Brucella abortus S19ワクチン接種ラクダにおけるサイトカインプロファイル解析

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Cytokine Responses in Camels (*Camelus bactrianus*) Vaccinated with *Brucella abortus* strain 19 Vaccine

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ABSTRACT. In the present study, we determined the levels of cytokines produced by camel (*Camelus bactrianus*) peripheral blood mononuclear cells (PBMCs) in response to live attenuated *Brucella abortus* (*B. abortus*) S19 vaccine. Seven camels were vaccinated with commercial *B. abortus* S19 vaccine, and their cytokine responses were determined using a real-time PCR assay. Cytokine responses to *B. abortus* S19 were examined at 6 hr, 48 hr and 1, 2 and 3 weeks post-vaccination. Serological tests were performed to further confirm these immune responses. The results revealed that IFN-γ and IL-6 were upregulated during the first week post-vaccination. Low level expressions of IL-1α, IL-1β, TNFα and IL-10 and no expression of IL-2 and IL-4 were observed compared with the control camels. The findings showed that *B. abortus* stimulates cell-mediated immunity by directly activating camel Th1 cells to secrete IFN-γ. This quantification of cytokine expression in camels is essential for understanding of *Camelidae* disease development and protective immune responses. This is the first report of *in vivo* camel cytokine quantification after vaccination.
KEY WORDS: *Brucella abortus* S19, camel, cytokine.

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Cytokines are key molecules that play a major role in determining protective immune response. Cytokine response to a wide variety of infectious diseases and its important roles in the regulation of immune and inflammatory responses are now clearly achieved. The main stimulators of immune response, T-helper (Th) lymphocytes, are subdivided into Th1 and Th2 subsets according to the range of cytokines they secrete [7]. Th1 cytokines such as interferon-gamma (IFN-γ) and interleukin (IL)-2 are mostly involved in protection against intracellular pathogens through cell-mediated immunity (CMI). Th2 cells secrete IL-4 and IL-10 and are mainly responsible for protection against extracellular pathogens by mediating antibody production [16]. Inflammatory cytokines play important roles in the outcome of infectious diseases and inflammation [9]. The inflammatory cytokines IL-1, IL-6 and TNF-α are the principal mediators of natural immunity against gram-negative bacteria and are key mediators of inflammatory response and septic shock [14]. Susceptibility or tolerance to various infectious diseases is associated with the expression of particular cytokine profiles. Analysis of cytokine profiles helps to clarify functional properties of immune cells, both for research and for clinical diagnosis [24]. Studies on the role of Th1/Th2-type and proinflammatory responses are of great importance for our understanding of how the host's immune system deals with an infectious agent. The balance in Th1, Th2 and inflammatory cytokines could determine the disease outcome [16].

Brucellosis remains a significant human health threat in many areas of the world. It has also been reported in one-humped (*Camelus dromedarius*) and two-humped camels (*Camelus bactrianus*). Among the six different species of *Brucella*, *B. abortus* and *B. melitensis* are the most common infectious species that cause abortion in camels [1]. Other diseases have also been reported; however, the resistance or susceptibility of camel has not been elucidated, which makes it necessary to learn more about their immune system. Moreover, no information is available about cytokine profiles related to cell-mediated immunity (CMI) in *Camelidae*.

The importance of cytokine responses in the pathogenesis of brucellosis has been studied previously. Live bacterial vaccines are considered essential for effectively inducing appropriate protective CMI responses [10, 19]. In particular, Th1 immune responses characterized by production of IFN-γ are associated with protective immunity to *Brucella* [8, 27]. *In vitro* experiments have demonstrated that *B. abortus* induces human and murine monocytes to secrete proinflammatory cytokines, such as TNFα and IL-1β, where TNFα is a costimulator in IFN-γ production [4, 29]. IL-1α, IL-1β, IL-6 and TNFα are key cytokines in inflammatory processes and mediate many of the clinical signs associated with endotoxemia due to gram-negative bacterial infection [17, 26]. Vaccination of cattle with *B. abortus* S19 induces antigen-specific T lymphocyte responses, which can be demonstrated *in vitro* and *in vivo* [21, 25].

