Basic and clinical aspects of mucosal inflammation and healing in Crohn's disease

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Chapter 5

TNF-α assessment during infliximab treatment for Crohn's disease: interference and cellular mechanisms

Short title: TNF-α assessment during infliximab treatment

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Abstract

Background: Infliximab, a chimeric IgG1 anti-tumor necrosis factor (TNF)-α antibody, has been shown to be a very effective bio-therapeutic agent for Crohn's disease (CD), particularly treatment-refractory or fistulizing disease.
Aim: To study potential interference of infliximab in immunosorbent assays for the measurement of TNF-α in plasma and to assess the mechanism(s) of infliximab affecting the TNF-α producing cells in the circulation of CD patients.
Methods: Blood from CD patients, pre- and 2 hours post-infliximab infusion, and blood form healthy volunteers, with or without infliximab, were incubated for 1.5-24 hours with or without lipopolysaccharide (LPS). TNF-α in plasma was assessed by two kinds of assays (Quantikine, R&D system, and BioSource, BioSource Europa S.A.), TNF-α mRNA was semi-quantified by reverse transcription polymerase chain reaction (RT-PCR).
Results: The TNF-α protein levels in blood samples were almost completely inhibited by infliximab. In the presence of infliximab and stimulation with LPS for 24 hours the rate of inhibition for both patients and volunteers was 100% with the Quantikine kit and 60% and 88% with the BioSource kit, respectively, in comparison to the samples without infliximab. With in vitro interference tests, we confirmed that the presence of infliximab severely impaired the determination of TNF-α with both assays. Furthermore, whole blood samples after infusion/incubation of infliximab for 4 hours, washed to remove infliximab, and stimulated with LPS in neat autologous plasma, revealed a TNF-α protein production which was similar to the samples without exposure to infliximab. In addition, TNF-α mRNA levels were 3- to 6-fold higher in cells from the CD patients, and 13- to 50-fold higher in cells from healthy volunteers, compared to control incubation, but unaffected by the presence of infliximab. Pre-exposure to both LPS and infliximab rendered TNF-α producing cells hardly to express any TNF-α protein and mRNA. Inactivation of the complement system did not affect this process.
Conclusions: These results show that the determination of TNF-α by immunosorbent assays is strongly interfered by infliximab. Furthermore, infliximab does not exert its efficacy by impairing the cellular capability to produce TNF-α.

Inflammatory bowel disease (IBD) is comprised of two forms of chronic intestinal inflammation, ulcerative colitis (UC) and Crohn's disease (CD) [1;2]. Their precise etiology remains poorly understood. Genetic factors, enteric microflora and immunity have been implicated in the pathogenesis of the disease. Numerous inflammatory mediators are involved in the pathophysiological process of IBD, including lipid mediators, neuropeptides, oxygen metabolites, enzymes, chemokines and cytokines. Among the proinflammatory cytokines, tumor necrosis factor (TNF)-α plays a central role in the mucosal inflammation in CD [3]. TNF-α is a member of the TNF and TNF-receptor superfamilies [4]. There are two forms of TNF-α: the transmembrane form (mTNF-α) and a soluble form (sTNF-α). The 26 kD mTNF-α is a TNF-α precursor protein which anchors its unusually long signal peptide to the plasma membrane. Monocytes/macrophages are believed to be the largest source of TNF-α, although T lymphocytes and other kinds of cells are also able to produce a significant amount of TNF-α [5;6]. In response to a variety of inflammatory stimuli, TNF-α-producing cells can be activated, at both the transcriptional and translational level, to express TNF-α. Lipopolysaccharide (LPS), a molecule present in Gram-negative bacterial cell walls, is the most potential stimulatory factor for monocytes/macrophages to express TNF-α [7]. The signal of LPS is transduced into the cytoplasm of monocytes/macrophages through the Toll-like receptor (TLR)-4, cooperated by CD14. The intracellular transduction of the LPS signal results in the activation of the transcriptional factor, nuclear factor kappa B (NFκB), which is transferred into the nucleus to trigger mRNA transcription of TNF-α and other inflammatory mediators [8]. Enteric bacteria or bacterial products are proposed to prime the immunoinflammatory response in the alimentary tract [1;9]. In the intestinal mucosal inflammation of CD, TNF-α is relevant to the disruption of the epithelial barrier, coagulation and fibrinolysis, as well as formation of inflammatory granulomas. TNF-α can also activate macrophages, stimulate B lymphocytes, transmit signals between immune cells and other cells, and also promotes the migration of circulating neutrophils to inflamed sites by upregulating the expression of adhesion molecules in endothelial cells [6;10;11].

