

Mast cell typing in aortic onchocerciasis and the immunohistochemical demonstration of *Wolbachia* bacteria

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Abstract: In this study, the presence of endosymbiotic *Wolbachia* bacteria was determined by immunohistochemical methods in 8 out of 43 paraffin-embedded bovine aortic tissues that were macroscopically and microscopically diagnosed with aortic onchocerciasis in the archive of the Harran University Faculty of Veterinary Medicine's Department of Pathology. By means of the enzyme histochemical and immunohistochemical staining methods, it was ascertained that the tryptase-containing subtype of mast cells (MC_T) was involved in the inflammatory response in the bovine aortae with onchocerciasis. The aim of this study was to elucidate the pathogenesis of aortic onchocerciasis lesions caused by *Onchocerca armillata* in Turkey and to demonstrate the presence of endosymbiotic *Wolbachia* bacteria in these lesions.

Key words: *Onchocerca armillata*, *Wolbachia*, mast cell, immunohistochemistry, cattle

1. Introduction

Parasites of the genus *Onchocerca* belonging to the superfamily Filarioidea are nematodes that occur in humans, ruminants, equidae and canidae (1). Filarioidea species have an indirect life cycle and are transmitted by black flies (*Simulium* spp.) and mosquitoes (*Culicoides* spp.), which serve as their intermediate hosts (2). These parasites occur mostly in South Asia, in the equatorial regions of Africa and in Turkey (1,3,4). *O. armillata* may be found in the proximity of the aortic arch as well as in the brachiocephalic trunk, costocervical and brachial arteries, and the section of the abdominal aorta up to the iliac bifurcation (5), and also in the pericardium (6). Viable and mature microfilariae pass into the blood circulation (7) and cause endothelial damage in the tunica media and intima, which may result in the development of thrombosis (2). Complications include ruptures in the wall of the aorta due to weakening and aneurysms caused by calcified nodules in elderly animals (2,5,6). The tunica intima of the affected aortae has a rough appearance and the tunica media presents several tunnels and nodules. Microscopically, necrotic cells and tissue debris, cholesterol clefts, hemorrhage, degenerate or dead parasites, and microfilariae may be observed in the center of the nodules.

In the course of time, fibrous tissue and net-like collagen accumulations may be observed in the granulomas in the tunica media and adventitia (2). The parasitic tunnels are surrounded by a thin layer of connective tissue in the absence of inflammatory cells. Dead, degenerate, or calcified parasites are surrounded by subacute or chronic focal granulomatous inflammation dominated by eosinophil leukocytes (2,8,9). An obligate symbiotic relationship exists between the *Rickettsia*-like *Wolbachia* bacterium and filarial nematodes (10). *Wolbachia* is found in the hypodermal cells on the lateral surface of mature *Onchocerca* spp., and also in the embryonic development stages of the parasite and in the microfilariae (1). It has been observed that the antibiotic treatment of *Wolbachia*-positive cattle infected with *Onchocerca* spp. reduces the number of *Wolbachia* bacteria and eventually kills the filarial parasites. This has shown that the viability of this parasite depends on the *Wolbachia* bacteria (11,12). Normally, mast cells are located near the epithelium in the surfaces of the skin, the respiratory system and digestive system, etc. The location of the filarial parasites in these areas causes the stimulation of mast cells by immunoglobulin E, which results in an increased number and activation of mast cells. Some mast cell-derived inflammatory

