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Quantitation of Rabbit Cytokine mRNA by Real-Time RT-PCR

Charmie Godornes¹, Brandon Troy Leader², Barbara J. Molini¹, Arturo Centurion-Lara^{1,2},
 and Sheila A. Lukehart^{1,2,*}

¹ Department of Medicine, University of Washington, Seattle WA 98195

² Department of Pathobiology, University of Washington, Seattle WA 98195

Abstract

Fundamental understanding of rabbit immunology and the use of the rabbit as a disease model have long been hindered by the lack of immunological assays specific to this species. In the present study, we sought to develop a method to quantitate cytokine expression in rabbit cells and tissues. We report the development of a quantitative real-time RT-PCR method for measuring the relative levels of rabbit IFN- γ , IL-2, IL-4, IL-10 and TNF- α mRNA. Quantitation was accomplished by comparison to a standard curve generated using plasmid DNA containing partial sequences of the relevant cytokines. Experimental studies demonstrate applicability of this assay to quantitate cytokine mRNA levels from rabbit spleen cells following mitogen stimulation. We have further utilized this assay to also examine cytokine expression in rabbit tissues during experimental syphilis infection.

Keywords

Rabbit; Cytokines; quantitative real-time RT-PCR; Syphilis

1. INTRODUCTION

The rabbit's value to biomedical research includes its use in the production of high quality antiserum, studies of immunoglobulin structure and regulation (Kindt, 1975), B cell development (Yang et al., 2005; Sinha et al., 2006), and antibody repertoire development (Rhee et al., 2004; Rhee et al., 2005), and as models of inflammation and infectious diseases. In the study of syphilis, the rabbit is the preferred experimental model because it develops clinical manifestations that closely resemble human disease (Turner and Hollander, 1957). Similarly, the rabbit is an important model for tuberculosis due to its formation of pulmonary cavities, a distinct characteristic of human tuberculosis that other small animal models do not develop (Helke et al., 2005). The rabbit is also used to study a variety of other diseases such as enteropathogenic and enterohemorrhagic *E. coli* infection (Zhu et al., 2006), meningitis and vasculitis caused by *Coccidioides immitis* (Zucker et al., 2006), and various ocular diseases including those caused by herpes viruses (Weisbroth et al., 1974; Morishige et al., 2006).

Knowledge of the rabbit's immune system is vital to its use as a model for infectious diseases and other inflammatory responses. However, relatively few immunological reagents exist for the rabbit, especially compared to those available for the human or mouse. In terms of cytokines, assays for measuring a small number of inflammatory mediators, such as IL-8 (Ivey

*Corresponding author: Mailing address: Sheila A. Lukehart, PhD, Department of Medicine, Box 359779, Harborview Medical Center, 325 Ninth Ave. Seattle, WA 98104, Phone 206 341 5361, Fax 206 341 5363, Email: lukehart@u.washington.edu

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et al., 1995), are commercially available. No methods have been described for assaying many of the important cytokines that mediate and regulate immune responses.

The sequences for the rabbit homologs of cytokines such as IL-1, IL-2, IL-4, IL-6, IL-8, IL-10, IFN- γ , TNF- α have been determined (Cannon et al., 1989; Shakhov et al., 1990; Isono et al., 1996; Perkins et al., 2000), thus enabling the development of new molecular assays for examining rabbit cytokine expression. While quantitative competitive RT-PCR can be useful in measuring cytokine expression, this method can be labor intensive and requires post-PCR manipulations such as agarose gel electrophoresis and ethidium bromide staining. This method has been supplanted by quantitative real time RT-PCR which offers advantages in terms of labor, sensitivity, and time. Zucker et al. very recently reported RT-PCR assays for relative quantitation of a number of pro-inflammatory molecules as well as a few cytokines (Zucker et al., 2006). In this study, we describe the development of a quantitative real-time RT-PCR assay for measuring mRNA for rabbit cytokines IFN- γ , IL-2, IL-4, IL-10, and TNF- α using the LightCycler system (Roche Applied Science, Mannheim, Germany). This quantification technique measures PCR product accumulated during the exponential phase of the PCR reaction. It is fast, sensitive, and accurate and requires no post PCR manipulations. This method was validated by quantitation of mRNA from Concanavalin A (ConA)-stimulated rabbit splenocytes and was applied to measurement of cytokine expression in biopsies of skin lesions from rabbits following experimental syphilis infection.