Analysis of cytokine profiles helps to clarify functional properties of immune cells, both for research and for clinical

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diagnosis. Real-time reverse transcription polymerase chain reaction (RT-PCR) is becoming widely used to quantify cytokines derived from cells and tissues [20]. We previously described a method by which llama inflammatory cytokine mRNAs can be quantified using real-time RT-PCR technology with the double-stranded DNA-binding dye SYBR Green I [13]. In the field of camel immunology, real-time PCR has not been used to measure cytokine mRNAs and has only been used in limited studies on camel brucellosis. In this study, quantification of Th1 type, Th2 type and inflammatory cytokine mRNA expression was performed by real-time PCR for two-humped camel vaccinated with *B. abortus* S19. Our study is the first attempt to investigate cytokine profiles in vaccinated camels.

Fourteen camels ages 4–9 years that serologically tested negative by rose Bengal test (RBT), tube agglutination test (TAT) and complement fixation test (CFT), as described previously [2, 11, 12], were used as experimental animals in Erdene, Tuv Province, Mongolia. These camels were kept under controlled conditions using the guidelines of the Institute of Veterinary Medicine of Mongolia. The commercial *B. abortus* S19 (attenuated live) vaccine (BIOCOMBINAT Biological State owned Enterprise, Ulaanbaatar, Mongolia) was injected once into the neck regions 7 camels subcutaneously with a dose of 10×10^9 viable organisms. No adverse effect was seen in any of the vaccinated camels. The other 7 camels were used as control. Blood samples were collected by jugular venipuncture before vaccination (time zero) and at 6 hr, 48 hr, and 1, 2 and 3 weeks after vaccination. Peripheral blood mononuclear cells (PBMCs) were purified by density gradient centrifugation on Percoll (Amersham-Pharmacia, Buckinghamshire, UK) from the heparinized venous blood of camels. The isolated PBMCs were washed 3 times in 10 m*l* of PBS, counted and incubated to a concentration of 1×10^7 PBMCs/m*l* in complete RPMI 1640 medium containing 10% fetal calf serum, 100 U/m*l* penicillin and 100 *n*g/m*l* streptomycin. PBMCs were grown on 12-well plates and stimulated with ConA (Sigma Chemical, St Louis, MO, U.S.A.) (5 μg/m*l*) for 24 hr. Total RNA was isolated from the ConA-stimulated PBMCs using TRIzol reagent (Invitrogen, Carlsbad, CA, U.S.A.). An aliquot of the total RNA (5 μg) was reverse-transcribed by RAV2 reverse transcriptase (20 U/μ*l*, TAKARA, Shiga, Japan) and oligo-dT primer (300 pmol) in a total volume of 40 μ*l* mixture according to the manufacturer's instructions. The cDNAs were analyzed immediately or stored at –20°C until use. The real-time PCR assay was performed using the same conditions and the oligonucleotide primers described by Odbileg *et al.* [13]. Other newly designed primer sequences used are as follows: AAACTCTCCAGGATGCTCAC, forward, and GGAACTGAAGGGATCTGAAA, reverse, for IL-2; CAAAGAACACAACTGAGAAG, forward, and GGCTAAAGAAGATTATGAAG, reverse, for IL-4; AAGCCTTGTCGGAGATGAC, forward, and AGCCATGAGTGAGTTCGACA, reverse, for IL-10; ATTGTCTCCTTCTACTTCAA, forward, and AGCGGAAGAGAAGTCAGAAT, reverse, for IFN-γ. To quantify the cytokine expression by real-time PCR, we used plasmid cDNA as standards. The standard curve was prepared for each target cytokine as described previously [13]. Serial dilution of plasmids encoding target cytokines or β-actin gene were used in standard curve quantification. Furthermore, calibration curves were made from the measured fluorescence of dilution series of the control cDNA to create the same amplification curves. Then, the concentrations of unknowns were calculated from the standards values. The results were confirmed by single peak after melting point analysis, and the result of sequence analysis is 100% homology with the camel cytokine. All data are presented in means + SD. The analyses were performed by obtaining ratios of the cytokine mRNAs concentrations based on the dose of β-actin mRNA.

