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Cytokine Profiles in Paraffin-Embedded Biopsy Samples of Lepromatous Leprosy Patients: Semi-Quantitative Measure of Cytokine mRNA Using RT-PCR[1]

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Molecular analysis of nucleic acids in paraffin-embedded tissue (PET) has many applications in experimental studies as well as in clinical practice ([13, 27]). *In vitro* amplification using reverse transcription polymerase chain reaction (RT-PCR) is an effective approach for retrospective analysis of gene expression since RT-PCR could be performed successfully using partially degraded RNA. In tissue samples, RNA that survives tissue fixation remains in a relatively stable state in the paraffin blocks. Hence, the paraffin-embedded hospital archives of human pathological biopsy materials are amenable to analysis of expressed RNA by RT-PCR. In addition, RT-PCR analysis of fixed tissues in paraffin allows the study of gene expression of field samples in the laboratory. this approach is also safer, since the handling of infectious biopsy material is minimized.

RNA being labile and ribonucleases (RNases) endogenous and ubiquitous in the tissues, the extraction of relatively undegraded RNA using solvents requires complex and time-consuming protocols ([1]). In earlier studies, RNA was extracted from 0.5–2 mg of paraffin-embedded tissue by incubation at 50°C–60°C for 6–18 hr in a lysis buffer containing Tris, EDTA, SDS and proteinase K followed by phenol extraction ([8, 18, 21, 31]). In addition, these methods require large amounts of tissue to produce enough RNA for RT-PCR analysis. In most published procedures, guanidium thiocyanate was used in the extraction buffers ([14, 28, 32]) except in one report ([2]) wherein deparaffinated tissue sections boiled in diethyl pyrocarbonate (DEPC)-treated water for 10 min were directly used for RT-PCR analysis. Under our conditions, none of the described methods gave reproducible results.

In this study we compared four different extraction methods to obtain RNA preparations from biopsies that could give reproducible RT-PCR data. The extraction conditions were optimized for RT-PCR analysis of cytokine mRNA in the dermal lesions of stable lepromatous leprosy patients.

Cytokines produced locally in response to pathogens play a critical role in the outcome of infection by directing the immune response toward the cell-mediated (Th1) or humoral (Th2) immune pathways ([25]). We used the techniques discussed in this paper for analyzing the cytokine mRNA from dermal lesions of LL patients. At present, limited data are available on cytokine mRNA expression in stable leprosy lesions, and the reported studies are restricted to either fresh or frozen tissue ([33, 34]) or peripheral blood samples of patients ([15, 17, 26]). Yamamura, *et al.* ([33]) reported that the immune response in tuberculoid patients is Th1, and presumably this cell-mediated response led to the control of intracellular infection. In the same study, the polar lepromatous form was demonstrated to have a Th2 response. It was argued that the Th2 response could not control the infection because of the intracellular nature of the pathogen. However, subsequent studies have shown that the pattern

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of cytokine production does not show clear Th1/Th2 dichotomy in TT and LL conditions, respectively ([15, 17, 23, 26, 30]). These studies predominantly focused on the cytokine mRNA profiles of peripheral blood cells ([15, 17, 23, 26]) and immunocytochemical analysis of cytokines in the dermal lesions of leprosy ([4, 5, 10]). Therefore, we re-examined the cytokine mRNA levels of biopsy samples using semi-quantitative RT-PCR (SQ-PCR).

We specifically examined the cytokine profiles of stable polar lepromatous leprosy patients closely following the methodology adopted by Yamamura, *et al.* ([33]). Our data using biopsy material from stable LL patients clearly showed the presence of transforming growth factor-beta (TGF-β) and tumor necrosis factor-alpha (TNF-α) mRNA in the lesions of LL patients, confirming the cytochemical data ([5, 10]). Interleukin-10 (IL-10) mRNA was found in all LL cases examined, but IL-5 mRNA was found in only 60% of the LL cases. The presence of gamma interferon (IFN-γ) mRNA was demonstrated in 30% of the LL cases. The results reported in this paper confirm and extend earlier observations and also indicate the usefulness of this simple methodology in dissecting the cytokine profiles of the lesions of various forms of leprosy.

MATERIALS AND METHODS

Preparation of paraffin-embedded tissues. Biopsy samples from patients were obtained from the Voluntary Health Services Hospital (Shakti Nager, Periyar District, Tamil Nadu, India). Leprosy patients were diagnosed and classified with respect to the leprosy spectrum on the basis of the clinico-pathological parameters of Ridley and Jopling ([20]). Punch biopsies were collected after obtaining informed consent as per the norms laid down by the ethical committee and the Indian Council of Medical Research. Table 1 gives clinical information on the patients from whom biopsies were taken. Tissue samples were cut into 3-ml-thick pieces, fixed in freshly prepared buffered formaldehyde for a period of four hr followed by dehydration in graded ethanol: 70% ethanol for 1 hr, 95% ethanol for 1 hr, and 100% ethanol for 4 hr (changed at 1-hr intervals). After dehydration, the tissue sections were immersed in xylene for 2 hr and then embedded in melted wax kept at 60°C for 2 hr. The paraffin blocks were stored at room temperature.

