It is still an endemic disease in most

* Contact address:
Doc. Dr. Frane Božić, Department of Pharmacology and Toxicology, Faculty of Veterinary Medicine, University of Zagreb, Heinzelova 55, 10000 Zagreb, Croatia, Phone: +385 1 2390 163; Fax: +385 1 2441 390; E-mail: bozic@vef.hr

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European countries, mainly induced by three *Trichinella* species: *T. spiralis*, *T. britovi* and *T. nativa* (POZIO, 1998). While *T. spiralis* is the aetiological agent of domestic trichinellosis, the two latter species survive only among populations of sylvatic carnivores. Although trichinellosis is most frequently associated with carnivores, with the rat-pig cycle being the most notable, mice have been extensively used as experimental hosts for *T. spiralis*. In mice, a primary infection is expelled after approximately 2-3 weeks (BELL, 1988).

Specific immune mechanisms responsible for the expulsion of primary Trichinella infections in rodents are not fully understood, but local T cell-dependent inflammatory responses are believed to be involved (WAKELIN, 1993; BELL, 1998; BOŽIĆ et al., 2000a). It seems that the $CD4^+$ T cells play an important role in regulating the immune and inflammatory responses against *T. spiralis* infection through their ability to secrete cytokines (WAKELIN and GOYAL, 1996). The role of $CD8^+$ T cells in mucosal defence against Trichinella infections is as yet unclear. According to a recent report, the commencement of expulsion of *T. spiralis* adults in mice infected with 200 muscle larvae coincided with a transient but significant increase in $CD8^+$ intestinal intraepithelial lymphocytes (i-IEL) (BOŽIĆ et al., 2000b). Surprisingly, a loss of i-IEL population from the gut epithelium of mice infected with 400 L1 *T. spiralis* larvae occurred early during infection, before the expulsion starts (DOMINIS-KRAMARIĆ et al., 2000). Thus, despite an initial proposition that the kinetics of intestinal intraepithelial T cells might correspond to the kinetics of worm expulsion, it seems that this may not be the case. By using the *T. spiralis*/mouse model, we and others have proposed that during the early phase of infection, *T. spiralis* rather stimulates T-cell-mediated immunity in other gut-associated lymphoid tissues (GALT) as well as systemic immune responses in the spleen (WAKELIN et al., 1994; BOŽIĆ et al., 1998).

It is generally believed that the mucosal immune reactions induced by a wide variety of invading pathogens can occur independently of systemic immunity, or they may act in concert. Peyer's patches (PP), also termed intestinal tonsils or aggregated follicles, play a central role in the induction of mucosal immune responses in the GALT (NEUTRA et al., 2001). This is the site where the first encounter between immune cells, such as T and B

cells and follicular dendritic cells, and environmental antigens takes place. The PP B cells are responsible not only for the local IgA production but also for the generation of immunological memory, and the T cells play a role in immunoglobulin isotype regulation (SÁNCHEZ CARRIL et al., 2002; MAKALA et al., 2002-03; MOSER and EBERT, 2003). Although both the PP and spleen of normal, uninfected mice are populated with different T cell subsets, their role in the generation of host protective immunity after *T. spiralis* infection remains unclear (BOŽIĆ et al., 1998). Thus, in this study we have analyzed changes in the PP and spleen T lymphocyte subpopulations of mice infected with *T. spiralis* to provide information about their activities during the intestinal stage of infection.

Materials and methods

Experimental animal. In the present study we used male C57BL mice aged 10-14 weeks obtained from the Ruđer Bošković Institute, Zagreb, Croatia. Mice were kept under standard conditions in the animal facility of the Faculty of Veterinary Medicine, University of Zagreb, Croatia and were fed with commercially prepared standard diets *ad libitum*.

Parasite and infection. The Trichinella used in the study was *T. spiralis* (MSUS/PO/60/ISS3), obtained from the Trichinella Reference Centre (Istituto Superiore di Sanitŕ, Rome, Italy). The parasite was maintained by serial passages in Wistar rats. Infective muscle larvae (L1) were obtained by 1% pepsin-HCl digestion of eviscerated ground rodent carcasses as described previously (WAKELIN and WILSON, 1977) and mice were routinely infected at day 0 with 200-300 viable L1 by using a ball-tipped feeding tube. In each of five separate experiments, *T. spiralis*-infected (experimental) and uninfected (control) groups comprised 4-5 animals.