2. MATERIALS AND METHODS

2.1. Animals

Adult male New Zealand White rabbits were obtained from R&R Rabbitry (Stanwood, WA). Rabbits were housed individually at 18°C to 20°C and given antibiotic-free food and water. The rabbits used in this study were screened for evidence of *Treponema paraluiscuniculi* infection by Venereal Disease Research Laboratory and fluorescent treponemal antibody tests before use. All protocols were approved in advance by the University of Washington Institutional Animal Care and Use Committee.

2.2. Preparation of rabbit splenocytes

Concanavalin A -stimulated splenocytes were used to develop our quantitative RT PCR assays. Total spleen cells were obtained from a normal adult male New Zealand White rabbit as described elsewhere (Lukehart et al., 1980b). Splenic lymphocytes were stimulated with 50 μ g/ml final concentration of ConA (Sigma, St. Louis, MO) and cultured in 24 -well culture plates at 1.25×10^6 cells/well in RPMI 1640 medium containing penicillin-streptomycin (Gibco) and L-glutamine (Gibco, and supplemented with 10% normal rabbit serum,). As baseline controls, replicate wells were similarly cultured, with phosphate buffered saline (PBS) in stead of Con A. After 3, 6, 11, 24, 32, 36, and 50 hours of incubation at 37°C, the cells from 6 wells for each condition were harvested and pooled in a 15 ml conical tube. The cells were pelleted at $180 \times g$ for 10 minutes, immediately resuspended in 3 ml of Ultraspec RNA (Biotecx Laboratory, E. Houston, TX), and homogenized by pipetting up and down vigorously. 500 μ l aliquots of these cell suspensions were placed in 1.7 ml siliconized tubes and stored at -80°C until RNA extraction.

2.3. Sample collection from early lesions during experimental syphilis

Four rabbits were infected intradermally at 12 separate sites on their backs with 0.1 ml of a suspension of Nichols strain of *Treponema pallidum* containing 1×10^7 bacteria/ml. On days 2, 6, 9, 13, 19, 29, 36, and 45 following inoculation, animals were anesthetized with ketamine and xylazine and 4 mm punch biopsies were obtained from 2 lesions on each animal; biopsies from each lesion were taken only once during the course of the experiment. Each biopsy was

thoroughly minced using a sterile scalpel blade, transferred to a 1.7 ml tube containing 0.5 ml Ultraspec RNA, and then further homogenized using a disposable pestle. Processed samples were immediately frozen in dry ice, and stored at -80°C until RNA extraction.

2.4. RNA Isolation and Reverse Transcription

Total RNA from ConA-stimulated splenocytes and lesion biopsies was isolated using Ultraspec RNA isolation reagent following the manufacturer's instruction. RNA was then treated with 5 U of amplification grade DNase I (Invitrogen, Carlsbad, CA) or 2 U of Turbo DNase (Ambion, Austin TX) according to manufacturer's specifications to remove any contaminating genomic DNA. To synthesize cDNA, 2 μl of random hexamers (Invitrogen, Carlsbad, CA), 2 μl of 2.5 mM stock of dNTPs (Invitrogen), and 16 μl of DNase-treated RNA in DEPC water were denatured at 65°C for 5 minutes and immediately put on ice for 3 minutes. The following reagents were added: 4 μl of 10x Superscript buffer (Invitrogen), 8 μl of 25 mM stock of MgCl_2 (Invitrogen), 4 μl of 0.1 M dithiothreitol (Invitrogen), and 2 μl RNase OUT (Invitrogen). The contents were mixed gently and incubated for 2 minutes at room temperature. The tubes were briefly centrifuged and incubated for another 10 minutes at room temperature after adding 2 μl of 50 $\mu\text{g}/\mu\text{l}$ Superscript II (Invitrogen) followed by incubation at 42°C for 50 minutes. Superscript II was heat inactivated for 15 minutes at 72°C . Four units of RNase H were added to the reaction and incubated at 37°C for 30 minutes. cDNA samples were diluted 1:5 in DEPC water (Biotecx) prior to amplification. All cDNA samples were stored at -80°C until use.

