Anti-Wolbachia Surface Protein Antibodies Are Present in the Urine of Dogs Naturally Infected with *Dirofilaria immitis* with Circulating Microfilariae But Not in Dogs with Occult Infections

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Abstract

Heartworm infection (*Dirofilaria immitis*) can cause kidney damage due to the presence of circulating microfilariae (mf) that contribute to the production and deposit of immune complexes. It has been shown that mf are a major source of Wolbachia antigen during active infection. Here the authors compared urine samples from 19 naturally infected dogs with (mf+) and 12 without (mf–) microfilariae for the presence of proteinuria and anti-Wolbachia Surface Protein (WSP) IgG in ELISA. Kidneys from 6 mf+ and 3 mf– dogs were also examined by anti-WSP immuno-histochemistry. All infected dogs showed proteinuria, but mf+ dogs had significantly higher values compared to mf– dogs. mf+ dogs had optical density values for anti-WSP IgG consistently higher than established cut-off values and were significantly higher than values for mf– dogs. Kidneys from mf+ dogs showed Wolbachia+ mf in glomerular capillaries. Results strongly suggest that *Wolbachia* associated with circulating mf may contribute to immune-mediated kidney disease in dogs with heartworm infection.

Key Words: *Dirofilaria immitis*—Kidney—Microfilariae—Urine—*Wolbachia*.

Introduction

DIROFILARIA IMMITIS is the causative agent of heartworm disease in dogs, cats, ferrets, coyotes, and others (McCall et al. 2008). It has long been recognized that infected dogs can present microscopic and/or ultrastructural alterations of the kidney that include increased number of mesangial cells, increased thickness of the matrix, infiltration of the small round and plasma cells into the interstitium, thickening of the glomerular basement membrane (GBM), and the presence of dense deposits in the GBM (Ludders et al. 1988, Nakagaki et al. 1990, Paes-de-Almeida et al. 2003). Heartworm-associated glomerulonephropathy has been shown to be at least partially due to the *in situ* production and deposit of immune complexes toward both somatic and excretory/secretory antigens (Grauer et al. 1988, 1989) and has been correlated to the

presence of microfilariae (mf) within the glomerular capillaries and the medullary vessels (Ludders et al. 1988).

It has been shown more recently that circulating mf are likely an important source of Wolbachia antigen in infected dogs (Kramer et al. 2008) and it has been postulated that Wolbachia released from dying mf can induce inflammation and specific immune responses (McCall et al. 2008). Wolbachia-positive mf have been observed in numerous tissue capillaries of infected dogs, including the kidney (Kramer et al. 2008).

The aim of the present study is to evaluate the possible role of Wolbachia in heartworm-associated glomerulonephropathy in *D. immitis*-infected dogs by comparing proteinuria, the presence of anti-Wolbachia Surface Protein (WSP) antibodies in urine, and the presence of Wolbachia in renal capillaries in dogs with or without circulating mf.

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Materials and Methods

Animals and sampling procedures

Thirty-one naturally infected *D. immitis*-positive dogs from an endemic area of Gran Canary Island were evaluated. Nine dogs were from the municipal humane shelter and 22 were privately owned. The owners gave their authorization for the use of data obtained in the present study. Urine and blood samples were obtained from all dogs. Peripheral blood was analyzed for the presence of circulating mf using the modified Knott test and for circulating *D. immitis* antigens with a commercial kit (SnapTM Canine Heartworm PF, IDEXX Laboratories Inc.), according to manufacturers' instructions. Nineteen dogs were classified as antigen positive/microfilaria positive (mf+) and 12 dogs as antigen positive/microfilaria negative (mf-). Sterile urine samples were obtained by cystocentesis. The nine dogs (6 mf+ and 3 mf-) from the municipal shelter were humanely euthanized (according to state law in absence of adoption) and kidneys were obtained for anti-WSP immunohistochemistry.

