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A reverse transcription-polymerase chain reaction method to analyze porcine cytokine gene expression

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Abstract

A reverse transcription-polymerase chain reaction (RT-PCR) method was developed in order to provide a highly sensitive, rapid, and simple means of simultaneously measuring the expression of porcine cytokines in immune cell populations. Oligonucleotide primers were designed to amplify porcine cytokine cDNA from genes encoding IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IFN-γ, TNF-α, TNF-β and the housekeeping genes β-actin and cyclophilin by PCR. Primers were chosen from different exons to detect for possible genomic DNA contamination of samples. To validate RT-PCR, unstimulated and concanavalin A (ConA) stimulated porcine peripheral blood mononuclear cells (PBMCs) were cultured from 2 h to 72 h, RNA was extracted and reverse transcribed, and cDNA was amplified using the different primer sets. Band intensities of PCR products were quantified by densitometric scanning and values were normalized against cyclophilin. For each of the cytokines, the kinetics of gene expression were similar among PBMCs isolated from different animals and could be grouped into two main patterns. Lymphocyte derived cytokines (IL-2, IL-4, IFN-γ, and TNF-β) exhibited low level expression in unstimulated cells and increased expression in ConA-stimulated PBMCs. IFN-γ and IL-2 mRNA levels peaked at 24 h and returned to baseline by 72 h, whereas IL-4 and TNF-β mRNA levels did not return to

Abbreviations: ConA, concanavalin A; dNTP, deoxynucleotide triphosphate; IFN-γ, interferon-gamma; IL, interleukin; O.D., optical density; PBMCs, peripheral blood mononuclear cells; PBS, phosphate buffered saline; RT-PCR, reverse transcription-polymerase chain reaction; TBE, tris-borate EDTA; TNF, tumor necrosis factor

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baseline by 72 h. In contrast, substantial mRNA levels for inflammatory cytokines (IL-1α, IL-1β, IL-6, IL-8, IL-12, and TNF-α) and IL-10 were detected from both unstimulated and ConA-stimulated PBMCs. Results indicate that RT-PCR is a sensitive and convenient method to monitor cytokine mRNA expression in porcine samples. © 1997 Elsevier Science B.V.

Keywords: Cytokine; RT-PCR; Porcine

1. Introduction

Further knowledge concerning the mechanisms of porcine cytokines would provide valuable information about the pathogenesis of economically important porcine diseases and the immune response of swine used in experimental research models (Murtaugh, 1994). At present, study of the cytokine network in swine is limited by the lack of immunological and biological assays for the detection of porcine cytokines. A number of bioassays have been adapted for detection of porcine cytokines such as IL-1, IL-2, IL-6, and TNF (Murtaugh, 1994; Vézina et al., 1995). However, assays for detecting the production of other porcine cytokines are presently unavailable. As many of the genes encoding porcine cytokines have been recently cloned and sequenced, the detection of cytokine mRNA levels provides an alternative approach to functional assays for the detection of porcine cytokines. The reverse transcription-polymerase chain reaction (RT-PCR) is a sensitive and convenient assay that is especially advantageous for detection of low levels of mRNA from limited amounts of tissues and/or cells, and allows the simultaneous study of the expression of many cytokines. In addition, RT-PCR will allow the detection of cytokine mRNA in tissue samples from experimentally infected animals, where the presence of infectious agents may greatly impair assessment of cytokine protein detection by bioassays or immunoassays.

RT-PCR has been successfully used to clone or analyze cytokine mRNA levels in immune cells from species of veterinary interest (Covert and Splitter, 1995; Rottman et al., 1995; Seow et al., 1995). To date, few studies have examined the RT-PCR-based detection of cytokine gene expression in the pig (Bailey et al., 1994; Vézina et al., 1995). Using an RT-PCR assay, Vézina et al. (1995) examined the expression of IL-1β, IL-6, and TNF-α in unstimulated and LPS stimulated porcine alveolar macrophages. Bailey et al. (1994) demonstrated the expression of IL-4 and IL-2 mRNA in vitro in concanavalin A (ConA) stimulated intestinal and spleen lymphocytes by using RT-PCR, however, a housekeeping gene was not included in this study.

In the present study, we describe an RT-PCR assay to measure the mRNA levels of 11 different porcine cytokines: IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IFN-γ, TNF-α, and TNF-β. The method was validated by analyzing in vitro mRNA levels in porcine peripheral blood mononuclear cells (PBMCs).

