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Ivermectin-dependent attachment of neutrophils and peripheral blood mononuclear cells to *Dirofilaria immitis* microfilariae *in vitro*

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ABSTRACT

The macrocyclic lactones are the only anthelmintics used to prevent heartworm disease, but it is very difficult to reproduce their *in vivo* efficacy against *Dirofilaria immitis* larvae in experiments *in vitro*. These assays typically measure motility, suggesting that paralysis is not the mode of action of the macrocyclic lactones against *D. immitis*. We isolated peripheral blood mononuclear cells (PBMC) and neutrophils from uninfected dogs and measured their adherence to *D. immitis* microfilariae in the presence of varying concentrations of ivermectin. We found that adherence of PBMC to the microfilariae was increased in the presence of ivermectin concentrations ≥100 nM and adherence of neutrophils was increased in drug concentrations ≥10 nM. Up to 50% of microfilariae had adherent PBMC in the presence of the drug, and binding was maximal after 40 h incubation. Neutrophil adherence was maximal after 16 h, with approximately 20% of the microfilariae having at least one cell adhered to them. Adherent neutrophils showed morphological evidence of activation. These results are consistent with a model in which the macrocyclic lactones interfere with the parasites ability to evade the host's innate immune system.

1. Introduction

The macrocyclic lactone (ML) class of anthelmintics, which includes ivermectin, moxidectin, selamectin and the milbemycins, are the only drugs currently used for the prevention of heartworm disease caused by *Dirofilaria immitis*. Ivermectin is also used for removing microfilariae following adulticide treatment (Bowman and Mannella, 2011). However, despite the importance and widespread use of ML drugs for preventing disease with *D. immitis*, the mechanism of action of these drugs against *D. immitis* is unknown.

There is very strong evidence from other species of parasitic nematodes and *Caenorhabditis elegans* that the pharmacologically relevant target of the MLs is the glutamate-gated chloride channel (GluCl) (Cully et al., 1994; Wolstenholme and Rogers, 2005), and an ivermectin-sensitive GluCl subunit has been identified in *D. immitis* (Yates and Wolstenholme, 2004). The GluCl gene family is less complex in *D. immitis* and other filarioid species than in *C. elegans* (Ghedin et al., 2007; Godel et al., 2012; Williamson et al., 2007) and it is likely that only a single gene, *Dii-avr-14*, (nomenclature according to Beech et al.,

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2010), encodes ML-sensitive GluCl subunits. GluCls control nematode movement, feeding and sensory processes (Wolstenholme, 2012) and the anthelmintic effects of the MLs and other anthelmintics are frequently assessed by measuring their effects on locomotion (Marcellino et al., 2012; Smout et al., 2010). *In vitro* migration assays are frequently used to detect ML-resistance in gastrointestinal nematodes (Demeler et al., 2012; Kotze et al., 2006; Wagland et al., 1992), and a similar assay has been developed for measuring the sensitivity of *D. immitis* L3 to the ML drugs (Evans et al., 2013). Reduced drug-induced paralysis of *D. immitis* microfilariae *in vitro* has also been taken as an indication of potential ML-resistant isolates (Bourguinat et al., 2011a, 2011b). However, the concentrations needed to produce 50% inhibition of L3 migration (Evans et al., 2013) or 95% paralysis of microfilariae (Bourguinat et al., 2011a) were approximately three orders of magnitude higher than those reported to be present in the plasma of dogs receiving either the preventative (6 μg/kg) or microfilaricidal (100 μg/kg) doses of the drugs (Daurio et al., 1992; McKellar and Gokbulut, 2012). This suggests that paralysis is unlikely to be the major mechanism of ML action against *D. immitis* L3 or microfilariae. This suggestion is supported by data from the human filarioid parasite *Brugia malayi*, where the GluCl were expressed solely around the excretory/secretory apparatus of the microfilariae (Moreno et al., 2010) and similarly high drug concentrations are required to paralyze *B. malayi* microfilariae *in vitro* (Tompkins et al., 2010). Consistent with this finding, *in vitro* treatment of *B. malayi* Mf with ivermectin reduced protein secretion (Moreno et al., 2010).

