EXPERIMENTAL TRICHINELLOSIS IN RATS – PERITONEAL MACROPHAGE ACTIVITY

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Abstract – The influence of Trichinella spiralis infection on macrophage activity in rats during the first 28 days of infection was examined by measuring the production of NO and IL-6, as well as the expression of mannose receptor on the surface of peritoneal macrophages. During the course of a dynamic shift in the 3 life-cycle stages of the parasite, intermittent variations in NO production were observed but ended with increased values that coincided with the highest values for IL-6 release in the final, muscle phase of infection. No change in mannose receptor expression was observed during the course of infection. These results confirm that the Trichinella spiralis infection provokes changes in macrophage activity that could influence not only the course of the parasitic disease but also the overall immune status of the host.

Key words: Trichinella spiralis, peritoneal macrophages, NO, IL-6

INTRODUCTION

Trichinella spiralis is a parasitic nematode which can infect many mammalian species. The parasite completes its life cycle within the same host infected by ingesting meat containing infective larvae. The larvae are released in the stomach, migrate and invade the small intestine epithelial wall where they mature into adult worms. After copulation, the released newborn larvae (NBL) penetrate into the submucosa, enter the blood vessels and are carried by the circulatory system to various organs, where they cause transient inflammation. They invade skeletal muscle fibers where they develop into infective, L1 larvae, whereby muscle cells are transformed into “nurse cells” (Despommier, 1998). Each life-cycle stage provokes an immune response by the host. However, since T. spiralis establishes itself in host muscle cells, it obviously manages to avoid host defense mechanisms, and/or to modulate the immune response.

Macrophages, among other cells of the immune system, form the first line of defense against pathogens. They are distributed throughout the body near points of entry of the invading organism. Each macrophage-antigen contact may induce defense mechanisms, such as a phagocytic uptake of a wide variety of microbes (yeasts, fungi, protozoa and bacteria) and endocytosis of soluble glycoconjugates. In addition, they can also determine the magnitude and nature of adaptive responses through a variety of cytokines, costimulatory molecules and antigen presentation. (Clark and Kupper, 2005; Janeway and Medzhitov, 2002; Tosi, 2005). The existence of three types of activated macrophages with different phenotypes and functions has been demonstrated over the past few years. They are classically activated macrophages, alternatively activated macrophages and type II activated macrophages.

The best-characterized cells are the classically activated macrophages, which develop in response to IFN-γ and tumor necrosis factor (TNF), together with exposure to microbes or microbial products, such as lipopolysaccharide (LPS). These cells can be identified by their ability to produce nitric oxide (NO) (Murray, 1990; Roach et al., 1991) combined with their increased expression of major histocompatibility complex (MHC) class II and CD86, and their enhanced antigen-presenting capacity. Upon activation classically activated macrophages release
inflammatory cytokines IL-1, IL-6, TNF, IL-12 (Yamamoto et al., 1997; Garner et al., 1994; Shibata et al., 1997) and microbicidal products such as lyso- somal enzymes (Lefkowitz et al., 1997).

Alternatively activated macrophages arise in a Th2 environment in response to IL-4, IL-13 and glucocorticoids (Gordon, 2003; Mosser, 2003). Alternative macrophage activation has been proposed as a mechanism by which Type 2 responses attenuate excessive inflammation. These cells fail to produce NO and consequently are compromised in their ability to kill intracellular microbes. They are also inefficient in antigen presentation. Alternatively activated macrophages display increased expression of mannose receptor (MR) and arginase I, and produce IL-10 and IL-1 receptor antagonist (Gordon, 2003). It was suggested that they down-regulate inflammatory responses, suppress T cell proliferation and participate in tissue repair and wound healing (Gordon, 2003; Mosser, 2003).

Type II activated macrophages were recently identified. They require ligation of Fcγ receptors or toll-like receptors (TLR) for their activation (Anderson and Mosser, 2002). Type II activated macrophages could release reactive molecules such as NO and O2-, and cytokines IL-6 and TNF that is no different from classically activated macrophages. However, these macrophages secrete large amounts of IL-10, which suggests their potent anti-inflammatory effect (Gerber and Mosser, 2001). They possess the ability to preferentially induce a Th2-type immune response.

Little is known about the macrophage - T. spiralis interaction and its relevance to the host defense mechanisms during primary infection with this parasite. It was shown that macrophages actively participate in the cell infiltration of intestinal mucosa in the lamina propria (Karmanska et al., 1997), and in the “nurse cell” complex in mouse muscles (Dabrowska et al., 2004).

