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Serum levels of interleukin-6 type cytokines and soluble interleukin-6 receptor in patients with rheumatoid arthritis

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We investigated the serum concentrations of interleukin-6 (IL-6) and two IL-6 family of cytokines (leukaemia inhibitory factor (LIF) and ciliary neurotrophic factor (CNTF) as well as IL-6 soluble receptor (sIL-6R) using an enzyme-linked immunosorbent assay (ELISA) in 66 patients with rheumatoid arthritis (RA) and 24 healthy controls. We examined a possible association between the serum levels of these peptides and RA activity according to the Mallya and Mace scoring system and Ritchie's index. We also evaluated the correlation between the serum levels of IL-6, LIF, CNTF and sIL-6R and duration of the disease and calculated sIL-6R/IL-6 ratio in RA patients and in the control group. IL-6 and sIL-6R were detectable in all 66 patients with RA and 24 normal individuals. LIF was also found in the serum of all patients with RA and in 16 (66.7%) normal individuals. In contrast CNTF was measurable only in 15 (22.7%) patients with RA and 24 (33.3%) normal individuals. The highest IL-6 and sIL-6R levels were found in the patients with Stages 3 and 4 of RA activity and the lowest in the control group. In contrast there were no statistically significant diferences between the LIF and CNTF levels in RA patients and normal individuals. We found positive correlation between IL-6 and sIL-6R concentrations and Ritchie's index and a lack of such correlation with LIF and CNTF. IL-6 serum level correlated positively with the disease duration, but sIL-6R, LIF and CNTF did not. Serum sIL-6R/IL-6 ratio was significantly lower in RA patients than in healthy controls. In conclusion, an increase in the serum levels of IL-6 and sIL-6R, but not LIF and CNTF concentrations, may be useful markers for RA activity.

Key words: Interleukin 6, Leukaemia inhibitory factor, Cilary neurotrophic factor, Soluble interleukin 6 receptor, Rheumatoid arthritis, Disease activity

Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disease localized preferentially in the synovial joints, resulting in joint destruction and permanent disability.[1,2]

There is growing evidence suggesting that some cytokines, particularly proinflammatory cytokines such as tumour necrosis factor α (TNF-α), interleukin-1 (IL-1), interferon γ (IFN-γ) and interleukin-6 (IL-6), play an important role in the pathogenesis of this disease.[3,4] These inflammatory cytokines are present in the rheumatoid synovial membrane and participate in cell proliferation as well as in the synthesis of prostaglandins, metalloproteinases and other cytokines.[5–7]

IL-6 is a pleiotropic, immunomodulatory cytokine produced by a variety of cell types, including fibroblasts, endothelial cells, monocytes and both benign and malignant lymphocytes of B and T cell origin.[8] It is a multifunctional cytokine, which plays a key role in the differentiation and growth of haematopoietic cells, B-cells, T-cells, keratinocytes, neuronal cells, osteoclasts and endothelial cells.[9] Moreover, IL-6 modulates the transcription of several liver-specific genes during acute inflammatory states. Pertinent to inflammation is the ability of IL-6 to induce acute phase protein synthesis in hepatocytes.[10]

IL-6 is a member of a family of cytokines which also includes leukaemia inhibitory factor (LIF), oncostatin M (OSM), ciliary neurotrophic factor (CNTF), and interleukin-11 (IL-11).[11,12] These cytokines are a group of evolutionary related proteins characterized by a common tertiary framework, with a distinctive, four helix bundle topology.[13,14] IL-6 type cytokines induces growth or differention via a receptor system that involves a specific receptor and the use of a shared signalling subunit, gp 130.[15]

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Cells isolated from the synovium of RA patients expressed mRNA for LIF, OSM and IL-11 at higher levels than did synovial cells from osteoarthritis patients, and spontaneously released greater quantities of this protein in culture.[16] Elevated levels of LIF, OSM and IL-11 were also found in the synovial fluids of RA patients.[16–18] However OSM, LIF and IL-11 have been reported to be undetectable in peripheral blood of these patients.[16] To our knowledge the level of CNTF in the serum and synovial fluid of RA patients has been not investigated, so far. However, we have found a detectable level of this cytokine in the serum of the majority of systemic lupus erythematosus (SLE) patients.[19]