2. Materials and methods

2.1. Swine and isolation of PBMCs

Healthy conventionally reared three- to four-month-old male swine from a production facility at Castelnaudary, France, were used to obtain blood for isolation of PBMCs.

From each pig, 40 ml of fresh blood was aseptically collected from the jugular vein into tubes containing 20 I.U. of sodium heparin (Panpharma, Fougères, France). The blood was diluted two-fold in phosphate buffered saline (PBS, pH 7.4) and PBMCs were isolated using Histopaque®-1077 (Sigma, St. Quentin Fallavier, France).

2.2. *PBMC culture and stimulation*

PBMCs obtained from each animal were adjusted to 5×10^6 cells ml^{-1} in complete medium (Oswald et al., 1992), distributed in 24-well plates in 1 ml aliquots and stimulated or not with 10 μg ml^{-1} Con A (Sigma). After 2 h to 72 h of incubation at 37°C with 5% CO_2, unstimulated and ConA-stimulated PBMCs were harvested for RNA isolation. RNA samples were also obtained from PBMCs collected immediately after isolation.

2.3. *RNA extraction*

At each sampling time, cultured PBMCs were washed with 1 μl of PBS. Cell pellets were resuspended in 1 ml of RNAzol B (Tel-Test, Friendswood, TX, U.S.A.), and RNA was isolated according to the manufacturer's instructions. RNA was resuspended in 100 μl of ultra-pure water containing 0.02% (w/vol) diethyl pyrocarbonate (Sigma) and 1 mM EDTA. The total RNA was quantified by using a spectrophotometer at $O.D._{260}$, and the purity was assessed by determining the $O.D._{260}/O.D._{280}$ ratio. All samples had $O.D._{260}/O.D._{280}$ ratios above 1.8.

2.4. *Reverse transcription of porcine mRNA into cDNA*

Reverse transcriptions and PCR reactions were performed in a Perkin Elmer (St. Quentin Fallavier, France) 480 thermocycler using 0.5 ml thin-walled tubes (Polylabo, Strasbourg, France). Reverse transcription of RNA into cDNA was performed in a 25 μl total volume containing: (1) 1 μg of sample RNA; (2) 0.5 U random hexamers (Boehringer Mannheim, Meylan, France); (3) 0.25 mM deoxynucleotide triphosphate (dNTP) mix of the four dNTPs; (4) 1X reverse transcriptase buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$; (5) 8 mM DDT; (6) 15 U of RNasin® ribonuclease inhibitor (Promega, Charbonnieres, France); and (7) 200 U Superscript II $RNaseH^-$ reverse transcriptase (Life Technologies, Eragny, France). Briefly, template RNA and hexamers were incubated at 70°C for 10 min and then placed on ice for 2 min. All components except the reverse transcriptase were added and the sample was incubated at 25°C for 5 min. Superscript reverse transcriptase was then added and the samples were incubated for 10 min at 25°C and for 50 min at 42°C. Enzymes were deactivated by heating to 70°C for 15 min. Two units of RNase H (Life Technologies) were added and samples were incubated at 37°C for 20 min. Samples were then heated at 70°C for 10 min, suspended to a final volume of 200 μl with ultra-pure water, and were stored at −70°C.

2.5. *Oligonucleotide primers for PCR amplification of porcine cytokine cDNA*

The oligonucleotide primers used for the detection of cDNA specific to porcine interleukins (IL): IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12 p40; interferon

gamma (IFN-γ); tumor necrosis factors (TNF): TNF-α and TNF-β; cyclophilin and β-actin were derived from published nucleic acid sequences or sequences available from the GenBank/EMBL databases (Table 1). The genes β-actin and cyclophilin were used as constitutively expressed 'housekeeping' gene controls to determine the uniformity of

Table 1
Oligonucleotides designed in this study to specifically detect porcine cytokine, β-actin, and cyclophilin cDNA

