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A SEMI-QUANTITATIVE REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION METHOD FOR MEASUREMENT OF MRNA FOR TNF- α AND IL-1 β IN WHOLE BLOOD CULTURES: ITS APPLICATION IN TYPHOID FEVER AND EXENTRIC EXERCISE



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Whole blood cultures are used to study cytokine stimulation and release *ex vivo*. In the present study this method was compared with a more direct approach and a quantitative reverse transcriptase polymerase chain reaction (RT-PCR) was used to assess mRNA expression for IL-1 β and tumour necrosis factor alpha (TNF- α) and mRNA in whole blood.

Stimulation of whole blood from normal donors with lipopolysaccharide (LPS) at various time intervals showed a parallel rise of immunogenic IL-1 β and TNF- α as well as a rise of mRNA expression for IL-1 β and TNF- α with peak levels for IL-1 β after 4–6 h stimulation and for mRNA TNF- α expression after 2 h stimulation. These methods were used to explore cytokine production during the course of typhoid fever and after a 5 km run.

In both conditions circulating cytokine concentrations were not influenced, but the TNF- α and IL-1 β mRNA gene expression in circulating whole blood cells was increased in patients with typhoid fever. The LPS-stimulated production of TNF- α and IL-1 β was decreased in both but there was no change for the mRNA content in whole blood for these cytokines. These findings demonstrate that RT-PCR is an attractive method to study the gene expression of cytokines in whole blood, an increased TNF- α and IL-1 β gene expression is present in typhoid fever, and that the LPS stimulated downregulation of cytokines in exercise and typhoid fever may be mediated by post-transcriptional processes.

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Whole blood culture (WBC) is a practical and reproducible method for assessment of cytokine production from human cells.^{1,2} Although a variety of stimulants have been used, the authors prefer the addition of bacterial lipopolysaccharide (LPS) which is added as an immunostimulus to venous blood. After a stationary incubation for 24 h at 37°C, the release of cytokines is measured and compared to the concentration in a control tube. Minimal amounts of blood are required and insight is provided in the capacity to produce cytokines.³ In healthy volunteers addition of LPS to

whole blood will lead to measurable cytokine concentrations within a few hours after incubation, certainly as a result of an increased production and or cellular release into plasma.³ The time course of *ex vivo* production of cytokines in WBC is comparable to that in healthy volunteers after a single LPS injection.⁴ This method was used to assess the cytokine response in various clinical situations. Application of WBC revealed that the LPS stimulated *ex vivo* production of the inflammatory cytokines tumour necrosis factor-alpha (TNF- α) and interleukin 1 β (IL-1 β) becomes down-regulated not only in the acute phase of infections such as typhoid fever,⁶ meningococcal sepsis,⁵ and *Pneumocystis carinii* infections,⁸ during attacks of familial Mediterranean fever¹⁰ and after major surgery⁹ but also after strenuous physical exercise.⁷ In all these clinical situations, the production capacity of these cytokines restores during convalescence.

The mechanism of the depressed cytokine production is incompletely understood. In LPS-stimulated whole blood from patients with sepsis, Northern blot analysis showed a decreased expression of mRNA for

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TNF- α and IL-6 and led to the conclusion that transcription is a decisive mechanism for the modulation of cytokine production.¹¹

Apart from this study in sepsis, the molecular mechanism of downmodulation of cytokines in the above mentioned conditions is not known. In addition, the kinetics of cytokine mRNA expression in WBC is not fully elucidated. The present study describes a simple and reproducible method developed to assess the IL-1 β and TNF- α mRNA in whole blood with a semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR). This method appears to be suitable to determine the kinetics of cytokine mRNA expression in circulating cells after stimulation with LPS. Furthermore, with this method it is possible to evaluate the transcription of mRNA in the down-modulation of cytokine production capacity in various clinical conditions such as physical exercise and typhoid fever.

