Serum TNF-α and IL-10 in *Ehrlichia* spp. Naturally Infected Dogs

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ABSTRACT

**Background:** Canine ehrlichiosis (CE) is a disease caused by *Ehrlichia canis*, transmitted by *Rhipicephalus sanguineus* and dogs can present variable clinical manifestations, ranging from subclinical, acute to chronic illness. This pathogen preferentially infects leukocytes. In Brazil illness is described nationwide with the seropositivity varying from 4.8% to over 50%. The control of *Ehrlichia* spp. infection was related to cellular immune response mediated by Th1 while the CE acute phase is related to humoral immune response mediated by Th2, with high production of antibodies. Th1 and Th2 balance can be measured by cytokine profile. TNF-α is associated with inflammatory responses while IL-10 inhibits mechanisms which prevent tissue damage caused by the intense inflammatory response. The aim of this study was to evaluate the TNF-α and IL-10 serum levels in naturally infected dogs by *Ehrlichia* spp.

**Material, Methods & Results:** Twenty five dogs, both sexes, one to eight years old, weighing 5 to 40 kg, undefined breed were divided in two groups: Control (CT, n = 14) and naturally infected by *Ehrlichia* spp. dogs (EH, n = 11). Naturally infected dogs were from Zoonosis Control Center. *Ehrlichia* spp. morulae was visualized in bone marrow aspirates. Blood samples were collected for hematological analysis and serum dosages of TNF-α and IL-10 (commercial kits by sandwich ELISA). The frequency of clinical signs was expressed as percentage. *t* test, Spearman correlation and linear regression were used when necessary (\(P \leq 0.05\)). Alopecia (45%), generalized lymphadenopathy (36%), onychogryphosis (27%) and conjunctivitis (27%) were the most frequent clinical signs in the EH group. No difference was found between studied groups in hematological and biochemical parameters. TNF-α concentration was 43.3 ± 16.9 pg/mL in CT and 41.1 ± 3.8 pg/mL in EH, while IL-10 concentration was 20.2 ± 8.4 pg/mL in CT and 21.0 ± 5.1 pg/mL in EH. No significant differences were found between serum levels of these cytokines. Weak negative correlation (\(r = -0.12\)) between cytokine in CT and weak positive correlation (\(r = 0.07\)) in EH were observed.

**Discussion:** Parasite morulae visualization in blood smears is conclusive for CE diagnosis; however, *E. canis* can be detected for a short period in monocyte. According to that, EH dogs were all in acute phase of disease. Animals did not present anemia, leukocytosis or thrombocytopenia, which can be justified by the early infection associated with the absence of clinical signs in EH. As reported, thrombocytopenia would be an important finding in endemic areas for CE. Our results did not show differences in serum TNF-α and IL-10 between the groups. Similar results were observed in mice infected by *E. chaffeensis*. TNF-α and IL-10 are cytokines related to Th1 and Th2 cells activities, respectively. Th1 response has been associated with ehrlichiosis protection in mice mediated by INF-γ, but not TNF-α. Lastly, the correlation and regression analysis between TNF-α and IL-10 showed that the presence of *Ehrlichia* spp. infection alter the regulation mechanism of these cytokines. It suggests that the interactions parasite-host modulates the specific immune response. Furthermore, we can infer that in early ehrlichiosis stage the serum cytokines TNF-α and IL-10 are not associated with clinical manifestations of the disease. Others mediators may be investigated to evaluate the host immune response. To our knowledge, this is the first report of TNF-α and IL-10 in naturally infected dogs by *E. canis*.

**Keywords:** canine ehrlichiosis, TNF-α, IL-10, biomarkers.
INTRODUCTION

Canine ehrlichiosis (CE) is a disease caused by microorganisms of the *Ehrlichia canis* species transmitted by *Rhipicephalus sanguineus* [1]. The seropositivity varies from 4.8% to 70% all over the world and many dogs in Brazil are affected by the illness [1,23,26,29,31]. This disorder has a variety of clinical manifestations that can present as acute, subclinical or chronic [27,33] with diverse clinical signs [3,18,21]. Hematological findings in experimental CE includes anemia, leukocytosis and thrombocytopenia [5,20]. In endemic areas for CE, thrombocytopenia can be a strong indicator of infection [4], since anti-platelet antibodies are found in the acute phase of the disease [11].

