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Wolbachia and its influence on the pathology and immunology of *Dirofilaria immitis* infection

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Abstract

Since the definitive identification in 1995 of the bacterial endosymbiont *Wolbachia* that resides in different tissues of the filarial worm *Dirofilaria immitis*, there has been increasing interest to understand whether and what role it plays in the pathogenesis of and immune response to heartworm infection. The present study evaluated the effects of treatments on lung pathology in 20 beagle dogs experimentally infected with *D. immitis*. Dogs in Group 1 were treated with doxycycline (10 mg/kg/day) orally from weeks 0–6, 10–12, 16–18, 22–26, and 28–34. Dogs in Group 2 served as infected, non-treated controls. Dogs in Group 3 were given doxycycline as described for Group 1 combined with weekly oral doses of ivermectin (6 mcg/kg) for 34 weeks and intramuscular (IM) melarsomine (2.5 mg/kg) at week 24, followed by two additional melarsomine injections 24 h apart 1 month later. Group 4 received only melarsomine as described for Group 3. Lung lesion criteria, scored by two independent blinded pathologists, included perivascular inflammation and endothelial proliferation. Doxycycline treatment alone had no effect on lesion scores, whereas the combination of doxycycline and ivermectin resulted in less severe perivascular inflammation. All lungs were evaluated for positive immunostaining for the *Wolbachia* surface protein (WSP). Control dogs showed numerous thrombi, intense perivascular and interstitial inflammation and, occasionally, positive staining for WSP. Interestingly, dogs receiving doxycycline/ivermectin/melarsomine showed significantly less severe arterial lesions and the virtual absence of thrombi.
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1. Introduction

Wolbachia is an intracellular, Gram-negative bacteria belonging to the order Rickettsiales. Many pathogenic filarial worms of humans and animals harbor *Wolbachia*, and evidence strongly suggests that there is an endosymbiotic relationship between the bacteria and its filarial host (Taylor et al., 2005). The dependence of filarial nematodes on *Wolbachia* for fecundity and long-term survival has made it an interesting target for therapy and control of infection (Hoerauf et al., 2000, 2002). Recent interest has also been directed toward the role *Wolbachia* might play in the inflammatory pathology characteristic of filarial infection. It has been shown that

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Wolbachia and *Wolbachia*-derived molecules can induce pro-inflammatory responses in vitro (Bazzocchi et al., 2003; Brattig et al., 2004) and in vivo (Saint André et al., 2002). Furthermore, studies have shown that specific antibiotic treatment aimed at reducing *Wolbachia* levels in worms can have a beneficial effect by reducing filarial-induced pathology (Debrah et al., 2006).

Dirofilaria immitis is a filarial nematode that resides in the pulmonary arteries of dogs, causing canine heartworm disease. Intimal proliferation occurs in arteries occupied by living worms, and embolic worm fragments trigger thrombosis, both of which may completely obstruct segments of the pulmonary arteries. Perivascular inflammation is also present, triggered by worm-derived molecules and damaged endothelium. Severe thromboembolism and acute inflammation are associated with adult worm death for natural attrition or following macrofilaricide treatment.

The objective of the current study was to determine whether reduction of *Wolbachia* levels through specific therapy in dogs experimentally infected with *D. immitis* could improve lung pathology when compared with untreated controls and/or could improve post-adulticide pathology when compared with dogs treated only with a macrofilaricide.

2. Material and methods

2.1. Animals and treatments

A total of 20 young adult beagles (male and female), born and raised in a mosquito-proof environment and did not have any prior exposure to natural infection with *D. immitis,* were used in the study. Adult heartworms were harvested from a donor dog approximately 8 months after infection; seven male and nine female heartworms were introduced by intravenous (IV) transplantation into each study dog. Approximately 6 weeks later (day 0), the dogs were ranked within gender by microfilarial count and randomly allocated to four groups of five dogs each. Beginning on day 0, dogs in Group 1 were given doxycycline (10 mg/kg/day) orally from weeks 0–6, 10–12, 16–18, 22–26, and 28–34. The dogs in Group 2 served as infected, non-treated controls. Dogs in Group 3 were given doxycycline as described for Group 1, combined with weekly prophylactic doses of ivermectin (6 mcg/kg) orally for 34 weeks, and were then treated with an intramuscular (IM) injection of melarsomine (2.5 mg/kg) at week 24, followed by two additional melarsomine injections 24 h apart 1 month later. Group 4 received only melarsomine as described for Group 3. Approximately 36 weeks after IV filarial transplantation, the dogs were humanely euthanized by barbiturate injection and were necropsied. The study protocol was approved by the University of Georgia Ethics Committee for Care of Animals.

2.2. Lung histology and anti-WSP immunohistochemistry

At necropsy, the right caudal lung of each dog was fixed in 10% buffered formalin, processed for routine histology, and stained with hematoxylin–eosin. Tissue sections also were treated with a specific, hyperimmune serum raised against the *Wolbachia* surface protein (WSP), according to Kramer et al. (2003). One of the authors (LK) blindly evaluated lesions and assigned a lesion score (0–4) to each tissue section, according to criteria described in Table 1, and then calculated a mean lesion score for each dog and for each group. The presence of positive staining for WSP was noted.

