CLINICAL AND DIAGNOSTIC LABORATORY IMMUNOLOGY, Nov. 1995, p. 665–671 Vol. 2, No. 6
1071-412X/95/$04.00+0

Cloning of Porcine Cytokine-Specific cDNAs and Detection of Porcine Tumor Necrosis Factor Alpha, Interleukin 6 (IL-6), and IL-1β Gene Expression by Reverse Transcription PCR and Chemiluminescence Hybridization

SOPHIE-ANNICK VÉZINA,[1] DOMINIQUE ROBERGE,[1] MICHEL FOURNIER,[1] SERGE DEA,[2] DANIEL OTH,[3] AND DENIS ARCHAMBAULT[1]*

Université du Québec à Montréal, Département des Sciences Biologiques, Montréal,[1] and Institut Armand-Frappier, Centre de Recherche en Virologie[2] et en Immunologie,[3] Laval, Québec, Canada

Received 12 May 1995/Returned for modification 14 June 1995/Accepted 1 August 1995

A reverse transcription PCR assay with porcine cytokine-specific primers was developed to clone cDNA fragments and generate cDNA probes that were specific for porcine tumor necrosis factor alpha (TNF-α), interleukin 6 (IL-6), and IL-1β. The specificities of the cDNA PCR products were confirmed by sequence analysis on the basis of known porcine cytokine gene sequences. The reverse transcription PCR assay was also used to study cytokine mRNA expression in lipopolysaccharide (LPS)-stimulated and control unstimulated porcine alveolar macrophages. The cDNA products were analyzed in ethidium bromide-stained agarose gels, and the transcription level of each cytokine was determined relative to the endogenous glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA level of each cytokine by measuring the intensity of the chemiluminescence hybridization signals by densitometric scanning. Various levels of cytokine mRNAs were detected in both LPS-stimulated and control unstimulated cells. Thus, TNF-α mRNA levels were enhanced in the cell cultures stimulated for 6 h with LPS compared with those in control cell cultures. No differences in TNF-α transcription levels between LPS-stimulated and control cells were observed after incubation for 24 or 55 h. Enhancements of IL-6 and IL-1β mRNA levels were also observed in the cultures stimulated with LPS for 6 and 24 h compared with the cytokine mRNA levels in control cell cultures. The presence of cytokine mRNA transcripts in the LPS-stimulated macrophage cultures correlated with the detection of these soluble cytokines by the bioassays. In contrast, no soluble cytokine was detected in control macrophage culture supernatants in the presence of cytokine mRNA transcripts.

A variety of immunocompetent cells such as macrophages and T and B lymphocytes play a central role in the humoral and/or cellular immune response to pathogenic infections and other antigens of various natures. These immune responses are regulated by soluble factors called cytokines (2, 22), which are polypeptide hormones with autocrine or paracrine activities released by various immune cell types.

Although progress has been made in recent years, the cascade of cytokines and its effect on the regulation of cells of the immune system are not well understood, especially in vivo (22). However, it is generally accepted that monocytes/macrophages play a major role in the activation of lymphocytes through the cytokines that they secrete (22). Among them, interleukin 1 (IL-1), IL-6, and tumor necrosis factor alpha (TNF-α), which are mainly produced by the monocyte/macrophage lineage (17, 28, 34), can act on a variety of cells, which, in turn, produce other cytokines that may be important in various physiological functions within the host (3, 16, 22, 23, 30).

The cloning and sequencing of porcine TNF-α, IL-6, and IL-1β gene cDNAs have been reported previously (11, 18, 25). However, precise information on porcine monokine gene expression is lacking. Cytokine mRNAs are generally expressed at extremely low levels and are therefore difficult to detect, especially from small numbers of cells (6). Moreover, conventional bioassays that are used to detect the presence of cytokines in activated porcine immune cell supernatants offer relatively low sensitivities and specificities (8, 27).

PCR is a technique by which the nucleic acid in any sample can be specifically amplified by up to 10^6-fold prior to attempting to detect it (33). Over the past few years, a reverse transcription (RT) step has been used in conjunction with PCR amplification for the detection of a variety of RNA molecules from different sources (4, 6, 9, 15, 18, 19, 21, 31, 32, 38). In the study described in this report, porcine-specific cytokine nucleic acid probes were generated, and an RT-PCR assay, standardized with the endogenous glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene, was developed and was used to measure the relative levels of expression of porcine TNF-α, IL-6, and IL-1β mRNAs in porcine macrophages.

MATERIALS AND METHODS

Cells. Porcine peripheral blood mononuclear cells (PBMCs) were separated by Ficoll-diatrizoate (specific gravity = 1.077 g/ml) gradient centrifugation of sodium citrate-treated peripheral blood from individual 6- to 8-week-old specific-pathogen-free piglets as described previously (1, 40). The PBMCs were then resuspended (15×10^6 cells per ml) in RPMI 1640 medium supplemented with 2 mM L-glutamine, 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), 15% heat-inactivated fetal bovine serum (FBS), and antibiotics. Five milliliters of the PBMC suspension was then plated in cell culture in 60-mm petri dishes, and the dishes were incubated overnight at 37°C in a 5% CO_2 atmosphere. Thereafter, the adherent cells were washed three times with a phosphate-buffered saline solution and were cultured for 2 h in serum-free cell culture medium. The medium was then removed and was replaced with 5 ml of cell culture medium containing 5% FBS, 5×10^{-5} M 2-mercaptoethanol, and, where appropriate (stimulated cells), 2.5 μg of lipopolysaccharide (LPS; *Escherichia coli* serotype O17-B8; Sigma Chemical Company, St. Louis, Mo.) per ml.

