Increased Tumor Necrosis Factor-α mRNA Expression in Whole Blood from Patients with Rheumatoid Arthritis: Reduction After Infliximab Treatment Does Not Predict Response

ALEXANDRE PACHOT, BÉATRICE ARNAUD, HUBERT MARROTE, MARIE-ANGÉLIQUE CAZALIS, JENNIFER DIASPARRA, AURORE GOURAUD, BRUNO MOUGIN, and PIERRE MIOSSEC

ABSTRACT. ***Objective.*** It has been suggested that patients with rheumatoid arthritis (RA) with abundant tumor necrosis factor-α (TNF-α) are more likely to respond to TNF-α inhibitors. We measured expression of TNF-α mRNA in peripheral blood of RA patients undergoing infliximab treatment in order to test its predictive value for treatment response.

Methods. Forty-four RA patients showing persistent disease activity and 27 healthy controls were studied. Peripheral blood TNF-α mRNA levels were measured before and 4 hours after the first infliximab infusion and at Week 22 using quantitative RT-PCR. Results were correlated to the treatment response at Week 22 in the whole RA cohort and a subset of patients showing high TNF-α mRNA levels at baseline.

Results. At baseline and at Week 22, TNF-α mRNA expression in RA patients was significantly increased compared to healthy controls. At both timepoints, no significant difference was observed between responders and nonresponders. Compared to baseline, infliximab treatment induced a decrease in TNF-α mRNA level at 4 hours and at Week 22, although this effect was significant only in patients with high TNF-α mRNA expression at baseline. Such variation compared to baseline was similar in responders and nonresponders.

Conclusion. Peripheral blood TNF-α mRNA expression is increased in RA, but its reduction with anti-TNF treatment is not associated with treatment response. (First Release Sept 15 2007; J Rheumatol 2007;34:2158–61)

Key Indexing Terms:
RHEUMATOID ARTHRITIS TUMOR NECROSIS FACTOR-α mRNA INFLIXIMAB

Tumor necrosis factor-α (TNF-α) plays a major role in the pathogenesis of rheumatoid arthritis (RA)[1], and TNF-α blockade is currently indicated for the treatment of patients with active RA[2]. This often results in rapid clinical improvement and decrease in inflammatory mediators[2,3]. Despite treatment with anti-TNF-α, 20%–40% of patients have persistent disease activity and some patients show no response at all[2,4].

It has been suggested that patients producing a large amount of TNF-α are more likely to respond to TNF-α inhibitors. However, the TNF-α situation in blood is unclear, since a simple determination of plasma TNF-α concentrations by ELISA seems to be of limited help in predicting treatment response[4,5]. This might be explained by a lack of sensitivity, and also because immunoassays measure only protein concentrations, but not function, which can be modified by inhibitors and additional cytokines. Results obtained with a bioassay are in agreement with this hypothesis[5].

Measurement of plasma TNF-α levels yields information about the protein secretion of circulating and some tissue-entrapped cells. To evaluate TNF-α associated to cells, either flow cytometry or quantification of mRNA expression can be used; to test this new option, we examined TNF-α mRNA in peripheral blood (PB) of patients with RA before and during the course of infliximab treatment.

MATERIALS AND METHODS

Patients and controls. Forty-four patients with RA who fulfilled the revised criteria of the American College of Rheumatology were enrolled in the study. The study selection criteria called for patients who were naive to anti-TNF treatment and eligible for infliximab treatment based on persistent RA disease activity despite therapy with methotrexate (MTX). Patients received infliximab at the recommended dose of 3 mg/kg at Weeks 0, 2, and 6 and thereafter every 8 weeks, in combination with MTX. Disease activity was assessed at baseline and before the fifth infusion at Week 22 using the Disease Activity Score (DAS28). Clinical response to treatment was assessed using the DAS28[6]. Responders were defined at Week 22 as those showing > 1.2

From the Joint Unit, Hospices Civils de Lyon – bioMérieux, Hôpital Edouard Herriot; and the Clinical Immunology Unit, Department of Immunology and Rheumatology, Hôpital Edouard Herriot, Lyon, France.

A. Pachot, PharmD, PhD; B. Arnaud; M-A. Cazalis; J. Diasparra; A. Gouraud, PharmD; B. Mougin, PharmD, PhD, Joint Unit, Hospices Civils de Lyon – bioMérieux; H. Marrote, MD, PhD; P. Miossec, MD, PhD, Joint Unit, Hospices Civils de Lyon – bioMérieux and Clinical Immunology Unit, Department of Immunology and Rheumatology, Hôpital Edouard Herriot.

