**Quantification of Llama Inflammatory Cytokine mRNAs by Real-Time RT-PCR**

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(Received 7 July 2004/Accepted 15 October 2004)

**ABSTRACT.** We have developed a method by which llama cytokine mRNAs can be quantified using real-time reverse transcription polymerase chain reaction (RT-PCR). Total RNA was extracted from lipopolysaccharide (LPS)-stimulated peripheral blood mononuclear cells (PBMCs) of llama, reverse transcribed to cDNA, and cytokine profiles for interleukin (IL)-1α, IL-1β, IL-6 and tumor necrosis factor (TNF) α were quantified by real-time PCR. The expressions of mRNAs of inflammatory cytokines IL-1α, IL-1β, IL-6 and TNFα were upregulated upon stimulation with LPS in a dose- and time-dependent manner. Incubation of PBMCs with 100 and 1,000 pg/ml of LPS for 3 to 6 hr resulted in the acceleration of the mRNA levels of inflammatory cytokines. Here, we describe a highly sensitive and reproducible method to quantify the transcription of llama cytokine mRNAs by real-time RT-PCR with the double-stranded DNA-binding dye SYBR Green I.

**KEY WORDS:** Llama, LPS, real-time RT-PCR.

The important roles of cytokines in the regulation of immune and inflammatory responses are now clearly recognized [10]. Inflammatory cytokines are known as some of the immune mediators at inflammatory sites and appear to play an important role in the pathological processes of inflammatory lesions. IL-1, IL-6 and TNFα are multifunctional cytokines and known as activators of lymphocytes and macrophages [1, 6]. The host response to gram-negative bacterial infections, mainly studied in mice, human beings and other mammals, includes production of inflammatory cytokines such as IL-1α, IL-1β, IL-6 and TNFα. Bacterial lipopolysaccharide (LPS) is a key inducer of the inflammatory response following gram-negative infection [9].

The llama belongs to the family Camelidae and they are specially suited to the harsh environment. Llama and camel are relatively healthy, although they can be infected with many pathogens [3, 7, 12, 13, 15, 17], which infect cattle, sheep and horse. However, the roles of inflammatory cytokines such as IL-1, IL-6 and TNFα in bacterial infections are not well understood in Camelidae. Previously, we reported the cloning of several llama cytokines and these sequences are now available for the design of llama-specific primers [11 and Genebank databases]. The aim of the present study was to characterize the immune response in llama, experimentally induced with LPS, by quantification of cytokine mRNAs in the PBMCs using real-time PCR.

PBMCs were purified by density gradient centrifugation on Percoll (Amersham-Pharmacia, UK) from heparinized venous blood of a healthy 3-year-old llama maintained in the experimental animal facility unit of our laboratory. PBMCs were cultured with two different concentrations of LPS (100 and 1,000 pg/ml) for three different incubation periods of early (3 and 6 hr), intermediate (12 and 24 hr) and late (48 and 72 hr). LPS derived from *E. coli* 055:B5, Bacto (Wako, Japan) was used for endotoxic bacterial stimulation. Total RNA was isolated from LPS-stimulated and control (without LPS) PBMCs using the TRIzol reagent (Invitrogen, U.S.A.). An aliquot of the total RNA (5 µg) was reverse-transcribed by RAV2 reverse transcriptase (20 U/µl, TAKARA, Japan) and the oligo-dT primer (300 pmol) in a reaction according to the manufacturer’s instructions. cDNAs were analyzed immediately or stored at –20°C until use. The oligonucleotide primers used for the detection of cDNA specific for llama cytokines (IL-1α, IL-1β, TNFα and IL-6) and β-actin were derived from published nucleic acid sequences [11] or sequence available from the GenBank databases. The details of all oligonucleotide primer sequences, predicted product lengths by amplification, and optimal amplification conditions in the real-time PCR are listed in Table 1. Real-time PCR assay was carried out with LightCycler (Roche Diagnostics, Germany), using LightCycler FastStart DNA Master SYBR Green I (Roche Diagnostics), in order to observe the levels of mRNAs. One µl of cDNA was added to a 19 µl of reaction mixture including MgCl2 at the optimal concentration, 20 pmol of each primer and 2 µl of a LightCycler FastStart DNA Master SYBR Green I, and incubated in LightCycler under the following conditions: at 95°C for 10 min for denaturation, followed by 50 cycles of 94°C for 1 second, each of the optimal annealing temperature for 5 seconds, 72°C for 10 seconds and finally cooling to 40°C.