RESULTS

Time course of IL-1 β and TNF- α gene expression

The induction of TNF- α mRNA in healthy volunteers was very rapid, and high levels were attained after 30 min. Maximum expression was found after 2 h of incubation with LPS. After this time point, the TNF- α mRNA levels declined and after 24 h incubation values were still higher compared to those at baseline. IL-1 β mRNA expression became increased 2 h after LPS stimulation, but maximum values were reached now until only after 4–6 h. Compared to TNF- α , IL-1 β mRNA decreased slower. (Fig. 1A) The kinetics of TNF- α and IL-1 β protein secretion production and release follow that of the mRNA transcription. (Fig. 1B)

Analysis of protein and mRNA from circulating and ex vivo production of cytokines

Typhoid fever

In patients with typhoid fever, circulating concentrations of IL-1 β and TNF- α remained unchanged regardless of the phase of the disease (Table 1). However, mRNA analysis from the circulating uncultured cells of

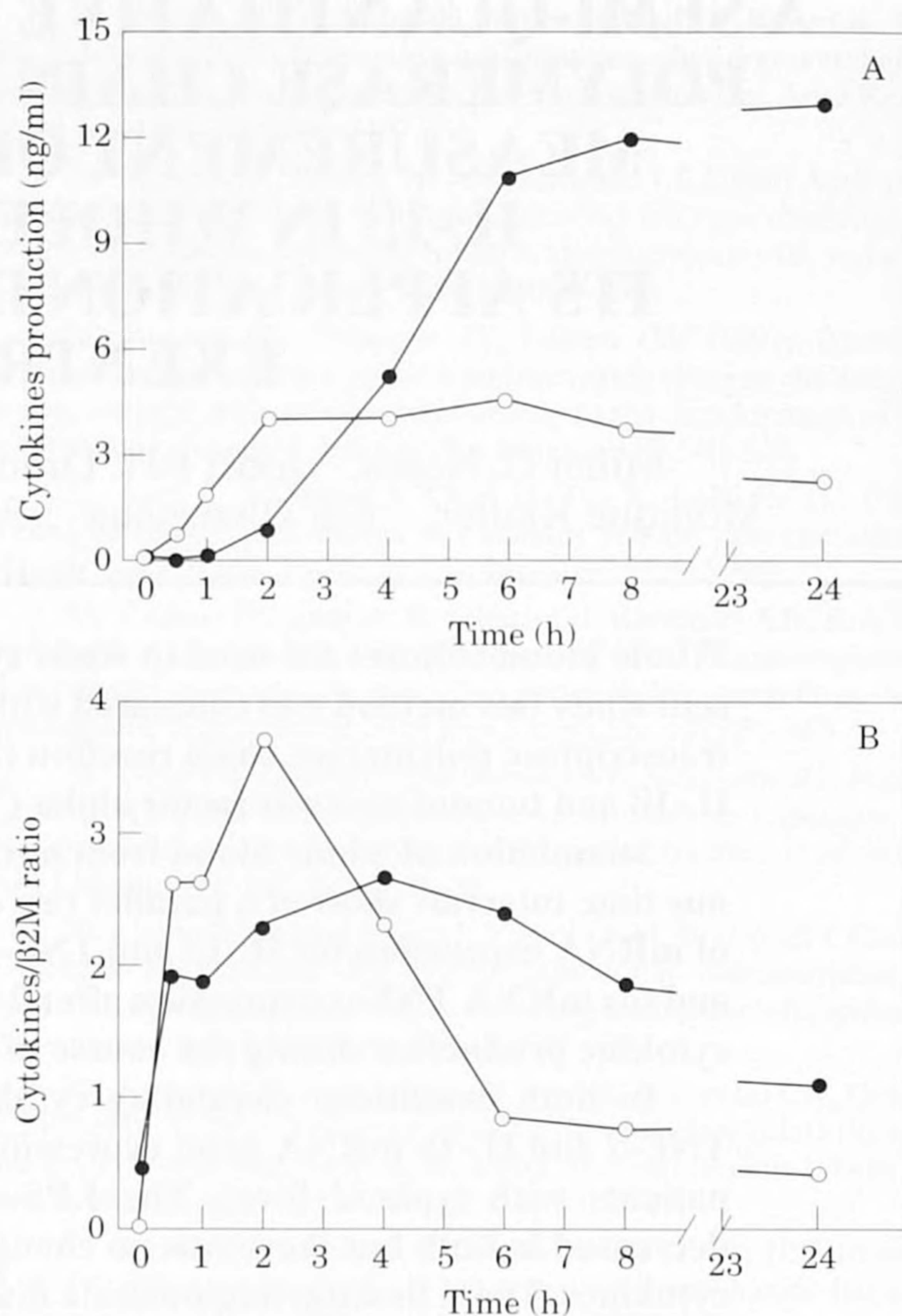


Figure 1. Kinetics of TNF- α (○) and IL-1 β (●) production (mRNA Fig. 1A; protein Fig. 1B) in LPS-stimulated human whole blood.

Blood was obtained from normal healthy volunteers and stimulated with LPS and incubated at 37°C for the indicated intervals. The plasma cytokine concentrations were then measured using specific RIAs. The values shown represent mean for four individual donors.