2.5. Primers for the RT-PCR

The sequences of rabbit IFN- γ , IL-2, IL-10, TNF- α , and IL-4 were obtained from GenBank (Accession numbers [D84216](#), [Z36904](#), [D84217](#), [M12845](#), [AF169170](#), respectively), as was the sequence for hypoxanthine phosphoribosyl transferase (HPRT) (Accession number [M31642](#)) (used as a housekeeping gene for normalization). Using these sequences, primers were designed for each of the cytokines (Table 1) for PCR amplification of cDNA for plasmid construction and for cytokine quantitation using real-time RT-PCR.

2.6. Construction of the Plasmid Standards

A single plasmid was constructed containing portions of the sequences of all the cytokines in Table 1 (Figure 1). Amplification of rabbit cytokines and HPRT by conventional RT-PCR, using the primers described in Table 1, was performed as follows using cDNA from Con A-stimulated splenocytes collected after 16 hours of culture: 100 μl PCR total volume containing 200 μM dNTPs (Promega, Madison, WI), 50 mM Tris-HCl (pH 9.0), 1.5 mM MgCl_2 , 200 mM NH_4SO_4 , 1 μM of each primer, 2.5 units of Taq Polymerase (Promega), and 2 μl of cDNA. The cycling conditions for all conventional cytokine RT-PCRs were as follows: initial denaturation of 94°C for 4 minutes, then 35 cycles of 94°C for 1 minute, 60°C for 2 minutes, 72°C for 1 minute and a final extension step of 72°C for 10 minutes. 10 μl of PCR products were separated by electrophoresis on high resolution TBE/1.5% NuSieve agarose gels to confirm the expected size. The remaining 90 μl of amplicon was purified on silica columns (Qiaquick PCR purification; Qiagen Chatsworth, CA) and cloned into TOPO II Dual PromoterTM vector (Invitrogen). Ligated products were transformed into TOP 10 *E. coli* competent cells (Invitrogen). Double-stranded plasmid DNA was extracted from clones containing all inserts using the Qiagen Plasmid Kit. The purified plasmid was sequenced using automated sequencing by the dye terminator method (Perkin Elmer); the TOPO vector's M13 FWD and M13REV primers were used to verify the sequences of all cytokine inserts. The cytokine plasmids were then linearized with *EcoRV* (NEB Biolabs, Ipswich, MA) and purified using Qiaquick spin columns. Plasmid DNA concentrations were measured by optical density using

a spectrophotometer (NanoDrop, Wilmington, DE) and copy numbers were calculated using the following equation:

$$\text{Copies} / \mu\text{l} = \frac{(6.02 \times 10^{23} \text{ copies}) \times (\text{plasmid concentration g} / \mu\text{l})}{(\text{number of bases}) \times (660 \text{ daltons} / \text{base})}$$

Ten-fold serial dilutions of the plasmid (10^6 copies to 1 copy) in 0.2 M cDNA cocktail, which contained enzymes and reagents necessary to synthesize cDNA, were prepared to create standard curves for each cytokine; these conditions best mimic the cDNA made from the experimental tissues. Single use aliquots of the standard dilutions were placed in siliconized tubes and stored in -80°C to ensure DNA stability. Real time RT-PCR quantification of *T. pallidum* levels in lesion biopsies during experimental syphilis was accomplished using the method for measuring the expression of the gene for the treponemal 47-kDa lipoprotein (TpN47) described extensively elsewhere (Giacani et al., 2007).