* Corresponding author. Mailing address: Université du Québec à Montréal, Département des Sciences Biologiques, C.P. 8888, Succursale Centre-Ville, Montréal, Québec, Canada H3C 3P8. Phone: (514) 987-4622. Fax: (514) 987-4647.

TABLE 1. Nucleotide sequences of PCR primers of target cytokine genes

Cytokine mRNA	Sense	Sequence (5′ to 3′)	Predicted product size (bp)
IL-1β	+	TCAGGCAGATGGTGTCTGTC	417
	−	GGTCTATATCCTCCAGCTGC	
IL-6	+	AACGCCTGGAAGAAGATGCC	532
	−	CTCAGGCTGAACTGCAGGAA	
TNF-α	+	GCTGTACCTCATCTACTCCC	291
	−	TAGACCTGCCCAGATTCAGC	

Cytochemical analysis (i.e., sodium fluoride-inhibited esterase activity) revealed that the adherent cell population consisted of >95% monocytes.

Pig alveolar macrophage (PAM) cells, which were used for the kinetic studies for cytokine gene expression, were obtained from bronchoalveolar washes of individual specific-pathogen-free piglets as described previously (5, 26). The viability of the cell preparation was >95%, as determined by the trypan blue exclusion test, and examination of Wright-stained smears revealed that 75 to 80% of the cells were macrophages. The cells were plated at a concentration of 5×10^6 cells per cell culture in petri dishes. The cell cultures were then treated as described above for the blood-derived adherent cells.

RNA isolation and analysis. Control unstimulated and LPS-stimulated cells were lysed at 6, 24, and 55 h after treatment, and total cellular RNA was isolated by a guanidinium isothiocyanate procedure with a commercial kit (TRIzol Reagent; Gibco/BRL, Gaithersburg, Md.). Aliquots of the cell culture supernatants were kept at −85°C and were used for cytokine detection in the bioassays. The resulting RNA preparation was resuspended in RNase-free diethyl pyrocarbonate-treated water, heated to 94°C for 5 min, and chilled on ice prior to RNase-free DNase treatment (2.5 U for 1 h) to remove possible contaminating genomic DNA (32). The RNA suspension was then heated at 94°C for 10 min to inactivate the DNase, adjusted to a concentration of 0.05 μg/μl (as measured by determining the optical density at 260 nm), and kept at −85°C until it was used. Cytokine mRNA levels were determined by RT-PCR (38), and the transcription level of each cytokine was determined relative to the endogenous GAPDH RNA level (15).

Oligonucleotide primers, cDNA synthesis, and PCR. The oligonucleotide primers listed in Table 1 and derived according to the published sequences of porcine IL-1β (18), IL-6 (25), and TNF-α (11) cDNAs were synthesized with a Gene Assembler Plus DNA Synthesizer (Pharmacia Biotech, Uppsala, Sweden). Primers were selected by using PRIMER analysis software (version 0.5; Whitehead Institute for Biomedical Research, Cambridge, Mass.) within the GCG system of the VAX (10). The GAPDH product was amplified with the commercial GAPDH Control Amplimer set of primers (Clontech Laboratories Inc., Palo Alto, Calif.) that is generally used to amplify human, rat, or mouse GAPDH sequences with an expected PCR product size of 450 bp. The melting temperatures of all oligonucleotide primers varied from 60 to 62°C.

For each cytokine to be tested, RT of mRNAs was carried out at room temperature for 10 min and at 42°C for 1 h in 1× *Taq* buffer (10× *Taq* buffer is 500 mM KCl, 100 mM Tris-HCl [pH 8.3], 1% Triton X-100) containing 1 mM (each) deoxynucleoside triphosphates, 5 mM $MgCl_2$, 15 U of avian myeloblastosis virus reverse transcriptase (Pharmacia Biotech), 60 U of RNAsin (Pharmacia Biotech), 10 μl of RNA sample, and 10 pmol of random hexadeoxyribonucleotides [pd$(N)_6$; Pharmacia Biotech] (21) in a final reaction volume of 40 μl. Then the mixture was incubated at 94°C for 5 min to inactivate the avian myeloblastosis virus reverse transcriptase. For PCR coamplification of DNA, 5 μl of 10× *Taq* buffer, 0.2 mM (each) deoxynucleoside triphosphates, 2.5 U of *Taq* DNA polymerase (Boehringer Mannheim, Laval, Québec, Canada), 10 pmol of each cytokine (IL-1β, IL-6, or TNF-α)-specific primer together with the GAPDH-specific sense and antisense primers were added to one-quarter of the cDNA mixture volume to a final reaction volume of 50 μl. The mixture was then overlaid with mineral oil and was incubated for 4 min at 94°C. The cDNA was then amplified by 20, 24, 28, and 30 successive cycles (to ensure that amplification was linear) of denaturation at 94°C for 1 min, primer annealing at 61°C for 1 min, and DNA chain extension at 72°C for 2 min by using a programmable thermal cycler (Temp.Tronic; Barnstead/Thermolyne, Dubuque, Iowa). An additional incubation step at 72°C for 8 min was allowed after the final amplification cycle. All reagents used in the RT-PCR procedure, including the buffer and the primer solutions, were routinely tested for possible cellular RNA contamination by running mock RT-PCRs. To control for possible DNA contamination, parallel PCRs (30 amplification cycles) were also run for each cytokine-specific primer set in which the RT step was omitted. The conditions used in the RT-PCR procedure described above were obtained from preliminary experiments in which RT-PCR assays were optimized by varying the annealing temperatures and the $MgCl_2$ and RNA template (from 32.25 to 250 ng of RNA) concentrations in the reaction mixtures (data not shown). The denaturation and extension times and temperatures described above for all primer pairs were also found to be suitable for the cDNA amplification step.