Filarioid nematodes are well known to secrete immunomodulatory molecules (Hewitson et al., 2008), and there have been previous reports that the killing of microfilariae by host granulocytes or peripheral blood mononuclear cells is enhanced, *in vitro*, by ivermectin (Rao et al., 1987; Zahner et al., 1997). This is consistent with evidence showing that clearance of microfilariae from dogs with occult *D. immitis* infections (*i.e.* infections where an adult nematode(s) are present but microfilariae are absent from the circulation) is mediated by neutrophils (Elsadr et al., 1983; Rzepczyk and Bishop, 1984). Since it has not been possible to reproduce the *in vivo* effectiveness of low concentrations of the MLs against *D. immitis* microfilariae by measuring their effects on motility *in vitro* (Bourguinat et al., 2011b), we examined the effect of ivermectin on the adherence of peripheral blood mononuclear cells (PBMC) and neutrophils to the parasites *in vitro*.

2. Materials and methods

2.1. Animals

All procedures involving animals were approved by the Institutional Animal Care and Use Committee of the University of Georgia.

2.2. Cell-free microfilariae

Microfilaraemic blood was drawn by jugular venipuncture into heparin-coated blood collection tubes (BD Vacutainer, BD, Franklin Lakes, NJ) the day before an assay was set up. Blood utilized was from two different *D. immitis*-infected dogs. The blood cells were lysed with 0.2% saponin (Tokyo Industry Chemical Co., Ltd., Tokyo, Japan) in 0.85% NaCl. Briefly, 1 or 2 mL blood were diluted in a ratio of 1:11 with the saponin in 15 mL centrifuge tubes and placed in a water bath at 37 °C for 15 min. The tubes were then centrifuged at room temperature at 850 × *g* for 5 min. The supernatant was siphoned off using a plastic Pasteur pipette, and the pellet was rinsed once with phosphate buffered saline (10 mM PBS, Boston BioProducts, Ashland, MA) and centrifuged again at 850 × *g* for 5 min.

Adapting the methods from Galal et al. (1989), the pellet was resuspended in 2 mL RPMI (BioWhittaker® RPMI-1640 with L-glutamine, Lonza, Walkersville, MD) and placed in Sephadex™-containing columns (PD-10 Columns, GE Healthcare, UK Limited, Little Chalfont, Buckinghamshire, UK) equilibrated with RPMI. The liquid was allowed to move through the column and the filtrate from the column was collected in a centrifuge tube. When the top portion of the column had emptied itself, this was topped up with RPMI and the next fraction of filtrate collected in a separate tube. This procedure was repeated approximately 11 times. Each fraction was examined under a standard compound microscope and the filtrate was qualitatively scored for cellular content and number of microfilariae. Fractions that did not contain cells or that contained microfilariae but negligible numbers of cells (estimated at less than 5 cells per microfilariae) were pooled and centrifuged at 850 × *g* for 10 min. The supernatant was siphoned off, the microfilariae rinsed with sterile RPMI and the tube centrifuged again at the same speed and time. This process was carried out once. The numbers of microfilariae in the suspension were estimated by counting three aliquots of 10 μL and stored in a tissue-culture incubator at 37 °C overnight.

2.3. Leukocytes

Blood leukocytes were prepared from blood from three specified pathogen-free beagles maintained in an insect-free indoor facility. For each assay, 6 mL of blood was drawn from an uninfected donor dog in EDTA-coated tubes (BD Vacutainer, BD, Franklin Lakes, NJ) and was allowed to cool to room temperature. Following the methods of Brinkmann et al. (2010), Histopaque®-1119 (Sigma-Aldrich, St. Louis, MO) at room temperature was used to separate the blood into mononuclear and polymorphonuclear cells, which were collected in separate centrifuge tubes. The cells were washed with PBS/1 mM EDTA, and each cell population was resuspended in 2 mL of PBS (Alam et al., 2006). The resuspended polymorphonuclear cells were centrifuged on a Percoll™ (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) gradient (Brinkmann et al., 2010), and the contaminating mononuclear cells were separated into a new tube, washed with PBS/1 mM EDTA and combined with the mononuclear cells collected previously. The combined mononuclear cells were washed two more times. The polymorphonuclear cells derived from the Percoll™ gradient were washed once with PBS containing 0.2% (w/v) glucose. The erythrocytes remaining in this suspension were lyzed in ice-cold 0.2% (w/v)