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mediators enable the migration of leukocytes to the site where the parasite is located (13). The release of histamine and proteinases, including tryptase and chymase, from mast cells may trigger inflammation. It has been reported that mast cell proteinases trigger inflammation. Reports indicate that mast cell proteinases vary among species as well as between individuals of the same species, which creates a 'heterogeneity of proteinases' both among species and between mast cell subtypes of the same species. While mast cells that are rich in tryptase and are found in mucosae indirectly in contact with the exterior environment are referred to as mast cell_{tryptase} (MC_T), mast cells that contain both proteinases in their granules and are found in body regions isolated from the exterior environment and in the proximity of nerve fiber ends and blood vessels are referred to as mast cell_{tryptase/chymase} (MC_{TC}) (5,14). It is considered that, owing to the significant differences in the chemical mediators contained by different mast cell populations, and thus due to the differences in the responses given by these cell populations to various pharmaceuticals, these cell populations have different roles in host defense mechanisms and diseases (15). It has been reported that, on the basis of their proteinase content, three subtypes of mast cells, namely MC_T⁻, MC_{TC}⁻, and MC_C⁻, exist in cattle (16). It is suggested that these enzymes play a major role in host defense mechanisms and homeostasis (17). Of these enzymes, tryptase has been shown to be involved in the proinflammatory stage of mast cells, while chymase has been demonstrated to be involved mostly in the inflammatory process (18). Literature reports indicate that mast cells are both more active and greater in number in *Onchocerca* lesions containing dead parasites in comparison to lesions caused by viable parasites (18–20). To the authors' knowledge, no detailed research is available yet on the mast cell subtypes found in tissues during the different stages of aortic lesions in cases of aortic onchocerciasis.

2. Materials and methods

Forty-three paraffin-embedded aortic tissue samples that were macroscopically and microscopically diagnosed with aortic onchocerciasis in 2011 and 2012 in the archive of the Harran University Faculty of Veterinary Medicine's Department of Pathology constituted the material of this study.

2.1. Histopathology

For the demonstration and histopathological evaluation of the structural changes in the aorta and the parasitic lesions, sections of 4 µm in thickness were cut from the paraffin blocks and stained first with hematoxylin and eosin (H&E) and then with Mallory's modified triple staining method.

2.2. Enzyme histochemistry

To demonstrate the chymase activity of the MC_C and MC_{TC} subtypes of the mast cells, the naphthol AS-D chloroacetate esterase enzyme histochemical staining technique was employed. Human intestine and skin sections known to contain the MC_C and MC_{TC} mast cell subtypes were used as positive controls (21).

2.3. Immunohistochemistry

2.3.1. Immunohistochemistry for mast cells

For the identification of the MC_T subtype of the mast cells, the streptavidin-biotin complex peroxidase (StrepABC) staining method was employed. The sections were incubated overnight in Mast Cell Tryptase Ab-2 (Clone AA1-Mouse Monoclonal MS-1216, Thermo Scientific) primary antibody solution at a dilution of 1:1600 at 4 °C. 3-3-Diaminobenzidine (DAB; TA-125-HD, Thermo Scientific) was used as a chromogen and Gill's hematoxylin was used for counterstaining. The tissue sections for the negative control were incubated with antibody dilution solution (TA-125-UD, Thermo Scientific).

2.3.2. Immunohistochemistry for *Wolbachia* surface protein

The StrepABC peroxidase staining method was used to demonstrate the presence of *Wolbachia* in the tissue lesions. The anti-*Wolbachia* surface protein (WSP) antibody used was obtained from Benjamin L Makepeace (University of Liverpool, UK). The sections were incubated in a rabbit polyclonal anti-WSP primary antibody solution at a dilution of 1:500 for 1 h at room temperature. DAB (TA-125-HD, Thermo Scientific) was used as a chromogen and Harris's hematoxylin was used for counterstaining. All of the stained sections were examined with an Olympus BX51 microscope and photographed with the Olympus DP71 digital imaging system.

3. Results

3.1. Histopathology

Results obtained with the H&E and Mallory's triple staining methods demonstrated that different histopathological pictures were observed on the basis of the filarial parasites in the aortae being viable or dead. It was observed that the viable parasite larvae were localized in tunnel-shaped cystic structures in regions near the tunica intima of the aorta. These cystic structures were surrounded by a thin connective tissue layer and no inflammatory cell infiltration was observed in the periphery of these structures. The death or degeneration of the filarial parasite in the chronic stage of the infection results in the breakdown of the surrounding cyst wall and the formation of an early granulomatous inflammatory focus. In some lesions, wide areas of hemorrhage were observed in the center and dense connective tissue proliferation was observed in the periphery. Lesions in this stage were

