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Cytokine Production in Canine PBMC after *Bartonella Henselae* Infection

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Abstract : *Bartonella henselae* is the causative agent of cat scratch disease. Although cats are the main zoonotic reservoirs of *Bartonella* spp., unusual cases of cat scratch disease caused by a domestic dog scratch have been recently reported. For the *in vivo B. henselae* infection, eight dogs were inoculated intradermally with 2×10^8 CFU of *B. henselae* Houston-1 suspended in 1 ml of phosphate buffered saline on day 0 and subsequent injections of the same amount given intradermally on days 21, 28, 36, 58 and 64. After *in vivo* canine *B. henselae* infection was confirmed by nested PCR, the IFN-γ levels of the culture supernatant of PBMC stimulated with *B. henselae* was significantly higher in the *B. henselae*-PCR positive group than the *B. henselae*-PCR negative group. Our results showed that the canine immune responses against *B. henselae* were different from those of cats. Th1 activation by *B. henselae* stimulation was characterized in dog peripheral blood mononuclear cells, whereas Th2 activation was reported in *B. henselae*-infected cats.

Key words **:** *Bartonella henselae*, dog, cytokine, cat scratch disease, Th1 immunity.

Introduction

Bartonella henselae is the causative agent of cat scratch disease (CSD). In immunocompetent patients, CSD caused by *Bartonella henselae* is mainly characterized by a benign regional lymphadenopathy, but a low proportion of CSD patients may develop atypical manifestations (19). In immunocompromised patients, bacillary angiomatosis and bacillary peliosis hepatitis or splenitis are the most common manifestations (10). CSD is commonly transmitted by scratches or bites from cats or kittens (6,8). But in 1998, Keret D *et al*. reported an unusual case of CSD caused by a scratch from a domestic dog in Israel (11). The first reported case of CSD in South Korea, confirmed by polymerase chain reaction (PCR) for *B. henselae* DNA, was also due to contact with a dog (3).

In our previous study, blood, saliva and/or nail samples were collected from 54 dogs to evaluate the prevalence of *B. henselae* and *B. clarridgeiae* in South Korea. Novel nested PCR detected the bacterium in 16.6% of blood, 18.5% of saliva and 29.6% of nail samples from dogs (12). Nine samples were infected with *B. henselae* and 2 samples were co-infected with *B. henselae* and *B. clarridgeiae*. These results indicate dogs can serve as potential reservoirs of *B. henselae* and *B. clarridgeiae*.

So far there are no data on the interaction between *Bartonella* and canine peripheral blood mononuclear cells (PBMC), although data on murine macrophage, human dendritic cell or HUVEC (Human Umbilical Vein Endothelial Cells) were reported (13,15,21). Moreover, little is known about cytokine responses against *B. henselae* in dogs. Therefore, this study was conducted to analyze cytokine production in dog PBMC after *B. henselae* infection.

Materials and Methods

Preparation of experimental animals

A total of eight male beagle dogs, determined to be healthy after a routine physical examination, complete blood counts, serum biochemistry analysis, and urinalysis were used under protocols approved by the Institutional Animal Care and Use Committee of Seoul National University. All efforts were made to minimize animal suffering. The dogs were housed separately in metal cages and maintained on commercial dry dog food and water *ad libitum*. The cages were kept at a constant temperature (22-25°C) and relative humidity (50-55%). The animals were acclimatized for at least 2 weeks prior to their use.

Inoculation of dogs with *B. henselae*

The dogs were inoculated intradermally with 1 ml of the bac-

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terial suspension containing 2×10^8 CFU of *B. henselae* Houston-1 suspended in PBS on day 0. Subsequent inoculations were given intradermally on days 21, 28, 36, 58 and 64.