The cellular IL-6 receptor complex consists of two different proteins, an 80-kDa ligand binding glycoprotein (IL-6R) and gp 130 involved in cellular signal transduction.[20] These two subunits of the IL-6R complex are proteolytically cleaved and released from the cell as soluble receptor proteins.[21] Soluble forms of the IL-6R (sIL-6R) and gp 130 are found in different body fluids in patients with various inflammatory diseases, including SLE and RA.[22,23]

In the present study we measured the serum concentration of IL-6, LIF, CNTF and sIL-6R in patients with RA using ELISA assay. We correlated the serum levels of these proteins with disease activity according to the Mallya and Mace scoring system and Ritchie's index as well as with disease duration and type of treatment. We also evaluated the correlation between the serum levels of these three cytokines and IL-6R as well as IL-6 with LIF and CNTF.

Patients and methods

Patients

The study involved 66 patients (57 females and nine males). Their mean age was 51.1 years (range 20–78 years). They fulfilled the revised criteria of the American Rheumatism Association (ARA), criteria for diagnosis of RA.[24,25]

The mean duration of their disease was 8.7 years (range from 1 month to 30 years). Forty-six of them were found positive for the rheumatoid factor (RF) and 20 were negative. Most of the RA patients were receiving non-steroidal anti-inflamatory drugs (NSAID). In addition, 26 of the patients were treated with prednisone, eight were taking D-pencillamine, nine-gold salts, eight methotrexate and eight sulfasalazine. On the day of blood sampling, each patient's history was recorded and a physical examination was performed. Clinical variables included disease duration, applied treatment and a joint count for pain/tenderness. Ritchie's articular index (RI),[26] duration of the morning stiffness and visual analogue pain score (VAS) were recorded by the same observer (A.G.).

Table 1. Clinical characteristics of patients with rheumatoid arthritis

Symptom	Number of patients	%
Total	66	100
Age		
Mean ± SD	53.6 ± 12.4	
Range	20–78	
Sex (male/female)	9/57	13.6/86.4
Disease activity according to Mallya and Mace		
Grade 1	1	1.5
Grade 2	15	22.7
Grade 3	34	51.6
Grade 4	16	24.2
Ritchie point index		
Mean ± SD	15.2 ± 6.9	
Range	1–30	
RE positive	44	66.6
Fever	12	18.2
Renal disorder (creatinine > 1.2 mg/dl)	10	15.2
Pulmonary rheumatic disease	2	0.3
Vasculitis lesions	8	12.1
Weight loss (>5 kg/6 months)	18	27.3
Treatment during the study		
NSAID	48	12.1
Prednisone	24	36.4
Methotrexate	7	10.6
D-penicillamine	8	12.1
Gold salts	8	12.1
Sulfasalazine	9	18.2
Laboratory parameters		
ESR		
Mean ± SD	54.9 ± 32.2	
Range	10–136	
Haemoglobin (g/dl)		
Mean ± SD	11.9 ± 1.5	
Range	7.6 ± 11.9	
Platelets ($\times 10^9$/1)		
Mean ± SD	286.4 ± 85.4	
Range	152–614	
White blood cells ($\times 10^9$)		
Mean ± SD	7.8 ± 1.9	
Range	4.8–12.5	

Disease activity was scored during visits to the outpatient clinic according to the method described by Mallya and Mace.[27] Our group of patients included 16 patients in Stage 1 or 2, 34 in Stage 3 and 16 in Stage 4 of the disease activity, according to this classification. The clinical and laboratory features of RA patients are presented in Table 1.

The control group consisted of 24 healthy individuals, 19 women and 5 men, aged from 24 to 68 years (mean 51.2 years). Each underwent a thorough physical examination performed by one of the authors (A.G.).

Laboratory tests

On the day of blood sampling for cytokines the following laboratory parameters were analysed: complete blood cell count (CBC), Westergren erythrocyte sedimentation rate (ESR), urinalysis blood urea

nitrogen and creatinine levels, fibrinogen level, liver function tests (GOT, GPT, bilirubin), C-reactive protein (CRP), serum iron and rheumatoid factor (RF). Chest radiographs, abdomen ultrasonography and EKG were also performed.

