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Identification of immunoreactive proteins from the dog heartworm (*Dirofilaria immitis*) differentially recognized by the sera from dogs with patent or occult infections

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ABSTRACT

Heartworm disease caused by *Dirofilaria immitis* affects canine and feline hosts. Moreover, the parasite can infect humans, causing pulmonary dirofilariosis. Most affected dogs have patent infections with circulating microfilariae in peripheral blood, although infected dogs sometimes develop occult infections characterized by the absence of microfilariae. Microfilaremic infections (mf+) are associated with a predominant Th2-type immune response, whereas a Th1-type response predominates in amicrofilaremic infections (mf−), suggesting a role for this response in the suppression of circulating microfilariae. However, nothing is known about the molecules involved in the immune regulation of these infections. The objective of the present work was to identify the parasite proteins recognized differentially by the immune response of dogs with patent or occult infections, using two-dimensional electrophoresis and mass spectrometry. Nineteen proteins of *D. immitis* were identified, of which 6 were immunoreactive against serum samples from both mf+ and mf− dogs, while another two groups of 6 and 7 different proteins were differentially recognized by sera from mf+ or mf− dogs, respectively. The results point to the existence of differential antigen recognition in patent and occult infections due to *D. immitis*. Several proteins that could be involved in the immune regulation of these infections are identified. Additionally, the findings seem to suggest that some antigens of *D. immitis*, together with *Wolbachia* antigens, could contribute to the stimulation of the Th1-type response.

1. Introduction

Dirofilaria immitis is the causative agent of canine and feline cardiopulmonary dirofilariosis (heartworm disease) in temperate and tropical areas throughout the world [1]. The parasite is transmitted by different species of culicid mosquitoes, some of which feed on both animal reservoirs and humans [2], also causing human pulmonary dirofilariosis [3]. Like other filarial species, *D. immitis* harbours the endosymbiont bacterium *Wolbachia* [4], which plays a key role in the biological processes of filariae-like embryogenesis and moulting.

The development of *D. immitis* in each of its hosts, and consequently the relationship established with them, is different [5]. The dog is the best adapted host, while in cats and humans the development of *D. immitis* shows some important modifications in comparison with the developmental pattern that occurs in dogs. In canine hosts, adult worms can survive for years, the females producing circulating microfilariae (microfilaremic or patent infections). However, in some cases no microfilariae are observed (amicrofilaremic, or occult infections), this situation being attributed to different causes such as unisexual infections, the age of the adult worms, and the immune response of the hosts. From the pathological point of view, canine cardiopulmonary dirofilariosis is a chronic disease that initially affects the walls of the pulmonary arteries, from where it progresses to the lung parenchyma, finally affecting the heart [6]. Moreover, acute reactions caused by the simultaneous death of many worms, either natural or induced by filaricide treatment, may appear [7,8]. The persistence of both adult worms and microfilariae in the circulatory system of immunocompetent hosts and the slow progress of the pathology suggest that *D. immitis* has developed complex mechanisms for long-term survival in

Abbreviations: 2-D WB, two-dimensional Western blot; 2-DE, two-dimensional electrophoresis; DiSB, soluble extract of proteins from *D. immitis* adult worms; GAPDH, glyceraldehyde-3 phosphate dehydrogenase; IEF, isoelectric focusing; MALDI-TOF, Matrix Assisted Laser Desorption/Ionization Time-of-Flight; mf+, microfilaremic; mf−, amicrofilaremic.

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dogs. Recent studies have indicated that *D. immitis* stimulates a Th2-type anti-inflammatory response in microfilaremic dogs, while the endosymbiont bacterium *Wolbachia* seems to stimulate a predominant Th1-type pro-inflammatory response in amicrofilaremic canine infections [9,10].

In lymphatic filariae and *Onchocerca volvulus*, many molecules have been identified and characterized, and different roles in the parasite/host relationships have been proposed [11–13]. By contrast, to date very few *D. immitis* antigens have been characterized. A polypeptide of 35 kDa that was immunodominant in L3, but not in L4 or adult worms [14], a group of cationic non-glycosylated proteins of 20–22 kDa [15,16], and an aspartyl protease inhibitor of 33 kDa [17] have been related to the diagnosis of infection in animal reservoirs and/or humans. Recently, it has been demonstrated that humans exposed to *D. immitis*, without pulmonary nodules, produce a predominant specific response of IgE antibodies against both a galectin and an aldolase of the parasite. In other helminth species, these molecules have been related to survival mechanisms, such as the up-regulation of the Th2-type response and interference in oxidative stress. [18].

Study of the mechanisms and molecules involved in immune regulation in patent and occult infections due to *D. immitis* should allow advances to be made in our knowledge of the relationships of this parasite and its hosts. With a view to such avenues of enquiry, the aim of the present work is to identify the proteins differentially recognized in a soluble antigen extract from *D. immitis* adult worms, by the immune response induced in microfilaremic and amicrofilaremic canine infections. The research was performed with a combination two-dimensional gel electrophoresis (2-DE) separations with antigen identification, using antibodies from dogs with microfilaremic (mf+) and amicrofilaremic (mf−) infections, and mass spectrometry in order to identify the *D. immitis* adult worm proteins that are immune targets for the host. We show that the combination of 2-DE and mass spectrometry enables the identification of selected *D. immitis* proteins through cross-species protein identification, and that this can be performed in the absence of a complete parasite genome sequence.

