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A SEMI-QUANTITATIVE REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION METHOD FOR MEASUREMENT OF MRNA FOR TNF-\(\alpha\) AND IL-1\(\beta\) IN WHOLE BLOOD CULTURES: ITS APPLICATION IN TYPHOID FEVER AND EXCENTRIC EXERCISE

Mihai G. Netea, Joost P.H. Drenth, Natasja De Bont, Anneke Hijmans, Monique Keuter, Edi Dharmana, Pierre N.M. Demacker, Jos W.M. van der Meer

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\(\beta\) and tumour necrosis factor alpha (TNF-\(\alpha\)) 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\(\beta\) and TNF-\(\alpha\) as well as a rise of mRNA expression for IL-1\(\beta\) and TNF-\(\alpha\) with peak levels for IL-1\(\beta\) after 4–6 h stimulation and for mRNA TNF-\(\alpha\) 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-\(\alpha\) and IL-1\(\beta\) mRNA gene expression in circulating whole blood cells was increased in patients with typhoid fever. The LPS-stimulated production of TNF-\(\alpha\) and IL-1\(\beta\) 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-\(\alpha\) and IL-1\(\beta\) 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-\(\alpha\)) and interleukin 1\(\beta\) (IL-1\(\beta\)) becomes down-regulated not only in the acute phase of infections such as typhoid fever,\(^5\) meningococcal sepsis,\(^5\) and Pneumocystis carinii infections,\(^6\) during attacks of familial Mediterranean fever\(^7\) and after major surgery\(^8\) 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...
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-1β 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-1β 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-1β 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-1β mRNA decreased slower. (Fig. 1A) The kinetics of TNF-α and IL-1β 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-1β and TNF-α remained unchanged regardless of the phase of the disease (Table 1). However, mRNA analysis from the circulating uncultured cells of typhoid fever patients in the acute phase of the disease revealed an increase of both IL-1β/β-M and TNF-α/β-M 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-1β 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-1β and TNF-α proteins, the IL-1β/β-M and TNF-α/β-M mRNA ratios did not differ significantly

<table>
<thead>
<tr>
<th>Clinical situation</th>
<th>TNF-α (ng/ml)</th>
<th>IL-1β (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Acute</td>
<td>Recovery</td>
</tr>
<tr>
<td>Typhoid fever</td>
<td>0.16 ± 0.02</td>
<td>0.19 ± 0.12</td>
</tr>
<tr>
<td></td>
<td>4.4 ± 1.1</td>
<td>7.1 ± 3.4</td>
</tr>
<tr>
<td>Physical exercise</td>
<td>0.09 ± 0.02</td>
<td>0.09 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>3.0 ± 1</td>
<td>6.9 ± 1.8</td>
</tr>
</tbody>
</table>
Acute circulating β,M
Acute circulating TNF-α
Acute circulating IL-1β