Preparation of cytokine- and GAPDH-specific cDNA probes. The amplified cDNA fragments from the IL-1β (417 bp), IL-6 (532 bp), TNF-α (291 bp), and GAPDH (450 bp) nucleotide sequences were cloned into the PCR II TA cloning vector (Invitrogen Corporation, San Diego, Calif.) for DNA probe preparation as described previously (38). The cloned cDNAs were then sequenced by the chain termination sequencing method (35) and were analyzed to confirm the porcine cytokine sequence. The sequence of the porcine GAPDH cDNA clone was confirmed after comparison with the published sequence of human GAPDH cDNA (13). The cDNA fragments were excised from the DNA vector with the appropriate restriction enzymes and were purified by using a low-melting-temperature agarose gel as described previously (38). The cDNA bands were recovered and used as templates in the random-primed DNA labeling technique in the presence of digoxigenin-11-dUTP by using a DNA labeling and detection kit (Boehringer Mannheim).

Analysis of amplified cytokine cDNAs, Southern blot hybridization, and RNA measurement. A 10-μl sample of the 50-μl reaction mixture was fractionated by electrophoresis through 2% NuSieve and 1% SeaKem agarose gels in TBE buffer (90 mM Tris-borate, 2 mM EDTA [pH 8.0]). The DNA was visualized under UV light by ethidium bromide (1 μg/ml) fluorescence.

For Southern blot hybridization, the RT-PCR products were transferred onto nylon membranes and were subjected to prehybridization and hybridization with 50 pmol of each digoxigenin-labeled cytokine cDNA probe essentially as described previously (38). The membranes were then subjected to chemiluminescence analysis with a commercial digoxigenin luminescence detection kit (Boehringer Mannheim). The chemiluminescence-treated membranes were exposed to X-ray film (X-OMAT AR-5; Eastman Kodak Company, Rochester, N.Y.), usually for 5 to 20 min. Preliminary experiments under the same hybridization conditions described above have shown that the DNA band intensities obtained with each cDNA probe, including that obtained with GAPDH, increased linearly for film exposure times up to 30 min (data not shown). Thereafter, the membranes were stripped off to remove the cytokine cDNA probe and were then subjected to hybridization with the GAPDH cDNA probe and autoradiography as described above. The intensity of the hybridization signal for each PCR product was determined by densitometric scanning (model PDS1-MAC 375A; Molecular Dynamics, Sunnyvale, Calif.). The relative levels of IL-1β, IL-6, and TNF-α RNAs were determined relative to the endogenous GAPDH RNA level of each cytokine and were expressed as the cytokine/GAPDH ratio (32). The given cytokine/GAPDH ratios of the LPS-stimulated cells were compared at each posttreatment time with those for the mRNA transcripts from unstimulated control cells for each PCR amplification cycle. Constant amplification in reaction tubes was assessed by coamplifying exogenous DNA (equine arteritis virus ORF-4 sequences) (38) in some samples.

Cytokine bioassays. The secretion of IL-6 and TNF-α by PAMs was detected by measuring the proliferation of IL-6-dependent B9 cells and the cytotoxicity of TNF-α-sensitive L929 and WEHI 164 cells, respectively (27), as described elsewhere (39).

RESULTS

Cytokine RNA amplification and cloning of the RT-PCR products. Following the RT reaction with random hexamers, the oligonucleotide primers specific for porcine IL-6, IL-1β, and TNF-α amplified, after 30 PCR amplification cycles, well-defined DNA products, as observed on an ethidium bromide-stained agarose gel with total cellular RNA extracted from porcine blood-derived monocytes stimulated for 12 h with LPS. These DNA bands corresponded to the expected size of 532 bp (IL-6), 417 bp (IL-1β), or 291 bp (TNF-α) (Fig. 1, lanes 1 to 3, respectively) on the basis of the published cDNA sequences specific to each porcine monokine. The primer pair used for GAPDH detection amplified a cDNA product corresponding to the expected size of 450 bp (Fig. 1, lane 4). The RT-PCR cDNA products were then excised from the agarose gels, cloned into the PCR II TA cloning vector, and sequenced for IL-1β, IL-6, and TNF-α sequence confirmation. Sequence analysis of the amplified porcine GAPDH product showed a 91% homology with the human GAPDH sequence (data not shown). The plasmid constructs were also used as positive control DNA templates in subsequent RT-PCRs and to generate cDNA probes specific to each cytokine and GAPDH gene product.

Detection and analysis of cytokine gene expression. After we demonstrated that the selected oligonucleotide primers and

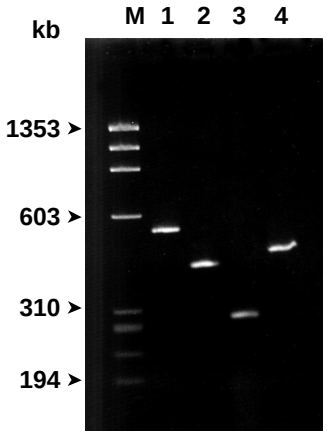

FIG. 1. Amplification products of porcine IL-6 (lane 1), IL-1β (lane 2), TNF-α (lane 3), and GAPDH (lane 4) mRNAs by RT-PCR. Total cellular RNAs isolated from porcine blood-derived adherent cells stimulated for 12 h with LPS were reverse transcribed with random hexamers and were then amplified for 30 cycles with each cytokine-specific sense and antisense oligonucleotide primer set described in Table 1. A 10-μl sample of each RT-PCR mixture was subjected to gel electrophoresis as described in Materials and Methods. Lane M, *Hae*III-digested ϕX174 replicative form DNA as molecular size markers.

assay conditions were suitable for IL-1β, IL-6, TNF-α, and GAPDH sequence amplification, we wished to evaluate the sensitivity of the RT-PCR for cytokine gene expression and semiquantitation. To fulfill this objective, alveolar macrophages were cultured and exposed to LPS, as described in Materials and Methods, for 6, 24, or 55 h. Total cellular RNA was then extracted and subjected to RT with the random hexamer primer as described above. cDNA aliquots were then coamplified with various numbers of PCR amplification cycles by using a primer pair specific to each cytokine together with the GAPDH-specific primers in the same tube reaction.