Serum sampling and cytokine determination

Venous blood samples for IL-6, IL-6 related cytokines (LIF and CNTF) and sIL-6R were collected at the time of clinical assessment into pyrogen free tubes, allowed to clot at –4°C for 1 h and centrifuged at 2000×***g*** for 10 min. The obtained serum was divided into aliqots and stored at –25°C until assayed for IL-6, LIF, CNTF and sIL-6R. The sera were randomly coded and testing was carried out without knowledge of the clinical status of the subject or of related laboratory data. The cytokine serum concentration was assayed by specific, commercially available, enzyme linked (ELISA) assay kits (Quantikine, R&D Systems Inc, USA) in accordance with the manufacturer's instructions and analysed with an ELISA reader at 492 mm. The procedure was described in details elsewhere.[19,28] In brief, the monoclonal antibodies, specific for the cytokines were placed onto the microtitre plates provided with the kits. Standard and the samples were pipetted into the wells and any present cytokines were bound by the immobilizing antibody. After rinsing enzyme – linked polyclonal antibodies, specific for cytokines, were added to the wells to sandwich the cytokines immobilized during the first incubation. After the next rinsing the substrate solution was added to the wells and the colour developed proportionally to the amount of cytokines bound in the first step. In each assay the appropriate recombinant human cytokine was used to generate the standard curve. The concentration of cytokines and sIL-6R in the samples were determined by interpolation from the standard curve. Serum sIL-6R concentration measurement was diluted 40 times and its level was measured between 7.8 and 500 pg/ml. Sensitivity of the assay for IL-6 was 0.7 pg/ml, for LIF 2.0 pg/ml and for CNTF 8.0 pg/ml.

Statistical analysis

The mean values were compared in Kruskal Wallis and Mann-Whitney tests. Differences in parameters between the groups were evaluated with Student's *t*-test. Statistical analysis for the frequency of detectable cytokines was performed using chi-squared test. The linear correlations between serum interleukin levels compared with each other or with Ritchie's index were evaluated using the Spearman rank-sum correlation coefficient and linear regression calculated with the least-squares method. Results are presented with R^2 coefficients. Comparison and correlation were considered significant when $P < 0.05$.

Results

Table 2 shows the results of measurement of IL-6, LIF, CNTF and sIL-6R in the serum of 66 patients with RA and 24 normal individuals. IL-6, LIF and sIL-6R were detectable in the serum of all 66 patients with RA. In contrast, CNTF was measurable only in 15 (22.7%) out of 66 patients. In the control group IL-6 and sIL-6R were detectable in all 24 individuals, LIF in 16 (66.7%) and CNTF only in eight (33.5%). The levels of IL-6 and sIL-6R were higher in RA patients than in healthy persons ($P < 0.001$ and $P < 0.03$ respectively). The concentrations of IL-6 and sIL-6R were higher in the patients in Stages 3 and 4 of RA activity than in Stages 1 and 2 RA activity. A detectable level of LIF was also found in all 66 patients with RA and in 16 out of 24 (66.7%) normal individuals. In contrast CNTF was measurable only in the sera from 15 out of 66 (22.7%) patients with RA and in eight out of 24 (33.3%) healthy subjects. The mean values of LIF and CNTF in RA patients and normal individuals were not statistically different ($P > 0.05$). However, LIF was more frequently detectable in RA patients than in the control group ($P < 0.03$). We found a positive correlation between IL-6 as well as sIL-6R concentrations and Ritchie's index ($R^2 = 0.1404$, $P < 0.002$ and $R^2 = 0.0952$, $P < 0.02$, respectively) but no such correlation between LIF and CNTF serum levels with Ritchie's index ($R^2 = 0.0035$, $P > 0.05$ and $R^2 = 0.002$, $P > 0.05$ respectively) (Fig. 1).

We also analysed the relationship between serum concentrations of IL-6, sIL-6R, LIF and CNTF with the duration of the disease (Fig. 2). We observed a positive correlation between these parameters only in the case of IL6 ($R^2 = 0.1401$, $P < 0.002$), but no such correlation in the case of sIL-6R, LIF and CNTF ($R^2 = 0.0420$, $P > 0.05$, $R^2 = 0.0001$, $P > 0.05$ and $R^2 = 0.0020$, $P > 0.05$ respectively).

The relationship between the serum levels of sIL-6R with evaluated cytokines, as well as between particular cytokines alone was also analysed (Fig. 3). We observed no correlation between any of the compared parameters. The calculation of the ratio of sIL-6R to IL-6 in patients with RA according to disease activity is presented in Table 3.