TABLE 1. *Patient clinical information and leprosy spectra of the biopsies analyzed in this study.*[a]

Sample no.	Sex	Bacterial index	Disease status
107	M	5	MB/LL
113	F	3.7	MB/LL
124	F	5+	MB/LL
132	M	4.2+	MB/LL
138	M	4.5	MB/LL
140	F	4	MB/LL
142	M	3.5	MB/LL
145	M	5.5+	MB/LL
146	M	5+	MB/LL
172	M	4+	MB/LL

[a] Biopsies were taken as described under Materials and Methods. Disease states were classified according to Ridley and Jopling ([20]).

Extraction of RNA. Five-micron sections of the paraffin-embedded wax blocks were cut using a microtome (Yorco India Ltd.). The blade was cleaned with RNAase Away (Molecular BioProducts, San Diego, California, U.S.A.) before each sample. The sections (normally 15 sections) were placed in a sterile 1.5 ml Eppendorf tube, mixed with 200 µl of octane and incubated at 37°C for 30 min. Tubes were centrifuged for 3 min at 12,000 rpm, and the octane layer was removed using a sterile Pasteur pipette. Deparaffination with octane was repeated once, after which 1 ml of ethanol was added to each tube, mixed well and followed by centrifugation at 12,000 rpm for 5 min. An ethanol wash was repeated to remove any remaining traces of octane. The samples were dried under vacuum, and the RNA was extracted using different methods as described below.

In method A, deparaffinated samples were incubated in 100 µl of digestion buffer (10 mM Tris HCl, 25 mM EDTA, 100 mM NaCl, 0.5% SDS, proteinase K at a final concentration of 300 µg/ml) (Sigma Chemical Co., St. Louis, Missouri, U.S.A.) at 52°C with constant shaking for 24 hr. At the end of the incubation period 1 ml of Trizol (Gibco BRL, Grand Island, New York, U.S.A.) was added, mixed well by aspira-

TABLE 2. *List of primers used in this study and the expected size of PCR products.*[a]

Primers	Sequence	Primer length	Expected size: CDNA/genomic DNA
	β-actin set I		
Forward primer	5′ GTGGGGCGCCCCAGGCACCA	20 bp	540 bp/1112 bp
Reverse primer	5′ CTCCTTAATGTCACGCACGATTTC	24 bp	
	β-actin set II		
Forward primer	5′ CAAGATAGAGATTGGCATGGC	21 bp	1116 bp/1688 bp
Reverse primer	5′ GTTCTACTCTAACCGTACCG	20 bp	
	IFN-γ		
Forward primer	5′ TGCAGAGCCAAATTGTCTCCTTT TAC	26 bp	299 bp/2724 bp
Reverse primer	5′ TTACTGGGATGCTCTTCGACCTC GAAACAGGAT	33 bp	
	IL-5		
Forward primer	5′ CAAACGCAGAACGTTTCAGA	20 bp	137 bp/624 bp
Reverse primer	5′ GCAGTGCCAAGTTCTCTTTC	20 bp	
	IL-10		
Forward primer	5′ TGGTGAAACCCCGTCTCTAC	20 bp	163 bp/1068 bp
Reverse primer	5′ CTGGAGTACAGGGGCATGAT	20 bp	
	TNF-α		
Forward primer	5′ GTGGACCTTAGGCCTTCCTC	20 bp	235 bp/968 bp
Reverse primer	5′ ACGGAAAACATGTCTGAGCC	20 bp	
	TGF-β		
Forward primer	5′ CTAGCACTTTTGGGAGGCAG	20 bp	197 bp/830 bp
Reverse primer	5′ GATCCTCCCACCTTAGCCTC	20 bp	

[a]Nucleotide sequence of forward and reverse primers and the expected size of the amplicons obtained with cDNA and genomic DNA as templates. Accession numbers of sequences used for the calculation of the amplicon size: β actin—X000351, IFN gamma—M29383, Il-5—NM_000879, IL-10—M57627, TNF-α—X01394, TGF-β—X05850.

tion and incubated at room temperature for 5 min.

In method B, samples were incubated in digestion buffer as described in method A for 24 hr followed by phenol : chloroform extraction instead of Trizol extraction.

Method C is similar to method A except for the omission of incubation in digestion buffer.

The samples prepared using methods A, B and C were treated as below for further extraction. Chloroform (200 µl) was added to the samples, mixed well and incubated on ice for 5 min. After centrifugation at 12,000 rpm for 15 min at 4°C the supernatant was carefully removed and 0.5 ml of isopropyl alcohol was added. Transfer RNA (10 µg) (Sigma) was used as the carrier and RNA was precipitated by overnight incubation at –20°C. RNA was pelleted by centrifugation at 12,000 rpm for 30 min at 4°C, and the RNA pellet was washed with 75% ethanol and dried under vacuum. The RNA pellet was dissolved in 20 µl of DEPC-treated water by incubation at 65°C for 10 min.