Nested PCR for detection of *B. henselae*

On days 36 and 64, nested PCR for detection of *B. henselae* (Bh) was conducted. Genomic DNA was extracted from blood samples with Genomic blood DNA kit (iNtRoN BIO TECHNOLOGY, Sungnam, Korea). A novel nested-PCR with increasing sensitivity (17) was used for the detection of *B. henselae.*

Collection of specimens and assay of cytokine levels in PBMC culture supernatants

On days 36, PBMC were harvested from approximately 6 ml of venous blood taken from each of the eight dogs to assay the cytokine levels in the PBMC culture supernatant; PBMC were harvested from approximately 6 ml of venous blood taken from eight dogs, which was supplemented with sodium heparin as anticoagulant. Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden) gradient centrifugation was performed at $400 \times g$ for 30 min. PBMC were washed twice with PBS and their concentrations were adjusted to 10^6 viable cells per milliliter of complete endotoxinfree RPMI 1640 medium (Invitrgen, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (Boehringer Mannheim, Australia), 100 U/ml of penicillin, and 100 μg/ml of streptomycin. A total of 5×10^5 cells/well was cultured in the presence or absence of *B. henselae* (MOI: 10, 5×10^6 CFU/well) in 96-well plates at 37°C and in a humidified atmosphere containing 5% CO_2. After 3 days, the supernatant of the culture was collected and assayed using canine cytokine ELISA Kits for tumor necrosis factor (TNF)-α, interferon (IFN)-γ, interleukin (IL)-10 and transforming growth factor (TGF)-β1 (R&D Systems, Minneapolis, MN, USA).

Because it was not possible to assess the canine IL-4 expression by ELISA, a semiquantitative reverse transcriptase PCR (RT-PCR) was used to evaluate the induction of IL-4. mRNA from 48 h cultured PBMC in the presence of Bh was used as template. The design of primers and the RT-PCR condition were conducted as described in a previous study (7).

Serum chemistry

On days 0 (before Bh infection) and 64 (after Bh infection), about 2 ml of blood was collected between 9 AM and 10 AM from the jugular vein. Serum biochemistry was assessed using an automatic blood chemistry analyzer (Seletra 2; Merck, Dieren, Netherlands)

Statistical analysis

All results are expressed as means with the standard error of the mean. Comparisons between groups were assessed by independent Student's t test with prior Levene's test for equality of variances and Shapiro Wilks test for the test of normality, whereas comparisons within a group were analyzed by a paired t test. Differences with a confidence level of 95% or higher were considered to be statistically significant ($P < 0.05$). All statistical analyses were performed using SPSS version 12.0.

Results

Nested PCR for detection of *B. henselae*

Four dogs were positive for *B. henselae* in the first PCR and five dogs were positive for *B. henselae* using nested PCR for detection of *B. henselae* on day 36 after *B. henselae* infection. Six dogs were positive for *B. henselae* in the first PCR and all eight dogs were positive for *B. henselae* with nested PCR on day 64 after *B. henselae* infection (Fig 1).

Assay of cytokine levels in PBMC culture supernatant

After *in vivo B. henselae* infection in dogs, TNF-α and IL-10 levels were not significantly different between the Bh-PCR positive (n = 5) and negative (n = 3) groups, even though the IFN-γ level of Bh-PCR positive group was significantly higher than the Bh-PCR negative group in *B. henselae*-stimulated wells (Fig 2, $p = 0.021$, Bh-PCR negative group: 3.03 ± 3.03 pg/ml, Bh-PCR positive group: 1259.35 ± 341.90 pg/ml). IL-4 mRNA expression was not detected in any of the samples.