Necropsy procedures and lymphocytes isolation. Infected mice were sacrificed at 1, 4, 7 and 14 days after infection and uninfected C57BL mice were always examined together with the infected group. After euthanasia of mice, the PP and spleens were aseptically excised for the isolation of PP cells and splenocytes for immunophenotyping. Lymphocytes from PP and spleens were prepared by perfusing and teasing tissue in modified Eagle's medium with Hanks (MEM-H; Institute of Immunology, Zagreb, Croatia), as previously described (BOŽIĆ et al., 1998).

Counts of PP cells and splenocytes. Following their isolation by density gradient centrifugation at 750 g for 25 min over Lymphoprep (Nycomed, Oslo, Norway), the collected cell-pellets were washed once with MEM-H medium. The freshly obtained cells from the PP and spleens of individual animals were enumerated under a light microscope in a haemocytometer and their viability was assessed by trypan-blue dye exclusion test. Number of PP and spleen cells in infected mice was expressed as percentage of cell numbers recovered from either of the two lymphatic tissues of uninfected mice (% of day 0).

Analysis of cells by flow cytometry. Single cell suspensions were prepared and incubated with monoclonal antibodies (mAbs) (10 mg/10^6 cells) used in one-colour flow cytometry to determine the percentage of positive staining. All mAbs used in the present study were obtained from Dianova/Pharmingen (Hamburg, Germany): fluorescein isothiocyanate (FITC)-conjugated anti-TCR$\alpha\beta$ (H57), FITC-conjugated anti TCR$\gamma\delta$ (GL3), phycoerythrin (PE)-conjugated anti-CD4 (L3T4) and PE-conjugated anti-CD8α (Ly-2). Data acquisition and single-colour flow cytometry analyses were performed on a FACScan using the Cellquest program (Becton Dickinson, San Diego, USA). Data are presented as the mean ± SD percentage of the total number of cells in the lymphocyte gate expressing a specific antigen. The lymphocyte gate was set according to the light scatter properties of mouse lymphocytes.

Statistical analysis. Statistical analysis was performed using the computer program Microsoft Excel Ver. 5.0, (Microsoft Corporation, USA). Levels of significance of differences between the two groups of mice (infected and uninfected) were determined by two-tailed Student's *t*-test. A value of $P \leq 0.05$ was considered significant.

Results and discussion

The present study shows that early during the intestinal phase of infection, absolute number of PP mononuclear cells gradually declined in mice infected with *T. spiralis* from first p. i. day onward.

As can be seen in Fig. 1, absolute number of PP mononuclear cells decreased markedly on first day p. i., but at 4, 7 and 14 p. i. days the numbers of these cells were significantly lower than in uninfected mice

