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The Role of Anti-TNFα Therapy in the Amelioration of Disease Burden in Patients with Refractory Rheumatoid Arthritis

Madha Mohammed Sheet*, Laith Abdul-Elah Kamel, Jamal Nasser Farhood *****
**Dept. Medical Labs., College of Health and Medical Technology, Baghdad*
*** Al-Karama Hospital, Baghdad*
****Dept. Medical Labs, College of Health and Medical Technology, Baghdad*
E-mail: madhataiz2004@yahoo.com.

Abstract:

To evaluate the changes in anti cyclic citrullinated peptide (Anti-CCP) antibodies, rheumatoid factor (RF), interleukins 17 and 10 (IL-17 and IL-10), high sensitivity C- reactive protein (hs CRP) and T regulatory cells (Treg) count following an anti TNF-α biological agent (etanercept) therapy in patients with rheumatoid arthritis.

Refractory rheumatoid arthritis patients who failed treatment with DMARDs (disease modifying anti rheumatic drugs) were treated with etanercept for three months. Serum and blood samples were tested before and after therapy for six markers including ACCP, RF, IL- 10, IL 17, hs CRP, and Treg cell. A significant dropping down in the serum level of ACCP, RF and hs CRP was documented 3 months after etanercept therapy. On the other hand a non- significant elevation in IL-10 serum level and peripheral blood Treg cell count, as well as a non significant dropping down in the serum level of IL-17 were also reported. Etanercept is a successful anti-TNF-α therapy in refractory RA patients for the first three months of the course therapy as indicated by clinical improvement and amelioration in 6 disease activity markers.

Key words: *rheumatoid arthritis, IL-17, etanercept, Treg, TNF-α*

الخلاصة:

تقييم التغيرات في اضداد البيتيد الحلقي، العامل الرثوي، انترلوكين 10 و17، البروتين التفاعلي عالي التحسس نوع س والخلايا اللمفاوية التنظيمية بعد العلاج باستخدام العلاج بمضادات العامل المنخر السرطان نوع الفا (ايتانارسبت) في مرضى التهاب المفاصل الرثواني.

تم علاج مجموعة من مرضى التهاب المفاصل الرثواني المستعصين على العلاج التقليدي وذلك باستخدام ايتانارسبت لمدة ثلاثة اشهر. تم تقييم عينات دم ومصل الدم لهم قبل وبعد اعطاء العلاج لستة مؤشرات مرضية شملت اضداد البيتيد الحلقي، العامل الرثوي، انترلوكين 10 و17، سي ريكتف بروتين العالي التحسس، والخلايا اللمفاوية التنظيمية. بعد ثلاثة اشهر من العلاج بالاينارسبت تم توثيق انخفاض معنوي كبير لاضداد البيتيد الحلقي, العامل الرثوي ,و سي ريكتف بروتين العالي التحسس وارتفاع غير معنوي لمستوى انترلوكين 10 والخلايا اللمفاوية التنظيمية وانخفاض غير معنوي لمستوى انترلوكين 17. عقارالايتانرسبت كمضاد للعامل المنخر للسرطان كان ناجحا لدى استخدامه لمدة 3 اشهر في مرضى التهاب المفاصل الرثواني المستعصي بدلالة التحسن السريري والتحسن في مستويات ستة من العلامات النشاطية للمرض.

Introduction:

Rheumatoid arthritis is a systemic autoimmune disease characterized by chronic joint inflammation that eventually results in bone destruction and severe disability. Disease modifying anti rheumatic drugs (DMARDs) may retard disease progression [1]. However, not all patients with rheumatoid arthritis can tolerate or respond to the traditional DMARDs [2]. In recent years, advances in molecular technology have contributed to developing a direct specific treatment that antagonize certain pro-inflammatory cytokines, such as tumor necrotic factor α and interleukin 1. Etanercept, a soluble anti TNF-α receptor fusion protein can bind and neutralize extracellular TNFα. It has been shown to

have marked clinical efficacy with minimal toxicity in rheumatoid arthritis patients who have an inadequate response to conventional DMARDs [3].