mostly localized in the tunica media and adventitia of the aorta. It was ascertained that calcification and necrosis had developed in late granulomatous inflammatory foci. The majority of the cells surrounding these foci were macrophages. To a lesser extent, eosinophil and neutrophil leukocytes were present and peripheral cell infiltrations consisting of lymphocytes, plasma cells, and giant cells were also observed (Figure 1). These granulomatous foci were surrounded by a fibrous capsule. This type of lesion was mostly localized in the tunica adventitia. Of the 43 tissues with aortic onchocerciasis, the early granulomatous inflammatory stage was observed in 8, the late granulomatous inflammatory stage in 22, and both late and early granulomatous inflammatory stages in 13.

3.2. Enzyme histochemistry

Although chymase-containing mast cells were present in the control sections of human skin and intestine, chymase-containing mast cells were not observed in any sections of the bovine aortae.

3.3. Immunohistochemistry

3.3.1. Immunohistochemistry for mast cells

A few tryptase-containing mast cells, which were observed distant from the cysts containing viable parasites, were surrounded by a thin connective tissue layer and localized in the connective tissue of the aorta (Figure 2). In the early granulomatous stage, during which the parasite is either viable or dead and the cyst wall is broken down, the tryptase-containing mast cells were localized mostly in the peripheral regions characterized by connective tissue proliferation, rather than in regions with a dense infiltration of inflammatory cells. It was determined that the number of positively stained tryptase-containing mast cells had increased in these regions (Figure 3). In the

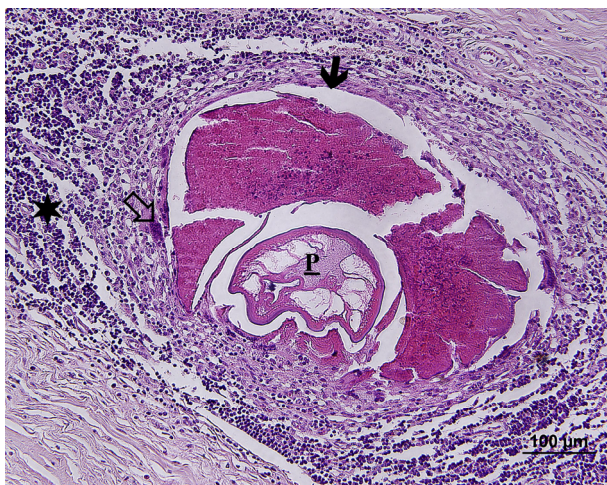


Figure 1. Fragmented parasite cysts and surrounding cell infiltration, parasites (P), parasitic cyst (black arrow), giant cells (white arrow), and mononuclear cell infiltration (star); H&E, 200 \times .

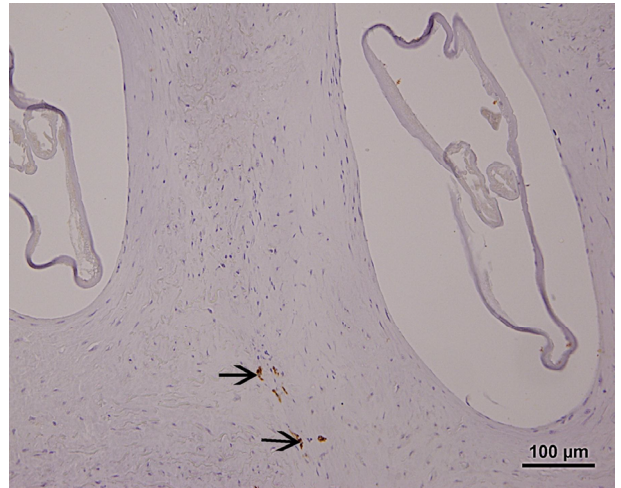


Figure 2. Tryptase-containing mast cells (arrows) distant from the parasitic cyst, immunohistochemical staining, 200 \times .