The relative expression values were normalized to (divided by) the expression value of β-actin. To quantify the results obtained by real-time RT-PCR, we used plasmids containing cDNA as standards. The cytokine cDNA of interest was amplified by RT-PCR using the same primers as for real-time RT-PCR. The PCR products were purified using the GeneClean II Kit (Bio 101, U.S.A.), and cloned into the pGEM-T easy vector (Promega, U.S.A.) with T4 DNA ligase. In each experiment, eight to 10 plasmid clones
containing cytokine cDNAs were inoculated into LB broth and incubated at 37°C overnight in a shaker. Plasmid DNAs were extracted from culture using a purification kit (QIAGEN, U.S.A.), and sequenced using the BigDye terminator cycle sequence kit and an automated DNA sequencer (PRISM™ 310 Genetic Analyzer, Applied Biosystems). Finally, plasmid DNA concentrations were measured spectrophotometrically (Beckman Du® 530, U.S.A.) and diluted to serve as standards for numerical quantification. The standard curve was prepared for each target cytokines (for example, TNFα in Fig. 1A and 1B) and while β-actin was used as the housekeeping gene in this study. The PCR products of the llama inflammatory cytokines and β-actin were sequenced to verify the analytical specificity using standard sequencing procedures. Melting curve analysis was also performed after PCR amplification to confirm that there was no primer dimer in the PCR products (Fig. 1C). Doses and time course studies with LPS were performed in triplicate. All data are presented as means ± SD.

Real-time RT-PCR reactions were performed under each cytokine specific condition to amplify four kinds of inflammatory cytokines, IL-1α, IL-1β, IL-6 and TNFα (Table 1). For each of the primer sets, non-specific amplification was not visualized after electrophoresis and ethidium bromide staining of agarose gels (Fig. 1D). This result indicated that real-time PCR conditions used in this study were suitable for the detection of cytokine mRNA expressed in llama PBMCs.

We then applied this technique to determine the kinetics of IL-1α, IL-1β, IL-6 and TNFα induction upon stimulation of llama PBMCs by LPS. The expression of IL-1α, IL-1β, IL-6 and TNFα mRNAs was obviously upregulated by LPS stimulation in a dose- and time dependent manner (Fig. 2) [16]. This observation was similar to what was noted with human monocytes and porcine epithelial cells stimulated with LPS, which strongly upregulated the secretion of IL-1α, IL-6 and IL-8 [8] in a dose dependent manner [2].

There was an appreciable increase in the IL-6 expression in the early stages of LPS-stimulation at the concentration of 1,000 pg/ml (Fig. 2). Under this condition, IL-1α, IL-1β, IL-6 and TNFα mRNAs were increased 6, 3, 25 and 1.4 fold, respectively, as compared with expression in the control groups (Fig. 2). The highest expressions of IL-1α, IL-1β, IL-6 and TNFα were demonstrated in the 100–1,000 pg/ml of LPS-stimulated PBMCs after 3 to 6 hr of stimulation. The increase in IL-6 mRNA expression after LPS stimulation was higher than that demonstrated for control mRNA (Fig. 2). The high concentrations of LPS used in this assay induce a high levels of inflammatory cytokines released from PBMCs of llama.

Many quantitative RT-PCR methods that use external standard and competitors are available [4]. However, all require considerable time and effort. We are interested in developing RT-PCR systems with the double stranded DNA-binding dye SYBR Green I [14] to estimate the expression level of llama cytokine mRNA. We investigated the mRNA expression of inflammatory cytokines, IL-1α, IL-1β, IL-6 and TNFα in llama PBMCs in vitro. PBMCs incubated with 1,000 pg/ml of LPS for 3 hr showed the most significant upregulation of the cytokine mRNAs. On the other hand, the expression level of IL-1α, IL-1β, TNFα and IL-6 were decreased at 12, 24 and 72 hr. The similar results were reported in previous study [5].

In addition, the mRNA expression of inflammatory cytokine profiles in this llama model will provide information useful for understanding the immunopathological mechanism of gram-negative bacterial infection in the Camelidae family. The methodology described here was easily and successfully applied for the quantification of several cytokine cDNAs from llama. Due to the high sensitivity, this assay is suitable for analyzing the cytokine mRNA present in small volumes of cDNA such as PBMCs collected from llama (camel). The present study demonstrated, for the first time, the determination of the kinetics of induction of cytokine mRNAs upon LPS stimulation of PBMCs from llama.

ACKNOWLEDGEMENT. This work was supported in part by a Grant-in-Aids (no. 15658095) from the Ministry of Education, Culture, Sports, Science and Technology, Government of Japan.

<table>
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REFERENCES


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Fig. 2. Kinetic study for cytokine mRNAs. Expression of llama mRNAs by PBMCs on stimulation with different concentrations of LPS in 0, 3, 6, 12, 24, 48 or 72 hr and analyzed by the real-time PCR as described in the text. The cytokines are follows: IL-1α, IL-1β, IL-6 and TNFα. (all data were standardized by β-actin and divided by control value)