Cytokine Gene Expression by Peripheral Blood Leukocytes in Dogs Experimentally Infected with a New Virulent Strain of *Ehrlichia canis*

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ABSTRACT: *Ehrlichia canis* (*E. canis*) is a lipopolysaccharide (LPS)-deficient obligatory intracellular bacterium that causes canine monocytic ehrlichiosis, a chronic febrile disease accompanied with hematological abnormality. This study analyzed temporal expression levels of IL-1β, IL-2, IL-6, IL-8, IFN-γ, and TNF-α mRNA by peripheral blood leukocytes from dogs experimentally infected with a new virulent strain of *E. canis* by using real-time RT-PCR. Relative levels of IL-1β and IL-8 transcripts normalized by the β-actin transcript levels, were significantly upregulated, whereas those of TNF-α and IFN-γ transcripts were only weakly upregulated in all three infected dogs, starting from 2 days up to 52 days post inoculation. The expressions of IL-2 and IL-6 genes were extremely low compared with the positive control (ConA-stimulated canine peripheral blood leukocytes). This study showed that *E. canis* can induce chronic expression of a subset of proinflammatory cytokine genes: balance, timing, and duration of these cytokine generations may contribute to the progression of canine ehrlichiosis.

KEYWORDS: *E. canis*; IL-1; IL-8; dog

INTRODUCTION

*Ehrlichia canis* (*E. canis*) is the causative agent of canine monocytic ehrlichiosis (CME) with tropism for monocytes and macrophages. CME has a worldwide distribution with a higher frequency in tropical and subtropical regions.1 The disease may be manifested by fever, depression, dyspnea, anorexia,
hemorrhage, edema, and slight weight loss accompanied with laboratory findings of thrombocytopenia, leukopenia, mild anemia, and hypergammaglobulinemia. The pathological and immunological pathways induced by *E. canis* in dogs are not well defined. Low levels of ehrlichemia and nature of clinical signs in canine ehrlichiosis suggest that the host immune response, including proinflammatory cytokine production, may play a pivotal role in the pathogenesis of ehrlichiosis. Overall, generation of cytokines and chemokines by the members of family *Anaplasmataceae* appears to be different from those by lipopolysaccharide (LPS), corroborating the recent report of absence of genes for biosynthesis of LPS among members of the family *Anaplasmataceae*. The involvement of cytokines in pathogenesis of canine ehrlichiosis has not been elucidated. Analyses of cytokines that mediate immune response and inflammation may provide better understanding of pathogenesis of canine ehrlichiosis. The purpose of this study was to analyze several cytokine gene mRNA expressions by PBLs from dogs experimentally infected with a virulent strain of *E. canis* by using quantitative RT-PCR.

**MATERIALS AND METHODS**

*Experimental Infection of Dogs*

Seven specific pathogen-free (SPF) female beagle dogs weighing from 6/7 kg to 12/13 kg were used. All dogs were tested free of infection with *E. canis* and other members of the family *Anaplasmataceae*. Three dogs (dog 1, 2, and 3) were inoculated with total of 0.5 mL *E. canis* New Mexico–infected canine blood (0.2 mL intradermally and 0.3 mL subcutaneously). Blood samples were collected periodically from the cephalic vein for indirect immunofluorescent assay (IFA) tests, RT-PCR assays, complete blood counts, and PCR assays. Rectal temperature, appetite, attitude, and any clinical signs were recorded daily and were used to monitor *E. canis* infection status with combination of PCR and IFA as previously described. Dogs 4 and 5 were kept as uninfected controls without any inoculation. Dogs 6 and 7 were inoculated with total of 0.5 mL uninfected canine blood (0.2 mL intradermally and 0.3 mL subcutaneously) and used as inoculation controls.