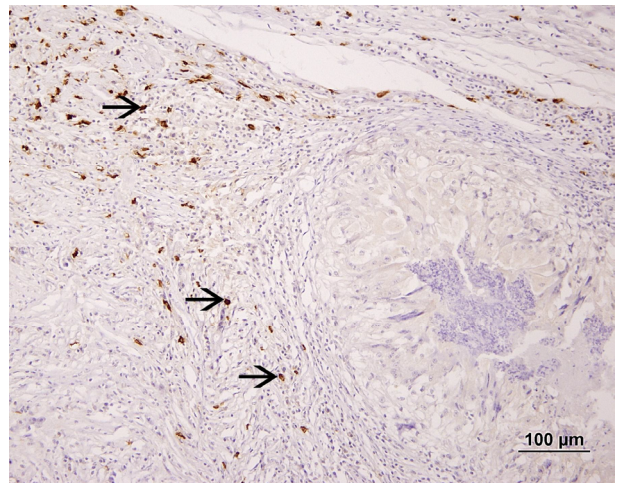


Figure 3. Tryptase-containing mast cells (arrows); immunohistochemical staining, 200 \times .

advanced granulomatous stage, tryptase-containing mast cells were observed in an even greater number, in the same region with the early granulomatous stage (Figure 4).

3.3.2. Immunohistochemistry for WSP

The immunohistochemical staining of the 43 sections resulted in positive staining for WSP in 8 (18.6%) of the aorta tissues. Positive staining was observed in sections containing the parasite surrounded by a thin layer of connective tissue. Positive staining was observed as a dark brown staining of the hypodermis in the parasites contained within cysts (Figure 5), the oocytes in the uterus of the adult female parasite, and microfilariae (Figure 6). Of the 8 tissues that were *Wolbachia*-positive in immunohistochemical staining, the early granulomatous

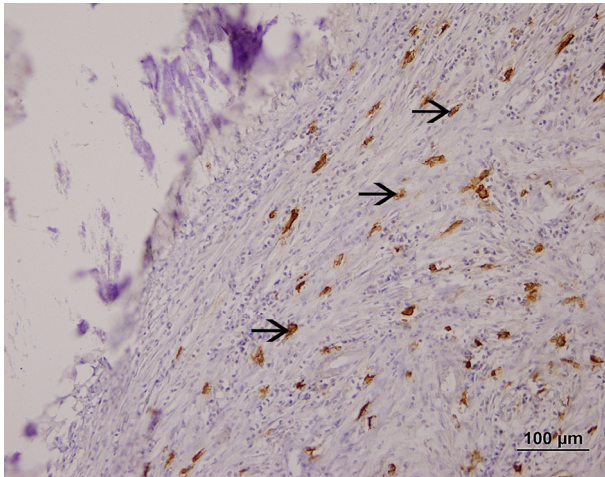


Figure 4. Tryptase-containing mast cells (arrows); immunohistochemical staining, 200×.



Figure 6. *Wolbachia*-positive microfilariae in the uterus (black arrows) and parasitic cyst membrane (white arrow); immunohistochemical staining, 200×.

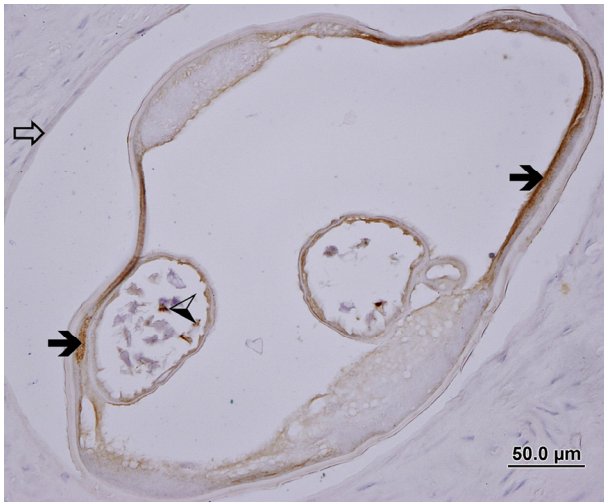


Figure 5. *Wolbachia*-positive parasite (black arrow) and parasitic cyst membrane (white arrow) in the hypodermis; immunohistochemical staining, 400×.

inflammatory stage was observed in 3 and both late and early granulomatous inflammatory stages were observed in 5.