2.7. Real-time RT-PCR

Real-time RT-PCR was performed with LightCycler Fast Start DNA Master PLUS SybrGreen Kit (Roche Applied Science) using 3 μl of a 1:5 cDNA dilution (in DEPC water) in a final volume of 20 μl with 0.2–1 μM final primer concentration. Quantitative PCR was performed using the LightCycler for 50 cycles at 95°C for 10 seconds, primer-specific annealing temperature for 5 seconds, and 72°C for 7–13 seconds (see detailed PCR conditions in Table 2). After each cycle, the temperature was raised to the primers' respective acquisition temperature (see Table 2) and fluorescence of SYBR green bound to double-stranded DNA was measured at 530 nm (LightCycler fluorescence channel F1). The crossing point, or the cycle number at which the fluorescence of the sample exceeded that of the background, was determined by the LightCycler software (version 3.5) using the second derivative method. A melting curve analysis was performed after the amplification phase to eliminate the possibility of non-specific amplification or primer-dimer formation.

3. RESULTS

3.1. Amplification efficiencies using plasmid standard curves

A plasmid containing the cytokine sequences amplified by our primer sets, as well as the sequence for the rabbit housekeeping gene, HPRT, was used to create external standard curves. Figure 2 shows the standard curves for HPRT, IL-2, IL-4, IL-10, IFN- γ , and TNF- α with their corresponding slopes, PCR efficiencies, and error values. An optimal standard curve should have a mean squared error of ≤ 0.05 and a slope near -3.32 to achieve a PCR efficiency near $2.0 E = (10^{-1/\text{slope}})$ (Kuhne and Oschmann, 2002). The correlation coefficients (R^2 value) for all cytokine standard curves were >0.99 , which validates the linear relationship between the crossing point value (Cp) and the logarithm of the DNA concentration.

3.2. Cytokine expression in ConA-stimulated splenocytes

We utilized real-time PCR analysis to analyze the kinetics of cytokine mRNA expression in splenocytes stimulated with ConA or PBS for 3, 6, 11, 24, 32, 36, and 50 hours. Cytokine values were normalized to 1000 copies of the rabbit housekeeping gene, HPRT. Gene expression for IFN- γ and IL-2 was detectable as early as 3 hours post-stimulation and gradual accumulation of mRNA for both cytokines was seen through 24–32 hours (Figure 3A and 3B). Peak expression of TNF- α and IL-4 was observed early in the culture period but declined to levels comparable to background thereafter (Figure 3C and 3E). IL-10 gene expression stayed at baseline levels throughout the culture period (Figure 3D).

3.3. Cytokine expression in syphilitic intradermal lesions

We examined cytokine expression in dermal lesions (chancres) of rabbits infected with the Nichols strain of *T. pallidum*, the causative agent of syphilis, to validate our assay in an experimental setting. We assessed bacterial burden during lesion progression by examining the mRNA levels of a treponemal gene, *tpN47*, that encodes for a highly-expressed lipoprotein unique to *T. pallidum* (Giacani et al., 2007). Lesions became clinically apparent on day 4 following intradermal infection and reached maximum size by day 13, averaging 11–15 mm in diameter. The lesions began to ulcerate on day 19 and were healing by day 29. IFN- γ mRNA was detected early in lesion development and reached maximum level on day 13, declining thereafter. The pattern of IFN- γ expression closely paralleled the burden of viable *T. pallidum* present in the lesions (Figure 4A). The level of IL-10 mRNA also correlated with bacterial burden except during the early stage of infection (uninfected [shown at day 0] through day 6) in which the level of IL-10 mRNA declined below baseline on days 2 and 6 (Figure 4D). This phenomenon was also observed following intradermal infection with the Chicago strain of *T. pallidum* (data not shown). Finally, IL-2 and IL-4 expression was not significantly higher than background at any timepoint (Figures 4B and 4C).