3. Results

Results of histopathologic lung scoring are reported in Table 2. Mean lesion score for the control group (1.45) was not markedly different from that of dogs in Group 1 treated with doxycycline intermittently for 20

Table 1
Lesion criteria for evaluation of lung pathology in 20 dogs experimentally infected with *Dirofilaria immitis* and allocated to different treatment regimens.

Pulmonary vasculature (5 arteries/lung)	Parenchymal lesions (10 fields/lung)
0 = normal	0 = normal
1 = slight thickening of the wall without obstruction	1 = slight inflammation and/or thickening of the alveolar wall/slight periarterial inflammation
2 = moderate thickening of the wall with endothelial proliferation	2 = moderate inflammation and/or thickening of the alveolar wall/moderate periarterial inflammation
3 = obstruction of the lumen due to intimal proliferation and/or severe thickening, sclerosis of the arterial wall/thrombi	3 = severe inflammation and/or thickening of the alveolar wall/severe periarterial inflammation

Table 2
Individual and mean lesion scores for individual dogs and group means for experimentally infected with *Dirofilaria immitis* and allocated to different treatment regimens.

Lesion score	Control						Doxycycline					
	782	870	877	883	889	Group mean	692	783	784	881	884	Group mean
Arterial wall	0	1	3	3	1		1	0	0	1	3	
Endothelium	1	0	1	0	0		0	1	2	0	1	
Alveolar wall	2	3	2	2	2		0	2	2	1	1	
Periarterial inflammation	1	3	2	2	2		1	2	2	2	2	
Mean/dog	1	1.25	2	1.75	1.25	1.45	0,50	1.25	1.25	1.50	2	1.3
Lesion score	Melarsomine						Doxycycline/ivermectin/melarsomine					
	874	875	888	890	893	Group mean	882	886	891	876	878	Group mean
Arterial wall	2	1	1	2	2		0	1	1	0	0	
Endothelium	3	2	3	1	3		1	0	1	1	1	
Alveolar wall	3	3	1	2	1		1	0	1	1	1	
Periarterial inflammation	3	3	2	3	3		1	2	1	1	1	
Mean/dog	2.75	2.25	1.75	2.0	2.25	2.2	0.5	0.75	1.25	0.5	0.5	0.7

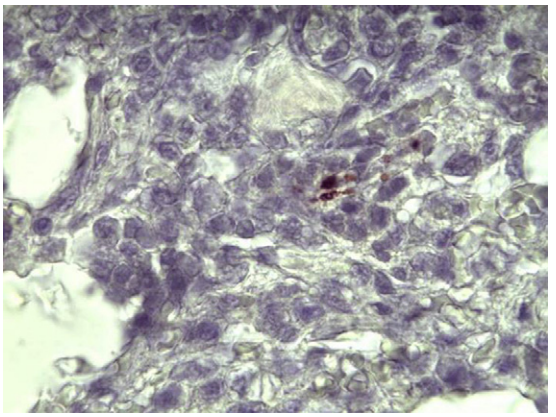

Fig. 1. Anti-*Wolbachia* surface protein immunohistochemistry. Lung from a control dog experimentally infected with *D. immitis*. Note positive staining for the *Wolbachia* surface protein within microfilariae (avidin–biotin–peroxidase complex–horseradish peroxidase, 60×).

weeks (1.3). Staining for the WSP was evident in lungs from control dogs and was consistently associated with microfilariae (Fig. 1). No positive staining was observed in dogs treated with doxycycline.

Adulticide treatment with melarsomine alone resulted in a mean group lesion score of 2.2, and the combination of ivermectin and doxycycline with melarsomine caused a dramatic decrease in lesion score (0.7). In particular, perivascular inflammation (Fig. 2) and endothelial proliferation (Fig. 3) were consistently less severe in dogs from Group 4 treated with doxycycline/ivermectin/melarsomine. Intense anti-WSP staining was observed in dogs treated with melarsomine alone and was particularly evident within the vascular endothelium near dying worms (Fig. 4). No staining for *Wolbachia* was observed in dogs from the doxycycline/ivermectin/melarsomine group.

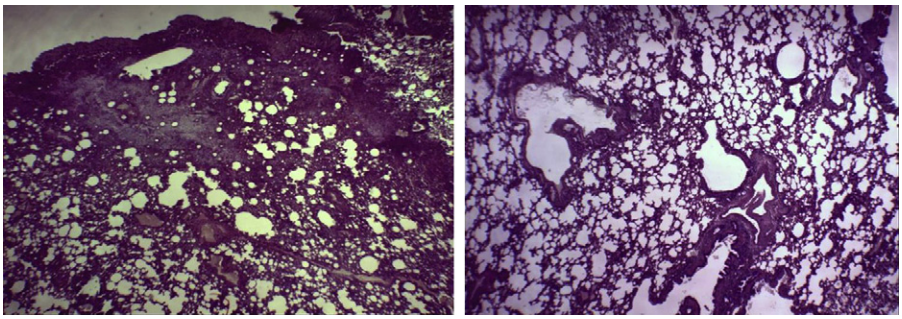

Fig. 2. Perivascular inflammation. (A) Lung from a dog experimentally infected with *D. immitis*. (B) Lung from a *D. immitis*-experimentally infected dog treated with doxycycline/ivermectin/melarsomine (hematoxylin–eosin stain, 40×).