Address reprint requests to Dr. P. Miossec, Clinical Immunology Unit, Department of Immunology and Rheumatology, Hôpital Edouard Herriot, 5 place d'Arsonval, 69003 Lyon, France.
E-mail: pierre.miosse@chu-lyon.fr

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improvement in the DAS28 from baseline. Nonresponders were patients with an improvement measure ≤ 1.2. Whole blood was collected at baseline immediately before and 4 h after the first infusion and at Week 22 before the fifth infusion. The 4 h sample was used to evaluate an acute effect immediately after the infusion. The 24-week sample was used to evaluate chronic administration, when an effect on disease activity can be measured. Twenty-seven healthy persons (mean age 49 ± 6 yrs, male/female ratio 0.65) served as controls.

The study protocol was approved by the local Ethical Committee for Clinical Research (CCPPRB Lyon B, 03122002), and all patients gave their informed consent.

RNA isolation and real-time PCR. Whole blood samples were collected into PAXgene™ blood RNA tubes and total RNA was extracted using the PAXgene™ blood RNA kit (PreAnalytix, Hilden, Germany). Residual genomic DNA was digested using the RNase-Free DNase set (Qiagen). Total RNA was reverse-transcribed into cDNA using the ThermoScript™ RT-PCR system (Invitrogen, Carlsbad, CA, USA) and TNF-α mRNA expression was quantified by quantitative RT-PCR using the LightCycler™ instrument and the Fast-Start™ DNA Master SYBR Green I real-time PCR kit (Roche Molecular Biochemicals, Basel, Switzerland) as described[7,8]. TNF-α mRNA level was normalized to that of the housekeeping gene peptidylpropyl isomerase B (PPIB) encoding for cyclophilin B as described[8]. Standards and primer mixes for PPIB and TNF-α were obtained from Search-LC (Heidelberg, Germany). Using the LightCycler software version 4, the results were expressed as a concentration ratio of TNF-α mRNA/PPIB mRNA.

Statistical analysis. Continuous variables were compared between groups with the nonparametric Mann-Whitney U test. Categorical variables were compared using the chi-square test. A value of $p < 0.05$ was considered significant.

RESULTS

According to the EULAR response criteria, 26 patients (59%) were responders and 18 (41%) nonresponders. At baseline, the mean DAS28 value was significantly higher in the responders (Table 1). Moreover, the percentage of women was significantly higher in the nonresponders (Table 1).

At baseline, the constitutive TNF-α mRNA expression in PB of RA patients before infliximab infusion was significantly increased compared to controls (median concentration ratio 4.38×10^{-3} vs 1.92×10^{-3}, respectively; $p < 0.0001$; Figure 1). At Week 22, before the fifth infliximab infusion, TNF-α mRNA remained significantly increased in patients compared to controls (median 3.48×10^{-3} vs 1.92×10^{-3}; $p < 0.0001$; Figure 1). Before treatment and at Week 22, when RA patients were stratified into responders or nonresponders, TNF-α mRNA expression remained significantly increased in each group compared to controls ($p < 0.05$; Figure 1), although no significant difference was observed between responders and nonresponders.

Considering the whole RA cohort, no significant difference was observed when comparing the baseline TNF-α mRNA level to the level observed at Week 22. However, a trend toward a decrease was observed over time, in particular in patients with high TNF-α mRNA expression at baseline. These high TNF-α mRNA patients were defined as those showing a level at baseline greater than mean ± 2 SD of TNF-α mRNA level of the controls (mean 1.84×10^{-3}, SD 0.43×10^{-3}; calculated cutoff 2.69×10^{-3}). This subgroup (n = 17) showed a significant decrease in TNF-α mRNA expression between Weeks 0 and 22 (median 7.61×10^{-3} vs 2.33×10^{-3}, respectively; $p < 0.01$; Figure 2). However, in this subgroup, as observed in the whole cohort, neither the level measured at Week 22 nor the variation compared to the baseline level showed significant difference between responders and nonresponders (data not shown).

Regarding the short-term effect of infliximab as measured 4 hours after the infusion, no significant change in TNF-α mRNA level was observed 4 hours after the infusion when the whole RA cohort was considered. The results were similar when RA patients were stratified into responders and nonresponders. However, selecting only the patients with high TNF-α mRNA level at baseline (n = 17), TNF-α mRNA expression before the infusion was significantly reduced 4 hours after the infusion (median 7.61×10^{-3} vs 4.57×10^{-3}; $p < 0.05$; Figure 2). As for the longterm effects, no significant difference was observed in this subgroup when RA patients were stratified into responders and nonresponders. Comparing patients who were high and those who were low TNF-α mRNA producers, none of the classical disease severity indicators (DAS28 score, C-reactive protein level) or joint destruction markers (Larsen score) showed statistically significant differences between the 2 groups (data not shown).

