

Serum Cytokines in Different Histological Variants of Rheumatoid Arthritis

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ABSTRACT. *Objective.* Rheumatoid arthritis (RA) is characterized by an invasive and tissue destructive infiltrate of lymphocytes, macrophages, and synoviocytes formed in the joints. Its etiopathogenesis and the role of the particular morphological components of synovitis remain unclear. There is evidence that its histological heterogeneity is correlated with synovium cytokine transcription. We investigated whether the serum cytokine profile is associated with the morphological appearance of the disease.

Methods. Tissue and serum samples were collected from 25 patients with clinically active RA and 25 with osteoarthritis (OA) as a control group. After histological analysis RA synovial biopsies were divided into 2 distinct types; 16 samples were characterized by diffuse lymphocyte infiltrates with no additional microanatomical organization. Lymphocytic aggregates with germinal center-like structures were found in 9 specimens. Serum concentrations of interferon- γ (IFN- γ), interleukin 12 (IL-12, p70 heterodimer), tumor necrosis factor- α (TNF- α), and IL-15 were measured by ELISA.

Results. Low concentrations of IFN- γ ($p < 0.01$) and IL-12 (NS) were found in RA patients' serum compared with OA controls. RA patients with follicular synovitis had lower serum concentration of IFN- γ ($p < 0.05$) and IL-12 ($p < 0.05$) than patients with diffuse infiltrates. High concentration of TNF- α and IL-15 characterized RA patient serum in comparison with controls (respectively, $p < 0.001$ and $p < 0.01$). In the serum of RA patients with follicular synovitis TNF- α was a dominant cytokine ($p < 0.01$) compared to patients with diffuse disease. At TNF- α level ≥ 44 pg/ml, 5 (56%) of 9 patients with follicular RA had such elevated values vs one of 16 diffuse patients ($< 10\%$; $p < 0.02$). Only serum concentrations of TNF- α could effectively differentiate between patients with OA and subgroups of RA. Analysis of clinical data suggested that activity of rheumatoid disease in patients with follicular synovitis was more severe than in those with diffuse infiltrates.

Conclusion. The association between distinct histological appearance of rheumatoid synovitis and serum cytokine profile and diverse clinical activity of disease seems to confirm its heterogeneity. (J Rheumatol 2001;28:1211-7)

Key Indexing Terms:

SERUM CYTOKINES
HISTOLOGY

RHEUMATOID ARTHRITIS

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Rheumatoid arthritis (RA) is a chronic inflammatory disease with progressive articular damage often associated with systemic manifestations. Invasive and tissue destructive infiltrates in the joint contain T and B cells, macrophages, and synoviocytes. Apart from dense synovial tissue infiltration by

mononuclear cells, histopathological changes also include neoangiogenesis and proliferation of the synovial lining layer¹⁻⁴. The disease etiopathogenesis and the role played by specific cellular elements infiltrating the synovium remain unclear. Macrophages and synovial fibroblasts mediate tissue destruction by several mechanisms including the production of monokines and metalloproteinases^{1,5,6}. There is also evidence that inflammatory reactions in the joint are regulated and controlled by T cells. The cytokines produced by these cells may enhance or suppress the inflammatory process⁷⁻¹⁰.

Studies have described genetic, histological, biological, and clinical heterogeneity of RA^{2,11-15}. However, the association between morphological changes and biological manifestations has not been studied in detail. The histological picture of rheumatoid synovitis is heterogeneous and the pathological appearance is not always useful in differentiating RA from other inflammatory arthropathies³. Only the formation of T-B cell aggregates, which sometimes form germinal-like centers, might be considered typical for RA^{16,17}. It has been shown that

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microscopic evaluation of rheumatoid synovium by conventional histology may confirm the existence of at least 3 distinct histological patterns¹⁸. The first was categorized as diffuse synovitis characterized by diffuse infiltrate of lymphocytes and macrophages with no additional microanatomical organization. The second, classified as follicular synovitis, shows demarcated lymphocytic aggregates with sparing of the intervening stroma. Sometimes they resemble germinal center-like structures with a central accumulation of B cells surrounded by T cells. The third pattern displays necrobiotic granulomas with a fibrinoid necrotic center lined by a collar of epithelioid histiocytes, sometimes with giant cells. The coexistence of follicular synovitis and granulomatous necrobiosis was not observed¹⁸.

