Values of Interleukin-4 (IL-4) and Interleukin-6 (IL-6) in the Synovial Fluid of Rheumatoid Arthritic Patients

1Ammal A.W Sallam, M.D. and 2Ayman M.H El-Sharawy, M.D.

1The Department of Clinical & Chemical Pathology, Research Institute of Ophthalmology, Giza, Egypt and the 2Department of Orthopaedic Surgery, Cairo University Hospital, Egypt

Abstract: To examine levels of interleukin-4 (IL-4) and interleukin-6 (IL-6) in the synovial fluid (SF) of adult patients already diagnosed with rheumatoid arthritis (RA) and to verify the relationships between IL-4, IL-6 and serum C-reactive protein (CRP). IL-4 and IL-6 were measured by enzyme-linked immunosorbant assay (ELISA) in the synovial fluid samples from 70 knee joints of 35 patients with RA compared to 35 non-rheumatoid arthritic subjects. IL-4 levels were significantly higher in the SF of the rheumatoid arthritic patients as compared with non-rheumatoid arthritic subjects. Meanwhile IL-6 levels were significantly higher in the SF of the rheumatoid arthritic patients as compared with non-rheumatoid arthritic subjects and SF levels of IL-4 in rheumatoid arthritic subjects. SF levels of IL-4 were not correlated to SF levels of IL-6 nor to the indicator of inflammation; C-reactive protein (CRP); while SF IL-6 were significantly correlated to CRP. The results of the current study revealed deficient production of synovial IL-4 and excessive production of synovial IL-6, suggesting an essential role of both interleukins in the pathogenesis of rheumatoid arthritis. Also our study demonstrated a strong correlation between the SF levels of IL-6 and the CRP in the RA patients was found.

Key words: Interleukin-4 and -6- Synovial fluid- Rheumatoid arthritis.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic autoimmune inflammatory disease of unknown aetiology characterized by synovial membrane hyperplasia and chronic synovitis with the invasion and destruction of cartilage and bone. The progressive destruction of the articular cartilage is one of the hallmarks of the disease and determines the outcome of RA in most of the affected individuals [1].

The mechanisms responsible for the development of these lesions are largely unknown, with both the T lymphocytes and the macrophages being considered as the pivotal cells involved in the pathogenesis of RA [2].

Park et al., 2001 [1] demonstrated that the decisive T cell role regarding the introduction of RA through antigen specific T cells mediating autoimmunity is still not clear. However a pathological process specific to RA with the imbalance of the T-helper (Th)1/Th2 in chronic RA was recognized.

While no definite biological marker exists for the diagnosis and prognosis of RA, the levels of biomarkers such as pro- and anti-inflammatory cytokines predominantly from macrophage lineage cells in the synovial membrane, play a major role in the initiation and continuation of the chronic inflammatory process in the RA synovial membrane. As well, the chemokines and the metalloproteinases can potentially reflect the inflammatory status in the joints [4].

The establishment of a chronic synovitis involves the traffic of circulating inflammatory cells into and through the synovial membrane, regulated by cell adhesion molecules which are in turn regulated by the pro-inflammatory cytokines[2].

The synovial fluid (SF) of rheumatoid arthritis (RA) patients contains a mixture of inflammatory mediators. In order to determine whether certain cytokines pattern is specifically related to the chronic inflammation in RA, the concentrations of synovial interleukin-4 and interleukin-6 were investigated [2].

Interleukin-4 (IL-4) is a pleiotropic T –cell derived cytokine that can exert suppressive or stimulatory effects on different cell types, and was originally identified as a B-cell growth factor and regulator of the humoral immune pathways [1].

Corresponding Author: Ammal A.W Sallam, M.D. The Department of Clinical & Chemical Pathology
Interleukin-6 (IL-6) is also a pleiotropic macrophage derived cytokine that coordinates both pro and anti-inflammatory activity during disease. Differential control of these properties is closely related to the ability of IL-6 to suppress innate immune responses and its capacity to promote acquired immunity[5].

**Aim of the Work:** We aimed to measure the concentrations of IL-4 and IL-6 in the synovial fluid of rheumatoid arthritic patients and to investigate the relationships between IL-4, IL-6 and serum CRP in RA patients.