It has been reported that the humoral immune response, mediated by Th2 cells, with high production of antibodies, characterize the acute phase of the disease [9,14,31], while the cellular immune response mediated by Th1 cells is related to control of *Ehrlichia* spp infection. [15]. Th1 and Th2 balance has been studied in many pathologies and their activities are measured through the cytokines.

Tumor Necrosis Factor (TNF-α) is a cytokine with inflammatory actions that modulates the immune response [7] while IL-10 is another cytokine that inhibits mechanisms that preventing tissue damage caused by exacerbated inflammatory responses [22]. TNF-α was detected in dogs with severe acute ehrlichiosis caused by *E. canis* and in mice infected by *E. chafeensis* had a TNF-α peak production two days prior to death caused by infection [15,16].

To understand the role of cytokines in CE, this study aimed to evaluate serum levels of TNF-α and IL-10 in naturally infected dogs by *Ehrlichia* spp.

MATERIALS AND METHODS

Study population

Dogs (n = 25), weighing 5 to 40 kg and aged one to eight years old, both sexes, undefined breed, were used and divided into two groups: Control (CT, n = 14) and Naturally infected dogs by *Ehrlichia* spp. (EH, n = 11). CT group was composed of dogs from private owners in Fortaleza, Ceará, submitted to clinical evaluation by a veterinarian, followed by laboratory tests. Previously, the owners were informed about the procedures to be performed according to responsibility term of the research.

Naturally infected dogs by *Ehrlichia* spp., from Zoonosis Control Center of Fortaleza-Ceará, were included in EH group. *Ehrlichia* spp. infection was confirmed by the parasite morulae visualization in bone marrow aspirates smears. All animals were negative for *Leishmania* infection in immunoassay Dual Path Platform (DPP®) and bone marrow aspirate.

Cachexia, onychogryphosis, conjunctivitis, alopecia, ulcerative lesions, dermatitis and generalized lymphadenopathy were the clinical signs evaluated. Scores were assigned from 0 to 2, where 0 = absent, 1 = mild and 2 = severe. Dogs with scores above six were considered symptomatic for CE [24].

Blood sampling

Blood sample from all animals was collected by venipuncture and distributed into vials containing anticoagulant ethylene-diamine-tetraacetic-acid (EDTA) for hematological evaluation that was performed at Laboratory of Clinical Pathology, Faculty of Veterinary Medicine of the State University of Ceará, using an automation hematologic veterinary machine². Peripheral blood smears were prepared for differential leukocytes count, hematozoa research and observation of cellular alterations. To obtain serum, blood sample was collected without anticoagulant then sera aliquots were stored at -80°C for total protein and cytokine dosages.

Cytokine dosages

TNF-α and IL-10 levels were detected in sera using commercial kits³ by sandwich ELISA following the manufacture instructions. Canine TNF-alpha DuoSet (DY1507)³ and Canine DuoSet IL-10 (DY735)³ kits were used.

Statistical analysis

The frequency of clinical signs was expressed as percentage. Statistical analysis was done by one way repeated measures analysis of variance (ANOVA). To detect differences between the groups (CT and EH), Student’s unpaired *t*-test was used for IL-10 and TNF-α concentrations and hematological parameters. The level of significance was set at *P* < 0.05. Statistical analysis were performed using the PRISM package (GraphPad Software Inc., San Diego, CA). The relation between different parameters was evaluated by correlation and linear regression.
RESULTS

The most frequent clinical signs in EH animals were alopecia (45%), generalized lymphadenopathy (36%), onychogryphosis (27%) and conjunctivitis (27%), while in CT group were observed alopecia (29%) and generalized lymphadenopathy (29%) (Figure 1).