Gene specificity	Oligonucleotide sequences (5′-3′)[a]	Gene sequence	
		Accession number	Reference
IL-1 α	(S) TGCCAGCTATGAGCCACTTCC	X52731	Maliszewski et al., 1990
	(AS) TGACGGGTCTCGAATGATGCT		
	(P) CAGAAGAAGAAATCATCACG		
IL-1 β	(S) AAAGGGGACTTGAAGAGAG	M86725	Huether et al., 1993
	(AS) CTGCTTGAGAGGTGCTGATGT	X74568	Vandenbroeck et al., 1993
	(P) TGACACGCCCACCCTGCAGC		
IL-2	(S) GATTTACAGTTGCTTTTGAA	X56750	Goodall et al., 1991
	(AS) GTTGAGTAGATGCTTTGACA		
	(P) CTCTGGAGGGAGTGCTAAAT		
IL-4	(S) TACCAGCAACTTCGTCCAC	X68330	Bailey et al., 1993
	(AS) ATCGTCTTTAGCCTTTCCAA		
	(P) GAACACGACGGAGAAGGAAA		
IL-6 (S)	ATGAACTCCCTCTCCACAAGC	M86722	Richards and Saklatvala, 1991
	(AS) TGGCTTTGTCTGGATTCTTTC		
	(P) CATCCTCGGCAAAATCTCTGC		
IL-8	(S) TTTCTGCAGCTCTCTGTGAGG	M99367	Goodman et al., 1992
	(AS) CTGCTGTTGTTGTTGCTTCTC	M86923	Lin et al., 1994
	(P) GCAGAACTTCGATGCCAGTGC		
IL-10	(S) GCATCCACTTCCCAACCA	L20001	Blancho et al., 1995
	(AS) CTTCCTCATCTTCATCGTCAT		
	(P) GACTTTAAGGGTTACCTGGG		
IL-12p40	(S) GATGCTGGCCAGTACACC	U08317	Foss and Murtaugh, 1995[b]
	(AS) TCCAGCACGACCTCAATG		
	(P) ACTCCGGACGTTTCACCT		
IFN-γ	(S) GTTTTTCTGGCTCTTACTGC	X53085	Dijkmans et al., 1990
	(AS) CTTCCGCTTTCTTAGGTTAG		
	(P) AACCAGGCCATTCAAAGGAG		
TNF-α (S)	ATCGGCCCCCAGAAGGAAGAG	M29079	Pauli et al., 1989
	(AS) GATGGCAGAGAGGAGGTTGAC	X54859	Kuhnert et al., 1991
	(P) CCCTCCTGGCCAACGGCGTGA		
TNF-β	(S) CCCATCCTCCTCCTCCTG	X54859	Kuhnert et al., 1991
	(AS) GCTCCAAAGAAGACGCTACTG		
	(P) GGATCGTGCCTTCCTCCGCCA		
β-actin	(S) GGACTTCGAGCAGGAGATGG	U07786	Baarsch, 1994
	(AS) GCACCGTGTTGGCGTAGAGG		
	(P) GCTACGAGCTGCCCGACGGG		
Cyclo-philin	(S) TAACCCCACCGTCTTCTT	F14571	Winteroe et al., 1995[b]
	(AS) TGCCATCCAACCACTCAG		
	(P) TGGTGACTTCACACGCCATA[b]		

[a]S = sense primer, AS = anti-sense primer, P = internal oligonucleotide probe
[b]Direct or unpublished submission to the GenBank/EMBL databases.

Table 2
Expected PCR fragment sizes and number of cycles used for PCR amplification of porcine cytokine, β-actin, and cyclophilin cDNA

Gene specificity	Temperature (°C) of primer set[a]	cDNA PCR product (base pairs)	Genomic DNA[b] (base pairs)	Number of PCR cycles
IL-1α	69	336	2272	27
IL-1β	57	285	2275	27
IL-2	54	337	4563	38
IL-4	60	323	8437	38
IL-6	63	493	4141	36
IL-8	62	269	2129	33
IL-10	60	446	3685	33
IL-12p40	59	377	3100	36
IFN-γ	59	410	4451	33
TNF-α	64	351	1423	27
TNF-β	62	557	856	38
β-actin	65	233	327	28
Cyclophilin	58	369	3125	30

[a]Melting temperature of the primer set under PCR conditions used.
[b]Approximate size of genomic DNA; deduced from porcine gDNA or estimated from consensus sequences of other species.

the reverse transcription reactions. Oligonucleotide primer pairs were selected from different exons, as determined from porcine or consensus DNA sequences from other species, to ensure that amplified cDNA could be distinguished from any amplified genomic DNA contaminants (Table 2).

