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Serum Levels Of Interleukin–12 And Tumour Necrosis Factor–α In Patients With Active And Inactive Rheumatoid Arthritis

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Abstract :

Seventeen patients with active rheumatoid arthritis (RA) (group I) and fifteen patients with inactive RA (group II), in addition to ten healthy control subjects were included in the present study. All patients were evaluated clinically and biochemically according to the American College of Rheumatology (ACR) core set measures, and a comparison was set between both groups of patients. Serum levels of interleukin–12 (IL–12) and tumour necrosis factor–α (TNF–α) were measured using enzyme linked immunosorbent assay (ELISA) in all patients and control subjects. It was found that there was no significant difference in age, sex, disease duration, degree of disability or physician's and patient's global assessments between both groups of patients ($P>0.05$), but patients with active RA had significantly higher tender joint score, swollen joint score, visual analogue pain scale, erythrocyte sedimentation rate and C–reactive protein compared to patients with inactive RA ($P<0.05$). Detectable levels of IL–12 in serum were found in 13 out of 17 (76.5%) active RA patients, 6 out of 15 (40%) inactive RA patients and 1 out of 10 (10%) healthy controls. TNF–α was also detected in the serum of 12 out of 17 (70.6%) active RA patients, 7 out of 15 (46.7%) inactive RA patients and 1 out of 10 (10%) healthy controls, with significantly higher detectability and significantly higher mean serum levels of IL–12 and TNF–α in patients with active RA compared to patients with inactive RA and healthy controls ($P<0.05$). However, patients with inactive RA had significantly higher detectability and significantly higher serum levels of IL–12 and TNF–α compared to the healthy controls ($P<0.05$) which may reflect the role of IL–12 and TNF–α in the pathogenesis of RA. Serum levels of IL–12 correlated positively with TNF–α levels in serum in case of active RA patients ($r=0.493$) and inactive RA patients ($r=0.474$). It was concluded that significantly elevated serum levels of IL–12 and TNF–α may be associated with clinical and laboratory markers of activity of RA; and measurement of serum IL–12 and TNF–α levels could be used for assessment of RA activity. IL–12 and TNF–α may play an important role in the pathogenesis and inflammatory activity of RA.

Introduction :

Rheumatoid arthritis (RA) is an autoimmune disease characterized by the proliferation of synovium and the infiltration of chronic inflammatory cells. Cytokines and inflammatory cells are thought to be important in the initiation and perpetuation of RA (Kim et al., 2000).

Cytokines are small proteins produced by immune and non immune cells in response to foreign antigens (Peters, 1996). Their central role include cell to cell communication, inflammatory response amplification and immune response regulation (Peters, 1999).

Interleukin–12 (IL–12) is recognized as a critical cytokine in terms of regulating the balance between T helper 1 (Th_1) and T helper 2 (Th_2) cells, as well as enhancing cytotoxic Tcell–mediated lysis and natural killer cell activity (Trinchieri, 1995). It has

been shown to play a critical role in the regulation of immune responses in various autoimmune disease models, and it has been suggested to play a role in the pathogenesis of Tcell mediated autoimmune diseases (Ehrhardt et al., 1997).

Tumour necrosis factor–α (TNF–α) is another cytokine that has been shown to play a pivotal role in the pathogenesis of autoimmune diseases as RA. Apart from exerting direct pathogenic effects, it induces the production of other proinflammatory molecules that may amplify the inflammatory reaction (Brennan et al., 1992; Van den Berg and Van Lent, 1996).

The aim of the present study was to evaluate the serum levels of IL–12 and TNF–α in patients with active RA and patients with inactive RA compared to healthy control subjects. A correlation between the serum levels of these two cytokines in active and inactive RA patients was also investigated.