In method D deparaffinated samples were boiled in DEPC-treated water for 10 min, and the lysate was directly used for RT-PCR analysis without further processing. RNA concentration and purity were assessed spectrophotometrically by absorbance at 260 nm and 280 nm. The presence of tRNA precludes the exact quantification of the mRNA as well as other RNA species from the tissue. However, this measurement allowed us to omit the preparations from old blocks that did not yield any RNA.

Preparation of cDNA. The reverse transcription reaction was carried out using a combination of oligo dT primers and random primers in a reaction mix containing the following: 1 µl of oligo dT_{15} primer (0.5 µg/µl) (Promega, Madison, Wisconsin, U.S.A.), 1 µl of random hexamer (0.5 µg/µl) (Promega), 5 µl or RNA (0.5 µg), 1 µl of DEPC-treated water (Sigma). The reaction mixture was incubated at 70°C for 10 min and chilled on ice at the end of the incubation period. First strand buffer (4 µl) (5 × buffer containing 250 mM Tris-HCl, pH 8.3, 375 mM KCl and 15 mM $MgCl_2$), 2 µl DTT (100 mM stock), 1 µl of RNAsin (40 U/µl) (Promega), 1 µl of dNTPs (10 mM each) and 200 U of MMLV reverse transcriptase enzyme (Gibco BRL) were added and incubated at 37°C for 1 hr. The enzyme was heat inactivated at 92°C for 2 min and the cDNA was stored at –20°C.

PCR amplification of cDNA. PCR amplification of cDNA templates was carried out using an MJ Research Thermocycler for 30 cycles with the following cycling parameters: denaturation at 94°C for 1.5 min, annealing at 54°C for 1 min, extension at 72°C for 1.5 min for 30 cycles followed by a final extension of 7 min. The primer sequences used in this study are described in Table 2. The primers were designed to target relatively short sequences since PET tissues yielded degraded RNA. Amplification of β-actin mRNA was used to normalize the quantity and quality of the template. PCR was performed in a 40 µl reaction mix containing template cDNA in a volume of 5 µl, 1.25 units of Taq Polymerase (Gibco BRL), 2 µl of $MgCl_2$ (final concentration 1 mM), 2 µl of dNTPs (final concentration 100 µM).

Semiquantitative PCR analysis was carried out by terminating PCR reactions at 18, 22, 26, and 30 cycles of amplification. PCR products were electrophoresed on a 2% agarose gel, and the bands were visualized after staining with ethidium bromide. The internal standard is β-actin mRNA amplified using β-actin-specific primers (Primer set I). Template concentration in the samples was adjusted such that the intensity of the β-actin amplicon at 18, 22, 26 and 30 cycles in the different samples analyzed are identical and linear. The intensity of the cytokine amplicons obtained were arbitrarily graded as +1 to +4 after the gels were documented using the UVP Gel Documentation System (UVP, Cambridge, UK). If a visible band appears even at 18 cycles of PCR amplification, the quantity is recorded as 4+; whereas if the amplicon appears only at 22 cycles of amplification it is recorded as 3+. Hence, 2+ and 1+ indicates that the signal is observed at 26 and 30 cycles of amplification, respectively. This grading corresponds well with the quantification based on the analysis of band intensity using the Imagequant program (Shabaana, *et al.,* unpublished). When no PCR product was observed at 30 cycles of amplification, the reaction was extended to 40 cycles under identical conditions to confirm the absence of the particular cytokine transcript.

RESULTS

It has been reported ([11]) that biopsy material fixed in mercury-based fixatives failed to yield usable RNA preparations. Therefore, in this study punch biopsies were fixed for 4 hr in freshly prepared, buffered formaldehyde followed by incubation in graded ethanol (as described under Materials and Methods). Samples extracted with phenol : chloroform following incubation in digestion buffer for 24 hr (method B) and samples extracted using Trizol only (method C) did not yield any usable RNA (Fig. 1a, lanes 3 and 4, respectively). These preparations also failed to give amplification in the RT-PCR reaction. However, material treated with digestion buffer improved the yield of RNA considerably when extracted with Trizol (method A), and these results are shown in Figure 1a, lanes 2 and 5. Boiling deparaffinated tissues without further extraction (method D) also failed to yield any RNA (Fig. 1a, lane 6). Figure 1b shows that RNA obtained using method A (lanes 2–5) alone gave amplifica-

