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Changes in the levels of eicosanoids in cats naturally and experimentally infected with *Dirofilaria immitis*

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Abstract

Feline heartworm (*Dirofilaria immitis*) infection is a severe, life-threatening disease. The eicosanoids are lipid mediators derived from the metabolism of the arachidonic acid, involved in the regulation of the immune response and of inflammatory reactions. In this study, naturally infected cats showed significant higher levels of prostaglandin E_2 (PGE2), thromboxane B_2 (TXB_2) and leukotriene B_4 (LTB4) than uninfected cats. Changes in the levels of eicosanoids during the infection were observed in experimentally infected cats. PGE2 increased significantly during the first 60 days post-infection, then progressively decreased until day 180 post-infection. At this time, PGE2 values are still significantly higher than those observed before the infection. TxB2 and LTB4 increased progressively from the beginning of infection and reached their maximum levels 180 days post-infection. In experimentally infected, ivermectin-treated cats, 15 days after treatment (45 days after infection) both PGE2 and LTB4 levels were similar to those observed in experimentally infected, untreated cats. No significant differences of PGE2 levels were found before the infection and at the end of the experiment (165 days post-treatment, 195 days post-infection). Increased levels of LTB4 were found 15 days post-treatment, afterward they progressively decreased. These data show that *D. immitis* infection influences the production of intravascular eicosanoids in cats. The high levels of PGE2 observed in the early phase of infection could be related to the survival of the worms, while those of TxB2 and LTB4 detected at the end of the study could mediate the inflammatory reactions and thrombi formation during the feline dirofilariosis.

Keywords: *Dirofilaria immitis*; Cats; Eicosanoids; Prostaglandin E_2; Thromboxane B_2; Leukotriene B_4

1. Introduction

Dirofilaria immitis is the causative agent of heartworm (HW) disease in both dogs and cats. The parasite can infect man causing benign pulmonary nodules that can be misdiagnosed with cancer or other pathological conditions (Simón et al., 2005). Heartworm is becoming increasingly diagnosed in endemic areas. Cats do not frequently develop patent infections (Dillon, 1986), most infected cats are asymptomatic and the appearance of clinical signs is mainly associated with the arrival of the preadult worms in the pulmonary arteries (5–6 months post-infection) and their subsequent death (Atkins et al., 1995; McCall et al., 1994; Genchi

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et al., 1995). Feline heartworm disease is characterized by thrombi and intense inflammatory reactions (endarteritis) as well as myo-intimal proliferation which results in arterial obstruction (McCall et al., 1994; Rawlings and Calvert, 1995). Adulticide treatment is not currently advisable since the death of worms dramatically increases inflammatory reactions and cats are at high risk of ''anaphylactic'' type reactions (Nelson et al., 2005). Monthly chemoprophylactic treatment with macrocyclic lactones, such as ivermectin, milbemycin oxime and moxidectin that eliminate infective (L3) and L4 larvae, thus preventing their development to adult parasites, is the only effective option for protecting cats against HW infection.

Among the causes of inflammatory reactions during infection, recent studies have implicated the endosymbiont bacteria of the genus *Wolbachia* that are present in many species of filarial worms, including *D. immitis* (Bandi et al., 2001; Bazzocchi et al., 2003; Simón et al., 2007). Antibodies against *Wolbachia* have been detected by Bazzocchi et al. (2000) and Morchón et al. (2004) in cats experimentally and naturally infected with *D. immitis*. Furthermore, a dramatic increase of specific antibodies against *Wolbachia* have been observed in experimentally infected, ivermectin-treated cats (Morchón et al., 2004). These data suggest that bacteriae and/or their products are released into the bloodstream during feline heartworm infection and following the death of worms.

The eicosanoids, that include prostaglandins, thromboxanes and leukotrienes, are lipid compounds produced during the metabolism of arachidonic acid and other polyunsaturated fatty acids. They locally modulate inflammatory and immunological responses in mammals (Liu and Weller, 1990). The effects of some of these eicosanoids antagonize the effects of others. PGE2 causes vasodilation and promote T-helper cell 2 (Th2) activity; thromboxanes (Txs) cause vasoconstriction, inhibit cyclic AMP and promote platelet aggregation; leukotrienes (LTs) are related to vascular permeability, chemotaxis and polymorphonuclear leukocyte activation (Betz and Fox, 1991; Sala and Giarcarlo, 2001). Some of these eicosanoids have been observed in lymphatic filariasis (Liu and Weller, 1990; Liu et al., 1992), in *Onchocerca volvulus* (Brattig et al., 2006) and in human pulmonary dirofilariosis caused by *D. immitis* (Morchón et al., 2006).