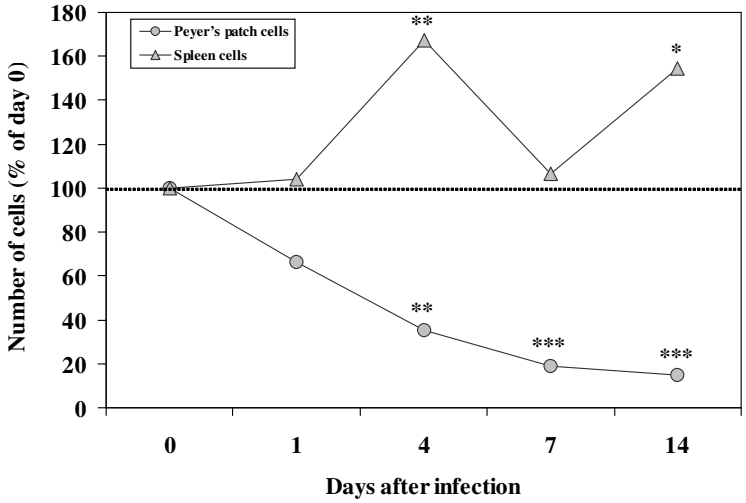
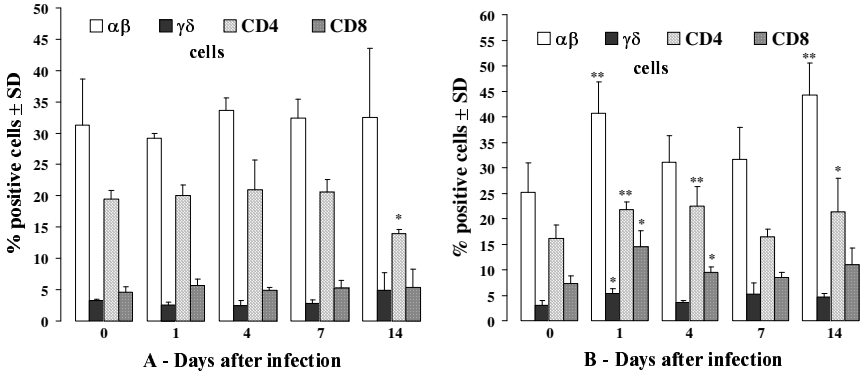


Fig. 1. Time course of the mean numbers of Payer's patch cells and spleen cells of mice infected with *Trichinella spiralis* expressed as a percentage of cell numbers recovered from the two lymphoid tissues of uninfected mice (day 0). Significantly different from day 0 values at *P ≤ 0.05, **P ≤ 0.01 and ***P ≤ 0.001.

(P≤0.01 for p. i. day 4 and P≤0.001 for 7 and 14 p. i. days, respectively). A significant reduction in the cellularity of ileal PP follicles was also observed in lambs infected with *Eimeria ovinoidalis* (ALEKSANDERSEN et al., 2002) as well as in pigs infected with enterotoxigenic *Escherichia coli* (data not shown). Taken together, these data suggest that mucosal pathogens indiscriminately induced loss of mononuclear cells from intestinal PP. This was not unexpected since a large number of lymphocytes normally emigrate from the PP upon oral stimulation with an antigen, reaching the blood circulation after expansion and maturation within the draining mesenteric lymph nodes (MLN) (ROTHKÖTTER et al., 1999). Although the time required for antigen-specific lymphocytes to migrate from the intestine into the blood is not known, it is worth noting that *T. spiralis*-derived antigens stimulate the draining MLN early during experimental oral infection (ZHU and BELL, 1989; KELLY et al., 1991; WAKELIN et al., 1994; ISHIKAWA et al., 1998). Activated, antigen-specific lymphocytes then immigrate into the blood, probably attracted by chemokines secreted during *T. spiralis* infection.

Indeed, there is a body of evidence showing that a quantity of chemotactic cytokines with chemoattractant properties for leukocytes could be found in the serum of mice infected with *T. spiralis* (FRYDAS et al., 1997; FRYDAS et al., 2002). It is important to stress that spleen has generally been considered as a peripheral, systemic lymphoid organ. Thus, it was not surprising that the absolute number of mononuclear cells significantly increased in the spleen of mice infected with *T. spiralis* 4 ($P \leq 0.01$) and 14 ($P \leq 0.001$) days following the infection (Fig. 1). The finding of the present study showing that absolute number of mononuclear cells did not increase in the spleen of infected mice at day 7 p. i., despite the fact that the absolute number of PP cells was found to be significantly decreased at that point, implies that the immune cells emigrating from PP could be retained in the MLN. Alternatively, these cells might directly immigrate into the effector sites of the gut mucosa or into the gut lumen (LARSH and RACE, 1975; STADNYK et al., 2000). According to our recent finding, this latter possibility seems to be more acceptable (data not shown). But it cannot be excluded that in C57BL mice infected with *T. spiralis* lymphoid cells also migrate back into the intestinal mucosa from the blood via blood vessels in the crypt area (McDERMOTT et al., 2001).