typhoid fever patients in the acute phase of the disease revealed an increase of both IL-1 β / β ₂M and TNF- α / β ₂M ratios when compared to the recovery phase (Fig. 2).

LPS stimulated WBC of patients in the acute phase of typhoid fever showed a decreased production of IL-1 β and TNF- α , but the production of these proteins restored during convalescence. In contrast to the decreased ex vivo LPS-stimulated production of the IL-1 β and TNF- α proteins, the IL-1 β / β ₂M and TNF- α / β ₂M mRNA ratios did not differ significantly

TABLE 1. Circulating concentrations (circ) and ex vivo production (ex vivo) of IL-1 β and TNF- α in supernatants of whole blood stimulated for 24 h at 37°C with LPS in patients suffering from typhoid fever and healthy volunteers subjected to a 5 km run. The concentrations of these cytokines were measured with specific radioimmunoassays

Clinical situation		TNF- α (ng/ml)		IL-1 β (ng/ml)	
		Acute	Recovery	Acute	Recovery
Typhoid fever	circ	0.16 \pm 0.02	0.19 \pm 0.12	0.17 \pm 0.03	0.14 \pm 0.02
	ex-vivo	4.4 \pm 1.1	7.1 \pm 3.4	3.5 \pm 2.8	5.2 \pm 4.8
Physical exercise	circ	0.09 \pm 0.02	0.09 \pm 0.03	0.05 \pm 0.01	0.06 \pm 0.18
	ex-vivo	3.0 \pm 1	6.9 \pm 1.8	17.2 \pm 9.9	22 \pm 8.5

