Intestinal mastocytosis in *Trichinella spiralis* infection: immunohistochemical study in murine model

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ABSTRACT

Background: *Trichinella spiralis* (*T. spiralis*) is a zoonotic nematode and food borne parasite. The intestinal phase is critical for trichinellosis as it determines both process and consequences of the disease. *T. spiralis*-induced mucosal mast cells (MMCs) hyperplasia in the small intestine may contribute to expulsion of adult worms. CD117 marker (C-kit) which is expressed by mast cells (MC) as well as a variety of neoplasms is considered a better marker for mast cell populations.

Objectives: Our aim was to clarify the contribution of MMCs in the effector phase of the immune response to intestinal phase of *T. spiralis* during the course of infection in mice.

Material and Methods: MMCs hyperplasia reaction was investigated in the intestine of mice infected with *T. spiralis* by immunohistochemical study using CD117 marker and analysis of results by ImageJ software.

Results: The results showed that MMCs continued to increase until 14 days post infection (dpi), coinciding with time of worm expulsion; and started to decline afterward. The minimal number of MMCs was detected 35 dpi.

Conclusion: Our findings suggest that *T. spiralis* infection causes a series of pathological and time-associated changes within intestinal immune cell populations in infected mice during the intestinal phase. MMCs hyperplasia is a clear contributor to the worm expulsion.

Keywords: DC117 marker, image J, immunohistochemistry, mast cells, *Trichinella spiralis*.

INTRODUCTION

*Trichinella spiralis* is the first identified and best characterized member of *Trichinella* genus. Its high infectivity for laboratory animals provides valuable *in vivo* models for basic immunological, biological and pathological studies[1]. Infection is acquired by consumption of infected undercooked or raw meat, mainly pork[2]. Under the influence of gastric juices, larvae are released in the stomach, molt, and develop into the adult stage inside the enterocytes of small intestine. After mating, newborn larvae are released into the circulation and spread throughout the tissues and organs; only those that enter striated muscles mature into muscle larvae[3]. In humans, infection could remain asymptomatic in the presence of a low number of larvae[4]; but in case of ingestion of few hundred larvae, gastrointestinal symptoms appear as soon as 2 dpi followed by development of a serious, and sometimes fatal, disease[5].

Unlike the case in humans, *T. spiralis* can reach a high worm burden in animals without causing clinical symptoms[5]. Mice are natural hosts for this parasitic nematode. First-stage larvae (L1) initiate infection in the intestine, colonizing the epithelium where they molt and develop into adults within 3–4 days. Adult worms are expelled between 10 and 15 dpi, simultaneous with the development of a pronounced intestinal mastocytosis[6]. During the intestinal phase of infection, the immune reaction involves both Th1 and Th2 responses. Initially Th1 responses are induced followed by a dominant Th2 type response, characterized by the production of high levels of cytokines IL-4, IL-5, IL-9, IL-10, and IL-13, as well as immunoglobulin E (IgE), and the mobilization of eosinophils, basophils, and mast cells[7].

Mast cell hyperplasia is highly dependent on the mucosal T-cells and is augmented by the cytokines IL-4 and IL-13. Mast cells are important sources of TNF-α and IL-4[8], suggesting a role for the mast cells in inducing immunity. Following exposure to larval glycoproteins, mice peritoneal mast cells have been shown to release histamine[9], and a hybrid cell that models connective tissue. However, MMCs have not been tested for activation in response to parasite products or their immune complexes. Mast cells are located in the external environment interfaces where they are involved in a broad variety of immunoregulatory roles, including inflammatory tissue responses to pathogenic agents. The functional and biochemical properties and the granule serine protease content of MMCs in the gastrointestinal and pulmonary tracts distinguish them from mast cells in other tissues[10]. Furthermore, Miller[11] stated that throughout multiple nematode infections, marked hyperplasia, activation, and distinction of MMCs occur...
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be done successfully by CD117 immunostaining antibody to the effector phase of an immune response. By immunohistochemistry analysis using CD117 our study, the goal was to determine the contribution expulsion and or inhibition of larval development. In the inflammatory responses evoked during worm However, it is not clear how the MMCs function in this case of cutaneous onchocerciasis., Also the diagnosis by immunohistochemistry with CD117 marker in a study by Fernandez-Flores and Alija[14] the population of mast cells by immunohistochemistry with CD117 marker in a case of cutaneous onchocerciasis., Also the diagnosis of *Giardia lamblia* in duodenal mucosal biopsies could be done successfully by CD117 immunostaining[13]. However, it is not clear how the MMCs function in the inflammatory responses evoked during worm expulsion and or inhibition of larval development. In our study, the goal was to determine the contribution of MMCs during the course of the intestinal phase of infection in mice by *T. spiralis*. This is to be determined by immunohistochemistry analysis using CD117 antibody to the effector phase of an immune response.