Anti-TNF-α biological therapy has been shown to be very effective in CD. Infliximab, a human-murine chimeric IgG1 anti-TNF-α monoclonal antibody, results in a dramatical and durable reduction of clinical signs and symptoms in patients with moderate to severely active disease and fistulizing disease [11-14]. The mode of action of infliximab is not only to immunoneutralize bioactive TNF-α but also to induce apoptosis of TNF-α-producing cells [15;16]. A reduction of activated macrophages and T lymphocytes in the intestinal mucosa of CD patients was found after therapy with infliximab [17;18]. In CD patients after infliximab infusion, Nikolaus et al. [19] did not find a detectable level of TNF-α in plasma of whole blood samples in response to LPS stimulation. The reason was believed to be the infliximab-induced death of activated TNF-α producing immune cells. In contrast, the study of Cornillie et al. [20] showed that infliximab did not affect the capability of peripheral blood mononuclear cells to produce TNF-α.

The present study was performed to elucidate possible mechanisms of infliximab affecting the TNF-α assessment and the capacity of circulating blood cells to produce TNF-α.

Materials and Methods

Patients, volunteers and blood samples

Patients with CD (n=11) were treated with infliximab for fistulizing and/or active disease. The patients had received either infliximab or immunosupressive therapy (corticosteriod and/or azathioprine), previously. Healthy volunteers (n=5) were recruited from the laboratory.

Heparinized blood samples were obtained from patients before and two hours after a single infusion of infliximab of 5 mg/kg over a 2 hour period. For the blood samples from the healthy volunteers a concentration of 75 μg infliximab per ml blood was used. At 37^{o}C and 5% CO_2, whole blood samples with/without infliximab were stimulated with/without LPS (Sigma, St. Louis, U.S.A) at 0.1 μg/ml blood for TNF-α mRNA and/or protein determination, similar to the experiments of Nikolaus et al [19]. The plasma was subsequently separated from the blood sample by centrifugation at 2800 rpm for 5 min. at 4^{o}C, and stored at -70^{o}C until further analysis. Leucocytes were isolated by the addition of erythrocyte lysis buffer, containing 0.16 M NH_4Cl, 10 mM $KHCO_3$, and 0.01 mM K_2-EDTA (pH 7.4 at 0^{o}C), to the cell pellets. After lysis of the erythrocytes the samples were centrifuged at 1500 rpm, 4^{o}C for 5 min., and the procedure was repeated to obtain pure leucocytes which were immediately used to isolate mRNA.

Experimental studies

Control group: Blood samples without LPS stimulation were incubated with 0.9% NaCl, at an equal volume to LPS and/or infliximab solutions under the same incubation conditions as for the stimulated samples.

According to the experimental design, the stimulated samples were divided into different groups: 1) blood samples from both patients and volunteers stimulated only with LPS, 2) samples first exposed to infliximab for 4 hours and LPS was then added for 1.5 to 24 hours, 3) to determine if exposure of infliximab obliterates TNF-α production, samples after 4 hours exposure to infliximab were washed, autologous neat plasma was added and stimulated again with LPS, and 4) to test whether the complement system is involved in the regulation of TNF-α production, blood cells were incubated with autologous heat inactivated complement plasma (56^{o}C, 30 min.) or non-inactivated plasma and stimulated with LPS in the presence of infliximab for 4 hours, then washed and neat plasma added for another LPS stimulation for 1.5 hours to determine TNF-α mRNA and for 24 hours to determine TNF-α protein.

TNF-α determination

TNF-α plasma levels were measured with the Quantikine ELISA kit (DTA 50, R&D system, Minneapolis, MN, USA) and with the BioSource EASIA kit, (KAC 1752, BioSource Europa S.A., Nivelles, Belgium). The measurements were performed according to the instructions of the manufacturers.