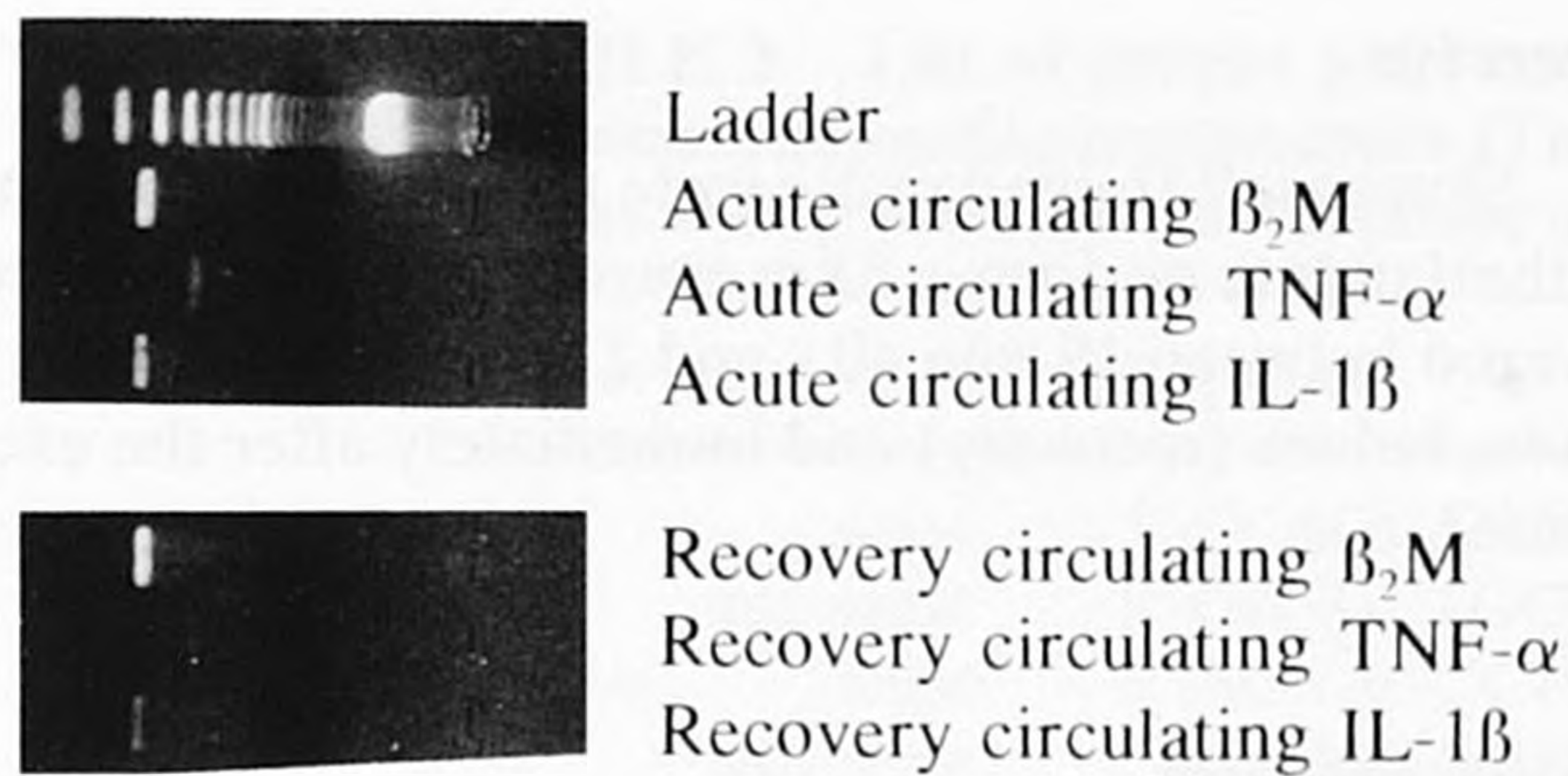


Figure 2. RT-PCR analysis of whole blood from a patient during and after the acute stage of typhoid fever. Whole blood was stimulated with LPS and at the 2 h time point mRNA was extracted and subjected to RT-PCR. The lanes show mean values for the ratio of TNF- α and IL-1 β to β_2 M expression for four volunteers.

between the acute and recovery phase of typhoid fever (Table 2).

Exercise

The 5 km run did not influence the circulating IL-1 β or TNF- α concentrations, nor was there any effect on mRNA content for these cytokines in uncultured whole blood (Tables 1 and 2). Similar to typhoid fever, an inhibition of LPS-stimulated *ex vivo* production of both IL-1 β and TNF- α in blood samples from athletes after a 5 km run was observed compared to pre-exercise values but the difference was larger for TNF- α . Remarkably, the IL-1 β production capacity obtained before exercise was substantially higher in the athletes compared to the recovered typhoid fever patients and to the values obtained from healthy sedentary controls for our laboratory. The mRNA values for IL-1 β and TNF- α were increased by the 5 km run, although these differences were not statistically significant (Table 2).