**Sample Collection:** Samples of synovial fluids were obtained by arthrocentesis of the knees for therapeutic purposes under aseptic conditions. All patients had synovitis at the time of arthrocentesis. The samples were centrifuged to remove the cells and debris and stored at -70°C for further analysis of Interleukin-4 and Interleukin-6. These cytokines were determined by ELISA methods[6].

**SUBJECTS AND METHODS**

**Subjects:** We involved 70 subjects to this study. Thirty five (35) rheumatoid arthritic patients (24 females and 11 males, mean age 59.61 ± 7.48 years) were compared with 35 non-rheumatoid arthritic subjects considered as control group (22 females and 13 males, mean age 50.23 ± 6.35 years). All arthritic patients were already previously diagnosed as chronic cases (the mean duration of RA was 7.63 ± 2.45 years). Clinically, they had one or more swollen joints and symptoms of inflammatory joint pain and/ or early morning stiffness and / or joint related soft tissue swelling and / or redness and warmth.

**Methods:** Both interleukin-4 and interleukin-6 were determined by an ELISA method[5]; C- reactive protein (CRP) by using immunoturbimetric assay was evaluated[5].

**Data Handling and Statistical Methods:** The statistical software package (SPSS version 10.0) was used for data management and analysis. Data were expressed as mean ± standard deviation. The data were subjected to the Kolmogorov- Smirmov test to determine the distribution and method of analysis. As most of the data was normally distributed continuous variables student’s t test was used. To exam the correlation, Pearson’s correlation coefficient’s (r) were calculated by linear regression analysis. p values < 0.05, < 0.01 and < 0.001 were considered significant, highly significant and very significant respectively.

**Results:** Characteristics of subjects investigated are present in Table (1). The mean duration of RA was 7.63 ± 2.45 years. The mean CRP values for the rheumatoid arthritic patients were 18.67± 1.53 mg/l and for the non- rheumatoid arthritic subjects were 0.83 ± 0.21 mg/l.

Table (2) demonstrates mean and ± SD values of IL-4 and IL-6 in the synovial fluid of the various studied groups. The synovial IL-4 levels were (14.97 ± 2.8 pg/ml) in the rheumatoid arthritic patients compared to the synovial IL-4 of non-rheumatoid arthritic subjects (2.49 ± 0.21 pg/ml, p < 0.001). While the synovial IL-6 levels were (418.51 ± 7.88 pg/ml) significantly higher in the rheumatoid arthritic patients compared to the synovial IL-6 of non- rheumatoid non-rheumatoid arthritic subjects (13.63 ± 1.23 pg/ml, p < 0.001).

Table (3) illustrates the correlation between SF levels of IL-4, SF levels of IL-6 and serum levels of CRP in RA patients. There was no significant correlation between the SF levels of IL-4 and IL-6 (r = 0.244, p = 0.158), also there was no correlation between SF levels of IL-4 and serum CRP (r = 0.27, p = 0.116).

A highly significant positive correlation was detected in the rheumatoid arthritic group between SF levels IL-6 and serum CRP (r = 0.962, p < 0.001). (Figure 1)

**Discussion:** The aetiology of RA is still unidentified and may be multifactorial. Viral pathogens as Epstein-Barr virus was alleged because of its possible role on B cells producing IgM rheumatoid factor (RF) and higher levels of EBV antibody titers in RA patients were also detected [8]. Genetic liability was strongly suggested with the HLA-DR4; however the triggering event that leads to RA in those susceptible individuals is yet to be recognized [9].

Emery et al., 2008 [10] described rheumatoid arthritis as a chronic auto-immune inflammatory disorder not only involving the synovial inflammation leading to the associated pain, stiffness and swelling, the cardinal features of RA joints, but also other compartments such as the bone marrow, cartilage and bone are strongly associated with tissue destruction.

The inflammatory process in RA is may be a reparative response to either localized cell death, or a consequence of infection, injury/wound healing, or due to the release of altered proteins due to hypoxia; this...
would result to the release of inflammatory mediators which would play an important role in the initiation and maintenance of an inflammatory lesion leading to chronic synovitis. Furthermore, host adhesion molecules which mediate the capture, rolling adhesion and transmigration of immunomodulatory cells from circulation may also be pivotal in the pathogenesis of RA. These assembled cells release many substances such as growth factors and pro-inflammatory cytokines (Interleukin (IL)-1, IL-6, IL-15, IL-18 and tumour necrosis factor-α (TNF-α), which cause a proliferative response in inflamed tissues.