As shown in Fig. 2A, we could readily amplify the TNF-α gene product in cells stimulated for 6 h with LPS, as demonstrated by the presence of a DNA band of the expected size on the ethidium bromide-stained agarose gel obtained with 20, 24, 28, or 30 PCR amplification cycles. The DNA band intensity increased with the number of amplification cycles (Fig. 2A, lanes 5 to 8). DNA bands of much less intensity were also detected in the nonstimulated control cell cultures after 24 to 30 PCR amplification cycles (Fig. 2A, lanes 2 to 4). These results suggest that mRNA for porcine TNF-α is constitutively expressed by macrophages at a low level and that its expression increases after LPS treatment. cDNA products specific to the constitutive GAPDH gene obtained from control and LPS-stimulated cells were somewhat similar in intensity when they were compared at each PCR amplification cycle. This shows that comparable amounts of cDNA templates were used in each PCR mixture. To further confirm that the PCR-derived products were TNF-α and GAPDH specific, the agarose gel was subjected to Southern hybridization with cDNA probes specific for the expected amplified sequences. As shown in Fig. 2B, positive signals of various intensities were detected for TNF-α in control and LPS-stimulated cells with either PCR amplification cycle number. The hybridization signals obtained with the TNF-α-specific cDNA probe (as well as for the IL-6- and IL-1β-specific cDNA probes; see below) were not observed with a control PCR II TA cloning vector containing no cytokine gene-specific DNA insert (data not shown).

It was apparent from the results obtained from the ethidium bromide-stained agarose gels and the corresponding autoradiograms that the level of TNF-α gene expression was increased in the cell cultures treated with LPS for 6 h compared with that in control unstimulated cells. Although it was possible to detect cytokine- and GAPDH-specific DNA bands in both control and LPS-stimulated cells, no significant difference in the cytokine transcription levels could be detected between these cultures when the cells were harvested for RNA analysis at 24 h (Fig. 2C and D) or 55 h (Fig. 2E and F) after starting cell stimulation. Variations in the intensities of the hybridization signals for both TNF-α and GAPDH at 6, 24, or 55 h after cell treatment were observed because of different film exposure times. This had no effect on the interpretation of the results, however, because the DNA band intensities, as mentioned above, were linearly proportional to film exposure time. Moreover, comparisons of cytokine gene expression between nonstimulated and LPS-stimulated cell cultures were always made on the same agarose gel and the same blotting membrane at each posttreatment time.

To generate quantitative data, the level of TNF-α transcription in LPS-stimulated cell cultures relative to the endogenous GAPDH RNA level was then determined and was compared with the cytokine/GAPDH ratio obtained in control cells at each PCR amplification cycle and at each posttreatment time. As shown in Fig. 2G, the differences in the TNF-α/GAPDH ratios between control cells and cells stimulated with LPS for 6 h were inversely proportional to the number of amplification cycles used in the PCR, with the highest differences being observed when 20 or 24 PCR amplification cycles were used. On the basis of these observations, there was a complete concordance between these ratio data and the results obtained with the ethidium bromide-stained agarose gel and the corresponding autoradiogram (Fig. 2A and B). In contrast, we observed no marked differences in the levels of TNF-α mRNA expression between the control and the LPS-stimulated cell cultures after 24 or 55 h of cell stimulation for any number of PCR amplification cycles (Fig. 2C to G).

Porcine IL-6 and IL-1β gene transcription levels relative to the endogenous GAPDH mRNA levels after cell stimulation were also evaluated. A noticeable increase in the level of IL-6 gene transcription was observed in cells stimulated with LPS for 6 h, as determined with the ethidium bromide-stained agarose gel, the corresponding autoradiogram, and the cytokine/GAPDH ratio determination, compared with the data for control cells after 20 to 30 PCR amplification cycles (Fig. 3A, B, and E). The IL-6 transcription level was still enhanced in LPS-treated cell cultures at 24 h posttreatment compared with that in the control cell cultures after 20 or 24 cDNA amplification cycles (Fig. 3C, D, and E), but the IL-6/GAPDH ratio differences between the LPS-stimulated and the control cell cultures were less than those obtained in the 6-h cell cultures. As shown in the cytokine/GAPDH histogram (Fig. 3E), no noticeable enhanced level of IL-6 transcription was observed in the cell cultures stimulated with LPS for 55 h compared with the level in the control cell cultures.