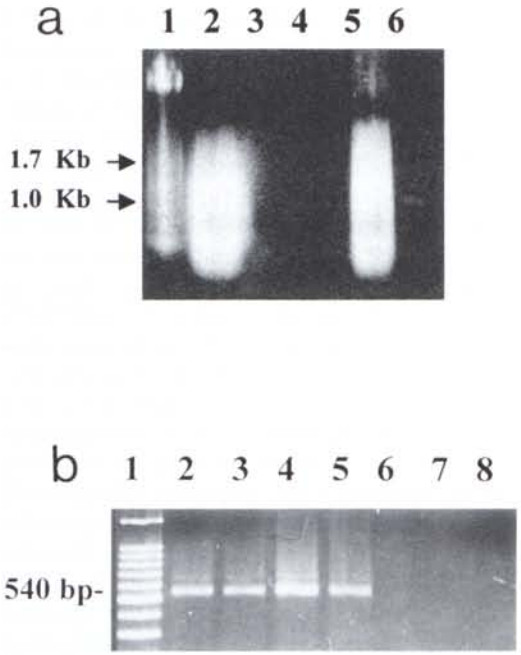

FIG. 1. **a** = Electrophoretic analysis of total RNA extracted using different protocols. Lane 1, total nucleic acids extracted from a cell line THP1 (fresh cells). RNA extracted using methods: A, lanes 2 and 5; B, lane 3; C, lane 4; D, lane 6. In some cases the two ribosomal rRNA bands were not visible, as in this case. Arrows indicate RNA molecular weight marker positions on a 1.5% agarose gel. **b** = RT-PCR amplification of RNA isolated using different methods. Primer set I (Table 2) for β actin was used. Lane 1, DNA ladder; lane 2–5, RNA extracted using method A; lane 6, RNA extracted using method B; lane 7, RNA extracted using method C; lane 8, RNA extracted using method D.

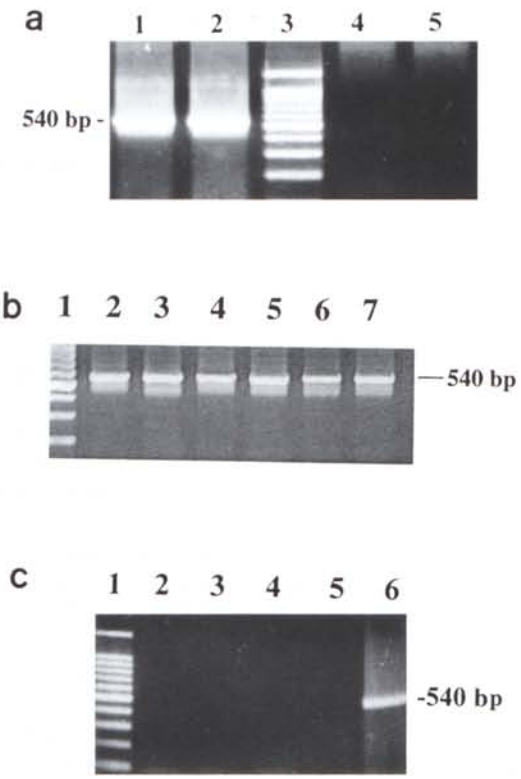

FIG. 2. Amplification of β-actin. **a** = First strand synthesis of RNA extracted from a 3-year-old block was carried out using oligo dT and random hexamers as primers. Lanes 1 and 2, oligo dT primers and random primers (ratio 1:1); lane 3, DNA marker; lane 4, oligo dT primer alone; lane 5, random hexamers alone. In all cases primer set I was used for PCR amplification. **b** = First strand synthesis from RNA extracted from freshly prepared blocks was carried out using oligo dT and random hexamers as primers. Lane 1, DNA marker; lanes 2 and 3, oligo dT and random primers (ratio 1:1) for samples 132 and 140, respectively; lanes 4 and 5, oligo dT primer for samples 132 and 140, respectively; lanes 6 and 7, random hexamers for samples 132 and 140, respectively. Primer set I was used in all cases. **c** = Amplification of β-actin locus using primer set I and II. The template described in Figure 2b was used. Lane 1, DNA marker; lanes 2–5, amplification using primer set II; lane 6, amplification using primer set I.

tion using β-actin primer set I (Table 2); whereas the preparations using other methods did not yield any PCR product. The optimal incubation period for the sample in digestion buffer was found to be 24 hr. Longer incubations resulted in degraded RNA preparations (data not shown). The absence of RNA or the contamination of RNA with other proteinaceous material could be responsible for the failure of PCR amplification in methods B, C and D (Fig. 1b, lanes 6, 7, 8). Inclusion of *Escherichia coli* transfer RNA (10 μg) as a carrier is essential for precipitation of RNA, as has been shown earlier ([28]).

RNA extracted from PET tissues is reported to be degraded and, hence, first strand cDNA synthesis is generally carried out with a combination of oligo dT_{15} and random hexamers as primers ([2, 7, 18, 22]). In order to confirm this observation, first strand cDNA synthesis was carried out from RNA extracted from older blocks (3 years old) using a combination of oligo dT primer and random hexamer, as well as individual reactions using oligo dT or random

primer alone. Amplification of β-actin could be observed only if both primers were used at a ratio of 1:1 for the cDNA synthesis in these blocks (Fig. 2a, lanes 1 and 2). Amplification of the β-actin locus was not detected when only one primer was used. However, when blocks were freshly prepared, oligo dT or random primers were sufficient for successful RT-PCR reactions (Fig. 2b).