The aim of this work is to investigate the presence of PGE2, TxB2 and LTB4 during feline HW infection as well as the changes in their levels when the death of worms is induced by treatment with ivermectin.

2. Materials and methods

2.1. Cats

Serum samples from 69 cats were analyzed. They were divided into four groups: G1, 20 serum samples from naturally infected, symptomatic cats living in an HW endemic area, echocardiography and/or antibody-ELISA positive to *D. immitis* (Prieto et al., 1997). G2, 10 serum samples from *D. immitis* experimentally infected cats, positive to the antibody-ELISA test. G3, 9 serum samples from experimentally infected cats, treated with ivermectin 30 days after the infection. G4, 30 serum samples from clinically healthy, antibody-ELISA-negative cats, living in a non-endemic area as control.

2.2. Serum samples, experimental infections and chemoprophylaxis

Serum samples from naturally infected, privately owned cats (G1) were taken following diagnosis of infection. Cats from groups G2 and G3 were infected by subcutaneous injection in the right inguinal region of 50 *D. immitis* infective larvae as previously described (Genchi et al., 2004). Serum samples from cats of the group G2 were taken before infection and 60, 120 and 180 days post-infection (p.i.). In group 3, ivermectin was administered 1 month after infection at 24 μg/kg (Cardotek FX^{TM}, Merial) and serum samples were taken before infection, 15 days post-treatment (p.t.) (45 days p.i.), 90 days p.t. (120 days p.i.), 135 days p.t. (165 days p.i.) and 165 days p.t. (195 days p.i.). Serum samples from clinically healthy cats (G4) were taken once during routine visits to a veterinary clinic located in a non-endemic area for *D. immitis*.

2.3. Tests

The levels of PGE2, TxB2 and LTB4 in serum samples were analyzed by commercial ELISAs (R&D Systems). Briefly, serum samples were tested at 1:10, 1:100 and 1:2 dilutions, respectively, for PGE2, TxB2 and LTB4. Optical densities (ODs) were measured at 405 nm in an Easy Reader (BioRad). The conversion of ODs to mg/ml was carried out following manufacturers' instructions. The intra- and inter-assay precision (coefficient of variation, CV) ranged from 9.8 to 3.1% and from 12.1 to 8.1%, respectively, for PGE2. For TxB2 the CVs were 3.6–1.6% and from 7.7 to 6.2%, respectively, and for LTB4, the CVs were 6.0–5.9 and 15.7–5.0%, respectively.

2.4. Statistical analysis

The non-parametric Kruskal–Wallis test was used for the multiple comparisons of the immunologic data (G1 versus G4). A significant difference was defined as a p-value of <0.5 for a confidence level of 95%. For the paired samples the test of Wilcoxon was used to compare values of each eicosanoid from groups G2 and G3 across time. In this case $p < 0.5$ was considered significantly different.

3. Results

3.1. Eicosanoid levels in naturally infected cats

PGE2, TxB2 and LTB4 values obtained from naturally infected, seropositive cats (G1) and from seronegative, uninfected cats (G4) are shown in Fig. 1. Each eicosanoid showed significantly higher values in seropositive cats than in seronegative ones (PGE2, $p < 0.05$; TxB2 and LTB4, $p < 0.01$). TxB2 was the eicosanoid with the highest mean values, followed by LTB4, while PGE2 showed the lowest values.

3.2. Eicosanoids in experimentally infected cats

The levels of PGE2, TxB2 and LTB4 found throughout the study in experimentally infected cats are shown in Fig. 2. PGE2 significantly increased after infection, reaching the maximum 60 days p.i. ($p < 0.01$), then progressively decreased until the end of the study. On day 120 p.i. and day 180 p.i., PGE2 values were significantly higher when compared with pre-infection values ($p < 0.05$). Significant differences were also found between day 60 and day 120 p.i. ($p < 0.01$) and day 60 and day 180 p.i. ($p < 0.01$). Both TxB2 and LTB4 progressively increased after infection and showed

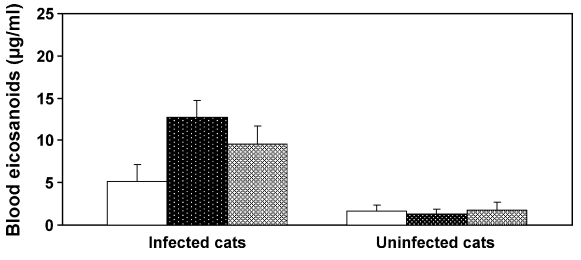

Fig. 1. Intravascular eicosanoid in *Dirofilaria immitis* naturally infected, seropositive cats and seronegative, uninfected cats. (□) Prostraglandin E_2. (■) Thromboxane B_2. (▨) Leukotriene B_4. Bars indicate the standard deviations. Statistical differences revealed by the non-parametric Kruskal–Wallis test: infected cats vs. uninfected cats: PGE2, $p < 0.05$; TxB2, $p < 0.01$; LTB4, $p < 0.01$.