In the present study, we demonstrated quantitative analysis of the mRNA levels of eight cytokines in vaccinated and unvaccinated camels. Antibody responses were also measured after vaccination, and the results were compared for the seven unvaccinated camels. In the vaccinated camels, antibodies were detected one week after injection, and the highest antibody titer was observed after two weeks. Camels that showed a titer of 1:40 in TAT or 1:5 in CFT or greater were considered positive. The results of CFT were scored positive when the serum gave a 2+ reaction in a 1:5 or higher dilution. Serum samples in the TAT tests were classified positive, negative or suspect on the basis of the standard criteria previously established [2, 11, 12].

The mRNA levels of IFN-γ, IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-10 and TNFα were determined by real-time PCR for all fourteen camels. The cDNA amplicons from the mRNAs of ConA-stimulated PBMCs derived from the experimental animals produced cytokine-specific bands for inflammatory (IL-1α, IL-1β, TNFα and IL-6), Th1 (IL-2, IFN-γ) and Th2 (IL-4 and IL-10) cytokines. The mRNA expression levels of IFN-γ, IL-6, IL-1α, IL-1β, TNFα and IL-10 of the vaccinated camels were as high as 6.5, 4.1, 2.1, 2.0, 1.9 and 2.7-fold, the levels of the unvaccinated camels, respectively (Fig. 1 and Fig. 2). The highest expressions of IFN-γ and IL-6 were demonstrated in the vaccinated camels 1 week after vaccination. On the other hand, a low level of IL-2 was detected in the vaccinated group (Fig. 1 and Fig. 2). Low levels of inflammatory cytokines such as IL-1α and IL-1β and a low level of TNFα were also observed (Fig. 1). The kinetic patterns of IL-4 and IL-10 were investigated, and it was found that a low level of IL-10 expression and no IL-4 expression were evident after vaccination (Fig. 2).

Immunity to intracellular bacteria, including *B. abortus* and *Mycobacterium tuberculosis*, are dependent on generation of a protective IFN-γ producing T-cell response [18]. In domestic animals, humans and mouse, a number of studies have demonstrated that IFN-γ has a crucial role in the control of brucellosis [8, 27]. The high level of IFN-γ produced by Th1 cells after whole *B. abortus* stimulation may be one reason why the commercially available *B. abortus* S19 live vaccine confers protection for domestic animals. Similar results have been found in the human and mouse models for