*Real-Time PCR and RT-PCR*

Real-time PCR was carried out to determine levels of *E. canis* DNA in canine PBLs for each sample by using Brilliant® SYBR® Green QPCR Core Reagent kit (Stratagene, La Jolla, CA, USA). The forward and reverse primers used in the assay were located at 95–115 and 181–202 nucleotide position of 16S rRNA gene of *E. canis* Oklahoma (GeneBank No: M73221), respectively. To
quantify the 16S rRNA gene copy numbers, 389-bp fragment of the 16S rRNA gene was cloned into a PCRII cloning vector (Invitrogen, Carlsbad, CA, USA) and 10-fold serially diluted to generate standard curve.

Total cellular RNA extraction, DNase treatment of RNA, and cDNA synthesis were performed as previously described. Expression of cytokine genes (IL-1β, IL-2, IL-6, IL-8, IFN-γ, and TNF-α) and β-actin gene was determined by real-time PCR as described by Fujiwara et al. To quantify β-actin mRNA levels, 81-bp fragment of canine β-actin gene (GenBank No. Z70044) was cloned into a PCRII cloning vector. As a positive control, PBLs from each dog collected prior to infection were stimulated with concanavalinA (Sigma, St. Louis, MO, USA) (5μg/mL) in RPMI 1640 medium at 37°C for 3 h.

RESULTS

All three dogs inoculated with *E. canis* New Mexico developed signs of an acute severe ehrlichiosis, such as high fever (>40°C), loss of appetite, edema, dehydration, depression and weight loss, and thrombocytopenia (<106 × 10^9 platelets/L, Fig. 1A). The control dogs (dogs 4–7) did not show fever, thrombocytopenia, or any other clinical signs of canine ehrlichiosis during the same period. Temporal patterns of IFA IgG titers using *E.canis* Oklahoma as antigen were similar among three dogs inoculated with *E. canis* New Mexico. The titer started to rise on day 14 PI and reached 1:10,240 on day 56 PI. The IFA titer was negative in all control dogs (dogs 4–7).

Real-time PCR was used to detect 16S rRNA genes of *E. canis* in experimentally infected dogs. β-actin DNA was used to normalize input DNA from canine PBL across specimens. The ratios of *E. canis* 16S rRNA genes to that

![FIGURE 1. Platelet counts (A) and ehrlichemia levels (B) in the peripheral blood cells from dogs infected with *E. canis* New Mexico (dogs 1–3) and uninfected control dogs (dogs 4 and 5) as determined by real-time PCR.](image-url)
FIGURE 2. Cytokine mRNA expression by PBLs from infected (dogs 1–3) and uninfected (dogs 4 and 5) dogs.

Temporal mRNA levels of six cytokines were examined by real-time RT-PCR in all specimens from day 2 PI (Fig. 2). Rapid and persistent upregulation of IL-1β and IL-8 and relatively low levels of IFN-γ and TNF-α expressions were observed in all three infected dogs (dogs 1–3). None of the cytokine mRNAs was detected in PBLs from uninfected dogs (dogs 4 and 5) and placebo inoculated control dogs (dogs 6 and 7, data not shown). DNA contamination in RNA preparation was negligible, since no PCR products were detected from the RNA samples processed without addition of reverse transcriptase. PBLs from all these dogs were similarly responsive to ConA in vitro prior to E. canis infection.
DISCUSSION

IL-1β and IL-8 cause inflammatory responses by attracting neutrophils and activating NF-kB-mediated inflammatory cytokine gene expression in a variety of cells. High levels of IL-1β and IL-8 expression may be responsible for clinical signs (fever, depression, anorexia, dehydration, and thrombocytopenia) observed in these dogs in this study. IL-1β and IL-8 are also major cytokines generated by human monocytes upon incubation in vitro with *Ehrlichia chaffeensis*. Clinical signs and cytokine responses in dogs infected with the New Mexico strain of *E. canis* were distinct from those of dogs infected with *E. canis* Oklahoma strain, suggesting the association of strain virulence with the pattern of cytokine induction in the host.

REFERENCES


