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LEUKOGRAM AND NEUTROPHIL OXIDATIVE METABOLISM OF DOGS WITH VISCERAL LEISHMANIASIS BEFORE AND AFTER TREATMENT WITH MEGLUMINE ANTIMONIATE AND ALLOPURINOL

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ABSTRACT

Leukogram and neutrophil oxidative metabolism from dogs with visceral leishmaniasis (VL) were compared before and after treatment with meglumine antimoniate (AM) and with an association of meglumine antimoniate and allopurinol (AMA). The results obtained demonstrated that neutrophils of dogs with VL do not lose their capacity of reducing NBT and that oxidative metabolism has been more active in the majority of the cases. After treatment with AM and AMA, dogs with VL presented a redution of neutrophils oxidative metabolism, suggesting that this decrease was related with the decrease in the number of parasites and a probable inhibitor effect of these drugs on neutrophils oxidative metabolism

KEYWORDS: Glucantime, Leishmania sp, nitroblue tetrazolium (NBT), neutrophil functions.

RESUMO

LEUCOGRAMA E METABOLISMO OXIDATIVO DOS NEUTRÓFILOS DE CÃES COM LEISHMANIOSE VISCERAL ANTES E APÓS O TRATAMENTO COM ANTIMONIATO DE MEGLUMINA E ALOPURINOL

Compararam-se o leucograma e o metabolismo oxidativo dos neutrófilos de dez cães com leishmaniose visceral (LV) antes e após o início do tratamento com antimoniato de meglumina (AM) e com a associação de antimoniato de meglumina e alopurinol (AMA). Os resultados obtidos comprovaram que os neutrófilos de cães com LV não perdem sua capacidade de reduzir o tetrazólio nitroazul (NBT) e, na maioria dos casos, o metabolismo oxidativo apresentou-se mais ativo que o normal. Cães com LV apresentaram uma diminuição do metabolismo oxidativo dos neutrófilos após o tratamento com AM e AMA. O conjunto dos resultados sugere que a diminuição da taxa de redução do NBT está relacionada com a diminuição da carga parasitária de leishmania e a um possível efeito inibidor dessas drogas sobre o metabolismo oxidativo dos neutrófilos.

PALAVRAS-CHAVES: Função neutrofílica, glucantime, Leishmania sp., tetrazólio nitroazul (NBT).

INTRODUCTION

Canine visceral leishmaniasis (VL) has as ethiologic agents protozoa of the gender Leishmania, which, after penetrating the vertebrate host´s organism, replicate inside macrophages, disseminating by hematogen and lymphatic vias (INIESTA et al., 2002).

Although there are many researches about the disease pathogeny, specially the ones directed to cellular immune response, a few studies pointed out the function of neutrophils during the infection, regarding particularly its oxidative metabolism. Neutrophils participate directly of the infection control, at the initial stage of the disease; however, they do not seem to have and anti-parasitic function at such phase (ROUSSEAU et al., 2001). Studies carried out in experimental models demonstrated that resistant animals eliminate the parasite quickly, at the beginning of the infection, due to the participation of a great number of neutrophils. Even though they are able to internalize promastigotes forms of Leishmania sp., they are not considered host cells, because the parasite does not multiplicate inside such polymorphonuclears (SMELT et al., 2000; AGA et al., 2002). Leukocytosis has been associated to the clinical improvement of dogs with leishmaniases (BOURDOISEAU et al., 1997), being monocytosis a common finding, usually with the presence of big active monocytes, some of them infected (BURACCO et al., 1997; IKEDA et al., 2003).

Neutrophils interact with macrophages (RIBEIRO-GOMES, 2007), and present fundamental function in regulating citokine balance in the infection site, contributing to the development of specific immunity and death of *Leishmania donovani* at the initial phase of the infection (MCFARLANE et al., 2008). Besides, experimental models and neutrophil depletion are associated to an increase of the parasitary load of *L. donovani* in the spleen and the liver (SMELT et al., 2000).

The mechanisms which affect the oxidative metabolism of neutrophils has not been completed explained; however, it is acknowlegde that the acid phosphatase isolated from the promastigote surface of *L. donovani* inhibits the neutrophil oxide production in humans (SAHA et al., 1985). In the same manner, dogs infected by *Leishmania* sp. present a significant reduction in the neutrophils oxidative metabolism (BRANDONISIO et al., 1996; VUOTTO et al., 2000). When the oxidative metabolism is reduced, *Leishmania major* may survive inside the neutrophils and the activation of this mechanism is associated to the parasite intracellular elimination (LAUFS et al., 2002).