Infliximab interference experiments

Potential interference of infliximab in the determinations of TNF-α was assessed by adding 150 μg/ml infliximab to plasma of LPS stimulated blood and to ELISA/EASIA standards followed by a 2 hours incubation and measurement of TNF-α. In addition, plasma of LPS-stimulated blood with/without infliximab were assessed separatedly and 1:1 combined.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Oligonucleotide primers (table 1) were derived from the DNA sequence database of NCBI (Bethesda, MD, USA). Primer sets were designed using the Primers3 Output computer program (Whitehead Institute for Biomedical Research, Cambridge, MA, USA). The design of

the primers was that the PCR product of TNF-α crossed two introns to prevent interference of contamination by genomic DNA. Specificity of the primers was checked with the NCBI BLAST program. β2-microglobulin (β2-M) was used as a control to normalize the PCR signals from the different samples. Total RNA was isolated from leucocytes by the method of Chomczynski and Sacchi [21]. The integrity and quality of the purified RNA were analyzed by 1.5% agarose gel ethidium-bromide staining and the 260/280 nm absorbance ratio. Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase was used for cDNA synthesis. The PCR was started at 94°C for 3 min. followed by 30 or 35 cycles for TNF-α and 28 cycles for β2-M. The reaction was performed in Whatman T Gradient cycler (Biometra, Goettingen, Germany). 15 µl of the amplified products were electrophorized on 1.5% agarose gel containing ethidium-bromide (0.5µg/ml) and visualized under ultraviolet light. A RT-PCR, which contained RNA but not M-MLV reverse transcriptase, was used to check contamination with genomic DNA. The Scion imaging program (Frederick, Maryland, USA. www.scioncorp.com) was used to quantify the band density in the gels.

Table 1. Oligonucleotide primers for RT-PCR

mRNA	Gene	Sense primer	Antisense primer	Product size
NM-000594	TNF-α	CCCCAGGGACCTCTCTCTAA	GGAAGACCCCTCCCAGATAG	413
NM-004048	β2-M	CCAGCAGAGAATGGAAAGTC	GATGCTGCTTACATGTCTCG	269

Results

Whole blood 24h LPS stimulation resulted in a 13- to 27-fold increase in the TNF-α plasma level of CD patients, as determined by the BioSource and Quantikine kits, respectively, compared to the control incubation (Table 2). The LPS-induced increase of TNF-α in the healthy volunteers was found to be even higher, i.e., >300-fold in both assays. The presence of infliximab during this LPS stimulation resulted in an almost 100% inhibition of the TNF-α level for both patients and volunteers with the Quantikine kit and in an inhibition of 60% to 88% for the BioSource kit. In addition, the TNF-α plasma levels as determined by the BioSource EASIA was found to be considerably higher than with the Quantikine ELISA (Table 2), and in the presence of infliximab low but still detectable.

Table 2. TNF-α in blood samples after exposure to LPS for 24 hours with/without infliximab

ELISA kits	Groups	Control	LPS 24h	Infliximab + LPS 24h	Inhibition Rate
Quantikine	CD (n=7)	14 (0-140)	381 (205-5056)	10 (0-31)	99.8 (85.2-100)
	VO (n=5)	0 (0-1)	4856 (521-10135)	0 (0-28)	100 (99.5-100)
BioSource	CD (n=7)	60 (8-2852)	807 (356-10295)	358 (128-527)	59.9 (0-95.3)
	VO (n=5)	30 (11-92)	10011 (1838-15044)	1027 (510-1249)	88.4 (72.3-91.7)

CD: patients with Crohn's disease, VO: healthy volunteers,
TNF-α values expressed in pg/ml (median, range).

To elucideate the underlying mechanism(s) of these lower TNF-α levels in the presence of infliximab, several experiments were performed. Firstly, infliximab was added to some 24h LPS stimulated plasmas of CD patients and healthy volunteers. Furthermore, also to some standards of the assays infliximab was added. The determination of TNF-α by the Quantikine ELISA was found to be completely inhibited in vitro by infliximab, whereas for the BioSource EASIA the inhibition was found to be approximately 80% (Table 3).