The synovial tissue, which normally consists of a layer of synoviocytes (macrophages and fibroblasts), one to three cells thick, undergoes hyperplasia and is about 6 to 10 layers thicker in the rheumatoid arthritic joint. In addition the massive infiltration of activated cells, such as macrophages, T cells, B cells and dendritic cells, into the synovium, has been detected. Joint fibroblasts will be activated and become rheumatoid arthritis synovial fibroblasts (RASFs).

Progressive destruction of cartilage and bone occurs, which is believed to be mainly mediated by cytokine induction of enzymes, such as collagenase and stromelysin, produced by RASFs.

Angiogenesis is another histological hallmark of inflamed synovial tissue. Several pro-angiogenic factors are expressed by RASFs, in particular IL-8, vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and transforming growth factor-β (TGF-β)⁴.

Santos and Morand,⁵ have also emphasized on the essential roles of macrophage and T lymphocytes in RA. Macrophages produce a diversity of local and systemic amplifiers for the disease severity and continuation. The main local mechanisms include; recruitment of inflammatory cells, activation of newly immigrated inflammatory cells, cell activation of neighboring inflammatory cells, secretion of matrix-degrading enzymes, activation of mature dendritic cells and cytokine-mediated differentiation of other macrophages (possibly B cells, T cells and mesenchymal cells) into antigen-presenting cells, neo-vascularization, (trans)differentiation of macrophage into osteoclasts involved in subchondral bone damage.

While at the systemic level, macrophages’ amplification of the disease are through; the acute-phase response network, systematic production of TNF-α, anomalies in bone marrow differentiation and, chronic activation of circulating monocytes.

On the hand, Raza et al.,⁶ explained that the synovial T cell population is maintained through active inhibition of apoptosis, mediated at least in part by fibroblast and macrophage derived type1 interferon’s (IFN) and active retention facilitated by fibroblast derived TGF-β. Contact dependent interactions between T cells and macrophages stimulate the production of proinflammatory cytokines; including TNF-α. In RA, the imbalance between the pro- and anti-inflammatory cytokines is directed towards continuous inflammation. The pro-inflammatory cytokines become chronically increased and cause prolonged disproportional inflammation.

This persistent inflammation leads to connective tissue, cartilage and bone destruction, resulting in the progression of structural damage}

A shift towards T cells with a Th1 cytokine profile has been reported since the early stages of RA. Since Th1 and Th2 cytokines are believed to have mutual regulatory interactions, the balance between T cells with Th1 or Th2 phenotype is considered to be an important regulatory mechanism of T cell function in RA.

Several cytokines produced during the initial presentation of antigen to naïve T cells regulates the development of Th1 and Th 2 cells. Interleukin-12 (IL-12) and interferon-γ (IFNγ) cause a shift towards Th1 responses, whereas IL-4 directs the development Th 2 cells.

Several scientists agreed that RA synovitis is a Th1- dominated disease.

The Th1 cytokines activate macrophage/synoviocytes to produce joint- destructive cytokines and inflammatory mediators, such as TNF-α, IL-1 and metalloproteases.

Our study was done to assess the SF levels of IL-4, an anti-inflammatory cytokine and SF levels of IL-6, a pro-inflammatory cytokine in rheumatoid arthritic patients. Our results showed that SF IL-4 concentrations were lower than SF IL-6 in the joints of chronic rheumatoid arthritic patients these results were in agreement with Joosten et al.,1999 and De Jager et al.,¹⁵.

These results suggest that RA is either a selective Th1 process or is not driven by all T cells. Another proposed explanation could be the fact that IL-1α and IL-1β specifically inhibit IL-4 synthesis by T cells, indicating the crucial role of IL-1 in RA. A dysregulated immune response secondary to the general cytokine environment (not only IFN-γ) in the joint, whereby different subsets of dendritic cells may play a key role through the production of growth factors, which would lead to recruitment of the peripheral blood T cells towards the Th1 phenotype, with a secondary imbalance of Th1/Th2 ratio.
The protective effect against joint damage of IL-4 seems to be exerted essentially by the combined action of IL-4 and IL-10; not only do the two cytokines act synergistically, but also they each protect against the harmful effect of the other\[16\].