4. Discussion

Bovine onchocerciasis is a widespread filarial disease that has been reported from various regions of the world. Bovines are natural hosts for many species of filarial nematodes. Forty-three of the aortic arch tissue specimens included in the present study were diagnosed with aortic onchocerciasis both macroscopically and microscopically. Microscopic examinations have revealed the localization of these parasites in the tunica intima, media, and adventitia of the aorta (1,2). In the present study, while

cysts containing viable parasites were observed in the tunica intima, early granulomatous nodules were localized in the tunica media and adventitia, and late granulomatous nodules were found in the tunica adventitia. The microscopic findings determined for the cysts containing viable parasites were in agreement with those reported in previous research, and no inflammatory cells were observed in the periphery of the parasite, which was surrounded by a connective tissue cyst wall (2,8,9). The microscopic findings obtained for early and late granulomatous nodules also coincided with those reported in previous studies (2). Accordingly, a typical granuloma structure was observed, with necrotic cell debris and calcification in the center, which were surrounded by eosinophil and neutrophil leukocytes and further circumscribed by giant cell, macrophage, lymphocyte, and plasma cell infiltrations encapsulated by connective tissue at the outermost region (1,2,8,9). *Onchocerca* and some other nematodes exist in association with *Wolbachia* bacteria throughout their lifespan, and literature reports suggest that these bacteria can be transmitted vertically (22). The survival of the bacteria being dependent on its intracellular localization was demonstrated in research conducted at the genomic level. As these bacteria lack the capability of synthesizing the amino acids required for protein synthesis, they obtain the required material from the parasite with which they coexist. On the other hand, while filarial parasites are incapable of synthesizing riboflavin and heme, it has been determined that *Wolbachia* possesses the gene sequences required for the synthesis of these cofactors (23). Some substances secreted by *Wolbachia* may affect certain components of the immune response against filariasis (24). The catalase enzyme contained by *Wolbachia* spp. protects both the bacteria

and the filarial parasite from oxidative damage. Thereby being protected from immune-mediated damage, the filarial parasite is able to survive for a relatively long time period. The *Wolbachia* bacteria, which are set free upon the death of the nematodes, pass into the blood circulation and become exposed to the immune system of the host (25,26). In previous research, it was observed that the antibiotic treatment of *Wolbachia*-positive cattle infected with *O. ochengi* resulted in the continuous decrease of the number of *Wolbachia* bacteria and the death of adult parasites. This demonstrated that the survival of the filarial parasite depends on this bacterium (11,12). The presence of endosymbiotic *Wolbachia* bacteria within several parasites, including *O. armillata* (1), *O. volvulus*, *O. ochengi*, *O. gibsoni*, and *O. fasciata* (25), has been demonstrated by immunohistochemical methods. In agreement with previous research, the present study also demonstrated the presence of the anti-WSP antibody in the hypodermis, uterine oocytes, and microfilariae of *O. armillata* by means of immunohistochemical staining. The secretion by mast cells of a high level of mouse mast cell proteinase-1 (mMCP-1), which defines the role of these cells in host defense mechanisms in parasitic infections, not only increases the inflammatory reaction in the organ in which the parasite resides but also accelerates the death of the parasite. However, in nematode species that lack mMCP-1, the deworming function of this enzyme does not apply for all parasites. Therefore, it has been suggested that some other proteases may have a more significant role in the protection of the host against parasites. For example, mMCP-4, which is a type of chymase, regulates the barrier function of intestines through the regulation of the intestinal permeability and the migration of epithelial cells. Therefore, available studies suggest that, in general, mast cell-derived chymase plays a more significant role in host defense mechanisms against intestinal parasitic infection. In the present study, while chymase-containing mast cells were found to exist in the control slides prepared from human skin and intestinal tissue, none of the different sections of the bovine aortae presented chymase-containing mast cells. This was attributed to the parasite species investigated and the type of tissue examined in the present study being different from those investigated and examined in previous research. In fact, while very few mast cells exist in only the tunica adventitia of normal blood vessels, it has been reported that a high number of mast cells accumulate in the periphery of the atherosclerotic plaque in the wall of atherosclerotic blood vessels (27). Research on the function of mast cells in the wall of atherosclerotic blood vessels has shown that mast cell-derived chymase causes apoptosis in the smooth muscle cells of blood vessels, which contributes to the detachment of the atherosclerotic plaque (27,28). Therefore, on the