PCR primers designed to amplify cDNA regions spanning one kilobase or more failed to work. Figure 2c shows the amplification of a 540 bp amplicon of β-actin (primer set I) (lane 6). However, primer set II designed to amplify a β-actin region of 1116 bp failed to show any PCR product (lanes 2–5) under identical conditions using the same cDNA preparation. This was found to be true with freshly prepared blocks also. Primers for β-actin and different cytokines were designed such that the PCR product length would be longer if the template cDNA had chromosomal DNA contamination due to the presence of intron sequence in the contaminating genomic DNA. We did not observe any extra bands of the expected sizes, indicating that the RNA preparations were free of DNA contamination. Further, in order to confirm the specificity of the PCR products, bands were electroeluted from the gel and their identity confirmed by restriction digestion analysis and nucleotide sequence analysis.

Optimized conditions of RNA extraction using method A and first strand cDNA synthesis were used to measure the level of cytokine transcripts in biopsy materials. Simultaneous amplification of cDNA for the β-actin gene was used as a control to normalize the quantity of the template and also to assess the efficiency of transcription. Figure 3, lanes 1–4 shows SQ-PCR for β-actin in all samples and indicates that the PCR product at 18, 22, 26 and 30 cycles for different samples analyzed are comparable in intensity. The linear increase in the intensity of PCR bands is critical for SQ-PCR analysis. Figure 3(a–d) shows the semi-quantitative amplification of various cytokine specific products in four LL patients. IL-10 mRNA was found in all LL patients (Fig. 3a–d, lanes 14–17; SQ-PCR at 18, 22, 26 and 30 cycles, respectively), and these data support earlier reports ([33, 34]). However, IL-5 mRNA (Fig. 3a–d, lanes 9–12; SQ-PCR at 18, 22, 26 and 30 cycles, respectively) could be detected only in samples 132, 142 and 145 but not in sample 140. The data in Table 3 show the summary of results for 10 LL patients; 40% of these LL patients show high levels of IL-5 mRNA and 20% show barely detectable levels. The interesting observation was that 40% of the samples do not show any IL-5 mRNA at all. This is in contrast to an earlier report which showed that all LL patients studied had high levels of IL-5 and IL-4 mRNA ([33]). In our present study, 80% of LL patients showed significant levels of TGF-β mRNA. TNF-α mRNA could be detected in 60% of the cases studied and IFN-γ could be detected in 30% of the cases. However, the quantity of IFN-γ mRNA in individual samples was variable.

DISCUSSION

Semi-quantitative RT-PCR determination of cytokine mRNA levels in archival biopsy materials as well as tissues freshly fixed and embedded were used to study the cytokine mRNA profiles of leprosy patients. Local concentrations of cytokines in the dermal lesions of patients, especially at the early stages of infection, would give valuable information about the outcome of the infection. In addition it has been shown that the polarization of Th1/Th2 response is determined to a large extent by the concentration of cytokines at the site of infection ([33]) even though the role of other factors, including the nature of antigenic peptides involved in the Th1/Th2 shift in leprosy, has not been examined so far ([9]). The classical study of Yamamura, *et al.* ([33]) showed the elaboration of Th1 cytokines such as IL-2 and IFN-γ predominantly in tuberculoid lesions and the Th2 cytokines IL-4, IL-5 and IL-10 appeared mainly in lepromatous dermal lesions. Their study also showed that the regulatory cytokines TGF-β and the proinflammatory cytokine TNF-α mainly were found in TT cases and not in LL cases. A subsequent study by Goulart, *et al.* ([5]) using the dermal lesions of the stable forms of leprosy showed a considerable amount of TGF-β protein in LL lesions by immunohistochemical analysis. These data were confirmed by Khanolkar-Young, *et al.* ([10]) using skin biopsies from LL and TT pa-