Hematological and biochemistry results were related at Table 1. The results of blood cells count of EH did not differ significantly from the CT group. EH animals showed neither anemia or leukocytosis/leucopenia nor thrombocytopenia. The parameters were evaluated according to reference values for the canine species [19].

Cytokines level were presented at Figure 2. The values of TNF-α concentrations for CT (43.3 ± 16.9 pg/mL) did not differ for EH (41.1 ± 3.8 pg/mL). In relation to IL-10, there was no significant difference between groups (CT = 20.2 ± 8.4 pg/mL; EH 21.0 ± 5.1 pg/mL). A weak negative correlation were observed in CT (ρ = -0.12) [Figure 3a] and a weak positive correlation in EH (ρ = 0.07) [Figure 3b]. TNF-α showed a negative influence on IL-10 in both groups (y = 22.4 – 0.04x and y = 26.32 – 0.13x).

<p>| Table 1. Hematological and biochemical parameters of control dogs (CT) and naturally infected dogs by Ehrlichia spp. (EH). |</p>
<table>
<thead>
<tr>
<th>Parameter (unit)</th>
<th>CT (X ± SD)</th>
<th>EH (X ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hematological</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Red blood cells (x10^3/dL)</td>
<td>7.06 ± 0.8</td>
<td>6.35 ± 1.42</td>
</tr>
<tr>
<td>Hemoglobin (g/dL)</td>
<td>16.88 ± 1.9</td>
<td>14.89 ± 4.2</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>48.07 ± 4.4</td>
<td>43.84 ± 11.9</td>
</tr>
<tr>
<td>White blood cells (x10^3/dL)</td>
<td>15.22 ± 5.2</td>
<td>14.99 ± 13.1</td>
</tr>
<tr>
<td>Neutrophil (%)</td>
<td>66.57 ± 10.5</td>
<td>76.27 ± 10.3</td>
</tr>
<tr>
<td>Lymphocyte (%)</td>
<td>20.79 ± 8.9</td>
<td>13.18 ± 8.2</td>
</tr>
<tr>
<td>Eosinophil (%)</td>
<td>6.79 ± 3.8</td>
<td>3.91 ± 5.7</td>
</tr>
<tr>
<td>Monocyte (%)</td>
<td>5.86 ± 3.06</td>
<td>6.55 ± 4.2</td>
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<tr>
<td>Platelets (x10^3/dL)</td>
<td>213.4 ± 64.8</td>
<td>172.5 ± 89.6</td>
</tr>
<tr>
<td>Biochemical</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total protein (g/dL)</td>
<td>6.87 ±1.10</td>
<td>7.11 ± 1.6</td>
</tr>
</tbody>
</table>

Figure 1. Frequency of clinical signs observed in control dogs (CT) and naturally infected dogs by Ehrlichia spp. (EH).

Figure 2. Serum concentrations of TNF-α and IL-10 in control dogs (CT) and naturally infected dogs by Ehrlichia spp. (EH).
DISCUSSION

Canine ehrlichiosis (CE) is a disease that affects many organs and tissues, with varied clinical manifestations [3,21,30]. In this study, the most frequent clinical signs in EH were alopecia, generalized lymphadenopathy, onychogryphosis and conjunctivitis (Figure 1). These data are consistent with other studies that showed apathy, anorexia, fever, vomiting, alopecia, ocular nasal secretion, splenomegaly, pale mucous membranes, petechiae, gastroenteritis, epistaxis and uveitis as CE clinical findings [3,5,13,18,20,21,28]. There are reports that apathy and reductions in ingestion of food are associated with the body temperature increased in dogs with ehrlichiosis [25]. Regarding the categorization of clinical signs, in the present study we found only one EH animal with symptomatic clinical scores equal to eight.