Subjects and Methods :

The present study was carried out on 32 RA patients who were attending the outpatient clinics of the Internal Medicine Department at Al–Zahraa University Hospital. They were diagnosed clinically and radiologically, and were subjected to laboratory investigations. Several clinical variables were evaluated in all patients according to the American College of Rheumatology (ACR) core set measures, including tender joint score, swollen joint score, visual analogue pain scale, physician's global assessment of disease activity, patient's global assessment of disease activity, degree of disability, erythrocyte sedimentation rate (ESR) and C–reactive protein (CRP). Rheumatoid factor (RF) was determined in all patients.

RA patients were divided according to rheumatoid activity into two groups : group I which included 17 patients (3 males and 14 females) with RA in the active stage (active RA) and with age range from 26 to 60 years (mean age 42.1 ± 8.3 years), and group II which included 15 patients (4 males and 11 females) with RA in the quiescent stage (inactive RA) and with age range from 25 to 62 years (mean age 40.4 ± 7.4 years).

Ten apparently healthy subjects (group III) (2 males and 8 females) with no rheumatic or rheumatoid disease, and with age range from 20 to 57 years (mean age 39.8 ± 5.1 years) were also included in the present study as a control group.

Blood samples were taken from all patients and control subjects, centrifuged at 1500 r.p.m. for 10 minutes, and serum samples were collected and stored at –20°C until use.

Determination of serum IL–12 and TNF–α levels :

Serum levels of IL–12 and TNF–α were determined by a solid phase sandwich enzyme linked–immunosorbent assay (ELISA) using commercial kits (Human IL–12 p70 ELISA Kit) and (TNF–α ELISA Kit) (Diaclone Research, France). According to the instructions of the manufacturer, standards of known IL–12 or TNF–α concentrations, control samples and tested serum samples were pipetted into the wells of the microtiter plates coated with a monoclonal antibody specific for IL–12 or TNF–α, then 50µl of a biotinylated monoclonal antibody specific for IL–12 or TNF–α were added to all wells and the plates were incubated for 3 hours at room temperature. After washing, 100µl of the enzyme streptavidin peroxidase were added to the wells and a second incubation for 30 minutes at room temperature was performed. After washing to remove all the unbound enzyme, 100µl of the substrate solution (Chromogen TMB substrate solution) were added to all wells and the plates were incubated in the dark for 12–15 minutes at room temperature. The enzyme–substrate reaction was stopped by adding 100µl of the stop solution (1.8 N sulfuric acid solution). The absorbance of each well was read using a spectrophotometer at 450 nm as the primary wavelength and 620 nm as the reference wavelength. The concentration of IL–12 or TNF–α in each serum sample was determined from a corresponding standard curve obtained by

assaying a dilution series of standard IL–12 or TNF–α in the same assays.

Statistical analysis :

Results were analyzed using standard statistical methods. Values were presented as mean ± standard deviation. The chi square test was used to determine the significance difference among the studied groups. The correlation coefficient r was also determined.

Results :

Comparison of personal, clinical and laboratory parameters between both groups of RA patients showed that there was no significant difference in age, sex, disease duration, degree of disability or physician's and patient's global assessments between the two groups of patients (P>0.05), but patients with active RA (group I) had significantly higher tender joint score, swollen joint score, visual analogue pain scale, ESR and CRP compared to patients with inactive RA (group II) (P<0.05). The rheumatoid factor was detected in all RA patients (Table 1).

Table (1) : Comparison of personal, clinical and laboratory parameters between patients with active RA and those with inactive RA.