4. DISCUSSION

Cytokines play important roles in regulating the host immune system from mediating protective responses against pathogens to suppressing inflammatory responses that may lead to unwanted tissue damage. The ability to examine cytokine responses in humans and experimental models such as mice has been vital to understanding both effector and regulatory responses elicited during infection and in other conditions (e.g. autoimmunity). While the rabbit has shown great value as a small experimental model, immunological studies, including those examining cytokines, have been impeded due to lack of rabbit-specific reagents and assays. Recently, real-time RT-PCR techniques have been developed as a quick, accurate, and relatively inexpensive method for quantitating cytokine responses in human and mouse tissues (Overbergh et al., 1999; Abdalla et al., 2003). Recently, real-time RT-PCR methods have also enabled the study of cytokines in many important veterinary species that, like the rabbit, lack commercially available immunological reagents (Konnai et al., 2003; Duvigneau et al., 2005; Budhia et al., 2006). In the present study, we report the development of a quantitative real time RT-PCR assay for measuring rabbit cytokine expression. We utilized our method to provide new information on the temporal patterns of cytokine expression within rabbit splenocytes following mitogen stimulation and to examine cytokine expression within chancres during experimental syphilis in the rabbit.

Previous studies using real-time RT-PCR to quantify cytokine expression in humans and veterinary species describe the kinetics of cytokine mRNA expression following stimulation, and demonstrate some variation among species (Abdalla et al., 2003; Konnai et al., 2003; Duvigneau et al., 2005; Budhia et al., 2006). Similar to our results with rabbit cells, IFN- γ and IL-2 expression in the cells of humans and other species display a sharp upregulation by 4 hours following mitogen stimulation and peaks between 12–36 hours (Abdalla et al., 2003; Konnai et al., 2003). IFN- γ and IL-2 mRNA levels return to the background levels in both rabbit and bovine cells by 48 hours, while human cells maintained highly elevated levels at 72 hours post stimulation (Abdalla et al., 2003). A low-level expression of IL-4 and IL-10 mRNA was seen in rabbit splenocytes early following ConA stimulation. Similarly, levels of IL-4 expression are much lower compared to other cytokines in other species (Budhia et al., 2006). These variations in the temporal patterns of expression may represent true species-specific differences in cytokine regulation, or they could also be the result of experimental conditions. For instance, the activation state and composition of the stimulated immune cell

population may account for some differences, as PBMC's were used in human studies while we examined in rabbit splenocytes.

The relationship between pathogen burden and the levels of cytokine expression can be assessed by quantitative real-time RT-PCR to examine the developing local immune response during infection. The detection of strong IFN- γ expression and little to no IL-4 mRNA in early lesions during experimental syphilis in the rabbit model mirrors the Th1 profile seen in human syphilis (Van Voorhis et al., 1996); thus providing further validation of the rabbit as an appropriate model of human syphilis. Moreover, the temporal pattern of IFN- γ expression closely correlates to bacterial burden within the lesions, and peak IFN- γ expression occurred when the levels of viable treponemes in the lesion were highest in the lesions (Figure 4A). This increase in local IFN- γ production within the lesion occurred directly prior to the dramatic decline in the number of viable treponemes, strongly suggesting a role for IFN- γ in bacterial clearance. Previous studies have shown that bacterial clearance in experimental syphilis occurs immediately following the peak infiltration of T lymphocytes and macrophages (Lukehart et al., 1980a). These phagocytic macrophages are likely activated by IFN- γ .

In conclusion, we have developed and validated a new real-time quantitative RT-PCR technique for examining cytokine responses in the rabbit. This method will enable future studies of both basic immune function in the rabbit and will also aid in studies of human diseases for which the rabbit is an important experimental model.