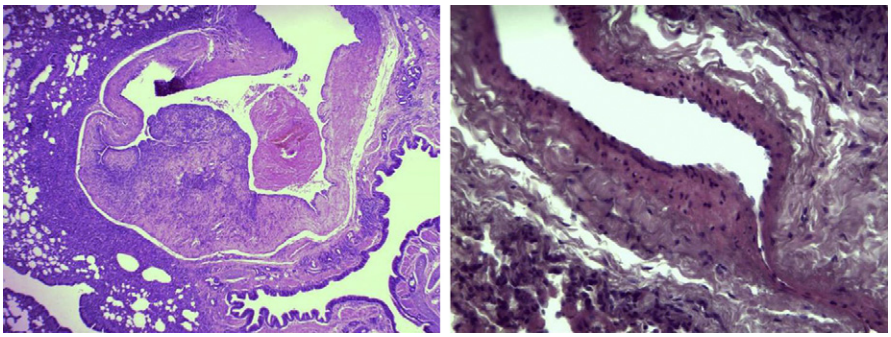

Fig. 3. Endothelial proliferation. (A) Lung from a dog experimentally infected with *D. immitis*. (B) Lung from a *D. immitis*-experimentally infected dog treated with doxycycline/ivermectin/melarsomine (hematoxylin–eosin stain, 40×).

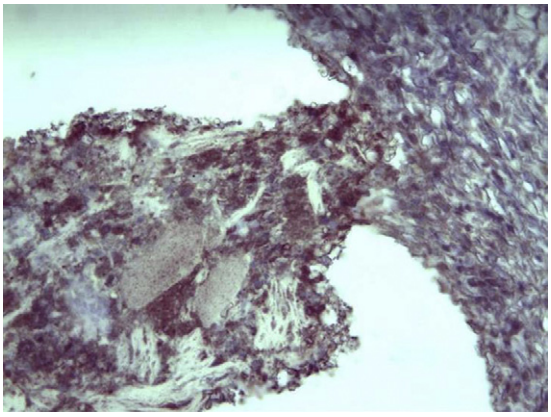

Fig. 4. Anti-*Wolbachia* surface protein immunohistochemistry. Lung from a dog experimentally infected with *D. immitis* and treated with melarsomine. Note positive staining for the Wolbachia surface protein within vascular endothelium (avidin–biotin–peroxidase complex–horseradish peroxidase, 60×).

4. Discussion

Filarial disease of both humans and animals features long-term survival of adult worms and chronic inflammatory reactions of the host tissue where adult and/or larval stages reside. The vasculatory alterations that characterize heartworm disease are due to complex immunomodulatory mechanisms that have yet to be clearly defined (Grandi et al., 2005). Doxycycline treatment was able to reduce *Wolbachia* levels in adult worms (data not shown), but this did not result in less severe pathology in these dogs when compared with that for controls. These results are similar to those reported by Chirgwin et al. (2003) in *Brugia pahangi*-infected gerbils treated with tetracycline, where lymph node lesions were comparable to control groups, despite a dramatic drop in *Wolbachia* in worms. These results suggest that there are other worm-derived factors that contribute to pathology during active infection. Debrah et al. (2006) recently reported that doxycycline treatment in humans with bancroftian filariasis significantly improved health status by reducing the number of patients with hydrocele and lymphedema. However, the beneficial effects of antibiotic treatment were apparent only 12–24 months after treatment and were associated with adult worm death. Here in our study, however, doxycycline did not cause adult worm death at 8 months from the start of the treatment (data not shown).

It is widely accepted that *Wolbachia* is released into the tissues of the filarial-infected host following worm death and that bacteria-derived molecules provoke innate inflammatory responses (Saint André et al., 2002). In the study reported here, when infected dogs were treated with melarsomine alone, there was an intense inflammatory reaction in the lungs and the presence of positive staining for a major surface protein of *Wolbachia* that were virtually absent when adulticide therapy was associated with the combination of doxycycline and ivermectin. Indeed, the treatment protocol of doxycycline and ivermectin has significant adulticide effects and is able to more efficiently reduce *Wolbachia* levels when compared with doxycycline treatment alone (Bazzocchi et al., 2008). These results suggest that reducing adult worm mass and *Wolbachia* levels in *D. immitis* before adulticide therapy could greatly improve the health status of dogs treated for heartworm disease.

Conflicts of interest

Authors L. Kramer, G. Grandi, M. Leoni, B. Passeri, J. W. McCall, C. Genchi, M. Mortarino and C. Bazzocchi have no financial or personal relationships with other people or organizations that could inappropriately influence or bias the paper.

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