We found a significantly lower ratio of sIL-6R to IL-6 in RA patients compared with normal persons (1406.3 and 8806.0 respectively). There was also significant difference between the sIL-6R to IL-6 ratio in Stages 1 and 2 and Stage 4 RA activity according to Mallya and Mace ($P < 0.05$).

The influence of the treatment schedule on the serum concentrations of IL-6, sIL-6R, LIF and CNTF has been also analysed (data not presented). However we found no statistically significant differences between the levels of detected cytokines or sIL-6R in the patients treated with NSAID only, prednisone, methotrexate, D-penicillamine, gold salts or sulfasalazine.

Table 2. Serum levels of IL-6, LIF, CNTF and sIL-6R in patients with RA according to disease activity (according to Mallya and Mace) and normal control group. Mean values in pg/ml ± SD, median and range in parentheses

Group cytokines	ALL RA patients $n = 66$ (a)	RA stages 1 & 2 $n = 16$ (b)	RA stage 3 $n = 34$ (c)	RA stage 4 $n = 16$ (d)	Normal control group $n = 24$ (e)	Statistical analysis for means
IL-6	$n_1 = 66$	$n_1 = 16$	$n_1 = 34$	$n_1 = 16$	$n_1 = 24$	
						a & e $p < 0.001$*
Mean ± SD	52.7 ± 53.2	22.5 ± 20.3	52.3 ± 42.2	83.6 ± 77.0	5.1 ± 3.0	b & e $p < 0.001$*
Median	34.1	12.3	40.9	37.5	5.3	c & e $p < 0.001$*
Range	1.5–234.0	2.3–58.0	1.5–138.4	5.8–234.0	0.5–16.6	d & e $p < 0.001$*
						b & c $p < 0.04$*
						b & d $p < 0.02$*
						c & d $p > 0.05$
LIF	$n_1 = 66$	$n_1 = 16$	$n_1 = 34$	$n_1 = 16$	$n_1 = 16$	
						a & e $p > 0.05$
Mean ± SD	5.1 ± 2.1	5.20 ± 2.1	5.0 ± 1.9	5.2 ± 2.7	3.8 ± 3.4	b & e $p > 0.05$
Median	4.8	4.8	4.8	4.8	4.0	c & e $p > 0.05$
Range	0.8–9.6	2.4–9.6	1.6–8.8	0.8–9.6	0.0–10.4	d & e $p > 0.05$
						b & c $p > 0.05$
						b & d $p > 0.05$
						c & d $p > 0.05$
CNTF	$n_1 = 15$	$n_1 = 3$	$n_1 = 7$	$n_1 = 5$	$n_1 = 8$	
						a & e $p > 0.05$
Mean ± SD	0.9 ± 3.0	0.49 ± 1.1	1.1 ± 4.0	0.93 ± 1.4	1.17 ± 2.30	b & e $p > 0.05$
Median	0.0	0.0	0.0	0.0	0.0	c & e $p > 0.05$
Range	0.0–19.5	0.0–3.9	0.0–19.5	0.0–3.2	0.0–8.5	d & e $p > 0.05$
						b & c $p > 0.05$
						b & d $p > 0.05$
						c & d $p > 0.05$
sIL-6R	$n_1 = 66$	$n_1 = 16$	$n_1 = 34$	$n_1 = 66$	$n_1 = 24$	
						a & e $p < 0.03$*
Mean ± SD	49756 ± 14503	40688 ± 10395	50436 ± 13564	57381 ± 15745	41683 ± 11497	b & e $p > 0.05$
Median	45366	40802	48066	50870	39674	c & e $p < 0.04$*
Range	17288–81760	17288–62904	31200–79248	40180–15744	23732–64512	d & e $p < 0.002$*
						b & c $p < 0.05$*
						b & d $p < 0.003$*
						c & d $p < 0.05$*

*Statistically significant difference; n = number of investigated individuals; n_1 = number of individuals with detectable cytokines.

