Modification of neutrophil function by plasma of rheumatoid arthritis patients treated with infliximab

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Abstract

Objective
To examine whether the release of superoxide anions from neutrophils of healthy donors was affected when incubated with plasma from infliximab-treated rheumatoid arthritis (RA) patients.

Methods
Fifteen consecutive seropositive RA patients were treated with 3mg/kg infliximab on weeks 0, 2, 6, and 14. Disease activity was assessed by DAS28 score and by IL-6 level. Neutrophils from healthy donors were incubated with plasma drawn before each infliximab treatment. PMA-stimulated superoxide release was measured by the ferricytochrome C reduction method.

Results
53% of the patients had a favorable clinical response. IL-6 levels showed a significant decline at week two, with a gradual increase thereafter. Treatment with infliximab did not change the superoxide production. However, when the group was divided retrospectively to responders ($\Delta$DAS28 > -1.2) and non-responders ($\Delta$DAS28 < -1.2), two different patterns were seen, although the pre-treatment levels were similar: Among the responders IL-6 remained low at its 2 weeks level till week 14, while in the non responders IL-6 increased 3 times ($P < 0.03$) from week 2 to 14. The responders showed mild, but continuous, reduction of superoxide release, while in the non-responders it increased significantly from week 2 on.

Conclusions
The reduction in IL-6 in RA sera following anti-TNF$\alpha$ therapy has little influence on the capacity of these sera to stimulate healthy neutrophils to produce superoxide, suggesting the existence of non-TNF$\alpha$ non-IL-6 dependent neutrophil-stimulating mediators in RA sera. The increasing level of IL-6 among the non-responders after initial dramatic decline might represent an escape phenomenon, possibly caused by alternative mediator(s). Clinically, this IL-6 "escape" might be used as a tool for early identification of responders from non-responders.

Key words
Anti-tumor necrosis factor-$\alpha$, neutrophils, rheumatoid arthritis, infliximab, superoxide anion production.

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Introduction

In rheumatoid arthritis (RA) the chronic inflammation of the synovial membrane eventually leads to the destruction of cartilage and the surrounding bone. The key cytokine is tumor necrosis factor-alpha (TNF-α), which was found in high concentrations in the serum and the synovial fluid of these patients (1), and its level has been found to have a strong relationship to the extent of the synovitis and to the erosion in joints (2). TNF-α promotes inflammation in a great variety of ways (3). It induces the production of other inflammatory cytokines such as IL-1, IL-6 and GM-CSF; it recruits inflammatory cells into the joints by induction of the expression of adhesion molecules in the synovial endothelium and by enhancement of the chemokine production; it also increases the formation of new blood vessels by inducing vascular endothelial growth factor, and by this it promotes the entrance of the inflammatory cells in large amounts to the joints. TNF-α increases reactive oxygen species (ROS) production from neutrophils (4, 5), which contribute to the destruction process. In addition, it stimulates chondrocytes and synovial fibroblasts to secrete matrix metalloproteinases; and it amplifies bone resorption by promoting osteoclast differentiation.

It is not surprising, therefore, that anti-TNF-α drug therapy shows a significant improvement in the clinical, laboratory and radiographic parameters of RA (6-7). The investigations of the mechanisms of action show that the drugs influence most of the above-mentioned pathways (8).

The effect of anti-TNF-α drugs on the activation of the inflammatory cells in RA was not yet clearly elucidated. The dominant cells in the RA synovial fluid are the neutrophils (9). In the active state they release various inflammatory factors, including proteolytic enzymes, arachidonic acid metabolites and ROS. Neutrophils in RA serum are in active state not only within the joints but in the serum as well, producing increased amounts of ROS (10). This activation is stimulated by TNF-α amongst other factors.

Several works have shown that ROS are responsible for many destructive processes, which cause damage in tissues with neutrophil accumulation (11). Free radicals are capable of destroying hyaluronic acid (12), collagen (13) and cartilage (14) and are also potentially chemotactic for neutrophils (15). Another important feature is their ability to change the synovial fluid in a way that can lead to IgG aggregation (16), which in turn is able to activate neutrophils to produce ROS, thus creating a vicious cycle within the joint. It was suggested that these IgG aggregates are the antigens for Rheumatoid Factor, and thus ROS have a double role in keeping the active inflammatory process, leading to its chronicity and destructive characteristics.