The level of IL-1β gene transcription was also enhanced in the LPS-stimulated culture after 6 or 24 h of cell exposure compared with that in control cell cultures. These enhanced IL-1β mRNA levels were observed at a low number of PCR amplification cycles (20 cycles), as determined with the ethidium bromide-stained agarose gel, from the intensity of the hybridization signals, and/or from the cytokine/GAPDH ratio differences (Fig. 4A, B, and C). The lower quantity of GAPDH cDNA PCR products compared with the quantity obtained for TNF-α or IL-6 gene transcription was due to the use of less starting cellular RNA material in the RT step prior to PCR amplification. Finally, as shown in the histogram of the cyto-

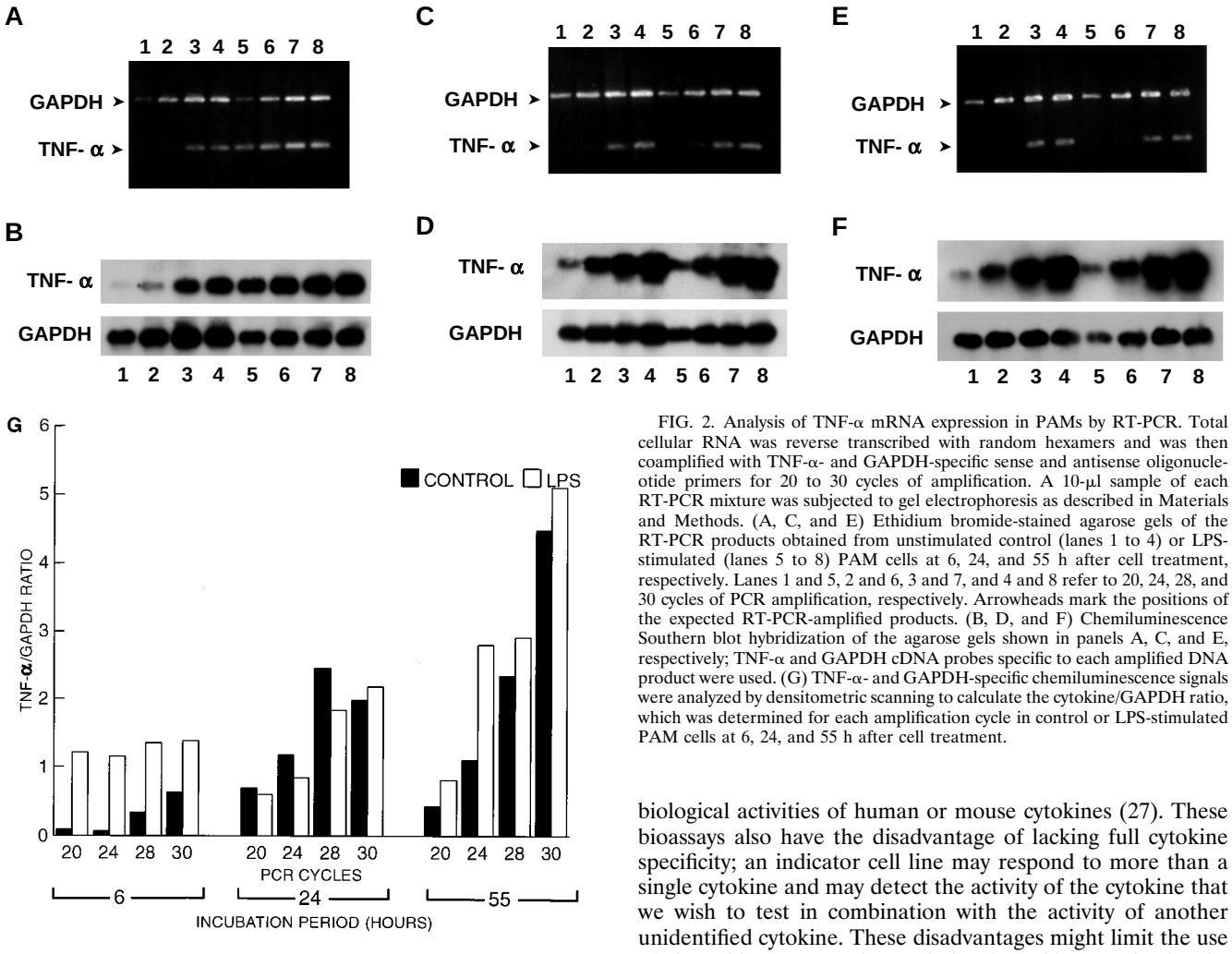

FIG. 2. Analysis of TNF-α mRNA expression in PAMs by RT-PCR. Total cellular RNA was reverse transcribed with random hexamers and was then coamplified with TNF-α- and GAPDH-specific sense and antisense oligonucleotide primers for 20 to 30 cycles of amplification. A 10-μl sample of each RT-PCR mixture was subjected to gel electrophoresis as described in Materials and Methods. (A, C, and E) Ethidium bromide-stained agarose gels of the RT-PCR products obtained from unstimulated control (lanes 1 to 4) or LPS-stimulated (lanes 5 to 8) PAM cells at 6, 24, and 55 h after cell treatment, respectively. Lanes 1 and 5, 2 and 6, 3 and 7, and 4 and 8 refer to 20, 24, 28, and 30 cycles of PCR amplification, respectively. Arrowheads mark the positions of the expected RT-PCR-amplified products. (B, D, and F) Chemiluminescence Southern blot hybridization of the agarose gels shown in panels A, C, and E, respectively; TNF-α and GAPDH cDNA probes specific to each amplified DNA product were used. (G) TNF-α- and GAPDH-specific chemiluminescence signals were analyzed by densitometric scanning to calculate the cytokine/GAPDH ratio, which was determined for each amplification cycle in control or LPS-stimulated PAM cells at 6, 24, and 55 h after cell treatment.

kine/GAPDH ratios in Fig. 4C, no enhancement of the level of IL-1β transcription was observed in the cell cultures stimulated with LPS for 55 h compared with that in control cell cultures.