2. Materials and methods

2.1. Parasites and selection of serum samples

D. immitis adult worms were obtained from the hearts of infected dogs through the jugular vein using Flexible Alligator Forceps.

Serum samples from 4 mf+ and 10 mf− *D. immitis* infected dogs, as well as 4 serum samples from healthy dogs were employed. The parasitological status of the dogs included in the study was assessed employing a commercial ELISA test for *D. immitis* circulating antigens (IDEXX Laboratory) and by the Knott technique for microfilariae detection [19]. A positive result in both tests indicated microfilaremic infections; samples positive in IDEXX and negative in Knott belonged to dogs with amicrofilaremic infections, and dogs negative in both tests were considered free from *D. immitis* (negative controls).

2.2. Collection of a soluble extract of proteins from adult D. immitis worms and preparation of samples for two-dimensional gel electrophoresis

D. immitis adult worms were homogenized in phosphate-buffered saline solution (PBS), pH 7.4, and sonicated on ice for six cycles of 1 min each at 75 kHz. A cocktail of protease inhibitors (1 mM EDTA, 1 mM N-ethylmaleimide, 0.1 μM Pepstatin A, 1 mM PMSF and 0.1 mM N-tosylamide-L-phenylalanine chloromethyl ketone) was added to this homogenate, as indicated by Maizels et al. [20]. The homogenate was centrifuged at 100,000 × *g* for 1 h at 4 °C and the supernatant was recovered and dialysed against water for 24 h. The protein concentration of the extract obtained, termed DiSB, was determined by the DC protein assay (Bio-Rad).

The DiSB samples to be electrophoresed were previously purified and concentrated with the ReadyPrep 2-D Cleanup Kit (Bio-Rad), following the manufacturer's instructions. Thus, samples of DiSB containing 400 μg of protein were cleaned and resuspended in 1250 μl of 2-D rehydration buffer (7 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 50 mM dithiothreitol (DTT)) without ampholytes. The sample was divided into 125 μl aliquots (containing 40 μg of protein) and these were kept at −80 °C until use.

2.3. Two-dimensional electrophoresis (2-DE)

Isoelectric focusing (IEF) was performed in 7-cm IPG strips (Bio-Rad) with linear pH ranges of 3–10, 5–8 and 7–10. All isoelectric focusings were accomplished using a Protean IEF Cell (Bio-Rad) with a surface temperature of 20 °C and a maximum current of 50 μA/strip.

The 125-μl sample aliquots were thawed and each of them was supplemented with 1.25 μl of 100× Bio-Lyte ampholytes (Bio-Rad) at the respective pH range to reach a final concentration of 0.2% ampholytes. Then, the samples were allowed to mix gently for 1 h at room temperature and centrifuged at 18,000 × *g* over 30 min to remove all particulate material. The supernatants were applied to the IPG strips by in-gel rehydration at 20 °C for at least 12 h, after which IEF was run for a total of 20,000 Vh.

Following IEF, the strips were reduced in equilibration buffer (6 M urea, 0.05 M Tris pH 8.8, 2% SDS, and 20% glycerol) containing 2% DTT over 15 min and then alkylated in equilibration buffer containing 2.5% iodoacetamide for 10 min. The second dimension was performed on 12% SDS-polyacrylamide gels using a Mini Protean cell (Bio-Rad). Running proceeded at 15 mA/gel for the first 15 min and then at 30 mA/gel. After running, the 2-D gels were either stained or electroblotted onto nitrocellulose membranes for immunoblot analysis.

The 2-D gels were routinely stained with silver stain or with a mass-compatible silver stain (when the gel was to be used for analysis by mass spectrometry), according to the protocols described by Stochaj et al. [21]. Additional 2-D gels were stained with Sypro Ruby florescent dye (Sigma) according to the manufacturer's instructions for quantitative analysis.

Silver-stained gels were scanned using an ImageScanner (Amersham Biosciences) and Sypro Ruby-stained gels were digitalized with the Fluor-S Max Multimager system (BioRad). Analysis of 2-D gels was accomplished using the ImageMaster 2-D Platinum Software v5.0 (Amersham Biosciences) and included the determination of the normalised volume corresponding to the area and intensity of all spots detected in Sypro Ruby-stained 2-D gels.

2.4. Immunoblot analyses

Proteins were electrotransferred from 2-D gels to nitrocellulose membranes at 400 mA for 90 min. Blots were blocked with 1% BSA in PBS for 1 h and then rinsed with washing buffer (PBS containing 0.05% Tween 20). Then, the nitrocellulose membranes were incubated for 1 h with a pool of 10 serum samples from mf− dogs, a pool of 4 serum samples from mf+ dogs, or a pool of 4 serum samples from negative control dogs at 1:200 dilution. After a further three washes, the blots were incubated with a horseradish peroxidase-labelled anti-dog IgG (Sigma) at 1:500 dilution for 1 h. All incubations were performed at 37 °C, and the washes were carried out at room temperature for 10 min per wash. Immunoblots were revealed with 4-chloro naphthol and images were digitalized