Rheumatoid factor (RF) is a partially specific serological marker for evaluating RA disease severity [4], whereas anti cyclic citrullinated peptide antibody (anti-CCP) is another highly specific and sensitive disease severity marker that is useful in the preclinical and early diagnosis of RA[5,6]. The role of Rheumatoid factor (RF) and anti cyclic citrullinated peptide antibody (anti-CCP) in the pathogenesis of RA is still poorly defined; however their titer levels may reflect the degree of responsiveness to certain therapies [7,8]. On the other hand, certain interleukins as IL-17 which is secreted by Th17 has a clearer role in the pathogenesis of RA. Its participation in tissue inflammation and destruction by inducing the expression of pro-inflammatory cytokines and matrix metalloproteinase may contribute to synovitis and bone destruction associated with RA [9]. Using of TNF-α inhibitors as etanercept and adalimumab for the treatment of patients with refractory RA has variable degrees of success. In recent literatures, the effect of these TNF-α inhibitors on the Th17and IL-17 in human is found to be with paradoxical results. One study found that TNF-α inhibitors, adalimumab, had reduced the frequency of circulating Th17 cells and their related cytokines including IL-6 in RA patients [10], whereas in another study it had been shown that an increased frequency of circulating Th17 cells after TNF-α blockers is accompanied by a decrease in Th17 specific chemokine receptors expression in RA [11]. Other interleukins as IL-10 which is a marker for T regulatory cell activity can play an important regulatory functions. It has been implicated to have a suppressive role for many inflammatory cytokines such as TNF-α, IL-6 and IL-1 by activated macrophages [12]. Many community-based and referral-population studies found that RA patients have a 1.5 to 3 fold increased risk for cardiovascular events compared with non RA controls[13,14].

C- reactive protein (CRP) may have a predictive capacity of future CV disease and may offer a prognostic advantage over measurement of lipid alone [15]. High sensitivity CRP (hs CRP) is one of the sensitive markers for evaluating the CRP level in serum. Measuring hs CRP in RA patients under certain therapeutic program can evaluate the inflammatory process of the disease which is highly associated with CV disease risk in those patients [16]. Few studies had evaluated the impact of TNF-α inhibitors on CV risk factors including hs CRP in RA patients. Finally, in RA, TNF-α may contribute to the failure of Treg in suppressing the proliferation of effecter cells by decreasing Foxp3 mRNA expression and this might be reversed by treatment with anti- TNF-α agents[17]. As the relationship between Treg and Th17 cells has become increasingly complex, knowledge of the relationship between these two subsets of T cells in the process of RA treatment will provide new insights into the pathogenesis of RA and the mechanisms of therapy–response with TNF-α inhibitors [18]. The aim of this study is to evaluate the effect of etanercept therapy in refractory RA patients on certain disease activity markers including anti-CCP, IL-17 , IL-10, hs CRP serum level and Treg cells ($CD4^+CD25^+$) counting level.

Materials and Methods:

Eighteen consecutive patient's ful-filling the American College of Rheuma-tology (ACR) classification criteria for RA[19] were included in the study. They introduced in three months course combined therapy with Etanercept plus Methotrexate (MTX) from July to November 2012 under the supervision of the rheumatologists in the

department of rheumatology/Baghdad hospital (Medical City). A limited number of patients (8 out of 18) had completed 12 weeks of therapy, whereas the other 10 patients were pulled out because of their unwilling to carry on. Any patient with infectious disease, active or latent tuberculosis, neoplastic disease, heart failure, cytopenia or a demyelinating disorder was excluded from the study. All candidates had failed previous therapy treatment with MTX and other DMARDs (hydroxychloroquine, azathioprine, cyclo - sporine, sulfasalzine, or lefunomide). Etanercept (at a dose of 25 mg) was administered subcutaneously twice weekly for three months. Serum and whole blood samples were collected twice from all patients; the first sample was just before starting the therapy (baseline) whereas the second sample was three months after starting the therapy. Sera were stored at –20°C until analyzed whereas blood was tested within 6 hours after collection. Twenty samples of serum and blood were also collected from healthy individuals and used as a control group.