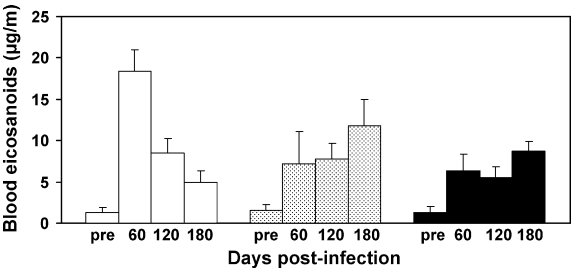

Fig. 2. Intravascular eicosanoids during experimental *Dirofilaria immitis* infections in cats. (□) Postraglandin E_2. (■) Thromboxane B_2. (▨) Leukotriene B_4; pre: before infection. Bars indicate standard deviations. Statistical difference revealed by Wilcoxon test for paired samples: PGE2, pre vs. 60 dyas p.i., $p < 0.01$; pre vs. 120 days p.i., $p < 0.05$; pre vs. 180 days p.i., $p < 0.05$; 60 vs. 120 days p.i., $p < 0.01$; 60 vs. 180 days p.i. TxB2 and LTB4, pre vs. 180 days p.i., $p < 0.05$. No significant differences were observed in other cases.

significant differences when pre-infection and 180 days p.i. values were compared ($p < 0.05$). On 180 days p.i. the mean values of each eicosanoid were similar to those found in naturally infected, seropositive cats.

3.3. Effect of pre-adult worms death on eicosanoids level

The effect of larval death on PGE2, TxB2 and LTB4 levels is shown in Fig. 3. The pattern of PGE2 in *D. immitis* infected, ivermectin-treated cats was similar to that found in infected, untreated-cats. PGE2 reached its maximum 15 days p.t. (45 days p.i.), afterwards the values progressively decreased until the end of the study. There were significant differences between values

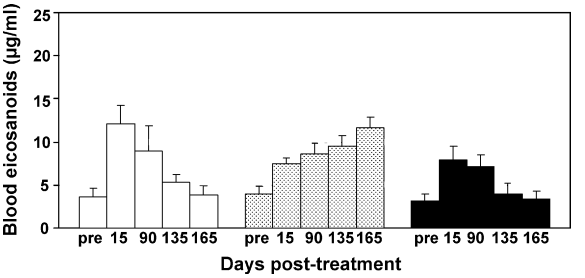

Fig. 3. Intravascular eicosanoids during experimental *Dirofilaria immitis* infections of cats treated with ivermectin 1 month after infection; pre: before infection. (□) Prostaglandin E_2. (■) Thromboxane B_2. (▨) Leukotriene B_4; pre: before infection; 15 days post-treatment (45 days post-infection); 90 days post-treatment (120 days post-infection); 135 days post-treatment (165 days post-infection); 165 days post-treatment (195 days post-treatment). Bars indicate standard deviations. Statistical differences revaled by Wilcoxon test for paired samples: PGE2, pre vs. 15 and 90 days p.t. (45 and 120 days p.i., respectively), $p < 0.05$; 15 days p.t. vs. 135 and 165 days p.t. (165 and 195 days p.i.), $p < 0.05$. TxB2, pre vs. 165 days p.t. (195 days p.i.), $p < 0.05$. LTB4, pre vs. 15 days p.t. (45 days p.i.), $p < 0.05$.

obtained before the infection and 15 and 90 days p.t. (45 and 120 days p.i., respectively) ($p < 0.05$), but not between 15 and 90 days p.t. Moreover, PGE2 levels observed at the end of the study (135 and 165 days p.t.) were significantly lower than those observed 15 days p.t. ($p < 0.05$). No significant differences were found between pre-infection values and values at the end of the study. TxB2 levels progressively increased until the end of the study (165 days p.t., 195 days p.i.; $p < 0.05$). A clear difference was found in LTB4 patterns betwenn treated cats and untreated cats. This eicosanoid significantly increased 15 days p.t. (45 days p.i.; $p < 0.05$), then decreased until the end of the study (165 days p.t./195 days p.i.). No significant differences were found between pre-infection values and values on day 165 p.t.