Parameter	Patients with active RA (group I) (n=17)	Patients with inactive RA (group II) (n=15)	P value
Age in years mean ± SD* (range)	42.1 ± 8.3 (26 – 60)	40.4 ± 7.4 (25 – 62)	P > 0.05 non significant
Sex (male:female)	3 : 14	4 : 11	P > 0.05 non significant
Disease duration in years mean ± SD (range)	11.6 ± 4.8 (4 – 21)	10.2 ± 4.2 (5 – 19)	P > 0.05 non significant
Tender joint score mean ± SD (range)	31.1 ± 6.9 (12 – 64)	12.7 ± 3.8 (0 – 20)	P < 0.05 significant
Swollen joint score mean ± SD (range)	27.6 ± 4.9 (15 – 42)	6.3 ± 2.4 (0 – 13)	P < 0.05 significant
Visual analogue pain scale in mm mean ± SD (range)	12.3 ± 3.5 (5 – 17)	5.1 ± 2.8 (2 – 11)	P < 0.05 significant
Physician's global assessment mean ± SD (range)	3.1 ± 1.4 (1 – 5)	1.9 ± 0.3 (1 – 3)	P > 0.05 non significant
Patient's global assessment mean ± SD (range)	2.4 ± 1.1 (1 – 4)	1.7 ± 0.7 (1 – 3)	P > 0.05 non significant
Degree of disability mean ± SD (range)	2.6 ± 1.2 (1 – 4)	1.5 ± 0.6 (1 – 2)	P > 0.05 non significant
Erythrocyte sedimentation rate (mm/h) mean ± SD (range)	59.2 ± 26.9 (16 – 130)	15.5 ± 6.7 (3 – 61)	P < 0.05 significant
C–reactive protein (mg/l) mean ± SD (range)	30.8 ± 8.7 (14.4 – 83.7)	13.1 ± 4.9 (6.2 – 44.8)	P < 0.05 significant
Rheumatoid factor Number of positive (percentage)	17 (100%)	15 (100%)	

* SD = standard deviation.

Determination of serum levels of IL–12 in the RA patients and the healthy control subjects showed that 13 out of 17 (76.5%) patients with active RA (group I), 6 out of

15 (40%) patients with inactive RA (group II) and 1 out of 10 (10%) healthy control subjects (group III) had detectable levels of IL–12 in serum (>5 pg/ml), with significantly higher detectability (positivity) of IL–12 in active RA patients compared to inactive RA patients and healthy controls (P<0.05). The mean level of serum IL–12 was significantly higher in patients with active RA than in patients with inactive RA and the healthy control (P<0.05). However, patients with inactive RA had significantly higher detectability of IL–12 in serum, and significantly higher serum IL–12 levels compared to the healthy controls (P<0.05) (Table 2).

Table (2) :Serum IL–12 in active RA patients, inactive RA patients and healthy controls.

IL–12	Active RA patients (group I) (n = 17)	Inactive RA patients (group II) (n = 15)	Healthy controls (group III) (n = 10)	P value
Number of positive (percentage)	13 (76.5%)	6 (40%)	1 (10%)	*P < 0.05 significant
Levels of IL–12 (pg/ml) [for patients : mean ± SD (range)]	57.9 ± 11.8 (16.6 – 98.3)	28.6 ± 8.5 (10.7 – 70.4)	7.5	*P < 0.05 significant

* Group I versus group II : P < 0.05
Group I versus group III : P < 0.05
Group II versus group III : P < 0.05

Tumour necrosis factor–α was found in detectable levels (>10 pg/ml) in the sera of 12 out of 17 (70.6%) patients with active RA (group I), 7 out of 15 (46.7%) patients with inactive RA (group II) and 1 out of 10 (10%) healthy control subjects (group III), with significantly higher detectability (positivity) of TNF–α in active RA patients compared to inactive RA patients and healthy controls (P<0.05). The mean level of serum TNF–α was significantly higher in patients with active RA than in patients with inactive RA and the healthy control (P<0.05). However, patients with inactive RA had significantly higher detectability of TNF–α in serum, and significantly higher serum TNF–α levels compared to the healthy controls (P<0.05) (Table 3).

Table (3) :Serum TNF–α in active RA patients, inactive RA patients and healthy controls.

TNF–α	Active RA patients (group I) (n = 17)	Inactive RA patients (group II) (n = 15)	Healthy controls (group III) (n = 10)	P value
Number of positive (percentage)	12 (70.6%)	7 (46.7%)	1 (10%)	*P < 0.05 significant
Levels of TNF–α (pg/ml) [for patients : mean ± SD (range)]	96.1 ± 33.2 (39.7 – 385.1)	75.7 ± 27.4 (29.8 – 216.3)	24.6	*P < 0.05 significant

* Group I versus group II : P < 0.05
Group I versus group III : P < 0.05
Group II versus group III : P < 0.05

Serum levels of IL–12 correlated positively with serum TNF–α levels in patients with active RA (r=0.493) and in patients with inactive RA (r=0.474) (Table 4).