2.6. PCR amplification of porcine cytokine cDNA and primer specificity

PCR was performed as previously described (Wynn et al., 1993) with slight modifications. Briefly, the PCR mixtures for amplification of cDNA were performed in a 50 μl total volume containing: (1) 0.25 mM dNTP mix; (2) 1X PCR buffer (50 mM KCl, 10 mM Tris-HCl, 1.5 mM $MgCl_2$); (3) 0.2 mM of sense and anti-sense primers; (4) 10 μl of the reverse transcribed sample containing template cDNA. Samples were heated at 94°C for 3 min as a 'hot start' and 1 U *Taq* polymerase (Life Technologies) was added to each tube. The temperature cycling consisted of denaturing at 94°C for 45 s, annealing at 54°C for 45 s and extension at 72°C for 45 s with a final extension at 72°C for 10 min. The number of cycles used for each of the cytokines is listed in Table 2. Twenty microliters of each PCR reaction sample were migrated on 1.2% TBE agarose gels. Gels were stained with ethidium bromide and photographed using Polaroid 665 film. Internal oligonucleotide probes (Table 1) were used to confirm the specificity of the oligonucleotide primers by Southern blot as previously described (Wynn et al., 1993).

2.7. Densitometric quantification of PCR products

To quantify the level of each of the PCR products, negatives of the photos were scanned on an Omni Media Scanner XRS 12cx (Bioimage, Roissy, France) and the

intensity of the bands was determined using Image Aquisition® and Whole Band Analyzer® software (Bioimage) on a Sun Sparc Station 5 (Cadrus, Ramonville St. Agne, France). To compare the relative mRNA expression levels from each of the samples, the values are presented as the ratio of the band intensities of the cytokine RT-PCR product over the corresponding cyclophilin RT-PCR product.

3. Results

3.1. Primer specificity and RT-PCR amplification of porcine cytokine mRNA transcripts

We first verified that our designed primer pairs specifically amplified the desired cytokine, β-actin, and cyclophilin cDNA sequences by testing them for PCR amplification of cDNA samples from ConA-stimulated PBMCs. As shown in Fig. 1, each of the primer sets amplified a single sharp band of the expected size for each cDNA amplification product (Table 2). For each of the primer sets, no amplification of non-specific or genomic DNA contaminants was visualized after electrophoresis and ethidium bromide staining of agarose gels (Fig. 1). Specificity of the bands for each of the cDNA products was further confirmed by Southern hybridization using internal oligonucleotide probes (data not shown).

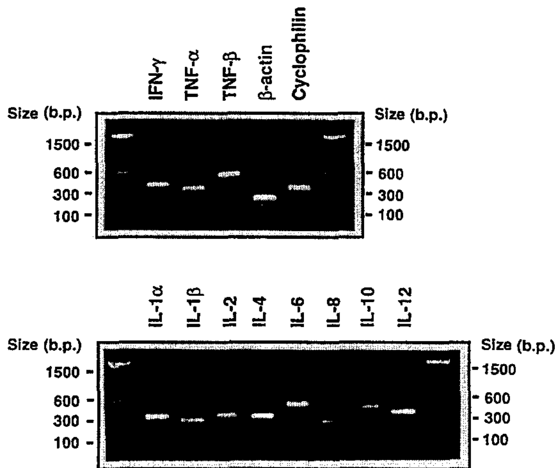

Fig. 1. Specificity of oligonucleotide primers for RT-PCR amplification of porcine β-actin, cyclophilin, and cytokine mRNA. Total RNA was isolated from 24 h ConA-stimulated PBMCs, was reverse transcribed and amplified by PCR using each of the primer sets for the number of cycles described in Table 2. PCR products were migrated in a 1.2% agarose gel and stained with ethidium bromide. Upper panel: INF-γ, TNF-α, TNF-β, β-actin, and cyclophilin RT-PCR products. Lower panel: Interleukin (IL) RT-PCR products. A 100-b.p. ladder DNA marker in the outer lanes was used for reference.

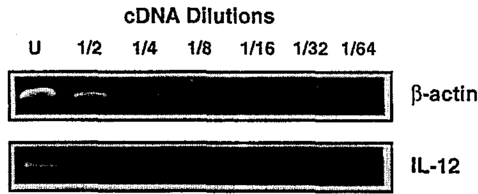

Fig. 2. Analysis of β-actin and IL-12 dilution series by RT-PCR. Serial two-fold dilutions of cDNA from ConA-activated PBMCs cultured for 24 h were amplified by PCR for 28 cycles (β-actin) or 36 cycles (IL-12) and migrated in 1.2% agarose gels. Dilution titers are indicated within the figure. Undiluted cDNA samples are labeled as (U).