IL-4 is a pleiotropic CD4+ T-cell derived cytokine that can exert suppressive or stimulatory effects on different cell types, and was originally identified as a B-cell growth factor and regulator of humoral immune pathways\[14\].

IL-4 inhibits the differentiation of naïve T cells to Th1 and cytokine production (as IL-2 and IFN-γ) by Th1 cells. This suppression has been suggested to be due to the inhibitory effect of IL-4 on IL-12 generation by antigen-presenting cells and macrophages. It is also known to be a potent anti-inflammatory cytokine by inhibiting the synthesis of pro-inflammatory cytokines such as IL-1, TNF-α, IL-6, IL-8 and IL-12 by macrophages and monocytes and through stimulating the synthesis of several cytokine inhibitors such as interleukin-1 receptor antagonist (IL-1Ra), soluble IL-1 receptor type II and TNF receptors\[14\].

IL-4 stimulates the proliferation, differentiation and activation of several cell types, including fibroblasts, endothelial cells and epithelial cells. While on human mononuclear phagocytes it suppresses metalloproteinase production and stimulates tissue inhibitor of metalloproteinase-1, a protective effect of IL-4 against extracellular matrix degradation; in addition to its inhibitory effect on osteoclasts. Deficiency of IL-4 in the joints of chronic RA patients explains findings attained that may be correlated to the pathogenesis of RA\[1\].

In our study there was no significant correlation between the SF levels of IL-4, SF levels of IL-6 and CRP.

De Jager et al., 2007\[15\] suggested that IL-4 production may be phase-dependent in RA; initial increased production of IL-4 in the joints of RA subjects followed later by reduced production of IL-4 which may be associated with the chronicity of the disease was reported.

Interleukin-6 (IL-6) is also a pleiotropic cytokine that coordinates both pro and anti-inflammatory activity during disease. Within the context of arthritis, IL-6 can affect the expression of inflammatory mediators, leucocytes trafficking, synovitis, cartilage degradation and joint erosion\[17\].

IL-6 is produced from macrophages and fibroblasts. The biological activities of IL-6 contribute to both systemic and local RA symptoms. IL-6 is a strong inducer of the acute-phase response, through activation of complement, induction of pro-inflammatory cytokines, stimulation of neutrophil chemotaxis and specific acute-phase proteins (as proteinase antagonists, opsonins, and procoagulants)\[17\].

Induction of the acute phase may result in fever, secondary amyloidosis, anaemia, and elevations of CRP. Serum amyloid A is the protein involved in secondary amyloidosis, which is frequently observed in patients with chronic diseases, particularly RA. IL-6 also induces the secretion of hepcidin, an iron regulatory peptide synthesized in the liver. Overproduction of hepcidin interferes with iron release from macrophages and with iron absorption; this dysregulation is believed to cause the development of anaemia in the chronic disease.

Peake et al.\[18\] explained that one of IL-6 most important activities is the induction of B-cell differentiation and antibody formation, which may stimulate the production of autoantibodies such as RF.

Other effects on immune cells include induction of T-cell activation and differentiation and macrophage differentiation. These activities may contribute to the inflammatory immune response that occurs in patients with RA.

The possibility that increased IL-6 levels have clinical importance in RA is supported by correlations between IL-6 concentrations and disease activity. In several studies, IL-6 levels have been found to correlate with surrogate markers of disease activity, including RF, erythrocyte sedimentation rate (ESR), and CRP. The last finding agrees with our results. Correlations between IL-6 levels and clinical manifestations, including morning stiffness and number of inflamed joints, have also been reported\[4\].

Locally, IL-6 activates endothelial cell production, leading to the release of IL-8 and monocyte chemotactic protein, expression of adhesion molecules, and recruitment of leucocytes to the inflammatory sites. In addition, IL-6 can stimulate synoviocyte proliferation and osteoclast activation, leading to synovial pannus formation\[17\].

IL-6 acts with IL-1 to increase production of matrix metalloproteinases, which may contribute to joint and cartilage destruction. Hence, IL-6 levels in the synovial fluid may also be correlated with the degree of radiological joint damage\[17\].