Table (4) :Correlation between serum IL–12 and TNF–α in patients with active and inactive RA.

RA patients	IL–12		TNF–α		r
	+ve (%)	mean ± SD (range)	+ve (%)	mean ± SD (range)	
Active RA patients (group I) (n = 17)	13 (76.5%)	57.9 ± 11.8 (16.6 – 98.3)	12 (70.6%)	96.1 ± 33.2 (39.7 – 385.1)	r=0.493
Inactive RA patients (group II) (n = 15)	6 (40%)	28.6 ± 8.5 (10.7 – 70.4)	7 (46.7%)	75.7 ± 27.4 (29.8 – 216.3)	r=0.474

Discussion :

RA is a chronic inflammatory disease with progressive articular damage often associated with systemic manifestations (Klimiuk *et al*., 2001).

Several clinical variables representing a set of disease activity measures were defined by the American College of Rheumatology (ACR) and were evaluated in several studies (Felson *et al*., 1993; Kim *et al*., 2000).

In the present study, two groups of RA patients [patients with active RA (group I) and patients with inactive RA (group II)] were evaluated according to the ACR core set measures of disease activity and were compared to each other, and it was found that there was no difference in age, sex, disease duration, degree of disability or physician's and patient's global assessments between both groups of patients (P>0.05). However patients with active RA (group I) had significantly higher tender joint score, swollen joint score, visual analogue pain scale, ESR and CRP compared to patients with inactive RA (group II) (P<0.05). These results are in agreement with the results obtained by Kim *et al*. (2000).

The aetiology and pathogenesis of RA are incompletely resolved (Klimiuk *et al*., 2001). Analysis of cytokines in RA has attracted a particular interest, as many cytokines are involved in the regulation of the immune and the inflammatory responses (Steiner et al., 1999).

The role of IL–12 has been addressed in the pathogenesis of RA. The administration of IL–12 enhanced disease expression and severity in an animal model of RA (Leonard et al., 1995). A blockade of IL–12 during the induction of collagen–induced arthritis markedly attenuated the severity of arthritis (Malfait *et al*., 1998). It has been documented that IL–12 is highly expressed by infiltrating macrophages and synovial lining cells in patients with RA (Sakkas *et al*., 1998).

Schlaak *et al*. (1996) found that patients with RA had significantly higher levels of IL–12 p 70, a biologically active form of IL–12, in serum compared with osteoarthritis patients and healthy controls. Similar results were obtained by Kim et al. (2000) who found detectable levels of IL–12 p 70 in the sera of 64 out of 152 (42.1%) RA patients, 1 out of 69 (1.4%) osteoarthritis patients and 5 out of 50 (10%) healthy controls with significantly higher levels of IL–12 in the sera of RA patients compared with osteoarthritis patients and healthy controls. They concluded that IL–12 levels reflect RA disease activity and an IL–12 blockade could be useful for the treatment of RA.

In the present study, IL–12 p 70 was determined in the sera of active and inactive RA patients and healthy controls, and it was found that 13 out of 17 (76.5%) active RA patients, 6 out of 15 (40%) inactive RA patients and 1 out of 10 (10%) healthy controls had detectable levels of IL–12 in serum, with significantly higher detectability of IL–12, and significantly higher serum levels of IL–12 in patients with active RA than in patients with inactive RA and the healthy control (P<0.05). So active RA was found to be associated with elevated serum levels of IL–12. These results agreed with the results obtained by

Yilmaz *et al*. (2001) who found that in juvenile RA patients, serum IL–12 levels during the active period of the disease were greater than in the controls, and there was a marked decrease in serum IL–12 levels when the patients entered the inactive phase of the disease. They concluded that IL–12 may play an important role in juvenile RA and may be used as a marker of disease activity. While Cordero et al. (2001) found that IL–12 levels were significantly higher in the sera of RA patients compared with the healthy controls, independently of disease activity.