We then determined the optimal number of cycles for β-actin, cyclophilin and cytokine cDNA amplification. For amplification of β-actin and cyclophilin cDNA, the number of PCR cycles was adjusted to non-saturated amplification conditions. For each cytokine, the number of PCR cycles was selected to optimize differences in the amounts of amplification product between cDNA samples from unstimulated and ConA-induced PBMCs. From 27 to 38 cycles were used for PCR amplification of cytokine or housekeeping gene cDNA from porcine PBMCs (Table 2).

To verify that the amplification conditions for each of the primer sets were non-saturated and could be used for semi-quantitative analysis of PCR products, two-fold serial dilutions of sample cDNA were amplified using each of the specific primer sets. For β-actin and IL-12, the band intensity of the PCR products decreased with each dilution, and detection on ethidium bromide gels was still visible at a 1/16 to 1/32 dilution of the initial cDNA sample (Fig. 2). Similar results were obtained for PCR amplification of diluted samples using each of the primer sets (data not shown). Hence, for each cytokine and housekeeping gene, the specific PCR amplification parameters utilized were within a suitable range for semi-quantitative analysis of cDNA levels on ethidium bromide stained agarose gels.

3.2. Uniform expression of porcine β-actin and cyclophilin

To determine whether the 'housekeeping' genes, β-actin and cyclophilin, were expressed to the same extent in all samples, cDNA obtained from unstimulated and ConA-stimulated PBMCs cultured for different times from 2 h to 72 h were amplified by PCR using β-actin and cyclophilin primer sets. A consistent level of β-actin and cyclophilin expression by both unstimulated and ConA-treated PBMCs was observed at all sampling times (Fig. 3). Moreover, in all PBMC samples obtained from three different pigs, the quantity of RT-PCR amplification products for β-actin and cyclophilin were uniform as determined by densitometric scanning. The mean intensity of the β-actin PCR product bands obtained from unstimulated PBMCs was not significantly different from the mean band intensity obtained from ConA-stimulated cells, 2.33 ± 0.18 ($n = 21$) versus 2.35 ± 0.28 ($n = 18$), respectively. Similarly, mean band intensities of the cyclophilin PCR products were not significantly different between

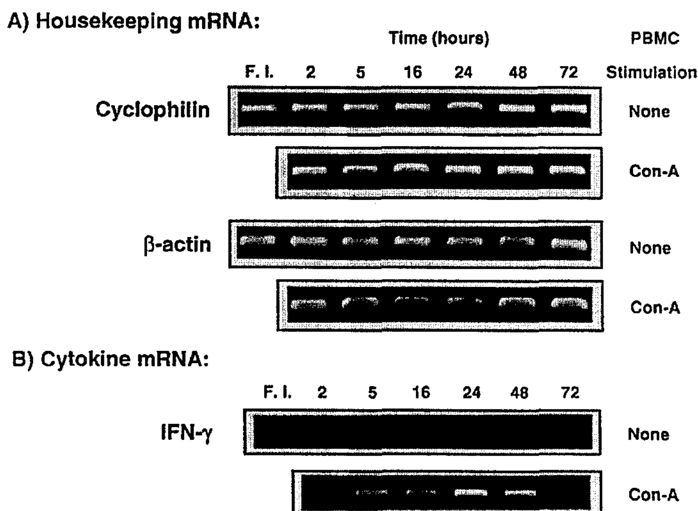

Fig. 3. RT-PCR analysis of cyclophilin, β-actin, and IFN-γ mRNA expression in PBMCs isolated from a representative pig. RNA was isolated from unstimulated and ConA-stimulated PBMCs at indicated sampling times. RNA from freshly isolated (F.I.) PBMCs obtained immediately after purification was included. Samples were reverse transcribed and amplified for 30 cycles (cyclophilin), 28 cycles (β-actin) or 33 cycles (INF-γ). RT-PCR products were separated in 1.2% agarose gels and stained with ethidium bromide.

unstimulated and ConA-stimulated PBMCs, 1.95 ± 0.13 ($n = 21$) and 1.92 ± 0.17 ($n = 18$), respectively. These results indicate that under the conditions used for RT-PCR, both β-actin and cyclophilin gene transcripts are suitable for evaluating the variation between samples. In both unstimulated and ConA-stimulated PBMCs, cyclophilin exhibited less sample to sample variability than β-actin, and cyclophilin therefore was chosen as the housekeeping gene for further analysis of cytokine gene expression.