Table 3. *In vitro* infliximab interference on the measurement of TNF-α

	Plasmas from 24h LPS stimulated samples	Same plasmas with added infliximab (150 µg/ml)	Inhibition rate (%)
Quantikine (n=6)[1]	966 (205-5945)	0 (0-70)	100 (98.8-100)
	750 (Standard)	0	100
BioSource (n=4)[2]	6859 (1558-9963)	789 (342-1889)	83.9 (78.1-92.1)
	460 (Standard 1)	103	77.6
	1430 (Standard 2)	302	78.9

TNF-α values expressed in pg/ml (median, range),
[1] 4 Crohn's disease patients and 2 volunteers,
[2] 2 Crohn's disease patients and 2 volunteers.

Apparently both these immunoassays are hampered by interference of infliximab. This was further illustrated by the in vitro addition of autologous infliximab plasma to the same amount of pre-infliximab plasma, both from LPS stimulated samples, of four individuals (2 patients and 2 volunteers) with the highest TNF-α level. This plasma combination resulted in a 97.3% (96.3-98.6%) reduction of the TNF-α level, from 3868 (1537-5945) to 111 (22-221) pg/ml, when measured with the Quantikine kit. Secondly, we assessed whether the presence of infliximab in the whole blood had affected the TNF-α producing capacity of the leucocytes. This evaluation was performed by washing cells which had been exposed to infliximab for 4h and replacing the plasma by neat plasma and LPS stimulation by 24h. As illustrated in Table 4, the TNF-α production was completely preserved or even elevated in both patients and volunteers. Also for the mRNA there was a 4- to 6-fold higher level compared to the control incubation without LPS, although the absolute levels were rather low (data not shown).

Table 4. Reversal of TNF-α levels in plasma of samples with/without exposure to infliximab

	LPS 24h	Infliximab + LPS 24h	Exposure to infliximab 4h-wash-LPS (in neat plasma) 24h
Patients (n=3)	287 (136-432)	11 (11-15)	245 (174-280)
Volunteers (n=4)	3372 (521-10135)	0 (0-28)	5254 (2460-8668)

TNF-α protein was measured with the Quantikine kit and values expressed in pg/ml (median, range).

Therefore, these experiments were expanded by short-term cultures in order to be able to optimally assess the TNF-α mRNA as well (Table 5). Also in these 1.5h LPS stimulation experiments we observed an impressive secretion of TNF-α compared to the control incubations, both in patients and healthy volunteers, accompanied by a 5- to 13-fold increase in the mRNA level, respectively. Once again infliximab was found to inhibit the detectable

TNF-α protein by more than 90%, but the induction of mRNA was not affected. In addition, preincubation with infliximab and replacement by neat autologous plasma with subsequent LPS stimulation restored the detectable TNF-α protein level and the mRNA level was comparable to the other LPS stimulation experiments.

We further assessed whether LPS stimulation did exhaust the TNF-α production by the cells and whether complement might be involved in the effects of infliximab. After incubation with both LPS and infliximab for 4h, samples from 4 volunteers were washed, neat plasma was added, and cells stimulated with LPS for another 24h. The TNF-α protein level in these samples was found to be very low, i.e., 103 (62-245) pg/ml. Apparently, the 4h LPS with infliximab had killed or exhausted the TNF-α producing cells. When this stimulation was performed in heat-inactivated complement plasma samples, the TNF-α mRNA level was found to be downregulated 27-fold (10-57) compared to that in samples which had been exposed only to infliximab for 4h and then to LPS for 1.5h. Similarly, however, in non-inactivated plasma samples the TNF-α mRNA level was downregulated 21-fold (6-64). Thus the complement system did not seem to be involved in exhaustion or killing of the TNF-α producing cell in the presence of LPS and infliximab.

Table 5. Results of TNF-α mRNA and protein after short-term incubation

		Control	LPS 1.5h	Infliximab 4h LPS 1.5h	Infliximab 4h wash-LPS (in neat plasma) 1.5h
Patients	mRNA	1	5 (1-73)	6 (1-8)	3 (0.2-13)
(n=7)	Protein	60 (8-2852)	2194 (210-8340)	215 (82-430)	902 (80-2333)
Volunteers	mRNA	1	13 (10-156)	50 (16-230)	36 (15-190)
(n=4)	Protein	30 (11-92)	4187 (1684-6347)	209 (140-261)	4793 (1608-6593)

TNF-α protein was measured with the Biosource kit and values expressed in pg/ml (median, range).