In order to reveal the influence of experimental T. spiralis infection on macrophage activity, the release of NO and IL-6, and the expression of mannose receptor (MR), as markers of macrophage activation, were examined. NO is an important effector molecule that can restrict pathogen growth in infected hosts, while on the other hand playing a role in immunomodulation and pathology (Murad, 1999). IL-6 participates in the generation of immunity against various pathogens, and is required for the induction of acute phase reactions (Hirano, 1998). MR is a carbohydrate-binding receptor which recognizes common structural and molecular motifs present on microbial surfaces and contributes to the induction of innate immune responses. These parameters were determined during the first 28 days of infection, which encompasses the intestinal, migratory and early muscle phase of parasites’ life cycle.

MATERIAL AND METHODS

Parasites and experimental infection

T. spiralis muscle larvae (TSL1) (ISS 161) were recovered from infected Wistar rats by the digestion of carcasses in pre-warmed gastric juice (Gamble, 2000).

Five groups of Wistar rats (2-6 months of age; five rats per group) were infected orally by submission of an individual dose of 2500 TSL1. Uninfected rats of the same age were used as controls. Animals were housed under standard conditions and fed ad libitum on a commercial diet.

Preparation of peritoneal macrophages

Each group of infected animals (5 per group) was successively sacrificed by cervical dislocation on the following post-infection days: 4, 7, 14, 21 and 28, and peritoneal macrophages were collected. Uninfected rats were also killed at all stages of the investigation and served as the negative control.

Peritoneal macrophages were isolated from infected and uninfected rats by peritoneal washing with 20 ml of ice-cold phosphate-saline (PBS). After centrifugation at 200 g for 10 min, cells were washed twice with PBS. Erythrocytes were lysed in
hypotonic solution. Macrophages were plated in Petri dishes in Dulbecco's modified Eagle's medium (DMEM) with 5% fetal calf serum (FCS) and allowed to adhere for 2 h at 37°C, 5% CO₂. Non-adherent cells were removed by repeated washing and adhered macrophages were detached by cold PBS containing 5mM EDTA. The collected macrophages were washed twice in DMEM, re-suspended in DMEM/5% FCS and counted. Macrophages were plated in 24-well plates (5 x 10⁵ cells/well) and after 48 h culture supernatants were collected for NO and IL-6 measurement. Macrophages in suspension (3 x 10⁶ cells/ml) were used for the determination of MR expression.

**Determination of macrophage activity**

Adhered macrophages were tested for the production of nitric oxide (NO) and cytokine IL-6. The culture supernatants were harvested after 48 h and the concentration of NO was determined by measuring the nitrate concentration in the culture medium using the Griess reaction (Green et al., 1982). Briefly, 50 μl of sample and standard reagent sodium nitrite reagent were added to individual wells of a 96-well plate, followed by 50 μl of Griess reagent (1% sulfanilamide, 0.1% naphthylenediamine hydrochloride in 2.5% H₃PO₄). After 10 min incubation, absorbance was read at 543 nm. IL-6 was measured using rat IL-6 ELISA kit (Quantakine M, R&D systems, UK).

**Determination of MR expression**

MR expression was determined in a binding assay using radioactively labeled Man-BSA, a ligand with high specificity for the MR (Stahl et al., 1978; Reading et al., 2000). Man-BSA was radiolabeled with ¹²⁵I by the chloramine-T method (Greenwood et al., 1963). The binding assay was performed as described previously (Gruden-Movsesijan and Sofronic Milosavljevic, 2006). Briefly, cells (3 x 10⁶ cells/ml) in a volume of 100 μl were incubated with ¹²⁵I-Man-BSA (200ng/ml) for 1h at 4°C. The reaction was terminated by the addition of an equal volume of cold DMEM. Cells were centrifuged, washed with cold PBS and the cell pellets were assayed for radioactivity. Nonspecific binding of ¹²⁵I-Man-BSA was determined in the presence of mannan (5 mg/ml) and was subtracted from total binding to calculate specific binding.

**Statistical analyses**

All experiments were repeated three times and evaluated by the nonparametric Mann-Whitney U test for statistical significance of differences between groups.