3.3. Time course of porcine cytokine gene expression

To analyze porcine cytokine gene expression over time, cDNA samples obtained from unstimulated and ConA-stimulated PBMCs cultured for different times from 2 h to 72 h were amplified by PCR using each of the primer sets (Table 2). As an example, Fig. 3 illustrates the induction of IFN-γ expression in PBMCs isolated from one pig. A basal constitutive level of IFN-γ gene expression was observed in unstimulated cells, whereas ConA-stimulated PBMCs exhibited increased expression of IFN-γ mRNA with peak expression at 24 h.

Fig. 4 and Fig. 5 illustrate the relative expression levels of 11 different cytokines from three different pigs over time in unstimulated and ConA-stimulated PBMCs. Values obtained for each cytokine specific PCR reaction were normalized against the respective cyclophilin bands to compensate for the variation among samples. For each of

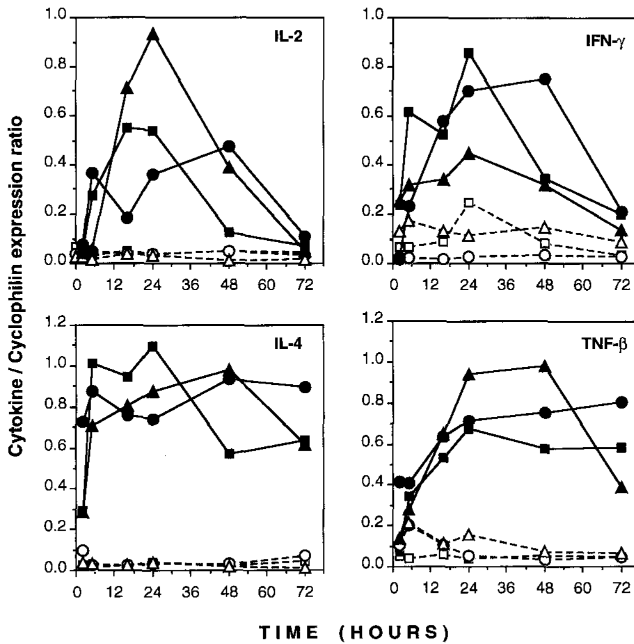

Fig. 4. Kinetics of mRNA expression of lymphocyte derived cytokines in PBMCs isolated from three different pigs. RNA samples obtained from unstimulated and ConA-stimulated PBMCs which had been cultured for different times (2 h, 5 h, 16 h, 24 h, 48 h, and 72 h) were amplified by RT-PCR. RT-PCR products migrated on ethidium bromide stained agarose gels were photographed and quantified by densitometry. Relative mRNA levels plotted against time are presented as the ratio of the cytokine RT-PCR product band intensity over the respective cyclophilin RT-PCR product band intensity. In each panel, unstimulated and ConA-stimulated PBMCs from the same individual pig are represented by the same symbol. Open symbols represent unstimulated PBMCs. Closed symbols represent ConA stimulated PBMCs.

the cytokines, the level of expression varied among samples, however, the kinetics of cytokine gene expression in PBMCs from the three different pigs were very similar.

The kinetics of cytokine gene expression after ConA stimulation of PBMCs could be grouped into two main patterns. For the cytokines produced mainly by lymphocytes, such as INF-γ, IL-2, IL-4, and TNF-β, mRNA expression was induced in ConA-stimulated PBMCs, whereas unstimulated PBMCs only expressed low levels of these cytokines (Fig. 4).

For the inflammatory cytokines tested, namely, IL-1α, IL-1β, IL-6, IL-8, IL-12 and TNF-α, and IL-10, substantial mRNA levels were observed in both unstimulated and ConA-stimulated PBMCs (Fig. 5). For TNF-α, IL-6, and IL-12, PBMCs stimulated with ConA demonstrated greater levels of gene expression than unstimulated PBMCs. However, for IL-12, the differences in mRNA levels were only discernable between unstimulated and ConA-stimulated PBMCs from the same animal. For IL-1β and IL-10, no differences in the expression of these cytokines were observed between unstimulated