Urine protein and anti-WSP antibodies

Urine samples from infected dogs were divided into two portions. One was frozen at -20°C for anti-WSP ELISA. The other was centrifuged at 1000 rpm for 5 min and protein concentration was determined by the Pyrogallol Red-Molybdate method. Urine from 20 clinically healthy dogs from nonendemic areas and negative for circulating *D. immitis* antigens was used as controls.

The Pyrogallol red-Molybdate method (SGM) was performed according to manufacturer's instructions. Briefly, 20 μL of urine was mixed with 1000 μL of reagent, and after 5 min at 37°C , the absorbance of the assay mixture was measured (A 600 nm) against distilled water. The measuring range was from 2 to 400 mg/dL.

For evaluation of the antibodies against Wolbachia in urine, recombinant Wolbachia surface protein (rWSP) was used as antigen according to Bazzocchi et al. 2000. Immunoglobulin G levels against rWSP were determined in ELISA following the protocol described by (Morchón et al. 2004), with several modifications. Briefly, microplates of Polivinyll chlorate (Corning Incorporated) were incubated overnight at 4°C with 200 μL /well of a solution containing 1 $\mu\text{g}/\mu\text{L}$ of rWSP. Urine samples were analyzed using a 1:1 dilution and the secondary antibodies with 1:2500 dilutions. Optical density values (ODs) were measured in an Easy-Reader (Bio-Rad Laboratories) at 492 nm. Cut-off points (ELISA rWSP 0.63) were obtained as OD arithmetical means $\pm$ three standard deviations of values obtained from the urine of 20 clinically healthy dogs from nonendemic areas.

Anti-WSP immunohistochemistry

At necropsy, both kidneys were isolated, fixed in 10% buffered formalin, and processed for immunohistochemistry. Five micro-thick sections were treated with a specific, polyclonal antibody raised against the WSP of *D. immitis*, according to Kramer et al. (2003).

Statistical analysis

Statistical analysis of values for proteinuria and urine anti-WSP IgG was carried out using the SPSS statistical package

(version 17.0. for Windows). Descriptive analyses of the variables were carried out using the test of proportions for qualitative variables and measurements of central tendency (mean), measures of dispersion (standard deviation; SD) for quantitative variables. Absolute means between groups were compared with the Student *t*-test for normally distributed variables. Bivariate analyses of proportionality of distribution of categorical variables were estimated using the χ^2 test. A *p*-value < 0.05 was determined as indicating significance.

Results

All infected dogs were positive for proteinuria. Indeed, healthy control dogs showed significantly lower levels of proteinuria (17.69 ± 7.25 mg/dL) than dogs with circulating mf and dogs with occult infections ($p < 0.01$, respectively). However, dogs with circulating mf showed significantly higher levels of proteinuria (68.57 ± 79.25 mg/dL) than dogs with occult infections (46.66 ± 28.75 mg/dL), showing significant differences between both groups ($p < 0.01$).

Results of anti-rWSP ELISA on urine samples is shown in Figure 1. Optical density values for anti-WSP IgG in urine from infected dogs with circulating mf were consistently above the cut-off value (mean O.D. 1.39) and were significantly higher when compared to both infected dogs without mf (mean O.D. 0.51; $p < 0.05$) and healthy control dogs (mean O.D. 0.11; $p < 0.01$). Further, mean O.D. values were significantly higher in infected dogs without mf when compared to healthy control dogs ($p < 0.05$).

Immunohistochemistry (Fig. 2a-d) confirmed the presence of Wolbachia+ mf within glomerular capillaries in all mf+ dogs. Interestingly, histology from dogs with occult infections showed limited kidney alterations with no signs of glomerulo- or interstitial nephritis, whereas dogs with circulating mf showed typical interstitial and glomerular inflammatory infiltrates and matrix thickening (data not shown).

FIG. 1. Optical density (OD) values of anti-recombinant Wolbachia Surface Protein values in ELISA in dogs naturally infected with *Dirofilaria immitis*. Bars indicate the standard deviation (S.D.); control: healthy dogs from nonendemic area for *D. immitis*; mf+: microfilaricemic dogs; mf-: amicrofilaricemic dogs.