All EH animals showed Ehrlichia spp. morulae in bone marrow aspirate smears. This visualization is conclusive for CE diagnosis [13]. It is known that E. canis morulae can only be detected in a short period of time in monocytes, not being found during the subclinical or chronic stages of the disease [21]. These data reflects that EH dogs were more likely in an early clinical stage of the disease, because the microorganism replication, with morulae formation, occurs 7 to 12 days following infection [2] and the incubation period is 8 to 20 days [20].

In this study, the values of red blood cell count (RBC), hematocrit (HT) and hemoglobin (HB) did not differ between EH and CT. Studies with E. canis experimentally infected dogs, were observed decrease of RBC, HT and HB two weeks after infection with posterior normalization on day 50 [5,25]. Normocytic and normochromic anemia was reported in 84% of naturally infected dogs [3], characterizing a chronic infectious disease. This situation was reported in other chronic diseases [8]. Anemia or pancytopenia are laboratorial findings in the chronic phase of CE, however mild decrease in the RBC can occur in acute stage [12]. Thus, this data indicates early stages of CE in EH group.

Our results showed that white blood cells count did not differ statistically between CT and EH. Leukocyte cells changes were reported only in symptomatic and naturally infected dogs by E. canis [18]. No changes were found in platelet counts in EH and this data can be justified by the recent infection associated to absence of clinical signs in EH group. As reported, thrombocytopenia would be an important finding in endemic areas for CE [4,6]. Some authors describe thrombocytopenia seven days after natural infection and two weeks after experimental studies [5,25].

TNF-α and IL-10 are cytokines related to Th1 and Th2 cells activities, respectively. In our study, TNF-α and IL-10 serum levels did not differ between groups. To our knowledge, this is the first report of TNF-α and IL-10 in naturally infected dogs by E. canis. The infection control of intracellular parasites has been strongly associated with cellular immune response [9,32]. TNF-α is important to control intracellular infections, however at high concentrations it may cause toxic shock syndrome [15]. In animal models of infection by Ehrlichia spp. the increase in TNF-α serum
is strongly associated with severe and fatal disease, while a lower production of this cytokine was associated with a prolonged survival of the animals [15]. In an experimental study, dogs showed a slight increase in the TNF-α production, with exception of one dog that peaked relevant production 21 days after infection and died after declining levels of cytokine on day 42 [25]. Ehrlichiosis protection in mice was associated with a Th1 response mediated by the increase of INF-γ but not TNF-α [15].

For *Ehrlichia* spp. infection control the body requires signaling for cellular immune responses, which cannot be produced in excess and consequently to be deleterious to the host. Then, we studied IL-10 serum levels, that is a key cytokine to limiting strong Th1 immune responses and it has central importance during the inflammation resolution [17,22]. In our work, IL-10 serum levels did not differ between groups. Similar results were observed in mice infected by *E. chaffeensis*, where their serum levels did not increase [10]. However, in experimental CE, it emphasizes an increase of IL-10 and TNF-α in the same dog [25]. As our animals were probably in the early stage of the disease the TNF-α levels were not increased, consequently the IL-10 levels were not increased as well.

The association between TNF-α and IL-10 showed that *Ehrlichia* spp. infection can induce alterations on cytokines regulation. It suggests that the microorganism, or the inoculation mechanism, interferes in the host immune response, favoring the disease establishment. Furthermore, this work demonstrates that CE early stages show no clinical manifestations, associated with changes in the TNF-α and IL-10 serum concentrations.

**CONCLUSIONS**

Based on these results, we can infer that in the ehrlichiosis early stage the cytokines TNF-α and IL-10 are not associated with clinical manifestations of the disease. In this view, additional studies should be performed, including the dosage of other cytokines and long term follow-up of infected dogs. Thus, cytokines can be used as a biomarker for evaluate the different stages in CE and hence contribute to understanding of the immunopathogenesis of disease.

**MANUFACTURERS**

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**Ethical approval.** This study was approved by Ethic Committee for Animal Use of the State University of Ceará (CEUA/UECE), protocol nº 12641308-8.

**Declaration of interest.** The authors declare that there is no conflict of interest.

**REFERENCES**