Table 1. Baseline clinical characteristics of responders and nonresponders.

Characteristic	Responders, n = 26	Nonresponders, n = 18	All, n = 44
Age, mean ± SD yrs	52 ± 14	55 ± 12	53 ± 13
Sex, % female	62	88	73*
Disease duration, mean ± SD yrs	15 ± 12	15 ± 12	15 ± 12
No. of DMARD, mean ± SD	2.9 ± 1.7	3.1 ± 1.5	3.0 ± 1.6
Rheumatoid factor-positive, %	73	78	75
Antinuclear antibody-positive, %	31	33	32
Right Larsen wrist index, mean ± SD	2.8 ± 1.6	1.7 ± 1.9	2.4 ± 1.8
DAS28 score	5.8 ± 1.1	4.7 ± 1.1	5.4 ± 1.2*
CRP, mg/l	32 ± 39	22 ± 25.5	28.0 ± 34.1
Methotrexate dose at baseline, mean ± SD mg/wk	14.6 ± 7.1	12.9 ± 3.5	14 ± 6
Corticosteroid dose at baseline, mean ± SD mg/day	5.4 ± 6.4	5.6 ± 7.0	5.5 ± 6.5

* Responders vs nonresponders, $p < 0.05$. DAS: Disease Activity Score, CRP: C-reactive protein.

DISCUSSION

To study the role and the regulation of TNF-α in the course of RA, we carried out an observational study of TNF-α mRNA expression in peripheral blood of RA patients undergoing infliximab treatment, and hypothesized that the TNF-α mRNA quantification in whole blood could provide an interesting marker of anti-TNF response.

It is well described that TNF-α is increased in the synovial fluid of RA patients[9,10]. In contrast, the serum level of TNF-α is not always detectable in RA, and levels are often similar in

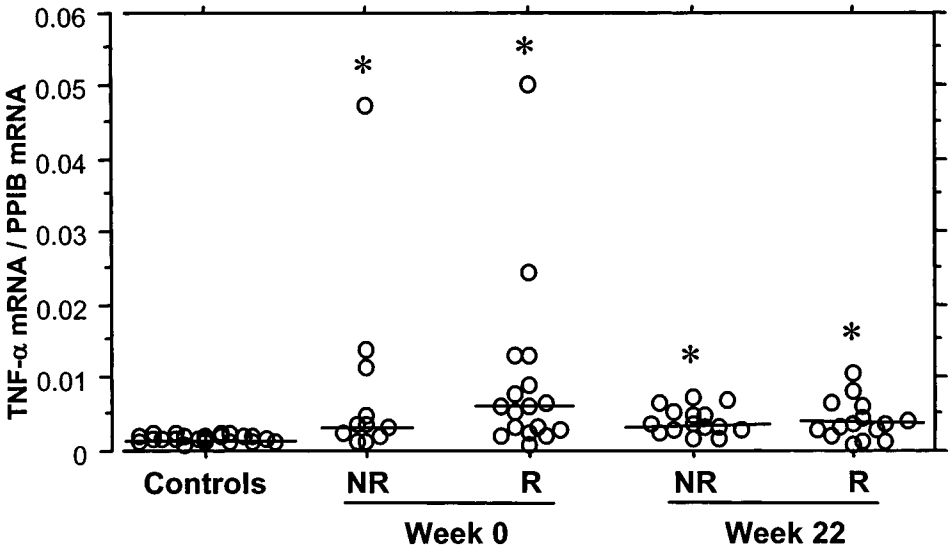

Figure 1. Peripheral blood TNF-α mRNA expression in RA patients measured before and 22 weeks after onset of infliximab treatment. Results are expressed as relative quantification ratio between TNF-α and the housekeeping gene PPIB. TNF-α mRNA level was measured in healthy controls (n = 27) and RA patients at Week 0 just before the onset of infliximab treatment, and before the fifth infusion of infliximab at Week 22. RA patients were subclassified into responders (R; Week 0: n = 17; Week 22 n = 16) or nonresponders (NR; Week 0: n = 11; Week 22: n = 15) according to EULAR criteria. Median values are indicated by a horizontal line. *p < 0.05 vs healthy controls.