Serum chemistry

After *in vivo B. henselae* infection, Alanine aminotransferase (ALT) was increased in seven of the eight dogs and the degree

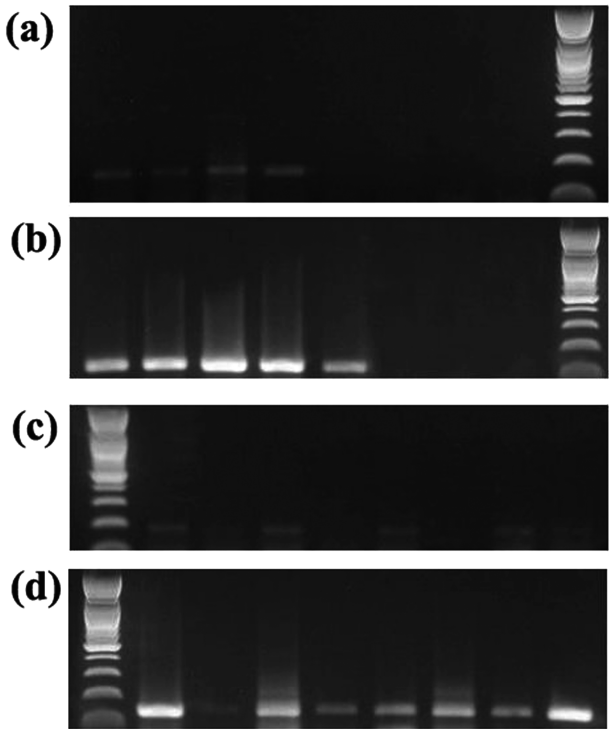

Fig 1. PCR performed on PBMC from experimental dogs after *Bartonella henselae* infection. (a) *B. henselae* 1st PCR on day 36 after *B. henselae* infection. (b) *B. henselae* nested PCR on day 36 after *B. henselae* infection. (c) *B. henselae* 1st PCR on day 64 after *B. henselae* infection. (d) *B. henselae* nested PCR on day 64 after *B. henselae* infection.

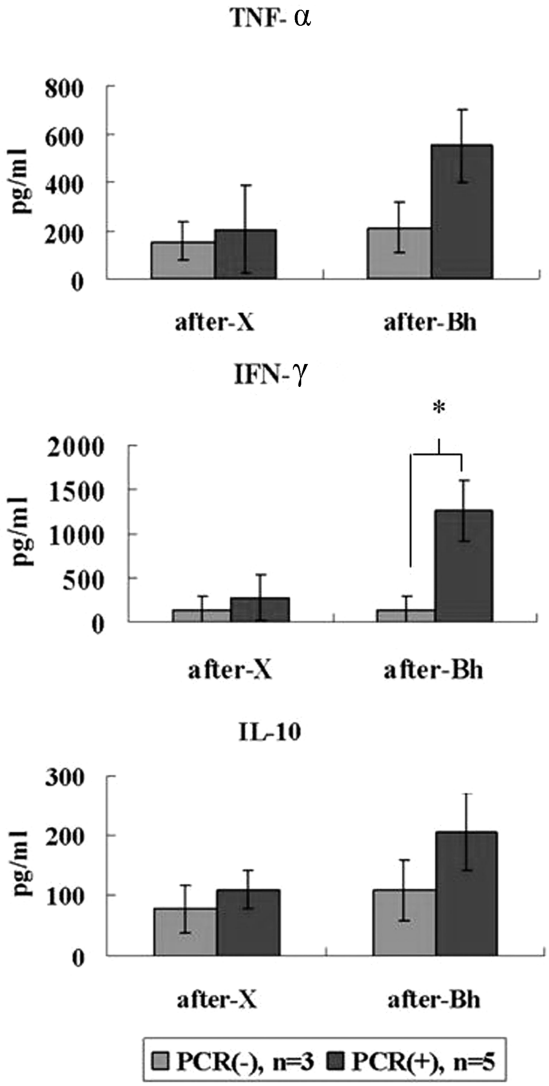

Fig 2. Comparison of cytokine production by cultured canine PBMC with/without *Bartonella henselae* stimulation between Bh nested PCR-positive and PCR-negative groups after *in vivo B. henselae* infection (after-X: after Bh infection/unstimulated well, after-Bh: after Bh infection/ *B. henselae*-stimulated well).

of increase was variable. ALT was dramatically increased in three dogs from 45.4 U/L to 784.8 U/L, 71.1 U/L to 171.8 U/L, and 38.5 U/L to 237.2 U/L. Aspartate Aminotransferase (AST) was dramatically increased in one dog from 21.2 U/L to 362.8 U/L. Alkaline Phosphatase (ALP), Blood Urea Nitrogen (BUN), creatinine, glucose, total bilirubin, total protein and albumin were not significantly changed.