**RESULTS**

**Nitric oxide production**

Cultured peritoneal macrophages, resulting from peritoneal washings performed between day 4 and day 28 post-infection (p.i.) showed a significant increase in NO production on days 14 and 28 p.i., compared to the control rates (Fig. 1). On day 4 p.i., NO synthesis was lower than control values. This decrease in NO synthesis was significant in one out of
three individual experiments. On day 7 p.i., an increase in NO production was observed, but the concentration of nitrite was not significantly different from that in the uninfected controls. On day 21 p.i., NO production had decreased almost to the level of the control. Although the values for nitrite concentration differed in all three independently performed experiments, the same trend in NO production was evident. When NO production values are presented as a NO production index (value of the control taken to be 1), the profile of NO release during infection can be clearly recognized (Fig. 2).

NO production after culture of peritoneal macrophages for 24 h in the presence of LPS (positive control; 10 μg/ml) was 30.92 ± 2.62 μM (determined according to the results of all four experiments).

IL-6 production

The activity of peritoneal macrophages during the course of infection was also followed by measuring the concentrations of IL-6 in the culture supernatants. Infection with *T. spiralis* caused an elevation in IL-6 production. A significant increase was observed on day 14 p.i., and with slight variations, the increased values for IL-6 concentration persisted till the day 28 p.i. (Fig. 3).

The expression of MR

To estimate the expression of MR on the surface of peritoneal macrophages during the course of infection, we performed a binding assay with a known ligand for this receptor – Man-BSA. Cells in suspension were incubated with 125I-Man-BSA, with or without a specific inhibitor of binding. The extent of binding of the specific ligand to peritoneal macrophages isolated on the days indicated above of *T. spiralis* infection is presented in Fig. 4. The increased production of NO was accompanied by a decrease in 125I-Man-BSA binding, and *vice versa*, meaning that the NO production and MR expression were in inverse correlation. However, except for day 14 p.i., when macrophages exhibit a significantly lower expression of MR, changes in the amount of bound labeled ligand were not statistically significant, compared to the controls.

DISCUSSION

The obtained profiles of NO and IL-6 production indicated that *T. spiralis* infection provoked changes in the activity of peritoneal macrophages. It is well known that NO has a protective effect in Th-1 inducing infections (Rajan et al., 1996), that it can cause immunosuppression (Dai and Gottstein, 1999), as well as pathology, but its role in Th2-inducing
helminth infections is not clear. The presence of the adult worms in the gut corresponded with the decrease in NO production on day 4 p.i., compared to the control values. Since the decrease was significant only in one out of three independent experiments, it could only be speculated that suppression of NO synthesis had occurred. Recently published data on the murine model have shown the suppression of macrophage ability to produce NO during early intestinal phase of *T. spiralis* infection (Kolodziej-Sobocinska et al., 2006a). After investigating the expression of inducible nitric oxide synthase (iNOS) in the mouse jejunum, ileum and surrounding tissues and organs during the intestinal phase of *T. spiralis* infection, Bian et al. (2001) hypothesized that the molecular products of the parasite (both adult and NBL) possess regulatory functions that directly or indirectly inhibit expression of iNOS at the local and systemic levels (Bian et al., 2001). Accordingly, it is possible that a similar mechanism is involved in the regulation of NO production in peritoneal macrophages on day 4 p.i. Then, from day 7 p.i. macrophages started to produce higher amounts of NO. The significant increase in the level of NO, observed on day 14 p.i., could be correlated with the stimulation of RES observed by other authors during the same infection period when NBL are present in the circulatory system of the host (Karmanska et al., 1997; Kolodziej-Sobocinska et al., 2006a). The drop of NO release on 21 p.i. could be a consequence of the “immunosuppressive effect” observed in *T. spiralis* infected rodents between 14 days p.i. and around 30 days p.i. It could be the adoptive mechanism for survival of the parasite and its successful invasion of muscle cells. It was previously shown that inhibition of iNOS (by aminoguanidine), and a consequent drop in NO concentration during the muscle phase of the infection, caused increases in muscle larvae numbers in experimental trichinellosis (Kolodziej-Sobocinska et al., 2006b). However, Hadas et al. (2002) reported that the administration of NO releasing drugs (which elevate the level of serum NO) increased the severity of *T. spiralis* infection. It was proposed that high levels of NO might suppress the host immune system (Oswald et al., 1994) and assist the parasite in establishing itself in the muscles. The significantly increased production of NO on day 28 p.i., which corresponds to the well-defined phenomenon of inflammation in infected muscles that exists in this period of infection (Li and Ko, 2001) could represent a mechanism provoked by the parasite which ensures its efficient settlement in the muscle cell.