Discussion

The role of cytokines in the pathogenesis of RA and their significance in clinical monitoring of the disease advancement has been attacting much attention since a few years.[3–6] In this disease the articular synovial membrane is infiltrated with inflammatory cells diffusing into the synovial fluid. The inflammatory process spreads from the synovial membrane to the cartilage and bone tissue causing their damage.[3] Cytokines play a crucial role in sustaining an inflammatory process within synovial membranese. So far, most attention has been paid to the role of inflammatory cytokines in RA pathogenesis, especially TNF-α, IL-1, IFN-γ and IL-6.[3,5,22] The activity of other cytokines constituting IL-6 group, like LIF, OSM and IL-11 in RA was a subject of single reports and the role of CNTF in this disease has not been investigated so far.[16–18,29] Unlike the soluble TNF receptors the role of sIL-6R in RA and the prognostic meaning of this receptor are also poorly investigated.[30,31]

In our studies we assessed serum concentration of IL-6, LIF, CNTF and sIL-6R in 66 patients with RA in different stages of disease advancement and duration as well as in 24 healthy controls. IL-6, sIL-6R were detected in the serum of all patients with RA and all healthy persons. Detectable levels of LIF were found in all RA patients but only in 16 (66.7%) healthy controls, and CNTF in 15 (22.7%) patients with RA and eight (33.3%) individuals from the control group.

The concentration of IL-6 in blood serum of RA patients was six-fold higher than in healthy persons and correlated with the disease activity and its duration. These results are consistent with the observations made by Madhok *et al.*[32] and van Leeuwen *et al.*[33] Moreover, high concentration of IL-6 in the synovial fluid of RA patients and the correlation between the concentration of this cytokine and the intensity of bone lesions seen in radiograms, as well as its significant role in the joint destruction, were demonstrated.[30,34] Also clinical improvement and the decrease of C-reactive protein in the serum of the

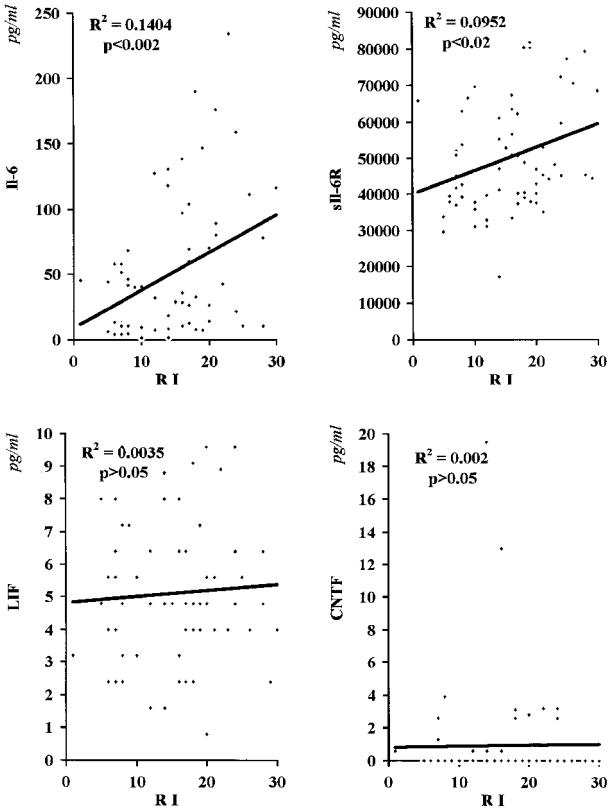

FIG. 1. Correlation between serum concentrations of IL-6, sIL-6R, LIF and CNTF with disease activity according to Ritchie's index (RI) in patients with rheumatoid arthritis (RA).