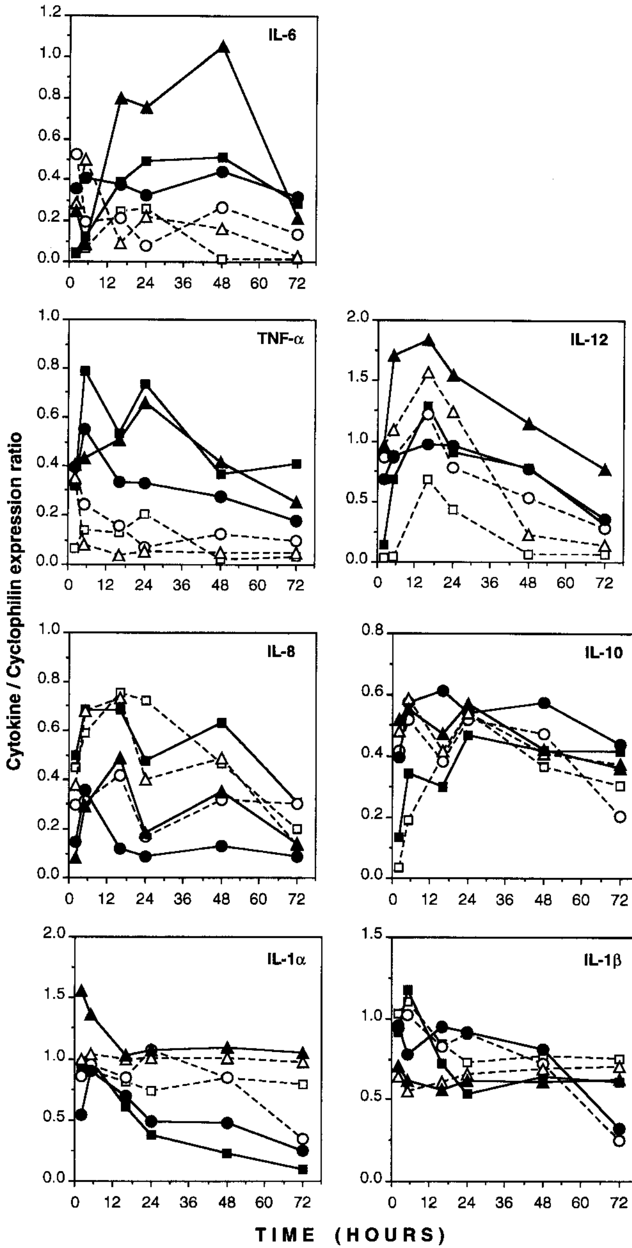

Fig. 5. Kinetics of mRNA expression of inflammatory cytokines and IL-10 in PBMCs isolated from three different pigs. Legend is the same as in Fig. 4.

or ConA-stimulated PBMCs. For IL-8 and IL-1α, mRNA levels in unstimulated PBMCs were slightly greater than those of ConA-stimulated PBMCs from the same pig.

4. Discussion

RT-PCR has proved to be a powerful method to study gene expression in mammalian tissues and has been successfully used to detect cytokine expression in a number of animal species including those of veterinary interest (Covert and Splitter, 1995; Rottman et al., 1995; Seow et al., 1995; Vézina et al., 1995). In the present report, we designed oligonucleotide primers from sequences specific to porcine cytokine genes (Fig. 1) and we used the RT-PCR technique to monitor in vitro the kinetics of expression of 11 different porcine cytokines in porcine PBMCs. The RT-PCR technique presented in this study has been successfully and repeatedly used in our laboratory with similar results obtained at different times with changes in the reagents (three different types of *Taq* polymerase, two different types of reverse transcriptase, and two types of ribonuclease inhibitor), the use of several thermocycler models (Perkin Elmer; MJ Research) and different workers. These experiments included analysis of cytokine expression in a number of porcine tissues and cell lines as well as tissues from piglets infected with pathogenic *Escherichia coli*. In addition, this RT-PCR method was used for semi-quantitative analysis of murine cytokines by two independent research groups (Wynn et al., 1994; Oswald et al., 1997). Thus, this RT-PCR technique provides a consistent means of analyzing porcine cytokine gene expression.