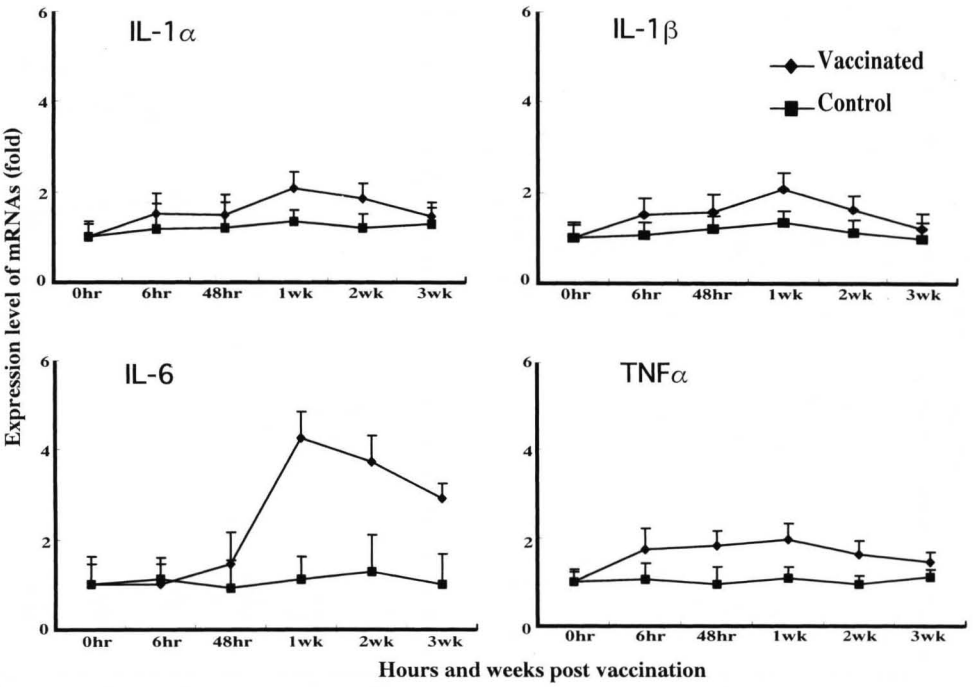

Fig. 1. Analysis of relative changes in inflammatory cytokine levels by real-time RT-PCR. The mRNA levels of the inflammatory cytokines were detected from ConA-stimulated PBMCs derived from *B. abortus* S19 vaccinated camels. The cytokines are as follows: inflammatory cytokines IL-1α, IL-1β, IL-6 and TNFα (all data were standardized by β-actin and divided by control value).

brucellosis. Some studies have suggested that IFN-γ and TNFα are involved in the pathophysiology of brucellosis and that they have a close relationship with inflammatory activation of the disease [3, 29]. Among the various cytokines, IFN-γ is the most relevant in generating macrophages with strong activity in killing intracellular Brucella [8, 19]. In our study, aside from IFN-γ, IL-6 and other inflammatory cytokines were expressed in the PBMCs of the vaccinated camels. Studies conducted to date have revealed that *B. abortus* can induce in a variety of cell types and the release of proinflammatory cytokines such as IL-1, IL-6 and TNFα [4, 22, 26, 28]. Our results confirm that IFN-γ and inflammatory cytokines are involved in the pathogenesis of brucellosis. Inflammatory cytokines show indirect antibacterial activity against *Brucella* and function as costimulators of IFN-γ expression [4, 22, 29]. In our study, the IL-6 levels were also found to be significantly higher in the vaccinated camels 1 week after vaccination, which makes it an early responder against bacterial infection.

B. abortus has also been shown to stimulate the production of IL-10 [6, 23], a cytokine that may eventually downmodulate the proinflammatory response elicited by the bacterium [6]. Interestingly, this previous finding was also observed in the present study taking into account the counter reaction between IL-6 and IL-10. The upregulation of IL-10 from the first to second week post vaccination resulted in downmodulation of IL-6. Moreover, the kinetic patterns of Th2 cytokines after vaccination revealed non-expression of IL-4. However, a low level of IL-10 was detected, which suggests its involvement in continuous expression of IFN-γ. These data indicate that *B. abortus* S19 is capable of not only activating camel T cells but also that it can induce B cells, probably of the Th2 phenotype, that secrete IL-10. Previous studies have indicated that both IL-10 and IFN-γ are produced following a *B. abortus* infection and that IL-10 induction does not downregulate IFN-γ production. This implies that in brucellosis, the effect of IL-10 on the immune response is to limit the consequences of an exaggerated proinflammatory response more than to counterbalance the production of Th1 cytokines. These findings are in agreement with other studies that measured IL-10 production following *Brucella* infection [5, 15, 23, 27]. Although there is evidence that IL-10 may promote protective immunity to brucellosis, it is reasonable to assume that optimal development and maintenance of a protective response against infection relies on a finely regulated balance of cytokines, rather than upon the level of a single cytokine. Based on the results and consistent with other studies, we conclude that *Brucella* infections induce Th1 response with weak Th2 response, despite some contradictory results in the literature. However, these findings should be addressed in further studies. Based on the result of high expression of IFN-γ and no expression of IL-4, we suggest that *B. abortus* S19 induces a cellular Th1 response in the camel. Cattle PBMCs express transcripts of the Th1 cytokine and IFN-γ (but not IL-2 and IL-4) in response to *B. abortus* [21]. Inter-