Secretion of porcine cytokines in macrophage culture supernatants. As shown in Table 2, the biological activities of TNF-α and IL-6 in LPS-stimulated alveolar macrophage culture supernatants increased progressively over time, reaching maximal levels at 24 or 55 h, respectively, after cell treatment. No biological activity of the cytokines was observed in control unstimulated cells. Similar results were obtained with macrophages from two different donors (data not shown).

DISCUSSION

Cytokines play a crucial role in the modulation of immunological and physiological reactions in animals under normal or stress reactions. IL-1, IL-6, and TNF-α are cytokines which are mainly produced by monocytes/macrophages in response to stressful conditions and which have a role in lymphocyte activation (22). In many animal species, the presence of IL-1β, IL-6, and TNF-α in stimulated immune cell supernatants can be detected by using bioassays that are performed with indicator cell lines such as D10.G4.1 cells, B9 cells, and L929 or WEHI 164 cells, respectively (27). However, these bioassays are laborious and time-consuming and, in swine, offer relatively poor sensitivity compared with those used to assess the biological activities of human or mouse cytokines (27). These bioassays also have the disadvantage of lacking full cytokine specificity; an indicator cell line may respond to more than a single cytokine and may detect the activity of the cytokine that we wish to test in combination with the activity of another unidentified cytokine. These disadvantages might limit the use of these bioassays to the analysis of cytokine production by cells in vivo. Therefore, the development of molecular reagents and methods to assess cytokine gene expression in the early stages of cell activation is needed to elucidate their biology. To this end, in swine, progress with the cloning and sequencing of various porcine cytokines has been made in recent years. In our study, we took advantage of these nucleotide sequence data to develop cytokine-specific cDNA probes and used the RT-PCR system for the rapid, sensitive, and specific detection of IL-1β, IL-6, and TNF-α gene transcription in porcine monocytes/macrophages.

The results presented here have indicated that all primer sets used in the RT-PCR procedure targeting the coding sequences of porcine IL-1β, IL-6, and TNF-α were successful in amplifying the cytokine cDNAs derived from reverse-transcribed monocyte or macrophage mRNAs. The specificities of the RT-PCR assays were confirmed when the amplified products of the expected size reacted positively with the corresponding cytokine-specific digoxigenin-labeled cDNA probes in the chemiluminescence assays.

To evaluate more adequately the modulation of cytokine gene expression, a quantitative or semiquantitative RT-PCR assay is needed. A relatively easy way to evaluate the transcription level of a particular gene is to use a semiquantitative PCR standardized with the endogenous GAPDH gene, whose level of expression is recognized to remain unchanged under most conditions (12, 24, 37, 41). Although quantitation of nucleic