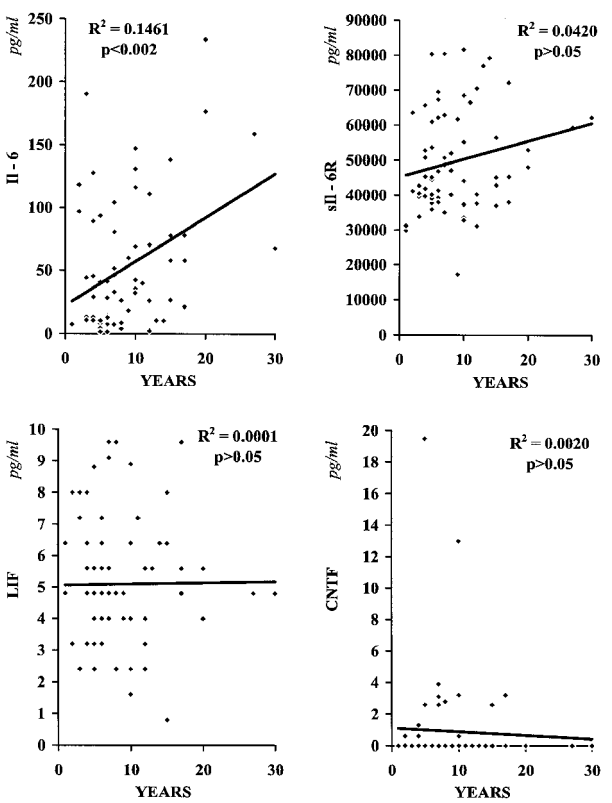

FIG. 2. Correlation between serum concentrations of IL-6, sIL-6R, LIF and CNTF with duration of disease.

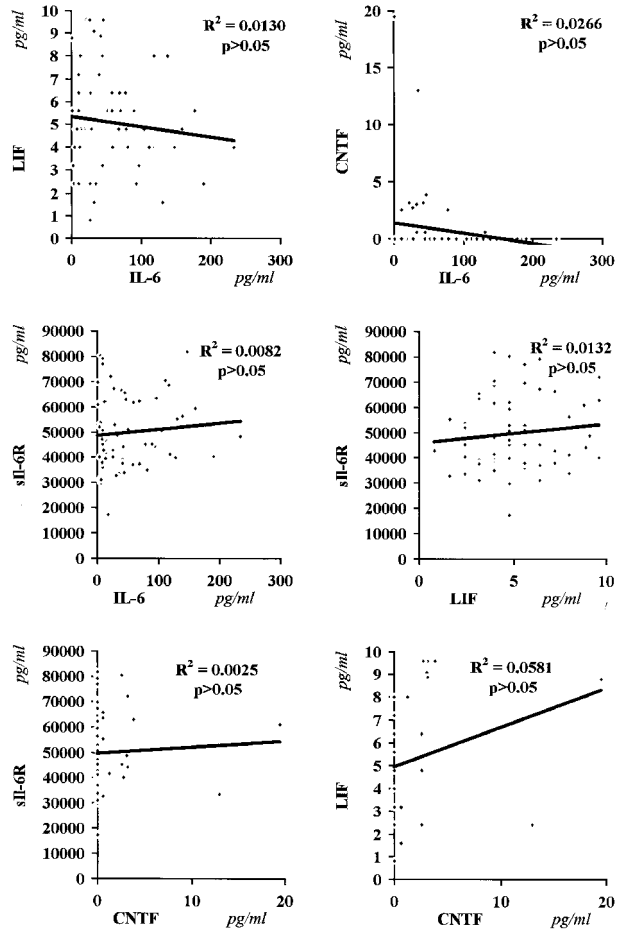

FIG. 3. Correlations between serum concentrations of IL-6R with IL-6, LIF and CNTF, IL-6 with LIF and CNTF and LIF with CNTF in patients with RA.

patients treated with antibodies against CD4+ lymphocytes, and then antibodies neutralizing IL-6, may prove the significance of IL-6 in the pathogenesis of RA.[35]

The results of our studies may indicate that two other cytokines related to IL-6 i.e. LIF and CNTF, unlike IL-6 are of smaller pathogenetic significance and of no use in determining RA activity. Their concentration in the serum of patients did not correlate with the degree of RA activity and was similar to their serum concentration in healthy individuals. Our results differ from those reported by Okamoto *et al.*[16] who showed that the isolated from the synovium of RA patients cell produce greater quantities of LIF than patients with osteoarthritis, nevertheless, they were unable to detect this cytokine in blood serum of patients with RA. These differences may stem from different sensitivity of ELISA tests applied in both studies. In the studies of Okamoto *et al.* the sensitivity reached 15 pg/ml, while in our tests the sensitivity was much higher and amounted to 2 pg/ml. High concentration of LIF in the synovial fluid of patients with RA was also observed by Waring *et al.*[17] It should be emphasized