Flow cytometry (Partec, Germany), ELISA reader (Biotech ELX 800, USA), and ELISA washer (Biotech, ELX 50, USA) were employed for sample analysis. The following kits were purchased and used in this study; Rheumatoid factor screen kit from Immuchem (Belgium), Human IL-10 kit from Cusabio (China), Human IL-10 kit from Cusabio (China), Anti-CCP antibodies kit from Human (Germany), hs-CRP ELISA kit from Demeditec diagnostics (Germany), and mouse anti-human $CD4^{+}CD25^{+}$ from US Biologica (USA).

Serum levels of IL-17, IL-10, hs CRP, ACPA were determined in 18 RA patients at baseline and three months after etanercept therapy, and in 20 healthy control group using ELISA according to the manufacturer's instructions. Total counts of circulating Treg cells in RA patients before and after anti TNF-α therapy and in healthy control were quantified using flow cytometry analysis. In order to detect circulating Treg cells, fluorescence isothiocyanate conjugated anti-CD4, and phycoerythrin conjugated anti-CD25 were quantified using flow cytometry according to the manufacturer protocol. Lymphocytes were gated on the basis of forward and side scatter properties and at least 10000 cells were analyzed using FCS 4 express flow cytometry software (USA).

Statistical analysis:

Statistical analysis was carried out using the SPSS 10 statistical package. The non-parametric tests were used to assess the statistical significance of difference between groups, like Mann-Whitney test for the difference between two groups and Kruskal-Wallis test for the difference between more than two groups, in view of the non normal distribution of the results. Differences were considered statistically significant at $P<0.05$.

Results:

The changes in the tested indices before and after etanercept treatment in the two groups (all patients and control) are summarized in table (1) and figure (1), whereas a comparison in these indices within the therapy responders only before and after therapy is summarized in table (2). For Anti-CCP, a significant decreased level ($P= 0.000$) in all patients of the study from 1087 U/ml before therapy to 19.1 U/ml after therapy in comparison with healthy control group (3 U/ml). Rheumatoid factor also decreased significantly from 378.9 U/ml before therapy to 229.1 U/ml after therapy in comparison with 14.6 U/ml in healthy control group ($P=0.000$).

The trend of IL-17 value between patients group (before and after therapy) and control was The cardiovascular risk marker hs CRP had also fallen significantly (P=0.028) from base line value of 11.8 µg/ml to 10.9 µg/ml after therapy in comparison with 3.5 µg/ml in healthy similar to that of hs CRP as shown in table (1) but with no statistical significant value. For IL-10, there was an elevation in the serum value when this value was compared between the baseline and after therapy in patients' responder group (from 11.6 pg/ml to32.8 pg/ml). However, this elevation was statistically non significant (p=0.18), and for IL-10 between baseline and after therapy in patients groups compared to their correspondent healthy control (P=0.228). These results indicate a partial amelioration in Treg function after therapy, despite that, Treg numbers didn't change significantly after therapy from base line value of 18.9% to 20.6% after therapy in comparison with 17.8% in healthy control(P=0.743).

Table- 1: Changes in the tested indices before and after etanercept therapy.

Study parameters	Before therapy		After therapy		P value*	Healthy control		P value**	P value***
	Mean	SD	Mean	SD		Mean	SD		
Anti-CCP U/ml	1087	1428.3	19.1	5.2	0.002	3	2.2	0.000	0.000
RF U/ml	378.9	414.7	229.1	474.7	0.461	14.6	32.2	0.000	0.000
IL-17 pg/ml	26.3	24.3	11.8	12.7	0.09	10.8	9.7	0.897	0.08
IL-10 pg/ml	26.6	39.2	17.3	15.6	0.615	7.9	7.8	0.364	0.228
hs CRP µg/ml	11.8	12.9	10.9	14.1	0.935	3.5	10.9	0.021	0.028
Treg %	18.9	8.4	20.6	5.5	0.612	17.8	9.9	0.375	0.743

*** P value for the difference between RA patients before therapy and after therapy**
**** P value for the difference between RA patients after therapy and control**
*** **P value for the difference between all groups of study**

Table -2: Changes in the tested indices before and after etanercept therapy in etanercept therapy responders group only.