basis of this information, it is considered that the absence of chymase-containing mast cells in aortae with onchocerciasis could be related to the lack of any tissue process that would require chymase activity. Research on the correlation of mast cells with other inflammatory cells in *O. gutturosa*, *O. tarsicola*, *O. ochengi*, *O. flexuosa*, and *O. armillata* has shown that intact mast cells are localized to the capsule and septa of *Onchocerca* nodules and to the fibrous and perivascular tissue associated with nonnodular parasites. Furthermore, inactive or active degranulated mast cells have been reported to be found in the infiltrate in the center of parasitic nodules or in the periphery of nonnodular filariae. In previous research, it was determined that success in the detection of tryptase was higher with the use of the immunohistochemical method than with the use of the toluidine blue technique, yet no method was tested for the identification of the subtypes of chymase-containing mast cells (29). Furthermore, in another study aimed at the investigation of the distribution of mast cells in parasitic nodules in patients infected with *O. volvulus*, the nodules formed by female parasites were classified into nodules containing and not containing microfilariae. Accordingly, it was detected that parasitic nodules formed by male parasites contained a greater number of mast cells compared to parasitic nodules formed by female parasites and nodules not containing microfilariae. On the basis of another type of classification, it was ascertained that nodules formed by dead parasites contained a significantly higher number of mast cells in comparison to lesions caused by viable larvae. It has been reported that the localization and number of mast cells may vary with the viability of parasites and the presence or absence of microfilariae, which is either directly or indirectly related to the secretion of *O. volvulus* antigens (13). Previous research on the role of mast cells during the treatment period of onchocerciasis patients with ivermectin revealed that following the death of the filarial parasites as a result of the effects of ivermectin, an increase was observed in plasma tryptase levels in parallel with the increase in the number of mast cells in the skin (20). In the present study, in agreement with previous research (13,20), it was determined that while the number of mast cells was higher in the periphery of the lesions containing dead parasites, only a very few mast cells were found in the periphery of the lesions containing viable parasites. The findings obtained in the present study showed that in the *O. armillata* lesions, which contained viable parasites within cystic tunnels that were in general surrounded by connective tissue and localized to the regions of the tunica media near the tunica intima, MC_T were observed only occasionally and as separate and independent cells. On the other hand, in lesions with intense hemorrhage and cell infiltration, which were in the early granulomatous stage,

it was observed that the number of MC_T had increased and formed the cell infiltration encapsulating the inflammatory focus at the outermost region. The present study demonstrated that the highest number of MC_T was observed in the fibrotic connective tissue surrounding the necrotic areas of resolving granulomatous lesions during the chronic inflammatory period of the late granulomatous stage. These findings demonstrate that tryptase-containing mast cells play an important role in collagen synthesis in aortic onchocerciasis lesions, and are in agreement with the report of Bot et al. (27), which suggests that tryptase stimulates the migration and proliferation of blood vessel smooth muscle cells and the synthesis of collagen. Available

literature data suggest that lipopolysaccharide-like substances secreted by *Wolbachia* spp., which are endosymbiotic bacteria contained by filarial parasites, are involved in the pathogenesis of *O. armillata* infection (30). Similarly, in the present study, it was observed that, like other inflammatory cells, mast cells can be activated upon the death of parasites and thus upon the death of filarial parasites containing *Wolbachia* bacteria. The present study demonstrated, for the first time, the mast cell subtypes found in aortic onchocerciasis lesions caused by *O. armillata* in cattle raised in Turkey and the presence of endosymbiotic *Wolbachia* bacteria in these lesions.

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