Although most other RT-PCR studies on cytokine expression in veterinary species did not present details of cytokine expression levels in unstimulated cells at different times, the results of the present study are in accordance with many other reports. Low levels of IL-2, IL-4, and IFN-γ mRNA were detected in unstimulated feline PBMCs (Rottman et al., 1995) and bovine immune cells (Covert and Splitter, 1995), whereas ConA stimulation increased the expression of these cytokines. In contrast, by using an RT-PCR assay, Vézina et al. (1995) reported constitutive IL-1β, IL-6, and TNF-α mRNA levels in unstimulated porcine alveolar macrophages that were often similar to those observed for LPS-stimulated macrophages. In addition, substantial levels of inflammatory cytokine mRNA were detected by RT-PCR in unstimulated immune cells from other species including cats (IL-10, IL-12, IL-6, and TNF-α) (Rottman et al., 1995), cattle (IL-1α, IL-1β, IL-6, IL-10, and TNF-α, GM-CSF) (Covert and Splitter, 1995), and monkeys (IL-1β, IL-10, and TNF-α) (Benveniste et al., 1996).

RT-PCR is advantageous for cytokine transcript analysis, because it can be used to quickly monitor the simultaneous expression of an array of cytokines from the same population of cells or tissues and requires only small quantities of starting template material. By using densitometric scanning, the RT-PCR method can provide semi-quantitative results when target mRNA levels are standardized with reference mRNA levels of housekeeping genes. The use of such reference genes as internal standards for mRNA levels has some limitations, as their expression may vary following cell activation or between different cell populations. In the present study we verified that in porcine PBMCs, expression levels of β-actin and cyclophilin were uniform throughout

the experiment. Thus, both of these genes were suitable housekeeping genes for our RT-PCR assay (Fig. 3). However, we chose cyclophilin as our internal mRNA standard, as it exhibited less sample to sample variation than β-actin in RT-PCR reactions. Cyclophilin has recently been used as a housekeeping gene for RT-PCR analysis of murine (Lukacs et al., 1995) and human (Burnet et al., 1994) tissues and is also constitutively expressed in rabbit osteoclasts (Sakai et al., 1995). To our knowledge, this is the first time cyclophilin has been used as a housekeeping gene to study porcine gene expression.

The RT-PCR assay we have developed provides an efficient means of screening simultaneously various porcine cells or tissues for differences in the mRNA levels of numerous cytokines. However, this technique has certain limitations. Firstly, the RT-PCR assay allows for relative quantitation of cytokine mRNA levels, but does not permit the precise determination of the quantity of target cytokine mRNA nor comparison of the amount of mRNA between different cytokines. Hence, only values obtained for the same cytokine can be compared among samples (Fig. 4 and Fig. 5). As an alternative, quantitative PCR analysis, also termed competitive PCR, using synthetic internal standards has also been used to study mRNA expression (Siebert and Larrick, 1992), and could be developed to more precisely determine cytokine expression levels in swine (Reddy et al., 1996). Secondly, relative mRNA levels determined by RT-PCR do not necessarily reflect the relative amounts of protein produced. Although for most cytokines mRNA expression correlates with cytokine protein production, post-transcriptional mechanisms prior to secretion as well as rapid utilization of cytokines by cells may explain some of the differences observed between gene expression and protein detection (Bailey et al., 1994; Vézina et al., 1995). We are currently comparing mRNA levels obtained by RT-PCR and cytokine protein production using available bioassays/immunoassays. Regardless of the limitations of RT-PCR, this technique provides a simple means of screening for cytokine gene expression in swine. Moreover, RT-PCR is advantageous for determining cytokine expression levels in cells or tissues from experimentally infected animals, where the presence of bacteria, viruses and/or their products may restrict the use of cytokine detection by bioassays or immunoassays. We are presently successfully using this method to analyze cytokine mRNA expression from intestinal tissues of piglets experimentally infected with pathogenic *E. coli*. Preliminary results have demonstrated increased levels of TNF-α and IL-1 expression in the jejunum and mesenteric lymph nodes of infected piglets as compared to uninfected controls.

In conclusion, an RT-PCR assay using newly designed oligonucleotide primers was developed to detect porcine cytokine expression in a specific and semi-quantitative manner. We validated the assay by using unstimulated and ConA-stimulated PBMCs. RT-PCR will provide a rapid and convenient means of determining cytokine expression profiles for porcine cells and tissues in vivo and in vitro. As more immunoassays and bioassays become available for the detection of different porcine cytokines (Murtaugh, 1994), the combination of a highly sensitive and specific RT-PCR assay and the use of such assays for the detection of secreted cytokines will lead to further elucidation of the porcine cytokine network and how it contributes to the immune response against infectious agents of economic interest such as *E. coli*.

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