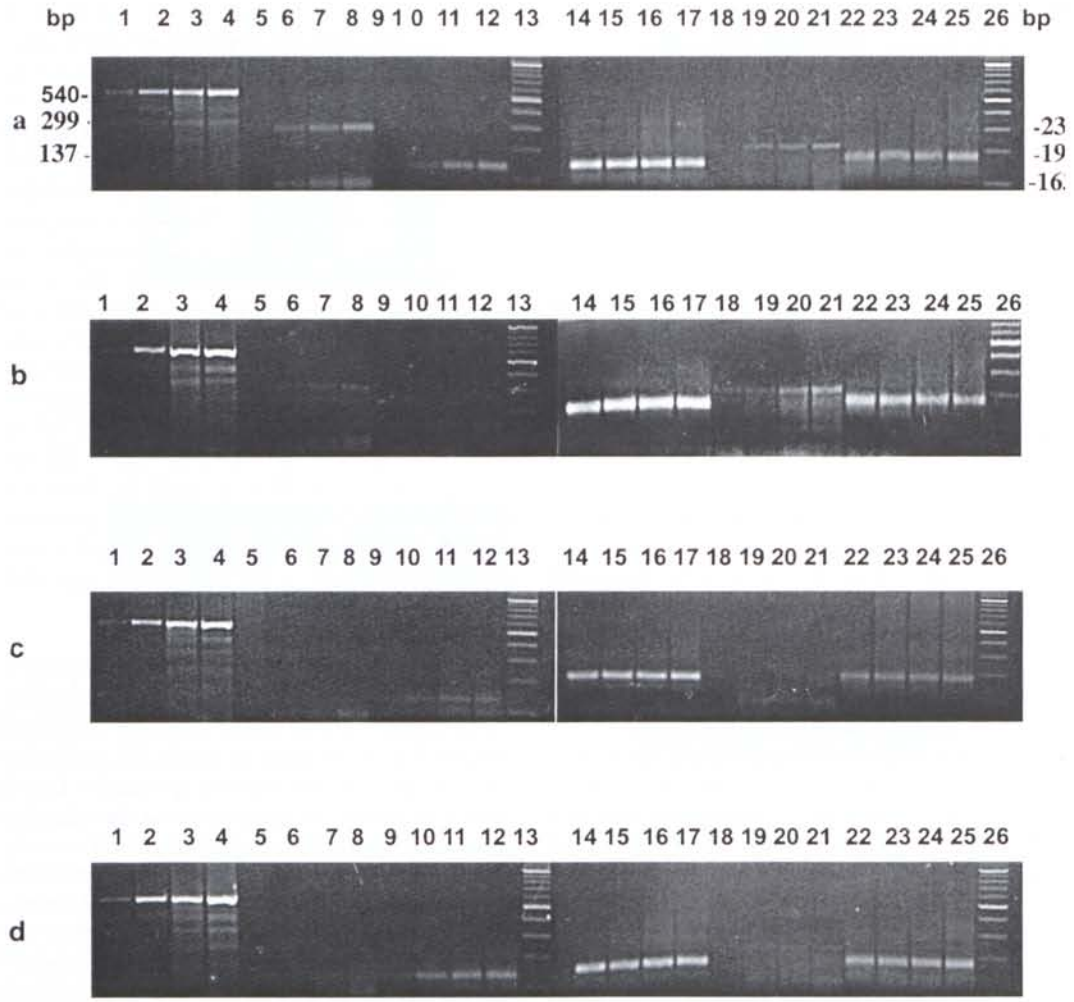

FIG. 3. Semi-quantitative RT-PCR analysis of cytokine mRNA from four LL patients. PCR products at 18, 22, 26 and 30 cycles of amplification were electrophoresed on 2% agarose gels. The amount of template cDNA used in all experiments was normalized to give a value of +4 with β-actin primer set I. **a** = Sample 132; **b** = sample 140; **c** = sample 142; **d** = sample 145. Lanes 1–4, SQ-PCR for β-actin; lanes 5–8, IFN-γ; lanes 9–12, IL-5; lane 13, DNA ladder; lanes 14–17, IL-10; lanes 18–21, TNF-α; lanes 22–25 TGF-β; lane 26, DNA ladder (100 bp ladder contains 2 intense bands—500 bp and 1000 bp; sizes of the PCR products are given in Table 2).

tients. However, they also reported the presence of TGF-β in TT lesions, although in significantly lesser quantities than LL lesions. The reason for the discrepancy between these two reports is unclear. We wished to examine the usefulness of RT-PCR analysis in measuring cytokine levels in skin biopsies from stable forms of leprosy since the mRNA data ([33]) were found to be contradictory to the immunocytochemical data ([5, 10]).

In order to examine the cytokine expression of a large number of biopsy samples taken in field hospitals and to examine archival material, we have developed a simpler method of preparation of biopsy material. A major hurdle in the extraction of RNA is the fibrous nature of the skin and the underlying tissue. The extraction buffers are effective only after the disintegration of the tissues using glass beads or cutting cryopreserved tissues into thin sections ([26]). The method using PET is inexpensive and could be used in field laboratories. Even though there are several reports using PET in other tissues, the repro-

TABLE 3. *Cytokine mRNA profiles of patients included in this study.*[a]

Sample no.	IL-5	IL-10	IFN-γ	TGF-β	TNF-α
107	ND	+++	ND	++	ND
113	ND	++	ND	+	ND
124	++++	+++	ND	ND	++++
132	+++	++++	+++	++++	+++
138	ND	+++	+	++	++
140	ND	++++	+	++++	+++
142	++	++++	ND	++++	ND
145	+++	++++	ND	++++	++
146	+++	+++	ND	++	++
172	+	++	ND	ND	ND

[a]Cytokine mRNA profiles of LL patients included in this study. Intensities of PCR bands were graded arbitrarily as +1 to +4, depending upon the appearance of the PCR product at 18, 22, 26 and 30 cycles, respectively. If a PCR product was seen at 18 cycles of amplification the quantity of the cytokine was expressed as +4. Lack of amplification of the desired product even after 40 cycles of PCR is indicated as ND (not detectable).