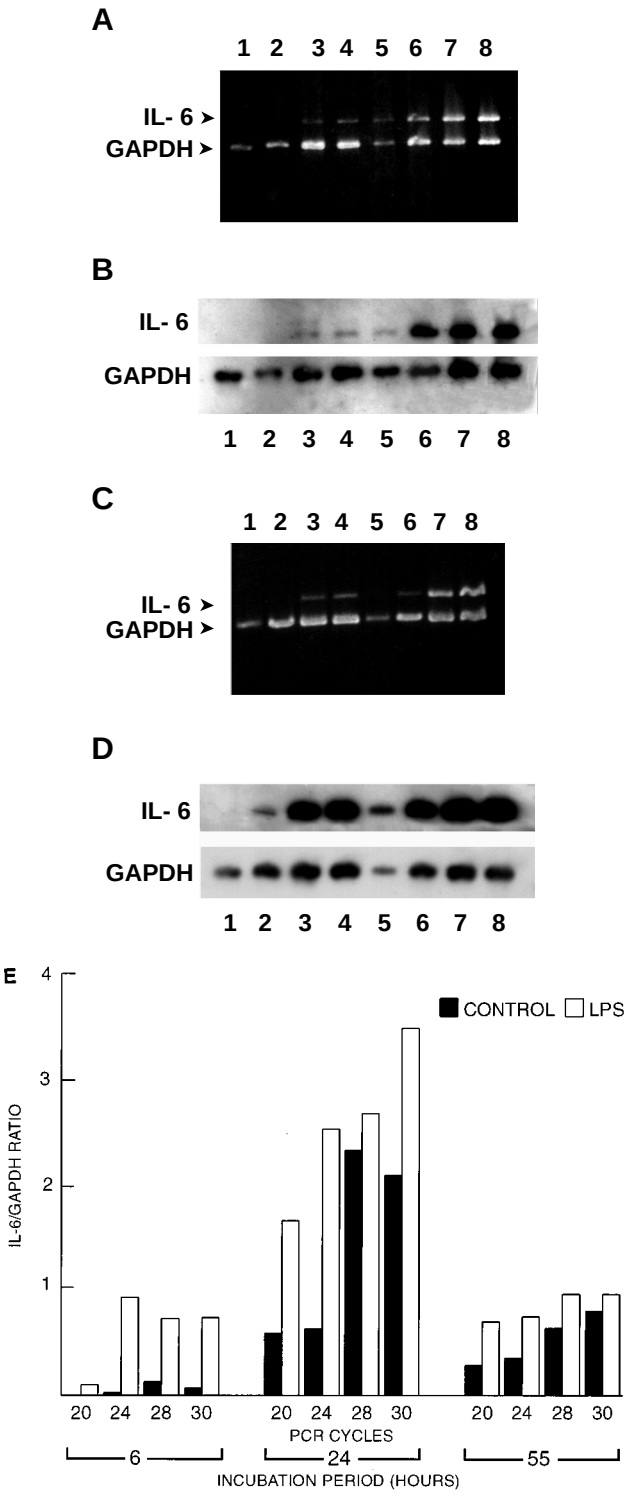

FIG. 3. Analysis of IL-6 mRNA expression in PAMs by RT-PCR. Total cellular RNA was reverse transcribed with random hexamers and was then coamplified with IL-6- and GAPDH-specific sense and antisense oligonucleotide primers for 20 to 30 cycles of amplification. A 10-μl sample of each RT-PCR mixture was subjected to gel electrophoresis as described in Materials and Methods. (A and C) Ethidium bromide-stained agarose gels of the RT-PCR products obtained from unstimulated control (lanes 1 to 4) or LPS-stimulated (lanes 5 to 8) PAM cells at 6 and 24 h after cell treatment, respectively. Lanes 1 and 5, 2 and 6, 3 and 7, and 4 and 8 refer to 20, 24, 28, and 30 cycles of PCR amplification, respectively. Arrowheads mark the positions of the expected RT-PCR-amplified products. (B and D) Chemiluminescence Southern blot hybridization of the agarose gels shown in panels A and C, respectively; IL-6 and GAPDH cDNA probes specific to each DNA amplified product were used. (E) IL-6- and GAPDH-specific chemiluminescence signals were analyzed by densitometric scanning to calculate the cytokine/GAPDH ratio, which was determined for each amplification cycle in control or LPS-stimulated PAM cells at 6, 24, and 55 h after cell treatment.

acids is not absolute, this technique was used in several other studies (9, 15) and has the advantage over competitive PCR methods (6, 31) of addressing the efficiency of the RT reaction prior to PCR amplification by using an invariant reference gene. Therefore, this approach combined with chemiluminescence hybridization performed on the RT-PCR cDNA products, as used in the present study, enabled us to evaluate and compare the levels of various monocyte/macrophage-derived cytokine mRNAs relatively to the those of GAPDH transcripts between LPS-stimulated and control cell cultures undergoing the same incubation times and PCR amplification cycle numbers.

In the current study, performing chemiluminescence hybridization with specific cDNA probes and varying the number of PCR amplification cycles appeared to be particularly valuable in light of the basal cytokine transcription levels detected in unstimulated cell cultures. First, for some RNA samples, the hybridization procedure allowed us to detect cytokine gene expression that was not observed by the ethidium bromide-stained agarose gel and to thereby determine differences in the levels of cytokine gene transcription between LPS-stimulated and unstimulated control cell cultures. Second, the most important differences between LPS-stimulated and unstimulated control cells were generally observed with the PCR-derived products obtained after 20 to 28 amplification cycles. After 30 amplification cycles, the differences between the cell culture types were generally much less than those obtained with lower amplification cycle numbers or, in most cases, were no longer observed, as analyzed with the ethidium bromide-stained agarose gel, by chemiluminescence hybridization, or from the calculated cytokine/GAPDH ratios. This observation may refer to different levels of reference and cytokine-targeted cDNAs prior to PCR amplification or to the plateau effect that is recognized to occur late during PCR cycles (20), depending on the conditions used in the PCR assays. To overcome these problems, analysis of reference and cytokine cDNAs must be performed with various numbers of PCR amplification cycles to ensure that both products are indeed analyzed during the exponential amplification phase. In most cases, our results showed comparable gene transcription levels in LPS-stimulated and unstimulated control cells when we used high numbers of PCR amplification cycles. Thus, it appeared to be important to use various amplification cycle numbers to provide assay conditions in which amplification is linear (9, 15) in order to detect cytokine gene expression differences between control and LPS-stimulated cell cultures.

Enhancement of porcine IL-1β, IL-6, and TNF-α mRNA transcription levels in alveolar macrophages, as determined by RT-PCR, was observed by 6 h after exposure of cells to LPS, returning to levels comparable to those of the control cell cultures by 24 h (TNF-α) or 55 h (IL-1β and IL-6) after LPS treatment. Similar results were obtained with cells from a different donor. Rapid enhanced levels of cytokine gene transcription followed by a decrease in the levels of cytokine-specific mRNA transcripts has also been reported for human and other animal species (4, 17, 19, 21, 28, 32). The enhanced cytokine gene transcription levels observed in our study were specific in the sense that they correlated with the detection of cytokine biological activity in corresponding macrophage cul-