**MATERIAL AND METHODS**

**Type of study:** This experimental descriptive study was conducted in Medical Parasitology Department, Faculty of Medicine, Tanta University, Egypt during the period from June 2019 to October 2019.

**Experimental animals:** Sixty male Swiss albino mice (6-8 week-old) weighing 18–25 g, were purchased from the animal house, Faculty of Medicine, Zagazig University, Egypt, and maintained on standard commercial pellet diet with free accessible water throughout the study in the animal house. Mice were divided into six main groups as follows: Group 1 comprised 10 healthy not infected control mice. Groups 2, 3, 4, 5 and 6 each comprised 10 infected mice. All groups except group 1 were infected orally with *T. spiralis* muscle larvae. The *T. spiralis* strain was obtained as laboratory bred infected albino mice from Medical Parasitology Department, Faculty of Medicine, Tanta University, Egypt. Group 2 was sacrificed at 7dpi, group 3 at 14 dpi, group 4 at 21 dpi, group 5 at 28 dpi and group 6 at 35 dpi. Infected mice were starved the night before sacrifice to minimize intestinal debris. Immediately after death, the abdominal cavity was dissected and the whole intestine was removed and washed rapidly under warm tap water to remove debris. Small portions (2 cm from the middle region of the small intestine) were immediately fixed in 10% formalin-saline for histopathological studies.

**Histopathology:** Samples from middle part of small intestine and femoral quadiceps muscle of sacrificed mice in different study groups were fixed in 10% neutral formalin for 24 h. The intestine was opened longitudinally and Swiss-rolled prior to fixation to obtain the longest surface area during evaluation[16]. The femoral quadiceps skeletal muscle was cut longitudinally. Two sections from each sample were processed and paraffin wax embedded into one block, sectioned on glass slides at 4 μm thickness as two serials per slide, stained with Hematoxylin and Eosin (H & E) and examined histologically. While small intestine tissue slices were immunohistochemically stained for evaluation of mast cells reaction.

**Immunohistochemistry:** According to Bancroft and Gamble[17], 4 μm thin intestinal paraffin sections were mounted on positively charged glass slides coated with poly-L-lysine. Sections were de-paraffinized by incubation in an oven at 56°C for 15 min, followed by insertion in xylene for 30 min. Slides were rehydrated in descending grades of 95%, 85%, 75% alcohol for 5 min each. Rinsed in distilled water for 5 min and then washed by phosphate buffer saline (PBS) for 5 min. For antigen retrieval, the mounted sections were immersed in 10 mM citrate buffer, pH 6.0 (Lab Vision) and then boiled in this solution in a microwave for 20 min. Excess liquid was strained off on a gauze pad. For blocking endogenous peroxidase, 3% Hydrogen peroxide was applied to cover specimens followed by incubation for 5 min, then a gentle rinsed with PBS. Immunohistochemical staining was performed using Universal kit Super sensitive™ link-table IHC detection system (Code No. QD000-5L Multilink® Detection kit, CA, USA) according to the manufacturer’s protocol. Each specimen was covered by 2-3 drops of CD117 primary rabbit polyclonal antibody (Thermo Scientific/ Lab Vision Corporation, dilution 1:200), and incubated in a humid chamber overnight at 4°C, then rinsed gently with PBS solution. Excess buffer was immediately tapped off as before. Sections were then incubated with secondary antibody, biotinylated anti-mouse immuno-globulins, for 15 min at room temperature followed by washing in buffer, incubation with streptavidin peroxidase solution for 10 min at room temperature and rinsing with PBS. Sections were then covered for 15 min by adding one drop of 3-30 diaminobenzidine-tetrahydrochloride (DAB) chromogen mixed with 2 ml of DAB substrate. Finally, sections were counterstained with Mayer’s hematoxylin, dehydrated in alcohol and mounted in distyrene-plasticizer- xylene (DPX). Negative controls had primary antibody replaced by buffer. The mean number of cells that were stained with the primary antibody was counted in all the sections.
and the cell numbers were expressed as visible mast cells per field. The distribution of mast cells in the epithelium, lamina propria, submucosa, and muscle layer was compared in equivalent areas of jejunum from ten mice per time point.