The most used protocol in the treatment of humans with VL is the administration of the meglumine antimoniate, which is distributed exclusively by the Brazilian Ministry of Health. This medicine is also used in Mediterranean countries to treat dogs with the infirmity as a single protocol, or in association with allopurinol. The exact activity mechanism of antimonials is not known; however, BERMAN et al. (1985) observed that its leishmanicidal activity is associated to the inhibition of glycolysis of the *Leishmania citric* acid cycle. It is possible that such mechanism may also compromise the glycolysis pathway of neutrophils, causing the inhibition of its oxidative metabolism. RAIS et al. (2000) observed that rats and humans treated with antimonial have the superoxide production improved. According to these authors, the increase of superoxide production in neutrophil, after the treatment, is associated to the decrease of the parasitary load and consequently the disappearance of the antigen inhibing acitivity on the oxidative metabolism of the host´s cells. In animals with visceral leishmaniasis, the life time of a neutrophil is smaller; nevertheless, studies indicate that the treatment with meglumine antimoniate increases the life time of these polymorphonuclears (GUARGA et al., 2002).

According to what was exposed, the present study aimed at evaluating the leukogram and the oxidative metabolism of the neutrophils of dogs with VL, before and after the treatment with meglumine antimoniate and allopurinol.

MATERIAL AND METHODS

After the research aproval by the Animal Ethics Comitee (Aproval 18/2 FOA-UNESP), twelve dogs positive for visceral leishmaniasis were selected. Leishmaniasis was confirmed by cytological exams of lymph nodes and bone marrow aspiration punction and serology by the use of the immune enzymatic method (ELISA). To avoid the risk of contaminating the other animals or human beings, all dogs wore an anti-parasitic collar of deltrametrine (Scalibor®, Intervet Production) and were kept in a closed, wire-netting cannel.

Dogs were randomly divided into two groups.

The first group (MAA), with six dogs, was treated with a combination of meglumine antimoniate (Glucantime®, Aventis Pharma), at the dose of 75 mg/kg, subcutaneously, each 12 hours for a period of 21 days, associated with allopurinol (Aluporinol, Hexal AG) at the dose of 10 mg/kg, orally, each 12 hours during three months. The second group (MA), six dogs, was treated with meglumine antimoniate, at the dose of 75 mg/kg, subcutaneously, each 12 hours for a period of 21 days. Blood samples were collected from all the animals in three different moments: M1 (before the treatment), M2 (30 days after the beginning of the treatment) and M3 (60 days after the beginning of the treatment).

A 10 ml blood sample was collected from each animal with hypodermic needles and disposable syringes. A 0.5 mL volume was acconditioned in sterile plastic tubes with 10 U of sodic heparin (liquemine®), to perform the test of NBT reduction, and a 4.5 mL volume of blood in glass flasks with 5 mg of sodic EDTA to perform the leukogram. The rest of the sample (5 mL) was used for leishmaniasis serologic test. Blood samples were kept refrigrated until the moment of laboratorial processing (until two hours after the collection).

The punction of the bone marrow in the animal´s iliac crest was also carried out. Aspirative punction was also performed in the popliteo lymph node or in the one which presented greatest volume.

For the research of the amastigote forms of *Leishmania* sp. in the sample collected from the bone marrow (iliac crest) and lymph node, smears stained with diff quick (Instant-Prov, Newprov) were performed. ELISA test was used according to prescriptions by LIMA et al. (2001) in order to observe the presence of anti-*Leishmania* sp antibodies in the serum.

Tests of NBT spontaneous and stimulated reduction were carried out according to description by CIARLINI et al. (2004). To do so, buffered NBT (NBT- vial, SIGMA diagnostic) and stimulant composed of bacterial extract (Stimulant, SIGMA diagnostic) were used. The percentage of NBT-reducing cells was established from the counting of 100 neutrophils.

Total leukocyte cells counting was carried out with an electronic automatic blood cell-counting device (CC530 vet, CELM), and differential leukocyte counting was performed in blood smears stained with diff quick (Instant-Prov, Newprov), according to the recommendations by the manufacturer and criteria by LASSEN & WEISER (2004).

At the end of the experiment, following recommendations by the Ministry of Health, all dogs were slaughtered with pentobarbital sodium (15 mg/kg intravenously), followed by 10 mL potassium chloride ampoule at 19.1%.

By the use of a statistical software (GraphPad InStat, v.3.05), after the study of the distribution of all variables regarding normality (Kolmogorov-Smirnov test) and homoscedasticity (Bartlett test), Friedman test and Dunn post-test were applied for the comparisons between the experimental moments

RESULTS AND DISCUSSION

Two dogs of the MA group were excluded and submitted to euthanasia because they presented severe pneumopathy, apparently not associated to VL or to the medication. The clinical and laboratorial evaluation of dogs in this study was recently described by IKEDA-GARCIA et al., 2007, showing that the treatment promoted the remission of the clinical signs and the retur of urea and serum albumin to normal levels, although there was a transitory hepatotoxicity.