DISCUSSION

Much of the knowledge regarding the gene expression of cytokines in humans has been obtained in isolated peripheral blood mononuclear cells. A whole blood culture system avoids possible confounding factors on gene expression that may be associated with the isolation of mononuclear cells such as the adherence-induced increase of TNF- α mRNA.¹² EDTA-coated

sampling tubes were used because coagulation of whole blood stimulates IL-1 β gene expression.¹³ Moreover, whole blood represents a more physiological environment for examining cytokine production to LPS because the cellular interactions are preserved and the presence of plasma factors such as the LPS-binding protein is maintained.¹⁴ The authors have developed a simple, sensitive and semi-quantitative RT-PCR for the detection of mRNA for cytokines in whole blood, which also could be applied in a country like Indonesia (where typhoid fever is studied by our group). The amount of mRNA of cytokines was compared to the amount of the housekeeping gene β_2 M mRNA. This allows IL-1 β and TNF- α gene expression to be assessed in whole blood. Because the presence of β_2 M was normalized our data express the mRNA content per individual cell which corrects for the increase of the number of leukocytes occurring after exercise. The kinetic data from this study confirm that incubation of LPS in whole blood leads to a very rapid induction of TNF- α .¹⁵ The TNF- α mRNA expression peaked at 2 h which is in agreement with other studies and corroborates the notion that TNF is a proximal mediator of the response to LPS.¹⁶ In contrast, the expression of mRNA for IL-1 β followed a different course with maximum mRNA levels following those of TNF- α at 4–6 h of incubation. In another study, the TNF- α mRNA expression in whole blood peaked at 1 h post-stimulation while IL-1 β reached its maximum after 2 h. Compared to this study, relatively low LPS concentrations were used to stimulate whole blood (10 μ g/ml vs 1 ng/ml).¹⁷ The cytokine mRNA expression in circulating whole blood cells in typhoid fever patients could be assessed. Despite the absence of differences of cytokine concentrations, there is an increase of mRNA expression for IL-1 β and TNF- α in patients with acute typhoid fever. This presence of cytokine mRNA in circulating cells of these patients suggests that the cytokine network is activated. The pathophysiological significance of these findings for typhoid fever are presently unclear. The absence of increased cytokine proteins in acute typhoid fever could be due to an increased turnover of protein for these cytokines. However, it remains possible that the increased expressed mRNA is not translated and thus does not

TABLE 2. mRNA for IL-1 β and TNF- α from circulating whole blood cells (circ) and from whole blood cultures (*ex vivo*) stimulated for 4 h at 37°C with LPS in patients with typhoid fever and healthy volunteers running 5 km. Results are expressed as a ratio of specific cytokine to the presence of the housekeeping gene β_2 M

Clinical situation		TNF- α / β_2 M		IL-1 β / β_2 M	
		Acute	Recovery	Acute	Recovery
Typhoid fever	circ	0.28 \pm 0.06	0.19 \pm 0.19	0.58 \pm 0.2	0.31 \pm 0.09
	<i>ex-vivo</i>	0.69 \pm 0.2	0.59 \pm 0.2	1.05 \pm 0.1	0.93 \pm 0.1
Physical exercise	circ	0	0	0	0
	<i>ex-vivo</i>	1.35 \pm 0.9	0.62 \pm 0.4	1.83 \pm 1.5	1.3 \pm 0.2

lead to the appearance of these proteins in the circulation. The absence of any change in the circulating TNF- α and IL-1 β concentrations in athletes is associated with absence of mRNA for these cytokines. Apparently, the stress related to a 5 km run is not intense enough to elicit detectable cytokines in the circulation of these athletes.

Study of the mRNA expression of cytokines in LPS-stimulated whole blood in patients with typhoid fever and athletes allowed us to make an important observation. Despite a decreased cytokine production in the acute phase of typhoid fever and post-exercise, both IL-1 β and TNF- α mRNA expression was similar in the acute and recovery phase. These findings suggest that under the conditions studied, the inhibition of the LPS-stimulated ex vivo cytokine production is a post-transcriptional event. In this respect the results here differ from those in a recent study in sepsis patients, where a decreased TNF- α and IL-6 mRNA expression in LPS-stimulated whole blood was found.¹¹ This suggests that the molecular mechanism of downmodulation of cytokine production may be influenced by type of infection.