The mechanisms responsible for high IL-6 levels in patients with chronic inflammatory diseases remain unclear. It has been suggested that dysregulated expression may involve promoter polymorphisms in the IL-6 gene; certain genotypes were associated with increased disease activity and lower age of onset, suggesting that promoter polymorphisms may influence disease severity\[18\].
There was no significant correlation between the SF levels of IL-4 and IL-6 in RA subjects to each other, however higher levels of SF of IL-6 than IL-4 was found in our study. Our results showed that the synovial levels of IL-6 were significantly correlated to the serum levels of CRP of RA subjects.

The acute-phase reactant C-reactive protein (CRP) is widely used as biological marker for assessing the disease activity and inflammation in RA\[^{19}\]. Veldhuijzen et al.,\[^{19}\] claimed that CRP levels correlate significantly with RA disease activity, radiological progression and response to treatment. Consequently, CRP is considered to be more specific marker of RA disease activity than the other acute-phase reactant ESR since the hepatic production of CRP is reflective of the effects of inflammatory cytokines on the liver.

The increases in CRP during inflammation are the result of an increase in the number of cells producing CRP, as well as an increase in CRP secretion rate. Not all the CRP produced by the liver is released directly into the blood; small amounts are bound to a hepatic carboxylesterase in the endoplasmic reticulum. During inflammation, however, this specific binding of CRP is diminished, resulting in more efficient secretion\[^{19}\].

Other sources of CRP, such as macrophages, lymphocytes, normal vascular tissue, and atherosclerotic plaques, have been reported. Patients with RA have increased rates of atherosclerosis; hence, the production of CRP from the atherosclerotic plaques cannot be discarded. This alternative source of CRP may provide another explanation for the stress- induced CRP increases in patients with RA\[^{20}\].

In addition, CRP status is predictive of functional disability, and suppression of elevated CRP levels is associated with improvement in functionality. Significantly, normalization of CRP by drug therapy may minimize further joint damage, supporting the immediate introduction of inflammation-suppressing therapy before the onset of erosive damage. In agreement with our results, scientists established a direct link between cytokine synthesis, particularly TNF-\(\alpha\) and IL-6 and CRP especially with the rapid suppression of IL-6 and CRP following TNF-\(\alpha\) blockade\[^{19}\].

![Fig. 1: The correlation between the synovial fluid levels of IL-6 and the serum CRP in the rheumatoid arthritic group.](image)

| Table 1: Clinical characteristics of studied subjects |
|---------------------------------|---------------------------------|
| **Control N= 35**               | **Patients with rheumatoid arthritis N= 35** |
| Gender :                        |                                                |
| Female                          | 22                                             |
| Male                            | 13                                             |
| Age (years)                     | 50.23 ± 6.35                                  |
| Duration of RA (years)          | 7.63 ± 2.45                                   |
| CRP (mg/l)                      | 0.83 ± 0.21                                   |

RA = Rheumatoid arthritis
Table 2: IL-4 and IL-6 concentrations in synovial fluid, of rheumatoid arthritic patients compared to non-rheumatoid arthritic subjects.

<table>
<thead>
<tr>
<th></th>
<th>Control N= 35</th>
<th>Patients with rheumatoid arthritis N= 35</th>
<th>p values</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-4 (pg/ml)</td>
<td>2.49 ± 0.21</td>
<td>14.97 ± 2.8</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td>IL-6 (pg/ml)</td>
<td>13.63 ± 1.23</td>
<td>418.51 ± 7.88</td>
<td>p &lt; 0.001</td>
</tr>
</tbody>
</table>

*p compared to control

Table 3: Relationships between the synovial fluid levels of IL-4 & IL-6 and serum levels of CRP.

<table>
<thead>
<tr>
<th>IL-4</th>
<th>IL-6</th>
<th>CRP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r = 0.244 p = 0.158 (NS)</td>
<td>r = 0.27 p = 0.116 (NS)</td>
</tr>
<tr>
<td>IL-6</td>
<td>r = 0.244 p = 0.158 (NS)</td>
<td>r = 0.962 p &lt; 0.001</td>
</tr>
</tbody>
</table>

Conclusion: We found that the relatively low synovial levels of IL-4 while high synovial levels of IL-6 were significantly pivotal in the pathogenesis of rheumatoid arthritis and may have an important role in the chronicity of the disease.

Recommendations: Further investigations of other causative cytokines and biomarkers in the pathogenesis of rheumatoid arthritis may be considered; for possible relief of symptoms, prevention of continuous articular tissue destruction and improvement in lifestyle of the RA patients especially during later phases of the disease.

REFERENCES