As a whole, our results on NO production differ from those obtained on the murine model (Wandurska-Novak and Wisnievska, 2002; Kolodziej-Sobocinska et al., 2006), who observed a constant increase in NO production by mouse peritoneal macrophages during trichinellosis.

Monitoring IL-6 release during the early stage of trichinellosis showed that infection led to an increase in IL-6 production, compared to the control, uninfected animals. IL-6 is commonly produced at local tissue sites by a number of cells including macrophages, dendritic cells, fibroblasts, T and B cells, endothelial cells, in almost all situations of homeostatic perturbations including endotoxemia, trauma and acute inflammations. The elevated production of IL-6 by peritoneal macrophages may be induced by the presence of NBL in
the circulation and their subsequent maturation into the infective L1 stage in host striated muscles. The obtained results correspond well with the previously reported significant increase of IL-6 production by antigen stimulated spleen cells in *T. spiralis* infected C57B1/6 mice (Sofronic Milo-savljevic et al., 1997). Since each life-cycle stage of the parasite has its own characteristic antigens, we assume that each of them *de novo* activates mechanisms of innate and adoptive immunity, including IL-6.

IL-6 has the potential to shift the Th1/Th2 balance towards Th2 (Diehl and Rincon, 2002). In our case, the elevated level of this cytokine could be one of the factors involved in skewing the immune response toward the Th2-type during *T. spiralis* infection. Much is known about the development and expression of these responses, but their induction is still very poorly understood.

Besides NO and IL-6, the expression of MR, as a marker of macrophage activation, was also monitored. We can discriminate between classically and alternatively activated macrophages according to the level of its expression, since the former down-regulates, while latter greatly up-regulates MR expression (Taylor et al., 2005). Although slight variations in the binding of specific ligand Man-BSA were observed during the course of the infection, changes were not significant compared to the control, healthy animals. The only exception was on day 14 p.i., when the elevated production of NO was accompanied by significantly lower MR expression, compared to control macrophages.

It is obvious that the immune response is very dynamic, changing with the ongoing infection. One type of response could be dominating in one moment, at a particular site, depending on the life-cycle stage of the parasite. A characteristic feature of helminth infections is the induction of a Th2 response (Maizels et al., 1993). *T. spiralis* infection is characterized by a transient Th1 response at the very beginning of the intestinal phase of infection that is switched to a mixed Th1/Th2 immune response at the muscle stage (our observation, unpublished data), rather than a dominant Th2 response. Macrophages possess great plasticity and the ability to react to a changing micro-environment (Mosser and Edwards, 2008) which makes them important for interaction between innate and adaptive immune systems and the polarization of immune response. According to the recently proposed macrophage classification, we could speculate that peritoneal macrophages during the early stage of *T. spiralis* infection acquired type II phenotype, with an elevated production of both NO and IL-6 and unchanged expression of MR, compared to the controls. A different phenotype that indicated classical activation was observed only on day 14 p.i., pointing to up-regulation of NO and IL-6 and down-regulation of the MR expression.

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**REFERENCES**


Sofronic Milosavljevic, Lj., Cuperlovic, K., Pejnovic, N., Kukic, Z., A. Dujic (1997). An excess of IL-6 production in the early muscle stage of Trichinella spiralis infection in mice is associated with strain susceptibility to infection. In: Immunoregulation in Health and Disease. (Ed. Lukic, M. L, Colic, M.,


**ЕКСПЕРИМЕНТАЛНА ТРИХИНЕЛОЗА КОД ПАЦОВА – АКТИВНОСТ ПЕРИТОНЕАЛНИХ МАКРОФАГА**

АЛИСА ГРУДЕН-МОВСЕСИЈАН и ЉИЉАНА СОФРОНИЋ-МИЛОСАВЉЕВИЋ

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Утицај инфекције паразитском нематодом *Trichinella spiralis* на активност макрофага пацова испитиван је током првих 28 дана инфекције праћеном продукције NO и IL-6, као и експресије манозног рецептора на површини макрофага. Наведени период инфекције обухвата сва три животна стадијума паразита, чија смена доводи до динамичке промене нивоа NO, при чему последњу, мишићну фазу, прати висок ниво NO продукције. Мишићну фазу такође карактерише и значајна продукција IL-6. Запажена активација макрофага није била праћена променама у експресији манозног рецептора. Може се рећи да инфекција са *Trichinella spiralis* доводи до промена у активности макрофага које могу утицати не само на ток паразитске болеснице, већ и на укупни имунизки статус организма до маћина.