FIG. 2. (a–d) Anti-Wolbachia Surface Protein immunohistochemistry of kidneys from dogs naturally infected with *D. immitis* with circulating mf. Arrows indicate positive staining mf within glomerular capillaries. (ABC-HSP, **a**, $\times 40$; **b**, $\times 100$; **c**, $\times 20$; **d**, $\times 40$). Color images available online at www.liebertonline.com/vbz

Discussion

In a ground-breaking study aimed at elucidating the pathogenesis of heartworm-associated nephropathy, Abramowsky et al. (1981) showed for the first time that mf caused alterations in kidneys of dogs experimentally infected with *D. immitis* through a filaria–antibody immune complex mechanism. The authors hypothesized that larval–tissue interactions occur to both live larvae and following larval death and subsequent release of immunogenic antigens. Thus, the presence of circulating mf within glomerular capillaries stimulates the production of specific antibodies that then form immune complexes that deposit on the GBM. Several later studies evaluated the nature of the antigenic stimulus responsible for IgG production in kidneys of infected dogs, identifying excretory/secretory antigens (Grauer et al. 1988), crude adult somatic antigens (Grauer et al. 1989), and crude mf antigens (Nakagaki et al. 1990). It is now known that all stages of *D. immitis*, including adults and mf, harbor the bacterial endosymbiont Wolbachia (Kramer et al. 2003) and that release of Wolbachia antigens from dead/dying worms stimulates a specific IgG response in infected dogs (Morchón et al. 2007). In the present study, the authors show that dogs with circulating mf of *D. immitis* have higher values of proteinuria and of anti-Wolbachia IgG in urine and Wolbachia-harboring mf in kidney capillaries, when compared to dogs without mf.

Loss of immunoglobulins (Ig) in urine mainly indicates glomerular injury, because Ig are larger than the diameter of the glomerular pore (Lulich and Osborne 1990). Thus, the presence of urinary anti-Wolbachia IgG observed in the present study could have been due to damage to the filtration activity of the glomerulus. However, it would be interesting to determine whether anti-Wolbachia antibodies are locally

produced in the urinary tract, as has been shown in dogs with visceral leishmaniasis due to *Leishmania infantum* (Solano-Gallego et al. 2003). It has been shown that while removal of Wolbachia has little or no effect on pathology due to the presence of live *D. immitis*, removal of the bacteria leads to improved pathology following worm death due to adulticide therapy (Kramer et al. 2008), suggesting that Wolbachia is pro-inflammatory only following its release from damaged worms. It has also been reported that cats experimentally infected with *D. immitis* and then treated with ivermectin showed increasing antibody titres to Wolbachia (Morchón et al. 2004), indicating that contact with exposure of the immune system to Wolbachia occurred following larval death. Results from the present study may suggest that mf destruction and turnover, which occurs in several organs, including kidneys (Wenk et al. 1993), is followed by an inflammatory response and by *in situ* production of specific IgG against Wolbachia, contributing to renal pathology during heartworm infection and perhaps to the production of immune complexes. Interestingly, kidney alterations have been reported for other filarial infections that feature Wolbachia-harboring mf. Dreyer et al. (1992) reported that over half of microfilaremic patients affected by lymphatic filariasis (*Wuchereria bancrofti*) had proteinuria and treatment with the microfilaricidal drug diethylcarbamazine induced the same abnormalities in almost all of the remaining microfilaremic patients. No proteinuria was observed in the amicrofilaremic patients. Kidney alterations associated with microfilaricidal therapy have also been described in heartworm-infected dogs treated with ivermectin, confirming the inflammatory role that dead/dying mf can have on renal tissue.

The authors of the present study conclude that the bacterial endosymbiont Wolbachia of *D. immitis* may contribute to mf-associated nephropathy. Further studies are needed to

determine the origin of the IgG and the possible role of Wolbachia in the production of immune complexes during *D. immitis* infection.

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Disclosure Statement

No competing financial interests exist.

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