It has been shown that each of the morphological patterns of rheumatoid synovitis may be characterized by unique cytokine mRNA expression in the synovium¹⁸. We investigated whether the profile of the serum cytokines might also correlate with histological forms of synovitis.

MATERIALS AND METHODS

Patients and controls. Synovial tissue and blood samples were taken from 25 patients with active RA that fulfilled the American College of Rheumatology 1987 revised criteria¹⁹. Tissue specimens were obtained during total arthroplasty of hip or knee joints. For a control, samples were taken from 25 patients with osteoarthritis (OA) during total hip arthroplasty. The characteristics of patient groups are shown in Table 1.

Clinical and laboratory assessment. The evaluation included duration of morning stiffness, number of tender joints (Ritchie index)²⁰, number of swollen joints, erythrocyte sedimentation rate (ESR), hemoglobin concentration, hematocrit, white and red blood cell count, platelet count, α_1 , α_2 and γ -globulin concentration, and rheumatoid factor (RF) level.

Histopathological analysis. All tissue samples were stained with hematoxylin and eosin. Cell infiltrate density and the topographical organization of lymphocytes and macrophages were determined and categorized as described¹⁸. One pathologist (BC), blinded to clinical and cytokine data, analyzed the specimens.

Serum sample preparation. Blood samples were allowed to clot for 30 min before centrifugation for 10 min at 1000 g. Aliquots of serum samples were frozen at -80°C immediately after sample collection.

Serum cytokine assessment. Serum concentrations of interferon- γ (IFN- γ), interleukin 12 (IL-12), tumor necrosis factor- α (TNF- α), and IL-15 were determined by quantitative sandwich ELISA (R&D Systems, Minneapolis, MN, USA) according to manufacturer's instructions. Analyses of assay reproducibility showed intraassay coefficients of variation (CV) for IFN- γ , IL-12, TNF- α , and IL-15 assessments were equal to or less than 4.0%, 1.5%, 5.2%, and 5.3%, respectively, while interassay CV were equal to or less than 7.5%, 7.1%, 7.4%, and 9.1%.

Statistical analysis. For statistical comparison of normally distributed clinical data the unpaired Student t test was used. The significance of the differences between cytokine values with abnormal distribution was assessed by Mann-Whitney U test. Fisher's exact test was used to assess probability of differences in frequency distributions. Correlations between variables were analyzed by Spearman rank test; p values < 0.05 were considered statistically significant.

RESULTS

Histopathological findings. Histological evaluation of synovial fragments from the OA control group revealed mainly mild mononuclear cell infiltrates. In contrast rheumatoid synovitis showed increased cellularity. Infiltrates varied in density, they were of perivascular and interstitial types, and consisted mainly of lymphocyte and macrophage-like cells. Sixteen RA synovium samples were characterized by diffuse lymphocyte infiltration with no additional microanatomical organization. These tissues were classified as diffuse synovitis. Lymphocytic nodular aggregates with germinal center-like structure formation were found in 9 specimens. Samples with such microanatomical organization were categorized as follicular synovitis. Other histological findings in rheumatoid synovium included mild edema, variable capillary neoangiogenesis, and synovial lining layer proliferation. Giant-like cells were rare. No formation of necrobiotic granulomas was found. Representative examples of OA and 2 different subtypes of rheumatoid synovitis are shown in Figure 1.

Demographic and clinical profiles. There was no statistically significant difference in sex profile, age, or disease duration between patients with both histological types of RA and OA. ESR was higher in diffuse and follicular RA than in OA (in both cases $p < 0.001$) (Table 1). Except for number of swollen

Table 1. Characteristics of patient populations. Data are shown as means \pm SD.