The removal of ROS from RA synovial fluid is impaired too. The inactivation of ROS takes place by the glutathione system. Glutathione reductase is one of the key enzymes in this process, and its activity is only moderately increased in RA synovial fluid. This limited increase in glutathione reductase activity is probably inadequate to neutralize the excessive production of ROS (17). The classic disease modifying anti-rheumatic drugs (DMARDs), like gold and sulphasalazine, were shown to decrease free radical production from neutrophils (18-23). It is thus surprising, that den Broeder et al. (24) could not demonstrate a significant decrease in superoxide anion release following Adalimumab (fully human IgG1 anti-TNF-α antibody) in RA.

The goal of the current work was to investigate the effect of plasma from infliximab (a chimeric monoclonal anti-TNF-α antibody) treated RA patients on release of superoxide from neutrophils from healthy donors. The rationale behind choosing healthy donor neutrophils lies in the fact that the isolation of neutrophils from peripheral blood might affect their capability to release superoxide in vitro. This effect is prevented when neutrophils isolated from healthy volunteers are incubated in parallel with blood from RA patients at various stages of infliximab treatment. The results are further normalized when expressed against super-
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oxide production when neutrophils are incubated with buffer.

Materials and methods

Study design

Sixteen patients with seropositive RA eligible to TNF-α therapy were included in the study. All had active disease, defined as a DAS28 > 3.8 (see later), and failed therapy with at least three DMARDs. Methotrexate therapy was continued in all patients in constant dose. Nonsteroidal anti inflammatory drugs (NSAID) and steroids, up to 10 mg daily, were kept stable during the study. Infliximab infusions (3 mg/kg) were given according to the usual schedule at weeks 0, 2, 6 and 14. All patients signed an informed consent form, and the trial was approved by the local Helsinki Committee (1663).

Clinical and laboratory evaluation

The assessment of RA activity was made before the first treatment (week 0) and at week 14, and is expressed by the disease activity score DAS28 (25):

\[
\text{DAS28} = 0.56 \times \text{tender joints} + 0.28 \times \text{swollen joints} + 0.7 \times \text{ln ESR} + 0.014 \times \text{visual analogue scale (VAS)}
\]

According to the score, DAS28 over 5.1 means a high disease activity, over 3.8 – an active disease, and a change of more than 1.2 is considered to be of clinical significance.

A sample of 10 ml venous blood was drawn before each infliximab infusion, i.e. at the minimal concentration of the drug, and the plasma was separated within 20 minutes. The separated plasma was stored at -70°C until the assays were carried out.

TNF-α and IL-6 were measured by Elisa, R&D systems (Minneapolis, Minnesota). Due to technical limitations (existence of anti-TNF-α antibody) TNF-α levels were measured at time 0 only, before the first anti-TNF-α treatment.

Measurement of superoxide release from neutrophils

The separation of neutrophils from the peripheral blood of healthy donors was carried out according to the method of Szucs (26). Ten ml peripheral blood of healthy donors was centrifuged for 30 minutes at 700xg at room temperature (18-26°C) on Histopaque gradient 1119/1077. After the separation the cells were washed twice with 10ml phosphate buffered saline. The viability of the cells was determined by 0.1% trypan blue.

The PMA (phorbol myristate acetate) activated superoxide production by neutrophils was carried out according to the method of Pick and MIZEL (27). The evaluation was made by an Elisa reading device. Every well included 10 µl of the neutrophils suspension (12.5 x 10⁶ cells to ml), 15µl SOD (300 units to ml) or 15 µl of Hank’s balanced salt solution (HBSS), 10µl of cytochrome C (800 µM) and 15 µl PMA (10 mg/ml). The incubation was made with 10µl plasma or 10µl of HBSS buffer (Bet Haemek, Israel) simultaneously in 37°C for 30 minutes. All the substances were purchased from Sigma.

The analysis of data was carried out with normalized results, i.e. superoxide production by neutrophils incubated with plasma divided by superoxide production by neutrophils of the same donor incubated with HBSS.

Statistics

Medians and interquartile ranges were calculated. Friedman test was used to compare data at different time points, followed by Wilcoxon signed rank test if significant. Differences between groups at the same time points were analyzed using the Mann-Whitney test. Spearman rank order correlation test was used to evaluate the correlation between variables. The results were considered statistically significant with p ≤ 0.05.