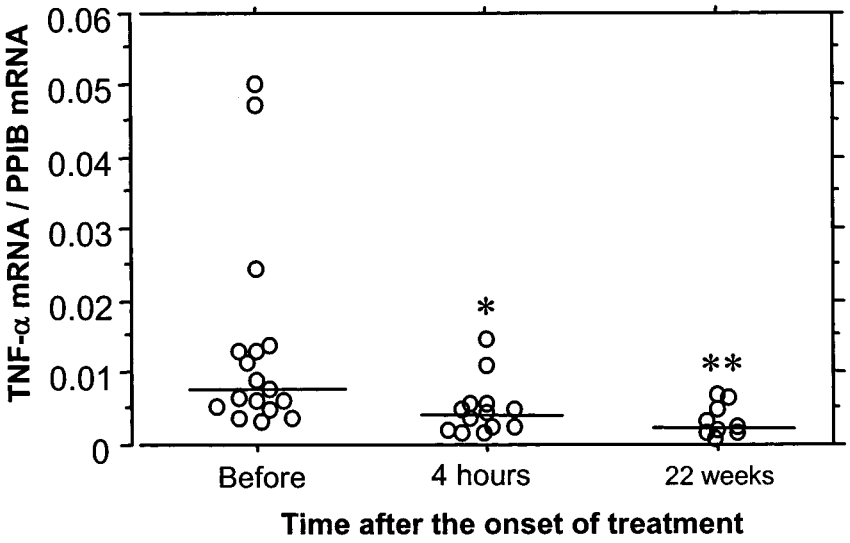

Figure 2. Short- and longterm effect of infliximab on TNF-α mRNA expression in peripheral blood of RA patients identified as high producers. mRNA level was normalized to that of the housekeeping gene PPIB. Individual values were taken into account when the TNF-α mRNA level at baseline was > 2.69×10^{-3}, corresponding to the mean ± 2 SD of levels in 27 controls. TNF-α mRNA level was measured before the onset of infliximab treatment (n = 17), 4 hours after first infusion (n = 13), and before the fifth infusion at Week 22 (n = 9). Median values are indicated by a horizontal line. *p < 0.05 vs controls; **p < 0.01 vs controls.

RA patients and healthy controls[3,9,11,12]. In accord with recently published data obtained from purified peripheral blood mononuclear cells of RA patients[13], we observed that the constitutive TNF-α mRNA expression in PB of RA patients before infliximab infusion was increased compared to healthy controls. However, these levels were very low even in patients with RA.

We examined gene expression patterns in PB cells as a whole, rather than specific cellular subsets. We used the PAXgene blood RNA system, which provides an efficient,

standardized method for whole-blood collection that reduces RNA degradation and prevents any postsampling stimulation[14]. This is critically important, because both previous studies and unpublished data from our institution show that blood collection in EDTA tubes and leukocyte purification are accompanied by profound changes in mRNA expression, particularly for cytokines with low level of expression such as TNF-α[14,15].

Previous results suggested that infliximab treatment can induce a rapid, dose-dependent, and persistent increase in immunoreactive, but not biologically active, TNF-α[3,11,16]. In contrast to results of previous studies investigating serum protein, we found that infliximab induced a decrease in PB TNF-α mRNA level 4 hours and 22 weeks after the onset of treatment, although this effect was most significant in the patients showing a high level of TNF-α mRNA expression at baseline. An effect on TNF-α expression and possibly production could contribute to an explanation of these results. Indeed, neutralization of TNF-α action by infliximab might result in interruption of the cytokine cascade. This effect would lead to the decrease of the proinflammatory cytokines interleukin 1 (IL-1) and IL-6 that are known to activate TNF-α promoter[17]. A reduction of the number of circulating TNF-α mRNA-expressing cells could be another explanation. However, in our study, no difference was detected in the total leukocyte, lymphocyte, and monocyte blood cell counts between the onset of treatment and Week 22 (data not shown).

As for the treatment response, our previous study measuring circulating TNF-α bioactivity suggested that high TNF-α producers are more likely to respond to anti-TNF treatment[5]. However, this study measuring TNF-α mRNA levels and others examining TNF-α protein levels[4,5] were unable to detect an association between blood TNF-α levels and treatment response.

In summary, quantitative RT-PCR is a reliable tool for monitoring levels of mRNA expression in PB of patients with RA. Our results showed that constitutive PB TNF-α mRNA expression is increased in RA, and that infliximab can induce an acute and chronic decrease in this measurement only in a subset of patients showing high levels at baseline. Neither TNF-α mRNA expression at baseline nor the magnitude of infliximab-induced TNF-α mRNA variation was associated to treatment response. This suggests the major contribution of anti-TNF-induced changes at other sites, mainly the synovium.

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