	Patients with OA	RA Patients with Diffuse Synovitis A	RA Patients with Follicular Synovitis B	p		
	OA	A	B	OA vs A	OA vs B	A vs B
Sex (F/M)	19/6	12/4	7/2	NS	NS	NS
Mean age, yrs	56.5 \pm 14.0	54.9 \pm 10.5	58.6 \pm 17.2	NS	NS	NS
Mean disease duration, yrs	14.2 \pm 10.8	17.7 \pm 7.6	15.6 \pm 6.4	NS	NS	NS
ESR, mm/h	17.0 \pm 11.3	47.1 \pm 10.5	58.7 \pm 18.7	< 0.001	< 0.001	< 0.06
Duration of morning stiffness, min	—	136.9 \pm 28.9	160.0 \pm 42.4	—	—	NS
Ritchie index	—	13.1 \pm 2.8	14.8 \pm 2.3	—	—	NS
No. swollen joints	—	13.2 \pm 3.2	16.2 \pm 3.5	—	—	< 0.05
RF positive	—	88	75	—	—	NS
Methotrexate use (last 3 mo prior to surgery), %	—	44	67	—	—	NS

joints ($p < 0.05$) no clinical disease activity variables or treatment regimens differed significantly between the distinct morphological variants of rheumatoid synovitis.

Serum cytokine concentrations. Sandwich ELISA specific for particular cytokines were used to determine amounts of IFN- γ in RA and OA serum. We were mainly interested in whether the concentrations of cytokines differed among patients with distinct histological types of synovitis. Results are illustrated in Figure 2. Serum concentrations of IFN- γ were lower in RA and in diffuse or follicular type synovitis in comparison with the OA control group ($p < 0.01$, $p < 0.05$, $p < 0.01$, respectively). The serum level of IFN- γ was lowest among patients with follicular synovitis differentiating them from those with diffuse type disease ($p < 0.05$).

As well, the serum concentration of IL-12, a macrophage derived cytokine promoting T helper cell selective expansion and IFN- γ production, was lower in RA in comparison with OA, but reached statistical significance only in the case of follicular synovitis ($p < 0.05$) (Figure 3). The lowest serum concentrations of IL-12 were associated with the presence of follicular synovitis in which the level of this cytokine was lower than in patients with diffuse infiltrate ($p < 0.05$), and differentiated between these 2 variants of rheumatoid synovitis.

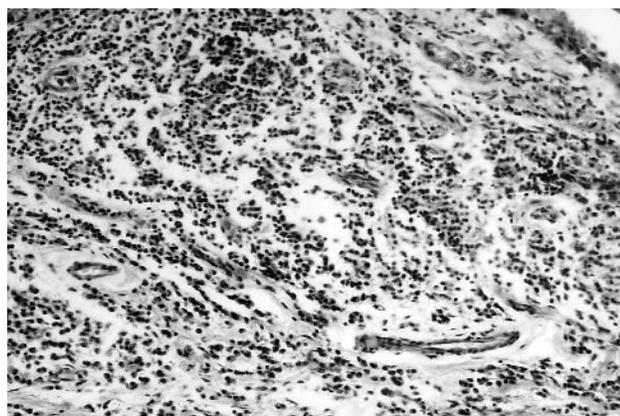
A completely different pattern of serum concentration was

seen for TNF- α and IL-15, which can induce TNF- α production through T lymphocyte activation (Figure 4). Serum levels of TNF- α were higher in RA and in both histological types of the disease as compared to OA (in all cases $p < 0.001$). In follicular synovitis TNF- α was a dominant cytokine and could be clearly distinguished from the lower serum level among patients with diffuse infiltrates ($p < 0.01$).