The rate of mRNA degradation is the most important mechanism of post-transcriptional deactivation.¹⁸ Several additional post-transcriptional processes are potentially able to influence the presence of cytoplasmic mRNA, including capping, splicing, polyadenylation, nuclear export and compartmentalization.^{19–23} Furthermore, kinetic influences may play a role and the rate of degradation of IL-1 β and TNF- α proteins may precede the degradation of mRNA. Nevertheless, our results are in line with ex vivo experiments with LPS-tolerant mice, showing that peritoneal macrophages did not produce TNF- α when restimulated, while its mRNA was still induced. This strongly suggests a post-transcriptional regulation of TNF downregulation.²⁴

MATERIALS AND METHODS

Controls

Four healthy volunteers participated in the study in order to obtain data for study of the kinetics of TNF- α and IL-1 β protein production and gene expression in LPS stimulated whole blood.

Typhoid fever

Four patients with culture proven typhoid fever were recruited in Dr Kariadi Hospital, Diponegoro University, Semarang, Indonesia as part of a project on cytokines in the pathophysiology of typhoid fever. Samples were obtained on admission (acute) and 7–10 days after defervescence (recovery).

Exercise

Seven well-trained males were recruited in Wijchen, The Netherlands to perform a 5 km exercise run. The distance was covered between 18 min 40 s and 22 min 16 s. Samples were drawn before (recovery) and immediately after the exercise (acute).

Blood samples

Blood for cytokine measurements was collected into 2-ml or 4-ml (ex vivo production) endotoxin-free tubes containing EDTA (Vacutainer Systems, Becton and Dickinson, Rutherford, NJ). Cytokine production was measured using a whole blood culture system as described elsewhere.³ Briefly, two 2-ml tubes containing 24 μ l EDTA-K₃ (Bayer, Leverkusen, Germany) (4 ml; 48 μ l EDTA-K₃) were drawn. One tube was incubated immediately, the other tube was incubated after addition of 25 μ l LPS (*Escherichia coli* serotype 055:B5; Sigma, St Louis, MO; final concentration 10 μ g/ml blood).

After 24 h of incubation at 37°C the tube was centrifuged at 2250 \times g for 10 min and secondly at 15 000 \times g for 5 min to obtain platelet-poor plasma. After 4 h (exercise volunteers) or 2 h (typhoid fever patients) after LPS-stimulation, aliquots of 500 μ l blood were taken, and after addition of an equivalent amount of guanidiniumisothiocyanate (GITC) stored at -70°C until RNA isolation. For studies on kinetics of mRNA in healthy volunteers the 4-ml EDTA tube was incubated for 24 h and after 0, 0.5, 1, 2, 4, 6, 8 and 24 h 500 μ l whole blood was taken and subsequently centrifuged at 2250 \times g for 10 min; plasma was used for cytokine protein determination and the cell pellet was dissolved in 500 μ l (4 M) GITC enriched with 7 μ l β -mercaptoethanol for isolation of total RNA. Aliquots were stored at -70°C until assay.

Materials

M-MLV Reverse Transcriptase, DTT, RT buffer, Agarose and Taq DNA polymerase were all purchased from Life Technologies (Breda, The Netherlands) RNasin was purchased from Promega (Leiden, The Netherlands). dNTPs and pd(N)₆ were purchased from Pharmacia (Woerden, The Netherlands). DNase I was purchased from Boehringer Mannheim. (Almere, The Netherlands) Ethidium bromide was purchased from Sigma (St Louis, MO). PCR primers for human TNF- α , IL-1 β and β_2 m were obtained from Dr E. Mensink (Department of Hematology, University Hospital St. Radboud, Nijmegen, The Netherlands). Quantitative densitometry of the gels was performed on a Molecular Analyst™/PC densitometer (Model GS-670, BIORAD, Veenendaal, The Netherlands).