Table 3. Serum sIL-6R/Il-6 ratio in the patients with RA according to disease activity according to Mallya and Mace

sIL-6R/Il-6	ALL RA patients $n = 66$ (a)	RA stages 1 & 2 $n = 16$ (b)	RA stage 3 $n = 34$ (c)	RA stage 4 $n = 16$ (d)	Normal control group $n = 24$ (e)	Statistical analysis for means
Mean ± SD	4114 ± 7829	4342 ± 4159	4716 ± 10200	2601 ± 4014	12976 ± 16309	a & e $p < 0.001$*
Median	1406.3	3381.5	987.5	1265.7	8806.0	b & e $p < 0.001$*
Range	206–6357	639–13524	331–46357	205–13852	2988–85816	c & e $p < 0.001$*
						d & e $p > 0.005$
						b & e $p < 0.005$*
						c & d $p < 0.05$

*Statistically significant difference.

that we detected LIF previously in the serum of some SLE patients and the concentration of this cytokine correlated with the disease activity when using the same ELISA method.[19]

Elevated levels of LIF were also observed in the serum of patients with non-Hodgkin's lymphoma, Hodgkin's disease, chronic lymphocytic leukaemia as well as in tissue shock.[36,37] Although it should be emphasized that in those studies the ELISA method with polyclonal antirabbit antibodies was employed, which is probably more sensitive than the commercially available tests used in our studies i.e. Quantikine, R&D Systems Inc. with monoclonal antibodies.[38]

To our knowledge CNTF has so far not been assessed in the serum of RA patients. In our studies this cytokine was detectable only in 15 out of 66 (22.7%) patients with RA and in eight out of 24 (33.3%) of healthy individuals. This cytokine was previously assessed by us in SLE patients, and was present in 52 out of 64 of the examined patients.[19] In SLE we also demonstrated a correlation between the concentration of CNTF and the activity of the disease. It may indicate that CNTF has a greater significance in the pathogenesis of SLE than in RA.

The determination of the role of IL-6 group cytokines in the pathogenesis of RA requires further investigation. However, the already available data indicate that the role of LIF, CNTF, OSM and IL-11 in the initializing and sustaining inflammation is less important than the role of IL-6 alone. Gabay *et al.*[39] showed that LIF and IL-11 stimulate human hepatocytes to produce the acute phase protein much poorer than IL-6, although in the experiments on mice it was found that CNTF stimulates production of the hepatic acute phase proteins to the same extent as IL-6.[40] It is not clear, however, whether this cytokine has a similar biological activity in experimental animals and humans. The results of our research are consistent with the observations of Okamoto *et al.*[16] and indicate that despite the functional similarity of IL-6 group cytokines, IL-6 is in this group the main mediator of inflammation in RA.

Soluble IL-6 receptor (sIL-6R), unlike other soluble cytokine receptors, has the unique property of acting agonistically with its ligand and enhances the stimulation of this cytokine on generating acute phase proteins (APP) by human hepatocytes,[41] as well as proliferation of myeloma cells[42] and synovial fibroblasts.[43] In our studies we found positive correlation between the serum concentration of sIL-6R in patients with RA and Ritchie's index. We also demonstrated significantly lower values of sIL-6R/IL-6 ratio in RA patients than in healthy subjects and lower values of this index in the most active form of the disease (Stage 4 of RA activity according to Mallya and Mace) than in less active forms (Stages 1 and 2). We did not reveal however, correlation between the concentrations of IL-6 and sIL-6R in the serum of RA patients. These observations indicate a complex and not very clear relation between these two proteins in this disease.

Studies of Kotake *et al.* showed higher concentrations of IL-6 and sIL-6R in the synovial fluid in RA patients than in patients with osteoarthritis and correlation of these peptides with the degree of joint destruction.[30] Though contradictory results were obtained in patients with juvenile rheumatoid arthritis, where negative correlation between the concentration of sIL-6R and IL-6 in serum was observed, and the concentration of sIL-6R in those patients was lower than in healthy persons.[44] However it should be emphasized that the concentration of sIL-6R in patients with SLE, AIDS and multiple myeloma was significantly higher than in healthy persons.[42,45] Nevertheless, like in the case of our RA patients, no correlation between the serum concentration of sIL-6R and IL-6 was found in multiple myeloma.[46] These data indicate that different agents influencing the production of both proteins, may be present in different diseases.

In conclusion we can state that the serum concentration of IL-6 and sIL-6R in patients with RA is higher than in healthy persons, and these peptides may serve as markers of this disease activity. The concentration of other cytokines of IL-6 group (LIF and CNTF) in serum of RA patients and healthy individuals neither differs significantly nor correlates with the activity of the disease.

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