Discussion

The Th phenotypes of CD4+ T cells involved in cell-mediated immunity induced by an infectious agent can be differentiated by their cytokine secretion patterns. CD4+ T cells of the Th1 type predominantly produce IFN-γ, while Th2 cells secrete large amounts of IL-4 and IL-5 (1). The studies on the mechanism of long-term bacteremia in *B. henselae*-infected cats by the examining host immune response were reported in the past (6,8). Their results showed increases of the IL-4 mRNA expression, but not IFN-γ, were observed in PBMC from infected cats (8). This selective induction of the Th2 immune responses might contribute to the establishment of persistent infection with *B. henselae* in cats. Although cats are the main zoonotic reservoirs of *Bartonella* spp. (16), cases of human cat scratch disease have been reported after contact with dogs (2,3,11,20). *Ctenocephalides felis*, the vector of *B. henselae* in cats, has a wide range of hosts, including dogs, making infection with *Bartonella* spp. possible (5). These facts indicate dogs can serve as potential reservoirs of *B. henselae*.

Our results showed that the canine immune responses against *B. henselae* were different from those of cats. In canine PBMC, Th1 activation by *B. henselae* stimulation was characterized. After *in vivo B. henselae* infection, the level of IFN-γ of the Bh-PCR positive group was significantly higher than the Bh-PCR negative group in *B. henselae*-stimulated wells. The level of IL-4 mRNA expression in PBMC was not detectable in any of the samples. Canine immune responses against *B. henselae* showed similar patterns as healthy human and mice, the immunocompetent host. Because *B. henselae* is an intracellular pathogen, immunity to *B. henselae* is mediated by Th1 cells in the immunocompetent host (9,14).

After *in vivo B. henselae* infection, ALT was increased in seven of eight dogs and the degree of increase was variable. AST was dramatically increased in only one dog. ALP, BUN, creatinine, glucose, total bilirubin, total protein and albumin was not significantly changed.

Peliosis hepatis was occasionally reported in dogs. In a murine study, liver tissue showed granulomatous inflammation reaching its highest degree of intensity during the fourth week of infection and resolving within 12 weeks post-infection (18). It is possible that the canine liver developed granulomatous inflammation after *in vivo B. henselae* infection such as that of murine study. We are planning to follow up the clinical signs, monitor the liver by imaging for the detection of peliosis hepatis by ultrasonograpy and then conduct RT-PCR to determine the gene expression concerning the pathogenesis of *B. henselae*.

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Bartonella Henselae 감염 후의 개 말초혈액단핵구에서의 사이토카인 양상

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요 약 : *Bartonella henselae* 는 고양이할퀴병의 원인체이다. 고양이가 *Bartonella* spp.의 주된 보유숙주이기는 하지만, 최근 애완견의 할퀴에 의한 고양이할퀴병 발생이 보고되었다. 8두의 개에 1 ml의 인산완충식염수에 부유한 2×10^8 CFU의 *B. henselae* Houston-1을 0일에 피내주사하고, 동량을 21, 28, 36, 58, 64일째에 추가로 피내주사 하였다. *B. henselae* 감염을 nested PCR을 통해 확인하였다. *B. henselae*-PCR 양성군이 음성군에 비해 *B. henselae*로 말초혈액단핵구를 자극한 후 얻은 배양상청액에서의 IFN-γ 농도가 유의성 있게 높았다. *B. henselae*자극시, Th1활성을 보이는 개 말초혈액단핵구의 면역양상은 Th2활성을 보인다고 알려진 고양이와는 다른 것으로 보인다.

주요어 : *Bartonella henselae*, 개, 사이토카인, 고양이할퀴병, Th1 면역