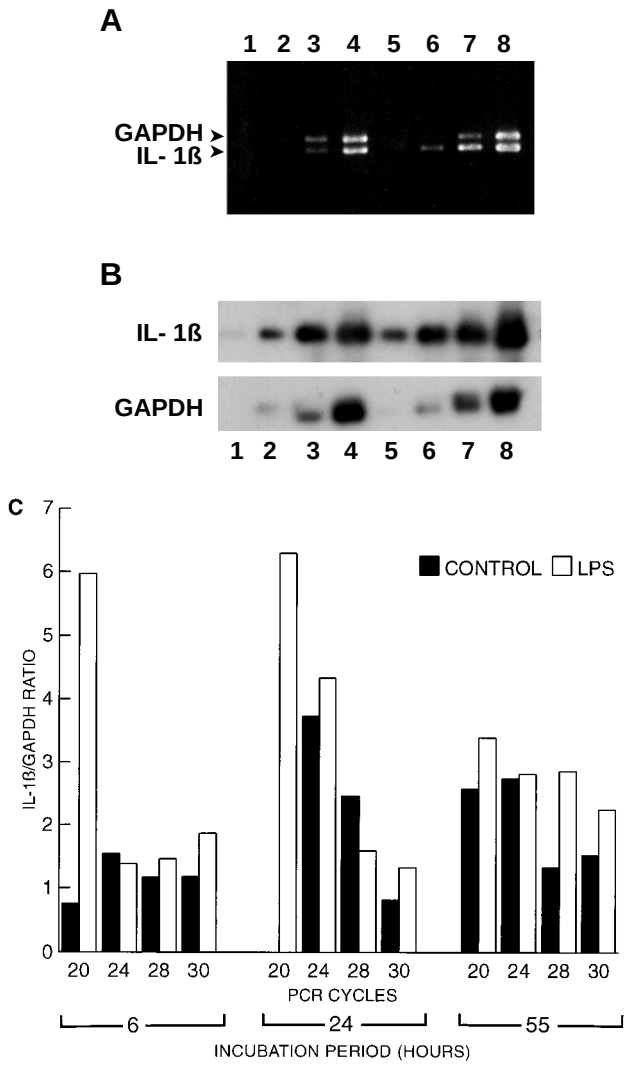

FIG. 4. Analysis of IL-1β mRNA expression in PAMs by RT-PCR. Total cellular RNA was reverse transcribed with random hexamers and was then coamplified with IL-1β- and GAPDH-specific sense and antisense oligonucleotide primers for 20 to 30 cycles of amplification. A 10-μl sample of each RT-PCR mixture was subjected to gel electrophoresis as described in Materials and Methods. (A) Ethidium bromide-stained agarose gel of the RT-PCR products obtained from unstimulated control (lanes 1 to 4) or LPS-stimulated (lanes 5 to 8) PAM cells at 24 h after cell treatment. Lanes 1 and 5, 2 and 6, 3 and 7, and 4 and 8 refer to 20, 24, 28, and 30 cycles of PCR amplification, respectively. Arrowheads mark the positions of the expected RT-PCR-amplified products. (B) Chemiluminescence Southern blot hybridization of the agarose gel shown in panel A; IL-1β- and GAPDH-specific cDNA probes specific to each amplified DNA product were used. (C) IL-1β- and GAPDH-specific chemiluminescence signals were analyzed by densitometric scanning to calculate the cytokine/GAPDH ratio, which was determined for each amplification cycle in control or LPS-stimulated PAM cells at 6, 24, and 55 h after cell treatment.

ture supernatants, as determined by bioassays with L929 or WEHI 164 (TNF-α) and B9 (IL-6) indicator cells.

Cytokine mRNA expression was also detected in unstimulated control cells. This shows that the RT-PCR technique is highly sensitive for the detection of minimal constitutive levels of cytokine mRNAs. However, by contrast to LPS-stimulated macrophage cultures, the presence of cytokine mRNA did not correlate with the release of detectable cytokines since no IL-6 or TNF-α activity could be detected in control cell culture supernatants. Similar observations were reported in unstimulated bovine (19) and human (36) PBMCs, whose adherence to glass or plastic resulted in the expression of cytokine (TNF-α or IL-1β) mRNAs without detectable cytokine synthesis. It is thus possible that control unstimulated cells did not receive the appropriate stimulation signals, which may have resulted in interference in the translation or secretion of the cytokines (7, 14, 29). A similar interpretation may also explain the enhancement of TNF-α or IL-6 activity in the cell cultures stimulated with LPS for 24 or 55 h without an apparent enhancement of RNA transcript levels compared with those in the control cell cultures. This might be due to the continued synthesis of cytokine proteins in supernatants from macrophages that have been primed with LPS, thereby receiving the appropriate stimulation signals. Finally, another possible explanation for the detection of mRNA transcripts without cytokine synthesis in the control cell cultures is that the biological assays simply lack sensitivity and/or specificity (19, 27).

TABLE 2. Secretion of TNF-α and IL-6 by LPS-stimulated porcine alveolar macrophages

Time in culture (h)	TNF-α level[a,b]		IL-6 level[b,c]	
	LPS-stimulated cells	Control cells	LPS-stimulated cells	Control cells
6	29 (3)	<2	31 (5)	<2
24	112 (15)	<2	141 (43)	<2
55	109 (19)	<2	331 (12)	<2

[a] The unit of TNF-α was defined as the reciprocal of the dilution giving 50% cytotoxicity. The cytotoxicity assay was performed on L929 indicator cells. Comparable results were obtained with WEHI 164 indicator cells (data not shown).
[b] Results are presented as the means (± standard deviations) for two aliquots of the same samples.
[c] The unit of IL-6 was defined as the amount inducing 50% maximal absorbance. The proliferation assay was performed on B9 indicator cells.

The results presented here demonstrate that the RT-PCR procedure based on coamplification of monokine and endogenous gene-specific sequences and then chemiluminescence hybridization with cytokine-specific cDNA probes can be used to analyze cytokine gene transcription in swine immune cells. The overall method is sensitive and allows RNAs to be analyzed when only limited number of cells are available for study. The RT-PCR and hybridization procedures developed in the present study are likely to have many applications such as in investigations of the modulatory effects of biotic or abiotic products (i.e., viruses, immunostimulanting agents, and other such products) on porcine IL-1β, IL-6, and TNF-α gene expression.

ACKNOWLEDGMENTS

This work was supported by an operating grant from the Conseil des Recherches en Pêche et Agro-Alimentaire du Québec (CORPAQ) (grant 3254). S.-A. Vézina is supported by a graduate student fellowship from the Fonds pour la Formation de Chercheurs et l'Aide à la Recherche (Fonds FCAR) of the province of Québec. D. Archambault is the holder of a research scholarship from the Fonds de la Recherche en Santé du Québec (FRSQ).

We are grateful to Mireille Varin and Louise Wilson for technical assistance. We also thank David Bird for reviewing the manuscript and Carole Villeneuve for secretarial work.

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