In this study, any amstigote form of *Leishmania* sp. internalized in the neutrophils was found, although it has been reported in vitro (LAUSF et al., 2002) and in vivo (IKEDA et al., 2003), confirming the statements by SMELT et al. (2000) and AGA et al. (2002), that the parasite does not multiply inside these polimorphonuclears.

Before the beginning of the treatment (M1), leukocyte counting of both groups (MAA and MA) was within the normality values considered by MEINKOTH & CLINKENBEARD (2000) for the canine species (Tables 1 and 2). The absence of remarkable leukocyte alterations in the dogs with leishmaniasis in this study confirm previous reports (IKEDA et al., 2003) that the leukogram is not frequently altered by CVL. Nevertheless, the absence of high rates of monocytes before the

treatment differes from provious study carried out in Araçatuba, SP, where monocytosis occured in 33.5% of dogs with CVL (IKEDA et al., 2003). Such finding contradicts the statements by BURACCO et al. (1997), that dogs with VL probably express differences regarding the infection phases, parasite-host relation, as well as they are a result of possible co-infections not diagnosed in the different studies.

The leukogram of dogs with VL submitted to treatment wih antimonial and allopurinol did not present significant differences among the initial treatment, 30 and 60 days after treatment (Table 1). This result contradicts the hypothesis by BOURDOISEAU et al. (1997) that leukocytosis is associated with the clinical improvement of dogs with leishmaniasis. On the other hand, 30 days after the treatment with MA, a significant increase of leukocytes, neutrophils ans monocytes total counting was observed (Table 2). The increase of the number of neutrophils in dogs treated with MA was also observed by GUARDA et al. (2002), supporting the hypothesis that the treatment with meglumine antimoniate raises the time of life of such polimorphonuclears. However, after 60 days, neutrophilia and monocytosis were no longer observed, suggesting that the leukocytes variation is a transitory response associated to the initial phase of higher destruction of *Leishmania* sp. Besides, the neutrophil directly and significantly acts on the infection control at the initial phase of leishmaniasis (ROUSSEAU et al., 2001; RIBEIRO-GOMES, 2007; MCFARLANE et al., 2008), the neutrophilia observed in the first thirty days of treatment suggests that these cells may also have an important role at the unespecific response during the initial phase of the leishmanicyde treatments.

TABLE 1. Mean values (x) and standard deviation (s) of the leukogram and the percentage (%) of NBT-reducing neutrophils at the non-stimulated (NS) and stimulated (S) test of dogs with visceral leishmaniasis, before the treatment with meglumine antimoniate associated with allopurinol (M1), 30 (M2), and 60 (M3) days after the treatment

	M 1	M2-	M3
	x ± s	x ± s	x ± s
NBT-NE (%)	21,4 ± 25,39 [a]	2,2 ± 2,17 [b]	5,0 ± 4,06 [ab]
NBT-E (%)	28,8 ± 12,78 [a]	9,0 ± 5,48 [ab]	6,6 ± 6,58 [b]
Leukocytes ($\times 10^9$/L)	8,96 ± 2,91 [a]	10,68 ± 2,91 [a]	9,72 ± 2,44 [a]
Segmented ($\times 10^9$/L)	5,05 ± 2,59 [a]	6,11 ± 2,43 [a]	5,51 ± 2,86 [a]
Lymphocytes ($\times 10^9$/L)	2,59 ± 1,01 [a]	2,81 ± 1,31 [a]	3,00 ± 2,03 [a]
Monocytes ($\times 10^9$/L)	0,58 ± 0,49 [a]	0,99 ± 0,54 [a]	0,57 ± 0,27 [a]
Eosinofils ($\times 10^9$/L)	0,58 ± 0,24 [a]	0,48 ± 0,42 [a]	0,69 ± 0,52 [a]

*Non-coincident letters at the same line indicate significant difference ($P<0.05$).

Before receiving the treatments with MAA or MA, 40 and 50% of the dogs with leishmaniasis, respectively (Tables 1 and 2), presented neutrophilic rates of NBT spontaneous reduction (non-stimulated test) superior than the rates considered normal for the species, as described by CIARLINI et al. (2004). At the initial phase, after being stimulated, 80% of the dogs from MAA group and 75% of the dogs from MA group presented an increase of NBT reduction rates (Tables 1 and 2). The association of the increase of the neutrophils oxidative metabolism with Leishmania sp. was also observed in vitro (LAUFS et al., 2002), and it suggests that the inhibition of the production of human neutrophils superoxide, caused by acid phosphatase of the membrane of *L. donovani* promastigote form (SAHA et al., 1985), does not occur in CVL. Such results contradic the observations by BRANDONISIO et al. (1996) and VUOTTO et al. (2000), who verified