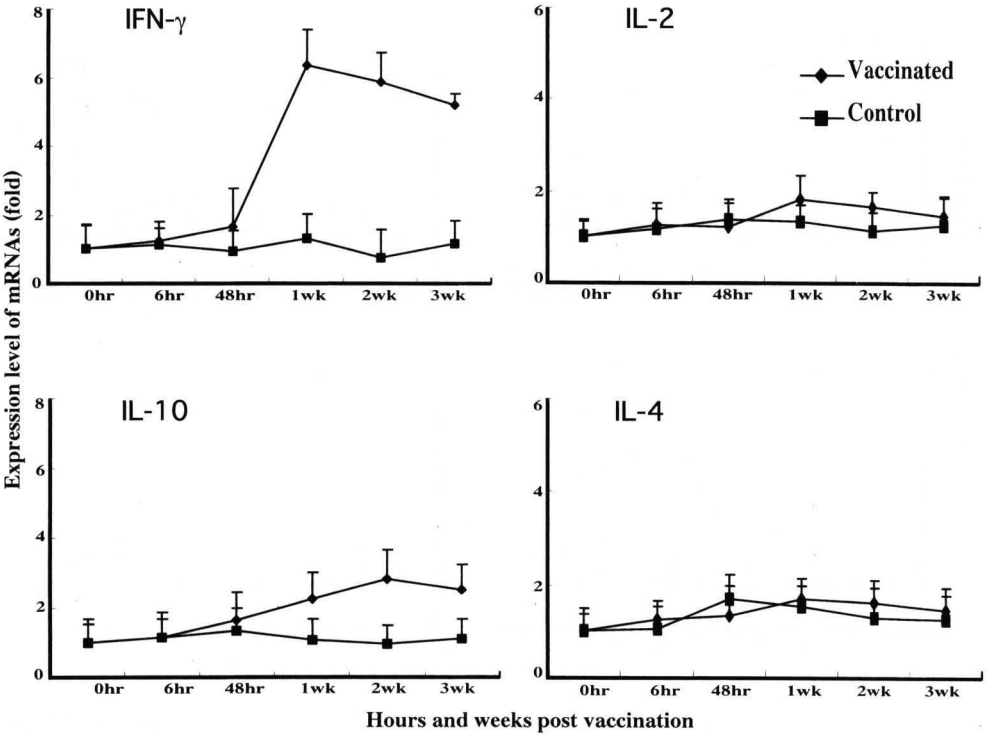

Fig. 2. Analysis of relative changes in Th1 and Th2 cytokine levels by real-time RT-PCR. The mRNA levels of the Th1 and Th2 cytokines were detected from ConA-stimulated PBMCs derived from *B. abortus* S19 vaccinated camels. The cytokines are as follows: Th1 (IFN-γ and IL-2) and Th2 cytokines (IL-4 and IL-10, all data were standardized by β-actin and divided by control value).

estingly, bovine, murine and camel T cells exhibit a similar cytokine profile following stimulation with *B. abortus*, suggesting a similar immune response in these three animal species, which affirms the usage of mice as a relevant model in the study of immunity against brucellosis. This suggests that Th1 cytokine responses might be essential in controlling intracellular pathogens [19]. In this report, it has been shown that *B. abortus* can activate camel Th1 cells to secrete IFN-γ.

Examination of cytokines simultaneously reveals functionally complex responses. We conclude that the most sensitive and complete description of immunization responses requires quantification of several cytokines, a process that will be critical to evaluation and discrimination of different vaccine regimens.

We therefore conclude that real-time PCR can be successfully applied for quantification of cytokine mRNA and for evaluation of the Th1/Th2 cytokine balance. This assay is suitable in analyzing cytokines from small amount of mRNA. Our results could be helpful for future research regarding immune response to *Brucella* infection in *Camelidae* (llama and camel). Hence, this is the first report in this field; further studies are needed to understand the complex interaction of cytokines in camel immune response to brucellosis. In summary, a highly sensitive real-time RT-PCR system was adapted for quantitation of *Camelidae* cytokine mRNA expression. With its high sensitivity, this assay is suitable for analyzing the cytokine mRNA present in small volumes such as PBMCs collected from camels (llama) or from cell culture systems, and for determination of early events in the kinetics of cytokine mRNA expression and Th1/Th2 differentiation.

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