ducibility of the published procedures was variable. In our hands, fixation using mercury-based fixatives did not give any usable RNA templates, and a similar observation was reported earlier ([11]). Tissues fixed in fresh buffered formaldehyde for 4 hr or less gave consistently good preparations. Longer incubations resulted in a decreasing yield as well as degraded RNA and, consequently, the templates failed to yield any amplification. Deparaffinated tissue treated with digestion buffer for 24 hr followed by extraction with Trizol reagent (method A) yielded expected bands after RT-PCR. Trizol treatment is essential since RNA precipitated directly after digestion in buffer did not yield any amplification product.

The extraction conditions proved to be effective even when 3-year-old blocks were used. However, random hexamers in addition to the oligo-dT primers also are needed to synthesize cDNA. On the other hand, when freshly embedded tissue blocks are used, random hexamers are not needed for cDNA preparation. Degradation of the polyA tail of the RNA could be responsible for the defect in cDNA synthesis with oligo dT alone. Earlier workers who also had used archival material ([2, 7, 18]) for RT-PCR reported the requirement of random hexamers. The PCR primers used in this study are different from the primers used in earlier reports ([15, 17, 33, 34]). The primers were designed in such a way that they frame the intron sequences of the genes and, hence, the genomic DNA contamination could be identified easily. In our experiments we did not detect any amplicons containing introns, and this indicates the absence of genomic DNA contamination.

Amplification of short segments ranging between 99 bp to 540 bp in length is highly reproducible under our experimental conditions. In addition, 30 cycles of PCR without any additional enzyme was sufficient for amplification of the fragment. This is a considerably improved method when compared to the earlier method wherein 50 PCR cycles were needed to amplify 100–200 bp targets prepared from paraffin-embedded tissues ([14]).

The polar forms of leprosy are perhaps the classical examples of Th1 and Th2 dichotomy in humans. The earliest report examining the cytokine profiles of biopsy samples using RT-PCR analysis showed the predominance of Th1 cytokines, IL-2 and IFN-γ in TT patients and the Th2 cytokines IL-4, IL-5 and IL-10 in LL cases ([33]). Also, in this study, TNF-α and TGF-β mRNA were found in TT cases only and not in LL cases. Subsequent studies reported by Goulart, *et al.* ([5, 6]) showed the presence of TGF-β protein in LL cases. This cytochemical data was confirmed by Khanolkar-Young, *et al.* ([10]); however TGF-β protein was detected throughout the spectrum of leprosy, although significantly higher amounts of TGF-β were found in LL cases. Our experimental conditions are comparable to the earlier study ([33]) since we could detect IL-10 in all cases and IL-5 in 60% of

the cases as was demonstrated. Under our conditions we could detect TGF-β mRNA in stable LL cases supporting the protein data ([5, 6, 10]). TGF-β levels get reduced in patients undergoing erythema nodosum leprosum reactions ([10]) and, hence, the patients reported on earlier ([33]) could be unstable LL cases.

TNF-α presumably has a role in the antimycobacterial defense in leprosy ([16, 29]). The serum level of TNF-α increases considerably during reactional states ([19, 23, 24]), indicating the role of this cytokine in immunopathology. Indeed, the LL patients who have more than a certain level of TNF-α, enter into reactional states ([19]). Our results also indicate that 60% of LL cases show the presence of TNF-α mRNA. Earlier reports of Nath and coworkers ([15, 26]) showed that the lepromatous pole is a mixture of Th0 and Th2 types rather than Th2 alone. Their observation is based on the analysis of cytokines in peripheral blood in reaction conditions as well as stable polar forms of leprosy. The presence of IFN-γ mRNA in 30% of the LL cases in our present study shows that the peripheral lesions also might be mixed Th0 and Th2 states. T-cell clones derived from LL lesions also showed coexpression of IFN-γ/TNF-α and IL-4/IL-5/IL-13 ([30]). However, we could not rule out the involvement of Th3-type condition wherein a subset of T cells predominantly producing IL-10 and TGF-β ([12]) might be contributing to the mixed immunological state, as has been in the case of antigen-specific cellular hypo-responsiveness in chronic helminth infection ([3]). Additional experiments are needed to clarify this point.

In conclusion, we have described an approach involving a reliable and reproducible method for fixation, RNA extraction, and RT-PCR from paraffin-embedded leprosy biopsies. The correlation of mRNA quantities to TGF-β, IL-10 and TNF-α protein levels reported by earlier workers also validates the usefulness of this method for analyzing mRNA levels as a means to evaluate cytokine levels. This approach would allow us to examine the immune modulation at the site of lesions.