Disease activity markers	Responder before therapy		Responder after therapy		P value
	Mean	**SD**	**Mean**	**SD**	
Anti-CCP U/ml	945.6	1369.3	19.1	5.2	0.018
RF U/ml	286.5	322.6	76.5	102.4	0.043
IL-17 pg/ml	30.1	14.1	10.5	14.1	0.003
IL-10 pg/ml	11.6	14	32.8	12.8	0.18
hs CRP µg/ml	6.5	9.1	0.2	0.2	0.18
Treg %	12.3%	11.1	21.9%	6.8	0.083

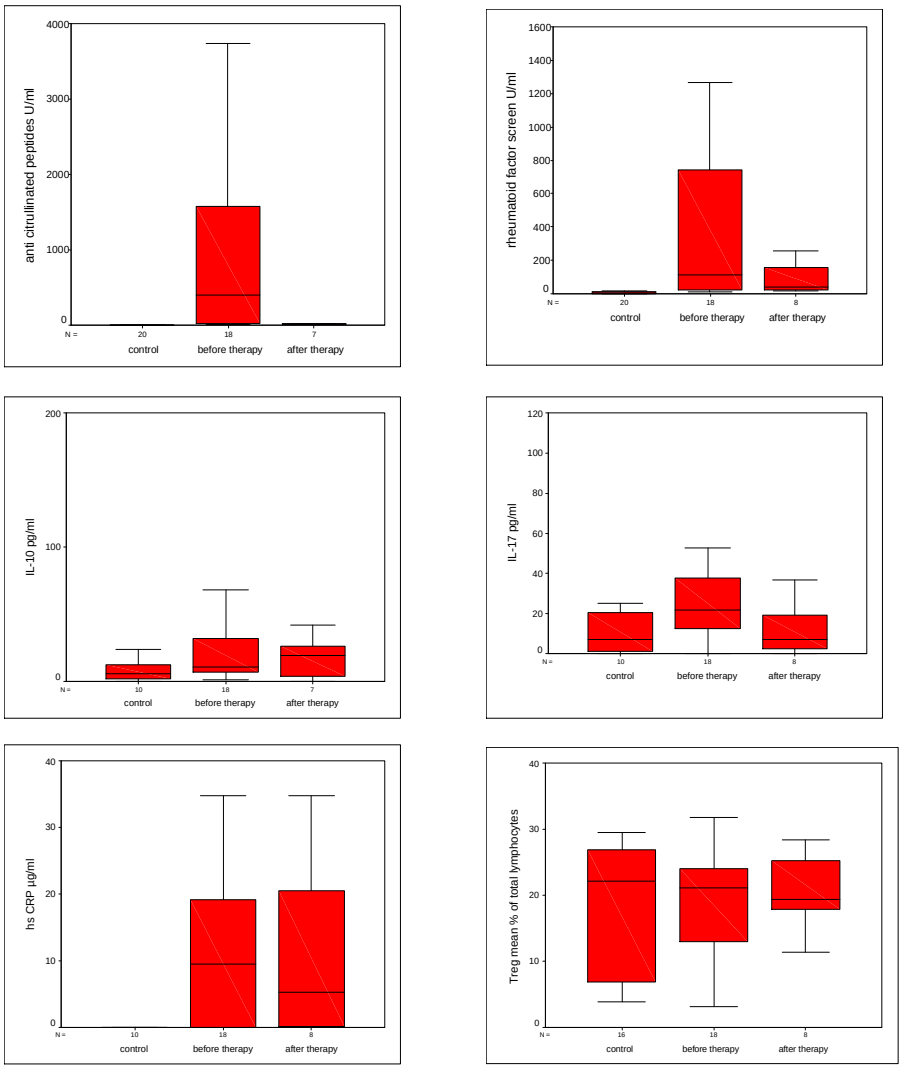

Figure-1: the effect of TNFα therapy on selected RA parameters (Anti-CCP, IL-17, hs CRP, and Treg) as compared with control group using SPSS10 version statistical program

Discussion:

This three months trial provides evidence for an obvious reduction in the RA disease activity and antibody production in response to TNF-α blocker (etanercept) combined with DMARDs. Using TNF-α blockers has dramatically changed the treatment of RA in recent years. The results of this study concerning the suppressive changes in ACCP and RF antibody titers were in a high consistent with many other studies that employed different types of TNF-α blockers as etanercept, infliximab, adalimumab and others[7,21]. whereas, other studies [20, 21] there was a paradoxical effect of TNF-α blockers therapy on the ACCP and RF titers of RA patients ranging from inhibitory to stimulatory effect. This discrepancy could be due to the fact that ACCP and RF antibodies are of different autoantibody system with different clonal origin of their B cell precursors [23].