RNA isolation

Total RNA was isolated by the method of Chomczynski and Sacchi with minor modifications.²⁵ Briefly, 500 μ l of whole blood was resuspended in 1 ml GITC, and sonicated for 10 min, followed by the addition of 2 M sodium acetate, phenol and chloroform/isoamylalcohol (49:1). After centrifugation, the RNA was precipitated twice from aqueous phase with

TABLE 3. List of primer pairs used for mRNA amplification, with data regarding sequence, annealing temperature (T_m°C), size of the PCR product and number of PCR cycles within the exponential phase of the reaction

Primer		Sequence	bp	T _m (°C)	Cycles
β ₂ m	sense	5'-CCAGCAGAGAATGGAAAGTC-3'	268	55	26
	anti-sense	5'-GATGCTGCTTACATGTCTCG-3'			
TNF-α	sense	5'-ACAAGCCTGTAGCCCATGTT-3'	427	55	29
	anti-sense	5'-AAAGTAGACCTGCCAGACT-3'			
IL-1β	sense	5'-GGATATGGAGCAACAAGTGG-3'	263	55	29
	anti-sense	5'-ATGTACCAGTTGGGGAAGT-3'			

acidified ethanol. The RNA was dissolved in RNAase-free sterile water. The amount and quality of RNA were determined by spectrophotometry and analysis by agarose gel electrophoresis.

RT-PCR analysis of cytokine mRNA and β₂-microglobulin (β₂m) mRNA

For each sample 0.5 µg of total RNA was reverse transcribed in a volume of 20 µl reverse transcriptase buffer (50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM MgCl₂) containing 10 mM dithiothreitol, 5 µM random Hexamers, 250 µM dNTPs, 20 U RNAsin, 200 U M-MLV Reverse Transcriptase. Reaction mixtures were overlaid with mineral oil. RT reaction was performed for 10 min at 20°C, followed by 45 min at 42°C, and finishing for 10 min at 95°C using a Mastercycler 5330 (Eppendorf, Hamburg, Germany), and the samples were stored at -20°C until PCR analysis was performed. Sequences of the PCR primers for human TNF-α, IL-1β and β₂M are listed in Table 3. Each primer pair was tested to determine the annealing temperature and the linear range of the reaction. PCR reactions consisted of 3 µl cDNA in 50 µl PCR buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM MgCl₂, 0.001% gelatin) containing 100 µM dNTPs, 0.3 µM of each primer and 1.25 U Taq polymerase. Reaction mixtures were overlaid with mineral oil. PCR cycles were performed (30 s denaturation at 92°C, 30 s annealing at 55°C and 90 s extension at 72°C) on the same Mastercycler 5330. The linearity of the PCR reactions was checked at different number of cycles. The plateau phase of the β₂M reaction became apparent after 30 cycles and of TNF-α and IL-1β after 32 cycles. Therefore, for serial determinations of mRNA we selected 26 cycles for β₂M and 29 cycles for IL-1β and TNF-α. PCR products were analysed by electrophoresis on 2% agarose gels stained with ethidium bromide in order to quantify the intensity of the banding pattern. Gels were scanned on a densitometer (GS-670, BIORAD, Veenendaal, The Netherlands) and analysed using Molecular Analyst™ software. Linearity of the densitometer and software was controlled in a separate experiment using consecutive dilutions of a sample with a known DNA content. To correct for fluctuations in leukocyte count, we corrected for the amount of TNF-α and IL-1β mRNA in a sample by expressing it as a ratio vs the amount of the housekeeping gene β₂M mRNA.

Cytokine measurements

Source of antiserum

Polyclonal antibodies for IL-1β were kindly provided by Sclavo (Siena, Italy) and antibodies for TNF-α were a gift of Dr C.A. Dinarello (Denver, CO).

Radioimmunoassay for cytokines

IL-1β and TNF-α in plasma were measured by non-equilibrium radioimmunoassays (RIA) as described extensively elsewhere.⁷ The sensitivity of the assay with 100 µl sample was 40 pg/ml (IL-1β) and 20 pg/ml (TNF-α). To minimize analytical errors, all samples from the same patients were analysed in the same run in duplicate. The inter-assay variation of our RIA is estimated at less than 15%, while the intra-assay variation is less than 10%, which are typical figures for these assays.

Statistical analysis

The non-paired non-parametric Mann-Whitney test was used for statistical comparison of results. Probability (*P*) values were calculated on the basis of two-tailed tests. *P* < 0.05 was considered to be the lowest level of significance. Data are given in mean ± SD.

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