**Mast cell count in the intestines:** CD117 positive mast cells in the epithelium and lamina propria were enumerated in 20 villus-crypt units (vcu), and in 20 vcu of the submucosa/muscle regions directly below. Numbers of mast cells in all regions were calculated and expressed per vcu[8,18].

**Image analysis:** Two sections from each tissue block of mice in the study groups were evaluated. The area percentage of color density of the marker was determined individually by Image J image analyzing system version # 1.52s. Images were first converted into grey scale via selecting 8-bit type of image, then thresholding was activated and adjusted to select region of interest as compared to the original colored image. The region of interest then became highlighted in red within the grey scale. Each colored red green blue (RGB) normal image was compared with a gray scale image using ImageJ software program, to better clarify the immunostaining of mast cells. In the original colored RGB image mast cells appeared brown. After transfer to gray scale image mast cells stain red against a gray background[19].

**Statistical analysis:** Values of the measured parameters were expressed as mean number of mast cell ± standard error. One way-ANOVA test (F value) was used to detect significance of the difference among more than two arithmetic means, followed by post hoc Tukey’s test to detect the difference between each two means. The difference was considered significant at values of P< 0.05. The statistical analysis was processed using the Statistical Program of Social Sciences (SPSS) for Windows, version 26.

**Ethical consideration:** The experiment was done following the ARRIVE guidelines and in accordance with the UK Animals (Scientific Procedures) Act, 1986 and associated guidelines; EU Directive 2010/63/ EU for animal experiments or the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978). The experiment was approved by Institutional Animal Care and Use Committee-Zagazig University (IACUC-ZU), approval no. 4297 on 4th February 2018.

**RESULTS**

**Histological (H & E) evaluation** (Fig. 1, a-e): Examination of intestinal sections retrieved from *T. spiralis* infected groups revealed mucosal and submucosal infiltration of mononuclear cells (Fig. 1-a), but no adult worms were detected. Examination of infected quadriceps thigh muscles sections revealed basophilic transformation of muscle cells, and coiled larvae surrounded with an eosinophilic well-formed capsule giving the typical nurse cell structure (Fig. 1-b, c, d, e). Nurse cells were surrounded with an intense cellular infiltrate that was also seen in sporadic foci within the muscle sheath (Fig. 1-c).

**Mast cell count** (Table 1; Fig. 2): Numerous positively stained mast cells could be seen in the small intestine tissues of *T. spiralis* infected mice by the 1st week of infection. Increased numbers of MCs appeared in the crypts at the base of the villi by the 2nd week reaching the highest number (262). After that the number of MCs decreased to reach the lowest record by the 5th week (75). The maximum number of MCs was seen in the mucosa (531) especially intra-epithelium (346), followed by lamina propria (185). The minimal number of MCs was seen in the muscle layer (100). The results were statistically significant (P<0.05).

**Fig. 1.** Histopathological intestinal and muscular features showing (a) dense mucosal infiltration of mononuclear cells and eosinophils (blue triangle) in the mucosa of intestine in *T. spiralis* infected mice; (b-e) thigh muscle sections from different *T. spiralis* studied groups, showing basophilic transformation of muscle cells (green triangle), and coiled *T. spiralis* larvae (black arrow) surrounded with an eosinophilic well-formed capsule (red triangle) representing the nurse cell wall; (b-e) intense cellular infiltrate surrounding the nurse cells and scattered within the muscle sheath (c). [H & E, x 200 (a, c, d, e), x 400 (b)].
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**Immunohistochemistry and image analysis**

(Figs. 3-6): Positively reacted mast cells appeared dark brown after immunohistochemical staining for CD117 antibody. The detected brown colored cells corresponded to the MMCs. The images reflected the expression of CD117 in all histopathological layers of duodenum of infected mice; figure 3(A) and (B): mast cell intra-epithelium; figure 4 (C) and (D): mast cell in the muscle layer; figure 5(E): mast cell in lamina propria, (F): mast cell in submucosa. The highest expression of CD 117 was found in mucosal epithelium layer (red color); figure 3 (A’’ and B’’). The lowest expression of CD117 marker was seen in the muscle layer; figure 4(C’’ and D’’). In figure 6, immunohistochemistry expression of MMCs (b-e) showed immunopositive reaction in infected mice: b, c, d, f and e at different time points, 7 dpi, 14 dpi, 21 dpi, 28 dpi and 35dpi respectively, compared with control (a).