4. Discussion

Filarial worms are able to transform host fatty acids in active mediators that have a key role in the regulation of inflammation and immune response (Liu et al., 1990). Further studies related to the presence and the role of different eicosanoids during human filarial infections have been published by Liu and Weller (1990), Liu et al. (1992) and more recently by Brattig et al. (2006). Studies of their presence in heartworm infections is more limited (Morchón et al., 2006) and there are no studies, to our knowledge, in feline dirofilariosis.

Our results show that *D. immitis* infection in cats causes significant increases of PGE2, TxB2 and LTB4 levels. Furthermore, the levels of these eicosanoids were similar in naturally infected, seropositive cats and in experimentally infected cats 180 days p.i., probably because cats from both the groups were in the same phase of infection. In fact, in feline natural HW infection clinical signs are manly associated with the arrival of preadult worms in the pulmonary arteries, about 180 days after the bite of an infected mosquito. The high levels of TxB2 and LTB4 found in both groups are consistent with the development of the inflammatory and obstructive reactions which characterise feline heartworm infection once the pre-adult worms arrive into the pulmonary arteries, 5–6 months p.i. In fact, these eicosanoids stimulate vasoconstriction, platelet aggregation, chemotaxis and they increase of vascular permeability. Recently we have also found high levels of TxB2 in humans with pulmonary dirofilariosis (Morchón et al., 2006), that features local inflammation and obstruction of small pulmonary arteries. PGE2 values, which were lower than TxB2 and LTB4 in naturally infected, seropositive-cats (G1), were higher than the other two eicosanoids in experimentally infected cats (G2) 60 days p.i., indicating a time-dependent regulation of eicosanoids during HW infection. Several studies have suggested that PGE2 is involved in the survival mechanisms of filarial worms in immunocompetent hosts. Prostacyclin and PGE2 produced by microfilariae of *Brugia malayi* are involved in the inhibitory mechanisms of platelet aggregation and the PGE2 produced by *O. volvulus* seems to affect the metabolism and immune response of the host in favour of filarial survival, promoting a Th2 type-response (Brattig et al., 2006). The high levels of PGE2 in experimentally infected cats 60 days p.i. could be involved in the survival of larvae during the first phase of the infection in the cat, a host that reacts strongly against *D. immitis*. The death of adult worms exacerbates inflammatory reactions during feline dirofilariosis. Nevertheless, the death of larvae after an ivermectin treatment changes the pattern of both PGE2 and LTB4 production. In experimentally infected, ivermectin-treated cats, PGE2 showed lower levels than those observed in experimentally infected, untreated cats and LTB4 progressively increased reaching its maximum on day 135 p.t., 165 days p.i., while TxB2 is not significantly altered. This is probably due to the balance of the eicosanoids metabolism catalyzed by the cicloxigenase (Wilson et al., 2007). Because LTB4 promotes the recruitment of inflammatory cells, the decrease of this eicosanoid could be related to the decrease of severe inflammatory reactions and thrombi once the dead larvae are cleared. Moreover, we cannot exclude a relationship between the ivermectin treatment and the changes observed in the eicosanoids production.

At present, we do not know the role of *Wolbachia* in the stimulus of the eicosanoids. Nevertheless, ongoing studies in our laboratory indicate that endothelial cell cultures stimulated with *Wolbachia* surface protein (WSP) increase the production of both cyclooxygenase (COX-2) and lipoxygenase (5-LO), enzymes related to the production these eicosanoids. Moreover, in a previous study, we reported high levels of anti-WSP antibodies in the same infected cats employed in this study, which demonstra

te a strong immune stimulus by *Wolbachia* derived antigens (Morchón et al., 2004). We can hypothesize that *Wolbachia* bacteria released into the arteries from dying worms play a role in the increased levels of the eicosanoids.

In conclusion, we have found significantly increased levels of eicosanoids (PGE2, TxB2 and LTB4) in natural and experimental feline HW infection. The high levels of TxB2 and LTB4 detected 6 months post-

infection are consistent with the inflammatory and obstructive reactions that characterize the HW disease in cats, while the increase of PGE2 2 month post-infection could be related to the survival of parasites in the first phase of infection. In spite of this, the early death of larvae as a consequence of the ivermectin treatment seems to cause a decrease of LTB4.

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