From the last comprehensive review of the generation and expression of immunity to *T. spiralis* in laboratory rodents it is obvious that *T. spiralis*-reactive T cells did not increase in the PP until day 4 of infection (BELL, 1998). In this context, it was rather surprising that, in general, no major changes in the phenotype of PP T cells were observed in the present study, nor in the14 days following the infection (Fig. 2A). The possible reasons for this are currently unclear, but together with evidence showing that the spleen T cell subpopulations exhibited significant increase in the infected versus uninfected mice (Fig. 2B) could be explained as follows. It is well known that the infective *T. spiralis* muscle larvae secrete excretory/secretory antigens within the first day following entry into the intestine and are then resynthesized (WAKELIN, 1984). These antigens could be presented by intestinal epithelial cells to IEL or lamina propria T cells, since there is an increase in the number of dividing T cells in the epithelium and lamina propria of experimental animals infected with *T. spiralis* as early as 12 h following infection (BELL, 1998; HERSBERG and MAYER, 2000). Despite the fact that *T. spiralis* infection of laboratory animals stimulates



*Significantly different values between the two groups at *P ≤ 0.05 and **P ≤ 0.01

Fig. 2. Flow cytometric analyses of the positively stained Payer's patch (A) or spleen (B) cells of mice orally infected with *Trichinella spiralis* and of the control, uninfected mice (day 0).

T cell-mediated immunity in their GALT during the intestinal phase of the infection, it seems that spleen could be stimulated prior to GALT (BOŽIĆ et al., 1998). The present study would suggest that this is indeed the case, because the spleen, but not PP T cells, up-regulated their markers on first day of infection, after being triggered by *T. spiralis*-derived antigens. The evidence showing that the two populations (αβ and γδ) and the two subpopulations (CD4 and CD8) of T cells promptly increased in the spleen, but not PP, of infected mice implies that *T. spiralis*-derived antigens could by-pass the PP, subsequently stimulating the induction of the systemic immune response. Early during experimental *T. spiralis* gut infection several type 1 cytokines, such as interleukin-2 and γ-interferon, are produced in the spleen as well as in the draining MLN (ZHU and BELL, 1989; KELLY et al., 1991; ISHIKAWA et al., 1998). A variety of cell types, including $CD4^+$, $CD8^+$, γδ TCR^+ and αβ TCR^+ cells may produce these cytokines. However, it is likely that they perform unprotective effects against *T. spiralis* infection as data from many sources indicate that rather type 2 cytokines elicited by *T. spiralis* infection are implicated in the protective immunity (VALLANCE et al., 1999; URBAN et al., 2000; HELMBY and GRENCIS, 2002). The exact role of the cytokine network in the PP during the infection is not completely understood and needs to be determined in the future, in

particular because of strong evidence indicating that the PP cells normally favour development of oral tolerance (TSUJI et al., 2001; SMITH et al., 2002; JUMP and LEVINE, 2002).

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SAŽETAK

Vjeruje se da su Peyerove ploče (PP) glavno mjesto indukcije imunosnog odgovora na uzročnike bolesti koji invadiraju crijevnu sluznicu, no fenotip T stanica u PP tijekom crijevne faze invazije oblićem *Trichinella spiralis* nije dovoljno istražen. Radi rasvjetljavanja tog problema , C57BL miševi bili su peroralno invadirani spomenutim parazitom 0. dana i žrtvovani 1., 4., 7. i 14. dana nakon invazije. Neinvadirani miševi rabljeni su kao kontrola. Uspoređen je ukupni broj mononuklearnih stanica, kao i ekspresija biljega na površini pojedinih subpopulacija T stanica u PP i slezeni invadiranih miševa. Rezultati su pokazali da se u PP broj mononuklearnih stanica postupno smanjuje veoma brzo nakon peroralne invazije, već od prvoga dana nadalje. Broj stanica u slezeni zamjetno se povećao 4. i 14. dana nakon invazije. Istodobno, nisu utvrđene znatnije promjene fenotipa T stanica u PP invadiranih u odnosu na neinvadirane miševe, no invazija je prouzrokovala značajne promjene u kinetici limfocita slezene.

Ključne riječi: *Trichinella spiralis*, imunofenotipiziranje, T stanice, protočna citometrija