The amino acid sequence of the presented peptides for cyclic citrullinated

peptide as well as for other autoantigens is under the influence of the genetic constitution of HLA class I and II system of the individual and this might result in variable levels in the degree of inhibitory effects of biological therapeutic agent used for the treatment of RA (and other autoimmune diseases) including TNF-α blockers. We have not to forget that ACCP and RF, however, may provide different but complementary information on RA [23].

In addition, the inconsistency in the literatures concerning the effect of TNF-α blockers on the autoantibodies of RA patients could be referred to the variation in the type and mode of action of the TNF-α blockers used in different studies.

Investigating of the IL-17 levels among RA patients under TNF-α blockers therapy (etanercept) to the best of our knowledge is the first trial in Iraq. The results of this study reveals that IL-17 level had dropped down 3 months after therapy with etanercept in RA patients, but this was statistically non significant result. In a recent study, it had been found that the beneficial effect of anti-TNF-α therapy might involve a decrease in Th17-related cytokines in responders, whereas rising levels of circulating Th17 cells and IL-17 were observed in patients with an inadequate response to anti- TNF-α [24]. This result was in partial coordination with the results of the current study as both TNF-α blockers responders and non responders (as evaluated by clinical improvement of RA symptoms) had expressed a decreasing trend (though statistically non-significant) after treatment for three months with etanercept.

On the other hand, one study [25] had provided evidence that blockade of TNF-α could exacerbate murine psoriasis like disease by enhancing Th17 function and decreasing the clonal expansion of Treg cells. However, the result of the current study is a full agreement with another recent study [26] which found that IL-17 had fallen to the baseline value without a statistically significant difference.

Although the etiology of RA remains unknown, it has been reported that a functional imbalance between a pro inflammatory cytokines and Treg is a key mechanism that underlies joint inflammation and disease progression in RA [27]. Treg cells have an immune-regulatory function through their effecter cytokines mainly IL-10 which is a pleotropic cytokine [28]. The immune-regulatory mechanism of IL-10 include the suppression of certain inflammatory mediators as TNF-α, IL-6, and IL-1 produced by activated macrophages[12]. In this study, IL-10 had an increased level (although non-significantly) after etanercept-3 months course therapy compared with the baseline level. This result was also applicable for the Treg cell count in RA patients after etanercept therapy, which indicates partial restoration in the count and activity of Treg by the effect of etanercept therapy in RA patients. In one study[29] , it had been found that IL-10 signaling in T cells may play a critical role in the pathogenesis of collagen induced arthritis (as an animal model of RA) by affecting the function of Treg through the stabilizing the expression of Foxp3, thus a more severe arthritis can develop in the absence of IL-10 signaling. In other study [30], it was documented that IL-10 may suppress IL-17 expression in RA, and hence, using of IL-10 as therapeutic agent may be useful in the treatment of many autoimmune diseases.

Highly sensitive CRP (hs CRP) had been validated as marker for CV risk in RA , as patients with RA have worse ”background atherosclerosis” than even subjects matched for classical CV risk factors. Therapeutic agents with suppressive effect on the hs CRP enable adequate approach in prevention of and treatment for CV diseases in RA patients [31]. In the current study, a significant

dropping down of the hs CRP serum level have been shown among RA patients with three months course etanercept therapy in comparison with the healthy control group. These results are in consistent with some other recent studies [32, 33].

In conclusion, treatment of refractory cases of RA patients with TNF-α blocker (etanercept) in combination with DMARDs ameliorate many disease activity serological markers including ACCP, RF, IL-10,IL-17 and hs CRP as well as Treg number as disease activity cellular marker.

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