Recovery circulating β,M
Recovery circulating TNF-α
Recovery circulating IL-1β

Figure 2. RT-PCR analysis of whole blood from a patient during
and after the acute phase of typhoid fever. Whole blood was stimu­
lated 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-1β to β,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-1β 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 inhi­
bition of LPS-stimulated ex vivo production of both IL­
1β 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-1β 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 lab­
oratory. The mRNA values for IL-1β 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 expres­
sion of cytokines in humans has been obtained in iso­
lated peripheral blood mononuclear cells. A whole
blood culture system avoids possible confounding fac­
tors on gene expression that may be associated with the
isolation of mononuclear cells such as the adherence­
induced increase of TNF-α mRNA. EDTA-coated
sampling tubes were used because coagulation of whole
blood stimulates IL-1β gene expression. Moreover,
whole blood represents a more physiological envi­
ronment for examining cytokine production to LPS
because the cellular interactions are preserved and the
presence of plasma factors such as the LPS-binding pro­
tein is maintained. 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,M mRNA. This allows IL-1β and
TNF-α gene expression to be assessed in whole blood.
Because the presence of β,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. 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. In con­
trast, the expression of mRNA for IL-1β followed a dif­
f erent 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-1β reached its maxi­
mum after 2 h. Compared to this study, relatively low
LPS concentrations were used to stimulate whole blood
(10 μg/ml vs 1 ng/ml). 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-1β 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>
<thead>
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<th>Clinical situation</th>
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<th>IL-1β/β,M</th>
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<tr>
<td></td>
<td>Acute</td>
<td>Recovery</td>
</tr>
<tr>
<td>Typhoid fever</td>
<td></td>
<td></td>
</tr>
<tr>
<td>circ</td>
<td>0.28 ± 0.06</td>
<td>0.19 ± 0.19</td>
</tr>
<tr>
<td>ex-vivo</td>
<td>0.69 ± 0.2</td>
<td>0.59 ± 0.2</td>
</tr>
<tr>
<td>Physical exercise</td>
<td></td>
<td></td>
</tr>
<tr>
<td>circ</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ex-vivo</td>
<td>1.35 ± 0.9</td>
<td>0.62 ± 0.4</td>
</tr>
</tbody>
</table>
lead to the appearance of these proteins in the circulation. The absence of any change in the circulating TNF-α and IL-1β 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-1β 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. 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. Several additional post-transcriptional processes are potentially able to influence the presence of cytoplasmic mRNA, including capping, splicing, polyadenylation, nuclear export and compartmentalization. Furthermore, kinetic influences may play a role and the rate of degradation of IL-1β 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 suggest a post-transcriptional regulation of TNF downregulation.

**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-1β 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. Briefly, two 2-ml tubes containing 24 µl EDTA-K3 (Bayer, Leverkusen, Germany) (4 ml; 48 µl EDTA-K3) were drawn. One tube was incubated immediately, the other tube was incubated after addition of 25 µl LPS (Escherichia coli serotype 055:B5; Sigma, St Louis, MO; final concentration 10 µg ml blood).

After 24 h of incubation at 37°C the tube was centrifuged at 2250 × g for 10 min and secondly at 15 000 × 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 µl blood were taken, and after addition of an equivalent amount of guanidinium isothiocyanate (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 µl whole blood was taken and subsequently centrifuged at 2250 × g for 10 min; plasma was used for cytokine protein determination and the cell pellet was dissolved in 500 µl (4 M) GITC enriched with 7 µl 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), were purchased from Pharmacia (Woerden, The Netherlands). DNase I was purchased from Boehringer Mannheim. (Almere, The Netherlands) E. coli was purchased from Sigma (St Louis, MO). PCR primers for human TNF-α, IL-1β and β2m 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 AnalystTM/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. Briefly, 500 µl 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/isooamylic alcohol (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 (Tm°C), size of the PCR product and number of PCR cycles within the exponential phase of the reaction

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>bp</th>
<th>Tm(°C)</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>β_m sense</td>
<td>5'-CCAGCACAGAGATGGGAAAGTC-3'</td>
<td>268</td>
<td>55</td>
<td>26</td>
</tr>
<tr>
<td>anti-sense</td>
<td>5'-GATGCTGCTTACATGTCTGG-3'</td>
<td>427</td>
<td>55</td>
<td>29</td>
</tr>
<tr>
<td>TNF-α sense</td>
<td>5'-AAAAGTATAGGAGGAACTG-3'</td>
<td>263</td>
<td>55</td>
<td>29</td>
</tr>
<tr>
<td>anti-sense</td>
<td>5'-ATGTACCCAGTTGGGAAGT-3'</td>
<td>286</td>
<td>55</td>
<td>29</td>
</tr>
</tbody>
</table>

acidified ethanol. The RNA was dissolved in RNase-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-microglobuline (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. 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.

Acknowledgements

J.P.H. Drenth is a recipient of a Dutch Organization for Scientific Research fellowship for Clinical Investigators (KWO 900-716-065). The authors wish to thank the athletes from ‘Athletiek Vereniging Wijchen’ (Wijchen, The Netherlands) for their cooperation with this study. R. Krebers and Dr S.H.M. van Uum are thanked for their help in performing the study.

REFERENCES