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**Table I.** Quantification of MMCs (data shows mean number ± S.E) in cross sections of the jejunum of non-infected and *T. spiralis*-infected mice.

| Days of infection | Mucosal epithelium | Mucosal lamina propria | Submucosa | Muscle layer | Total |
|-------------------|--------------------|------------------------|-----------|-------------|-------|
|                   | Mean (± S.E) of MMCs |                        |           |             |       |
| Non infected      | 5 (1)              | 4 (0.3)                | 3 (0.08)  | 12          |
| 7 dpi (n=6)       | 80 (3.5)            | 28 (3.1)               | 18 (0.2)  | 149         |
| 14 dpi (n=10)     | 120 (299)           | 38 (2.6)               | 30 (0.2)  | 262         |
| 21dpi (n=9)       | 66 (5)              | 25 (3)                 | 20 (1.5)  | 151         |
| 28 dpi (n=8)      | 45 (4)              | 15 (1.9)               | 17 (0.5)  | 105         |
| 35 dpi (n=6)      | 30 (3)              | 13 (1.5)               | 12 (0.4)  | 75          |
| Total             | 346                 | 185                    | 123       | 100         |

ANOVA  

\[ F = 3.16, \text{P value= 0.04}, \text{Significant vs control} \]

Post-hoc Tukey test results:

- Mucosal epithelium vs mucosal lamina propria \( P \) value = 0.08 (NS)
- Mucosal epithelium vs submucosa \( P \) value = 0.04 (S)
- Mucosal epithelium vs Muscle layer \( P \) value = 0.033 (S)

MMCs: Mucosal mast cells; \( n \) = Number of living mice/group, S: Significant, NS: Non-significant

**Fig. 3.** CD117 Immunohistochemistry expression of mucosal mast cells. (A-B) Representative microphotographs showing immunopositive cells in brown colour (corresponding to mucosal mast cells, MMCs) in the intestinal mucosal gland (A), intraepithelial mast cell infiltrate (B). A’ and B’ represent grey scale image by Image J. A’’ and B’’ represent red color in grey scale which is corresponding to the immunopositive cells by Image J software, version 1.52s. [Immunoperoxidase X400]. Scale bar: 20 µm.
DISCUSSION

In this study, we investigated the intestinal mucosa immune conditions during the intestinal phase of *T. spiralis* infection in mice. The results showed time-associated changes within intestinal immune cell populations in infected mice during the intestinal phase. The number of mast cells gradually increased during the entire intestinal phase and the highest numbers (120/vcu) were reached on 14 dpi. This was seen in the epithelium layer of intestinal mucosa. While the lowest numbers of mast cells were recorded on 35 dpi. Furthermore, the minimal numbers of mast cells (12/vcu) were seen in the muscle layer of intestinal mucosa and the result was statistically significant (*P*<0.5). The results are in accordance with the statement of Yu et al.[19] that *T. spiralis* infected mice showed mastocytosis in the intestinal mucosa on 10 and 15 dpi. It was reported that only few mast cells existed in the small intestine of wild-type mice, while more mast cells were observed in mice infected with *T. spiralis*[20]. Similarly, Pennock and Grencis[21] reported that mast cells hyperplasia coincides with worm expulsion.

Our study on the role of mast cells was prompted by the reported action of *T. spiralis* larvae secretion of tyvelose-bearing glycoproteins from their stichocytes while invading the host epithelium[22]. Shedding of the glycoproteins coincides with the first molt, about 8 h post infection, when the larvae are adjacent to the mucosal mast cells early in infection. The exposed mast cells are a contributing factor to the innate immune response[6]. Ding et al.[23] reported analogous results on the cellular reaction during the intestinal phase of infection indicating that the increase involved eosinophils, goblet cells, mucosal mast cells (which continued to increase up to the 17th dpi), and 33 D1+ dendritic cells (DCs). They concluded that numbers of mast cells are unchanged during early infection to later start to increase from the 11th dpi.
Contrary to the same time Roy et al.[18] evaluated the deletion of serglycin protein also utilizing immunohistochemistry staining with CD117/c-kit-antibody, and reported the lowering of mast cells per vcu at 12 dpi in infected mice than in infected wild mice. In view of the fact that mast cells become extremely influenced by the deletion of serglycin, Braga et al.[24] suggested that serglycin proteoglycans may directly support the recruitment of crypt mast cells. Moreover, the lack of serglycin proteoglycans was apparently found to increase susceptibility to T. spiralis infection either by delayed expulsion or increased establishment of the worms.