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Code Key

γ
gamma

α
alpha

μ

micro

Figure 1.
A diagram of the multi-gene plasmid with the locations of the cytokine genes in the TOPO II
TATM vector

Figure 2.

Standard curves for IFN- γ , TNF- α , IL-2, IL-4, IL-10 and the housekeeping gene, HPRT. Curves were constructed using 10-fold serial dilutions of the linearized plasmid ranging from 10^6 copies to 1 copy per microliter. The curves were obtained by plotting the mean values of log-calculated concentration versus the crossing point (Cp). Means $\pm$ SE from 4 replicate samples for each cytokine at each concentration are shown.

Figure 3.

Normalized (per 1000 HPRT copies) relative quantification of mRNA expression by real time RT-PCR for IFN- γ (A), IL-2 (B), IL-4 (C), IL-10 (D), TNF- α (E) in rabbit splenocytes stimulated with ConA for 3, 6, 11, 24, 32, 36, and 50 hours. Cells stimulated with PBS served as a negative control. Cytokine mRNA levels in PBS control cells were measured at each timepoint; all values were similar for each cytokine. The 24 hr control values are shown in the figures. All values represent means $\pm$ SE from triplicate samples for each cytokine at each time point.

Figure 4.

Real-time RT-PCR analysis of the changes in cytokine gene expression of IFN- γ (A), IL-2 (B), IL-4(C), IL-10(D), during progression and resolution of syphilis lesions in rabbits. Four mm punch biopsies of chancres resulting from infection with 10^6 *T. pallidum* were taken on days 2, 6, 9, 13, 19, 29, 36, 45 post infection. The letter "U" (at the 0 day location) indicates values obtained from skin biopsies taken before *T. pallidum* inoculation. Each cDNA sample was tested in the RT-PCR assay in triplicate. Values represent means $\pm$ SE of samples from one lesion per rabbit from each of four infected rabbits.

Table I

Primers* for PCR amplification

Cytokine	Amplicon Size (bp)	Sense Primer	Antisense Primer
HPRT	265	5'-TGATAGATCCATTTCCTATGACTGTAGA-3'	5'-GGGTCCTTTTCACCAGCAG-3'
IFN- γ	224	5'-TTCTTCAGCCTCACTCTCTCC-3'	5'-TGTTGTCACTCTCTCTTTCC-3'
IL-2	203	5'-TGAAACATCTTCAGTGTCTAGAAG-3'	5'-CATTGTAGAATTCTGAACAGAT-3'
IL-4	302	5'-GTCACTCTGCTCTGCCTCCTC-3'	5'-GGACTCGACAGGAACCTCTG-3'
IL-10	179	5'-GAGAACCACAGTCCAGCCAT-3'	5'-CATGGCTTTGTAGACGCCTT-3'
TNF- α	335	5'-GTCTTCCTCTCTCACGCACC-3'	5'-TGGGCTAGAGGCTTGTCAC-3'

* The primers used to construct the plasmid standards contain restriction tags at their 5' end to allow cloning into TOPO II Dual Promoter vector (Invitrogen). The following tags were used: Xho I (5'-ccgctcgagcgg) for IFN- γ ; Spe I (5'-gactagtc) for IL-2; Not I (5'-ataagaatggcgccgctaaactat) for IL-10; Bam HI (5'-cgggatcccg) for IL-4 and Nsi I (5'-ccaatgcattggttctgcagtt) for TNF- α . HPRT was inserted by direct cloning

Table II

Conditions used for real time PCR assays and standard curves

Cytokine	Primer conc	Annealing Temp	Extension Time	Acquisition Temp	Cycles
HPRT	0.5 μM	64°C	12 seconds	83°C	50
IFN-γ	1 μM	60°C	9 seconds	82°C	50
IL-2	1 μM	60°C	8 seconds	79°C	50
IL-4	0.2 μM	65°C	12 seconds	87°C	50
IL-10	1 μM	60°C	7 seconds	87°C	50
TNF-α	1 μM	64°C	13.4 seconds	89°C	50