Results

Patients and clinical course

Out of the recruited 16 patients one dropped out by week 6 due to severe side effect; his results were not included in the study. The baseline characteristics of the 15 patients who completed the study are given in Table I.

The clinical evaluation on week 14, 8 weeks after the third infliximab infusion, revealed a significant decrease in the average clinical parameters compared to the baseline: tender joints – 11 (5–14) with p < 0.012; swollen joints – 10 (4–13.5) with p < 0.01; ESR – 39 (21–40) with p < 0.03; and VAS – 5 (4–5.5) with p < 0.01. The decline in DAS was 1.22 (0.39–1.45).

IL-6 and superoxide levels (Fig. 1)

IL-6 was detectable in all but one of the sera tested. The overall results were found statistically significant by the Friedman test (p < 0.01). IL-6 levels at week 2 (2.2 (1.58–5.6) pg/ml) showed a significant reduction from baseline (12.3 (7.93–31.78) pg/ml, p < 0.0005), with a mild elevation four weeks later on week 6 (2.72 (1.6–5.6) pg/ml, p < 0.0005), and a relative stabilization thereafter (week 14 - 3.35 (1.83–11.83) pg/ml, p < 0.01).

At week 0, average superoxide release from neutrophils incubated in plasma from RA patients was 64.5 (56.9–68.75). Patients with a very high disease activity at baseline (DAS28 > 5.1) had a significantly higher median superoxide level compared to those with active disease (DAS28 < 5.1), 65.3 (62.5–76.45) vs. 39.6 (37.83–46.65) respectively, with p < 0.02. These two groups showed no significant differences in other

| Table I. Baseline characteristics of the patients (n = 15, medians and interquartile ranges) prior to infliximab therapy. |
|-------------|--------|--------|
| Age (yr)    | 57 (48.5–64.5) |
| F : M       | 10 : 5 |
| Duration of disease (yr) | 14 (9–15.5) |
| No. of tender joints (out of 28) | 13 (10.5–20.5) |
| No. of swollen joints (out of 28) | 13 (8–18) |
| Disease activity (VAS 0–10) | 7 (6.5–8) |
| Erythrocyte sedimentation rate (mm/hr) | 60 (25–76) |
| DAS28       | 5.99 (5.15–6.7) |
| TNF-α (pg/ml) | 3.2 (1.96–4.95) |
| IL-6 (pg/ml) | 12.3 (7.93–31.78) |
parameters, and did not differ in their response to anti-TNF-α therapy.

Superoxide level before treatment [64.5 (52.4–68.75)] was not statistically different from that at week 2 [68.1 (51.2–72.7)], at week 6 [64.55 (53.98–72.8)] and at week 14 [59.7 (52.25–69.3)] by the Friedman test. No correlation was found between superoxide levels at each point and the ESR, age or disease duration.

**Responders vs. non-responders**

Patients were divided into two groups based on the change in their DAS28 (ΔDAS28) (Table II): responders (ΔDAS28 > -1.2; 8 patients) and non-responders (ΔDAS28 < -1.2; 7 patients). The two groups were comparable at time 0 in their DAS28 score, TNF-α level, IL-6 level and superoxide level (Table III), but during the treatment course their IL-6 and superoxide levels took different patterns.

The initial IL-6 decline at week 2 was similar in the responders and the non-responders [3.3 (1.48–5.43) pg/ml vs. 2.2 (1.59–5.7) pg/ml, respectively], but then took a different direction (Fig. 2): in responders IL-6 remained stable from week 2 through week 6 [2.4 (1.38 – 4.63)] till week 14 [3.3 (1.7– 6.3)], while more than a three-fold increase from the trough of week 2 occurred in the non-responders [through week 6 – 3.4 (2.16–9.1) to week 14 – 6.6 (2.5–13.3), p < 0.03], although not reaching the pre-therapy level.

Superoxide production levels showed different patterns in the responders and non-responders (Fig. 3). In the former, a mild constant decline was noted [week 2 – 68.1 (53.8–86.55), with p < 0.03, week 6 – 67.4 (55.5-88.95), with p < 0.03, and week 14 – 58.1 (55.1–82), with p < 0.02.