a significant decrease in neutrophils oxidative metabolism of dogs naturally infected with *Leishmania* sp. It is noteworthy that differently from the cytochemical method adopted in this study, BRANDONISIO et al. (1996) and VUOTTO et al. (2000) used the method of superoxide quantification by cytochrome and the chemiluminescent method, respectively. These methods require the isolation of neutrophils from total blood and, thus, they alter the membrane receptors responsible for activating the oxidative metabolism, besides protecting the isolated cell from the influence of important cellular and plasmatic components contained in the total blood of dogs with VL. Considering that the animals in this experiment did not present signs of secondary infection, it can be assumed that the high percentage of NBT reduction observed before the treatment is due to the increase of neutrophil oxidative metabolism induced by the presence of the parasite. The results of the present study demonstrate that the neutrophils of dogs with VL have a functional oxidative metabolism capable of producing great quantities of superoxide and reducing the NBT, possibly suggesting that the immune suppressor capacity of *Leishmania* sp. is associated to other mechanisms.

TABLE 2. Mean values (x) and standard deviation (s) of the leukogram and the percentage (%) of NBT-reducing neutrophils at the non-stimulated (NS) and stimulated (S) test of dogs with visceral leishmaniasis, before the treatment with meglumine antimoniate associated with allopurinol (M1), 30 (M2), and 60 (M3) days after the treatment

	M1	M2	M3
	x ± s	x ± s	x ± s
NBT-NE (%)	10,3 ± 9,61[a]	2,3 ± 2,63[a]	1,8 ± 2,22[a]
NBT-E (%)	16,3 ± 12,61[a]	8,8 ± 13,6[a]	3,3 ± 2,99[a]
Leukocytes ($\times 10^9$/L)	6,5 ± 0,82[a]	20,5 ± 13,1[b]	9,2 ± 2,07[ab]
Segmented ($\times 10^9$/L)	4,9 ± 0,85[a]	14,2 ± 9,47[b]	5,5 ± 2,39[ab]
Lymphocytes ($\times 10^9$/L)	1,1 ± 0.17[a]	2,8 ± 0,58[a]	2,5 ± 0,58[a]
Monocytes ($\times 10^9$/L)	0,2 ± 0,07[a]	1,8 ± 1,78[b]	0,5 ± 0,15[ab]
Eosinofils ($\times 10^9$/L)	0.2 ± 0.22[a]	0.7 ± 1.30[a]	0.6 ± 0.40[a]

*Non-coincident letters at the same line indicate significant difference ($P<0.05$).

RAIS et al. (2000) observed that the use of anitomonial drugs in human beings and rats increase the oxidative metabolism of neutrophils due to the decrease of the parasitary load. However, in the present study, after 30 and 60 days of treatment with MA and MAA, no animal presented high values of NBT reduction in the non-estimulated test, and the lowest rates of NBT-reducing neutrophils coincided with the clinical improvement and the decrease of the parasitary load of the dogs in post-treatment (Tables 1 and 2). There are evidences that the neutrophil capacity to reduce NBT after treatment is related to other mechanisms, besides the decreasing of the parasitary load. Thirty and sixty days after the beginning of the treatment, 25% and 75% of the dogs treated with MA, respectively, and 20% and 40% of the dogs treated with MAA, respectively, presented spontaneous rates of NBT reduction (NBT-NS). The stimulated neutrophil capacity to reduce NBT (NBT-S) also decreased in both treatments, with lower values at sixty days in group MA. As a coincidence, 90% of the animals presented clinical improvement and became asymptomatic after treatment with MA and MAA. The low values of NBT reduction at the post-treatment period suggest that a possible inhibiting effect of MA and MAA on the neutrophil metabolism. It is likely that the inhibition of glycolysis and citric acid cicle of the parasite caused by antimonials (BERMAN et al., 1985) may also compromise the glycolisys of the neutrophil, causing the inhibition of its oxidative metabolism. Neutrophils undergo a "respiratory explosion" when they are activated, causing a great oxigen and glucose uptake (CIARLINI et al., 2001), so that the inhibiting activity of antimonials on the glycolysis of neutrophils may reduce the superoxide production

and, hence, the NBT reduction rates, as it could be verified in the present study.

Considering the adopted treatments contributed to the clinical improvement but not to the complete elimination of the infection, it is necessary to study whether the limits of the protocols currently prescribed for the CVL treatment are associated to a possible inhibiting effect of MA and MAA on the oxidative metabolism of the neutrophil.

CONCLUSION

Neutrophils of dogs with visceral leishmaniasis present a functional oxidative metabolism capable of producing great quantities of superoxide and reducing NBT. The treatment with meglumine antimoniate and allopurinol in dogs naturally infected by Leishmania sp. promote a reduction of the oxidative metabolism of neutrophils, which coincides with the clinical improvement and the decrease of the parasitary load.

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