Concerning TNF–α, it is a key mediator of inflammation and immunity, acting through its receptors expressed on all cells of the body. However, its overproduction may also lead to pathological changes. The latter situation occurs often in chronic inflammatory diseases as RA (Ziolkowska and Mastinski, 2003).

TNF–α is known to play a pivotal role in RA pathogenesis (Brennan et al., 1992), and there is an increasing evidence that implicates this cytokine, as well as IL–1, as contributing factors in the inflammatory, and perhaps the destructive manifestation of RA (Moreland, 1999). Apart from exerting direct pathogenic effects, TNF–α acts as a potent paracrine molecule inducing other proinflammatory molecules such as IL–1, granulocyte–monocyte colony stimulating factor, prostaglandin E_2 and platelet activating factor. These secondary mediators can amplify the inflammatory reaction as well (Van den Berg and Van Lent, 1996).

The successful introduction of anti–tumour necrosis factor treatment in clinical practice confirmed the biological relevance of TNF–α function in chronic inflammatory conditions in human, mainly in the pathogenesis of inflammatory bowel diseases and RA (Sfikakis and Kollias, 2003).

In the present study, TNF–α was detected in serum in 12 out of 17 (70.6%) active RA patients, 7 out of 15 (46.7%) inactive RA patients and 1 out of 10 (10%) healthy control subjects, with significantly higher detectability and significantly higher serum levels of TNF–α in active RA patients than in inactive RA patients and the healthy control ($P<0.05$). These results were consistent with the results obtained by Tetta *et al*. (1990) who detected TNF–α in the sera of most RA patients [9 out of 15 patients (60%)], with high levels of TNF–α in the sera of active RA patients.

Other studies investigated TNF–α in the serum of RA and osteoarthritis patients, and they found that serum TNF–α levels were higher in patients with RA than in those with osteoarthritis (Steiner *et al*., 1999; Klimiuk *et al*., 2001).

In our study, inactive RA patients, as well as active RA patients, had significantly higher detectability of IL–12 and TNF–α in serum, and significantly higher serum levels of IL–12 and TNF–α compared to the healthy control subjects ($P<0.05$), which may reflect the role of IL–12 and TNF–α in the pathogenesis of RA.

Correlating IL–12 with TNF–α in RA disease, Brennan et al. (1992) stated that IL–12 can induce the production of proinflammatory cytokines, including TNF–α, which contribute to the signs and symptoms of RA. Moreover, the levels of these cytokines correlate well with the activity markers of RA.

In the present study, serum levels of IL–12 correlated positively with serum TNF–α levels in patients with active RA ($r=0.493$) and those with inactive RA ($r=0.474$). This result agreed with the results obtained by Kim *et al*. (2000). In addition, in our study, elevated serum levels of IL–12 and TNF–α were associated with clinical and laboratory parameters of RA disease activity, and this agreed with the study done by Brennan *et al*. (1992).

In conclusion, significantly elevated serum levels of IL–12 and TNF–α were found to be associated with clinical and laboratory markers of activity of RA, so measurement of serum IL–12 and TNF–α levels could be used for assessment of RA activity. Serum levels of IL–12 correlated positively with serum TNF–α levels and both cytokines may play an important role in the pathogenesis and inflammatory activity of RA.