**Discussion**

The guidelines in Israel for anti-TNF-α therapy are substantially the same as in other western countries. Thus all patients in this study failed 3 DMARDs, had active disease and got infliximab in addition to the maximal tolerated dose of Methotrexate.

There are various scales for the evaluation of drug response in RA, such as ACR 20/50/70, EULAR and DAS28. In a large study of more than 200 patients, who were treated with infliximab, these three scales were used (28). DAS28 was found to be more sensitive for identifying responders. The rate of response in our study at week 14 was 53% based on the DAS28 score, and was similar to other studies: 54% and 67% reached ACR20 at week 12 and 14 respectively in two studies (29-30); in another approximately 60% achieved Paulus 20% after 16 weeks of treatment (6).

IL-6 is a proinflammatory multi-functional cytokine, which is involved in both physiological and pathological processes (30). During inflammation IL-6 induces the synthesis of acute phase proteins and promotes the differentiation of osteoclasts from precursor.
Higher levels of IL-6 have been found in the sera of RA patients compared to controls and in synovial fluid compared to serum, reflecting the local production of IL-6 by the synovium (31, 32). IL-6 levels correlate with disease activity in RA (33-34), and improvement of the disease after therapy with DMARDs is accompanied by a reduction in the serum IL-6 levels (34).

In our study we found that following the first infliximab infusion IL-6 decreased dramatically by week 2 to 1/6 of its pretreatment value in all patients. In the responders this low level was maintained till week 14. However, an "escape phenomenon" occurred among the non-responders, which was manifested as early as week 6. The level of IL-6 at week 14 was 3 times higher than that of responders. This "escape phenomenon" has not been addressed yet. Similar to other studies, we also could not identify responders from non-responders on the basis of their pretreatment data. Hence an early objective test, which could predict the outcome of anti-TNF-α therapy, is highly needed.

Superoxide production by neutrophils incubated with plasma of RA patients had a significant correlation with the severity of the clinical symptoms judging by DAS28. The relationship between superoxide level and the severity of RA is supported by the suppression of superoxide production with the clinical improvement following therapy with classical DMARDs.

A possible candidate for such pathway is lymphotoxin-α (LTA). TNF-α and LTA are related proinflammatory cytokines produced in response to various stimulations (35-36) and are known to induce ROS (37). Blockage of LTA/LIGHT (a receptor expressed on T-lymphocytes (38)) had a beneficial effect on murine collagen induced arthritis (39). The favorable response to Etanercept following infliximab failure was attributed to the presence of LTA in the inflamed synovium, as Etanercept but not infliximab blocks LTA in addition to TNF-α (40).

In recent years it became clear that RA is an independent risk for atherosclerosis (41), and that increased vascular wall thickness correlates with the severity of RA (42). Of the traditional cardiovascular disease (CVD) risk factors, only smoking increased CVD risk prior to the onset of inflammatory polyarthritis. Therefore an increased prevalence of CVD observed in these patients is likely to be a consequence of...
factors operating after the onset of the arthritis (43). There is growing evidence that chronic (and acute) overproduction of ROS is integral in the development of CVD. ROS release from various processes, including activation of macrophages, mediates different signaling pathways that underlie vascular inflammation in atherogenesis. Moreover, oxidative stress is a unifying mechanism for many CVD risk factors. As a result of oxidation of LDL to OXLDL, a variety of neoepitopes are formed, that are being recognized by macrophage scavenger receptors. The enhanced uptake of OXLDL by macrophages leads to foam cell formation and subsequently to atheromatous plaque.

If our observation of the lack of significant decrease of ROS release among responders will be verified in larger group of patients and for longer period, it might indicate the necessity for additional drug intervention to minimize the damage of chronic oxidative stress. In summary, the reduction in IL-6 in RA sera following anti-TNF-α therapy has little influence on the capacity of these sera to stimulate healthy neutrophils or to produce superoxide, suggesting the existence of non-TNF-α-onc-IL-6 dependent neutrophil-stimulating mediators in RA sera. The increasing level of IL-6 among the non-responders after initial dramatic decline might represent an escape phenomenon, possibly caused by alternative mediator(s). Clinically, this IL-6 “escape” can explain some of the failures and might be used as a tool for early identification of responders from non-responders.

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