Discussion

Infliximab has been approved for the treatment of patients with active CD that are resistant to conventional treatment and for CD patients with fistulas. About two thirds of patients with active, refractory CD were clinically responsive to a single intravenous infusion of infliximab [13;14]. The precise mechanisms of infliximab to modify the immuno-inflammatory process are being investigated. In a study on the clinical and immunological mechanisms of response and failure to treatment with infliximab, Nikolaus et al. [19] observed that the antibody was able to down-regulate the LPS-induced secretion of TNF-α by peripheral blood cells. However, when the authors ascribed their findings to the antibody-induced death of TNF-α-producing cells they did not realize that the ELISA kit they used (Quantikine, R&D system, the same as in the present study) shows a major interference by infliximab.

Our results showed that the determination of TNF-α by both the Quantikine and BioSource assay kits was strongly interfered by infliximab. The reason for this interference in the determination of TNF-α by immunosorbent assays might be that the binding-sites of infliximab and the antibodies of the immunoassay kits are at the same epitope(s) of TNF-α, or do partially overlap, so that the binding of TNF-α to infliximab completely or partly blocked the binding to the antibodies in the assays. In comparison to the Quantikine kit, we also

obtained relatively higher TNF-α levels in the same plasma samples with the BioSource kit and the inhibition by infliximab in the latter assay was not complete. These results may be related to the fact that the BioSource assay uses a blend of 4 monoclonal antibodies to capture TNF-α whereas the Quantikine kit has only one capture monoclonal antibody.

In the present study, the level of TNF-α in LPS-stimulated blood samples from CD patients was generally lower than that in the samples of the volunteers. This observation may be related to elevated soluble TNF-receptors and other TNF-α binding-proteins which have been found in the serum of CD patients, that might inhibit TNF-α measurements with immunosorbent assays and partially contribute to the difference in TNF-α levels between disease and normal conditions [22]. Another relevant factor in this study might be that the CD patients also received immunomodifying treatment, such as previous infusions of infliximab, steroids or azathioprine. These drugs may have caused repression of TNF-α synthesis in whole blood in response to the stimulation with LPS [6].

LPS stimulation increases TNF-α mRNA production up to several dozens-fold and simultaneously the translation of TNF-α protein is accelerated. The total efficacy of TNF-α can be increased up to several thousands-fold [23]. In our study, blood samples from both CD patients and healthy volunteers after 4 hours incubation with and subsequent removal of infliximab were capable of expressing normal TNF-α mRNA and protein levels in response to LPS stimulation. In fact, the mRNA level in the volunteers was even higher compared with the sample without infliximab incubation. These results demonstrated that after exposure to infliximab TNF-α-producing cells are still capable to express TNF-α at both the mRNA and protein level.

Besides the neutralization of the sTNF-α and mTNF-α, proposed mechanisms of action of infliximab are lyses of TNF-α-producing cells via binding transmembrane TNF-α, complement fixation and antibody dependent cellular cytotoxicity (ADCC) [16;18;24]. The blood samples that were simultaneously incubated with LPS and infliximab for 4 hours, and then restimulated with LPS in neat plasma showed that, independent from (in)activation of the complement system, the increment of TNF-α mRNA transcription was severely inhibited and TNF-α protein production level was very low. Although in this study we did not examine apoptosis of the TNF-α-producing cells these relative low mRNA and trace protein levels may be ascribe to two conditions: 1) the cells were exhausted to express both TNF-α mRNA and protein after 4 hours LPS stimulation, or 2) the activated TNF-α-producing cells were induced to undergo apoptosis by infliximab. LPS may have activated the TNF-α-producing cells to express more mTNF-α, which is known to be able to act as a receptor for infliximab, resulting in an increase in the Bax/Bcl-2 ratio in T cells or activation of caspases in monocytes and thereby inducing apoptosis [16;18]. Our results are also in agreement with recent studies [25;26] demonstrating that the complement system and ADCC do not seem to participate in the lysis of TNF-α-producing cells in the treatment of CD, although the role of the complement system in the mechanism of infliximab efficacy in IBD is still controversial [15;27].

In conclusion, the present study demonstrates that the determination of TNF-α with immunosorbent assays is severely interfered by infliximab and that the basis of anti-inflammatory and immunodulatory efficacy of infliximab in CD is not by directly affecting the TNF-α producing cells, unless a stimulatory agent like LPS is simultaneously present.

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