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مستويات الإنترلوكين 12 وعامل النخر–ألفا فى المصل فى مرضى الالتهاب المفصلى الروماتويدى النشط وغير النشط

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شمل هذا البحث اثنين وثلاثين من مرضى الالتهاب المفصلى الروماتويدى، والمترددين على مستشفى الزهراء الجامعى، وقد قُسم هؤلاء المرضى إلى مجموعتين : المجموعة الأولى وتشمل سبعة عشر مريضاً (ثلاثة ذكور وأربع عشرة أنثى) تتراوح أعمارهم بين ست وعشرين وستين عاماً، ويعانون من نشاط للمرض، والمجموعة الثانية وتشمل خمسة عشر مريضاً (أربعة ذكور وإحدى عشرة أنثى) تتراوح أعمارهم بين خمس وعشرين واثنين وستين عاماً، وهم فى فترة هدوء وعدم نشاط للمرض، بجانب عشرة من الأصحاء (اثنين من الذكور وثمانية من الإناث) تتراوح أعمارهم بين عشرين وسبعة وخمسين عاماً كمجموعة ضابطة.

وقد تم تقييم المرضى إكلينيكياً ومعملياً، كما تم أخذ عينات من المصل من المرضى ومن المجموعة الضابطة لقياس مستويات الإنترلوكين 12 وعامل النخر–ألفا باستخدام اختبار الإليزا.

وقد أوضح البحث عدم وجود فروق ذات دلالة إحصائية بين مرضى الالتهاب المفصلى الروماتويدى النشط وغير النشط من حيث العمر، والجنس، ومدة المرض، ودرجة الإعاقة، والتقييم العام للمريض وللطبيب، ولكن وُجدت فروق ذات دلالة إحصائية فى بعض النتائج المعملية والإكلينيكية بين مجموعتى المرضى مثل حساب ألم وتورم المفاصل، ومقياس الألم وسرعة ترسيب الدم ومستوى بروتين س المتفاعل حيث كانت كلها أعلى علواً ذا دلالة إحصائية فى مرضى الالتهاب المفصلى النشط عن غير النشط.

كما أوضح البحث أن ثلاثة عشر مريضاً بالالتهاب المفصلى الروماتويدى النشط (بنسبة 76.5%)، وست مرضى بالالتهاب المفصلى الروماتويدى غير النشط (بنسبة 40%)، وواحد من الأصحاء (بنسبة 10%) كان عندهم مستويات ملحوظة من الإنترلوكين 12 فى المصل.

وبالنسبة لعامل النخر–ألفا، فقد لُوحظ وجوده بمستويات ملحوظة فى المصل فى اثنى عشر مريضاً بالالتهاب النشط (بنسبة 70.6%)، وسبعة من مرضى الالتهاب غير النشط (بنسبة 46.7%)، وواحد من الأصحاء (بنسبة 10%)، وبذلك وُجد كل من الإنترلوكين 12 وعامل النخر–ألفا بشكل أكبر فى مرضى الالتهاب المفصلى الروماتويدى النشط مقارنة بمرضى الالتهاب غير النشط وبالأصحاء، كما وُجد أن متوسط مستويات الإنترلوكين 12 وعامل النخر–ألفا فى المصل أعلى علواً ذا دلالة إحصائية فى مرضى الالتهاب النشط عن مرضى الالتهاب غير النشط وعن الأصحاء.

ومن ناحية أخرى، وُجد كل من الإنترلوكين 12 وعامل النخر–ألفا في مرضى الالتهاب المفصلى الروماتويدى غير النشط بشكل أكبر وبمستويات أعلى علواً ذا دلالة إحصائية مقارنة بالأصحاء.

كما لُوحظ أن وجود الإنترلوكين 12 فى المصل بمستويات ملحوظة كان مرتبطاً ارتباطاً إيجابياً مع وجود عامل النخر–ألفا فى المصل بمستويات ملحوظة أيضاً، وذلك فى مرضى الالتهاب المفصلى الروماتويدى النشط وغير النشط.

وبذلك تم استنتاج إمكانية ارتباط المستويات العالية من الإنترلوكين 12 وعامل النخر–ألفا فى المصل لعلامات نشاط مرض الالتهاب المفصلى الروماتويدى، وأنه يمكن استخدام قياس مستويات الإنترلوكين 12 وعامل النخر–ألفا فى المصل فى تقييم نشاط المرض، وكذلك أن الإنترلوكين 12 وعامل النخر–ألفا قد يلعبان دوراً هاماً فى حدوث المرض ونشاطه.