Expulsion of worms from the small intestine is also governed by protease 1 (Mcpt-1) secretion by mast cells. This was confirmed in mast cells deficient mice (W/Wv) and Mcpt-1-null mice[8], and was supported by the report that bone marrow cells can reverse adult worm rejection[25]. This was verified by treatment of T. spiralis infected wild-type and IL-9 transgenic mice with anti-c-kit antibodies.[26] Also mast cell deficient mice are severely impaired and unable to develop a Th2 response to T. spiralis.[27]

Host-defense response between the stem cell factor (SCF) and the CD4C Th2 cytokines (IL-3, IL-4, IL-9) is essential in intestinal mastocytosis.[28] In addition, mast cells lead to type 2 cytokine-mediated reactions needed to establish defensive immunity against helminth parasites such as T. spiralis. This was confirmed by mast cell reaction inhibition resulting in associated reduction of inflammation and loss of protective immunity.[29] Strangely, an early study found that mast cell-triggered DC modulation promotes the activation of the Th1 and Th17 responses[28]. T. spiralis mediated mastocytosis relies on a dynamic connection between Th1 and Th2 reactions.[29]

The presence of rat mast cell protease II (RMCPII) in the serum of infected rats correlates with degranulation of mast cells and the rapid elimination of worms. This confirms that in the presence of intestinal antigenic threat, intestinal mast cells are the only source of RMCPII in the intestinal tissue of naive rats, despite the presence of RMCPII-positive cells also in the lung, liver and primary lymphoid tissues. It has been substantiated, that during adult worm elimination from primary infected rats MMCs produce RMCPII.[30] As shown by Miller and Pemberton[31], based on their location and degree of infection MCs in the intestinal mucosa and submucosa produce different types of proteases (Mcpt-1, Mcpt-2, Mcpt-5, Mcpt-6, Mcpt-7, Mcpt-9 trypases, the a-chymases, and b-chymases), of which, MMC-specific b-chymases, Mcpt-1 and Mcpt-2 are the most abundantly generated during infection by T. spiralis[31].

As with other gastrointestinal nematode infections, primary and secondary T. spiralis infections are associated with marked fluid accumulation in the intestinal lumen.[26] The resulting increased muscle contractility is supposed to promote the removal of adult worms; often referred to as ‘weep and sweep.’

Enhanced fluid retention is attributed to the combined rise in epithelial secretion induced by MMC mediators and enteric nerves, as well as the enhanced epithelial para-cellular permeability to macromolecules.[33] In addition to promoting parasite access to antibodies and other effector molecules, the increased para-cellular permeability has other unfavorable side effects such as diarrhea, loss of proteins and electrolyte disturbance.[34]

An important function of immune reaction is the quick expulsion of the intestinal worm.[32] Further study of the immune responses will facilitate the development of new prophylactic approaches to defend humans and animals from nematode infections.[27]. It has been known that MMCs and their special proteases are responsible for initially expelling adults T. spiralis from the intestine.[35].

Locally generated elements, such as SCF regulate numbers of mast cells, their subsequent differentiation inside tissues and the development of proteases. The dislodgement of worms from their intraepithelial niche and their subsequent elimination is governed by the wide range of MMC effectors. Evidently MMC hyperplasia is crucial for the expulsion of adult T. spiralis worms from the intestine. Further work is suggested to concentrate on the function of these effectors, particularly Mcp1 in adult worm expulsion.

Acknowledgement: We would like to thank Dr. Ahmed Bolbol, lecturer of Molecular genetics, Botany department, Faculty of Science, Zagazig University for critically reading the manuscript.

Author contribution: Both authors contributed significantly to this study. Mostafa E designed the study, and together with Atwa H performed the data analysis and interpretation, and prepared the manuscript, performed laboratory tests and processed the data. Both authors read and approved the final manuscript.

Conflict of Interest: The author declares no conflict of interest.

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