SUMMARY

A reproducible technique for fixation of tissue, RNA extraction and reverse transcription polymerase chain reaction (RT-PCR) analysis from paraffin-embedded leprosy biopsies, has been developed and used to study the mRNA profiles. This approach is valuable in retrospective analysis of gene expression, and the handling of infectious biopsy material is also minimized. Among the methods of RNA extraction compared, the most efficient method was found to be incubation of the tissue sections in digestion buffer followed by extraction with Trizol. The experimental conditions were optimized for first strand cDNA synthesis and PCR, and used to measure the quantity of cytokine transcripts in biopsy materials. Interleukin-10 (IL-10) mRNA was detectable in all cases examined, which correlates well with other earlier reports using frozen tissues. However, IL-5 transcripts were present in 60% of the biopsies, unlike the earlier reports which showed IL-5 mRNA in all LL cases. Transforming growth factor-beta (TGF-β) mRNA was detected in 80% of the biopsies, and this confirmed earlier immuno-cytochemical data which showed TGF-β protein in all cases. Tumor necrosis factor-alpha mRNA was found in about 60% of the biopsies; whereas interferon gamma mRNA was detected in 30% of the LL cases. In conclusion, the results obtained in our study confirm and extend earlier observations which examined cytokines in peripheral blood cells and dermal lesions of leprosy. The simplicity of this method would allow the examination of a large number of samples across the spectrum of leprosy.

RESUMEN

Se ha desarrollado una metodología para estudiar los perfiles de ARNm en cortes parafinados de biopsias de pacientes con lepra. La metodología incluye la fijación del tejido, la extracción del ARN y la reacción cadena de la ADN polimerasa (RT-PCR). La metodología, que minimiza el manejo del material infeccioso, es útil en el análisis retrospectivo de la expresión de genes. Entre los métodos de extracción del ARN probados, el más eficiente fue la incubación de las secciones de tejido en regulador de digestión, seguida pro extracción con Trizol. Las condiciones experimentales, optimizadas para la síntesis del ADN complementario (cADN) y para PCR, se aplicaron para cuantificar los transcritos de citocinas en el material de biopsia. El mARN de la interleucina 10 (IL-10) fue detectado en todas las biopsias examinadas, lo cual correlaciona con ontros reportes usando tejido congelado. Sin embargo, a diferencia de otros reportes que señalan la presencia de mARN para IL-5 en todos los

pacientes con LL, en este estudio, los transcritos para IL-5 sólo se encontraron en el 60% de las biopsias. El mARN para el factor beta del crecimiento transformante (TGF-β) se detectó en el 80% de las biopsias, confirmando los datos de estudios inmunocitoquímicos que reportan la presencia de TGF-β en todos los casos LL. El mARN para factor de necrosis tumoral alfa (TGF-α) se encontró en aproximadamente el 60% de las biopsias; mientras el mARN para interferón gamma se detectó en el 30% de los casos LL. En conclusión, los resultados de este estudio confirman y amplían observaciones anteriores donde se han utilizado leucocitos de sangre periférica y lesions dérmicas de la lepra. La simplicidad de este método permite el examen de un número grande de muestras a lo largo del espectro de la lepra.

RÉSUMÉ

Une technique reproductible de fixation des tissus, extraction de l'ARNm et d'analyse des produits de réaction de polymérase en chaine après transcription inverse (RT-PCR) a été développée et utilisée pour étudier les profils d'expression des ARNm à partir de biopsies inclues en paraffine de lésions lépreuses. Cette approche présente un intérêt pour l'analyse rétrospective de l'expressions génique et minimise la manutention de matériel biopsique infectieux non fixé. Parmi les méthodes d'extraction de l'ARNm, la méthode la plus efficace a été l'incubation des coupes de tissu dans un tampon de digestion suivie d'une extraction au Trizol. Les conditons expérimentales ont été optimisées pour la synthèse du premier brin d'ADN complémentaire (ADNc) et le PCR, et utilisé pour mesurer les quantités de transcrits codant pour les cytokines dans le matériel biopsique. L'ARNm de l'interleukine 10 (IL-10) étaient présente dans tous les cas examinés, ce qui corrèle bien avec d'autres rapports antérieurs utilisant des tissus congelés. Cependant, les transcrits de l'IL-5 étaient présents dans 60% des biopsies, à la différence des rapports précédents qui montrèrent l'ARNm de l'IL-5 dans tous les cas lépromateux (LL). L'ARNm du facteur béta de croissance et de transformation néoplasique (TGF-b) a été détecté dans 80% des biopsies, confirmant les données immunocytochimiques précédentes qui ont montré la présence de protéines TGF-b dans tous les cas LL. L'ARNm du facteur alpha de nécrose tumorale fut retrouvé dans environ 60% des biopsies; tandis que l'ARNm de l'interféron gamma fut détecté dans 30% des cas LL. En conclusion, les résultats obtenus dans notre étude confirment et étendent les observations déjà collectées à partir du sang périphérique et des lésions dermiques de la lèpre. La simplicité de cette méthode devrait permettre d'examiner un grand nombre de prélèvements provenant des diverses manifestations de la lèpre.

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