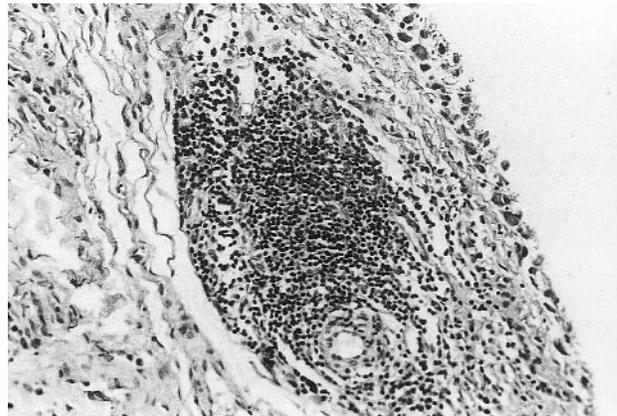
Similarly to TNF- α , serum concentration of IL-15 was higher in patients with RA and with diffuse or follicular histological forms of rheumatoid synovitis than in patients with OA ($p < 0.01$, $p < 0.01$, $p < 0.05$) (Figure 5). However, IL-15 concentration did not differ between the 2 distinct histological patterns of synovitis.

The cytokine pattern indicates that follicular in contrast to diffuse synovitis is associated with the lowest serum concentrations of IFN- γ or IL-12 and the highest concentrations of TNF- α . Thus serum levels of these cytokines seem to be associated with the morphological appearance of the disease.

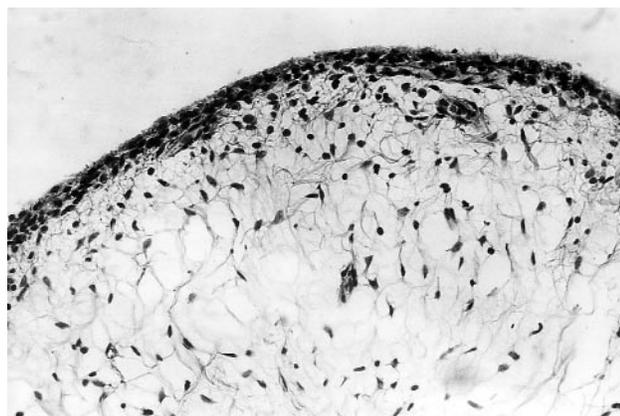
Correlations between clinical data and serum cytokine concentrations. Correlations among clinical disease activity variables or between them and cytokine concentrations in serum of all 25 RA patients are shown in Table 2. IFN- γ correlated negatively with ESR ($p < 0.05$) and IL-12 with ESR ($p < 0.01$), duration of morning stiffness ($p < 0.01$), Ritchie index



A



B



C

Figure 1. Histopathological findings in RA and OA synovia. All tissue specimens were analyzed for cell infiltrate density and the topographical organization of lymphocytes and macrophages. Representative examples are shown for the 3 groups of patients (original magnification $\times 200$). Sixteen RA synovium samples were characterized by diffuse lymphocyte infiltrates with no additional microanatomical organization (A). Lymphocytic follicular aggregates were found in 9 specimens (B). Twenty-five synovial fragments from the OA control group showed only moderate mononuclear cell infiltrates (C).

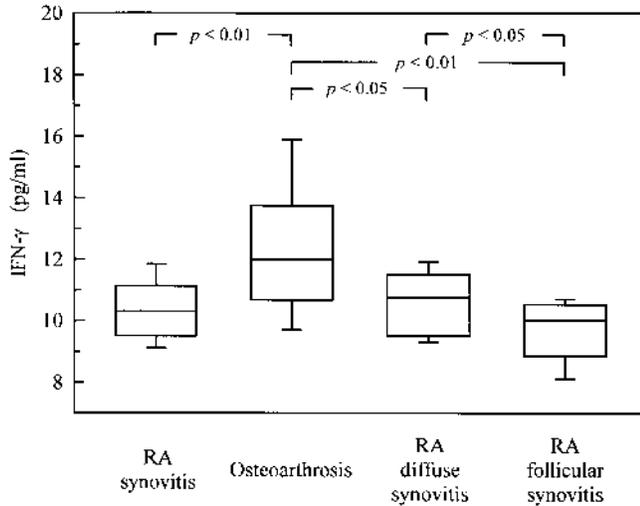


Figure 2. Serum concentration of IFN- γ in patients. IFN- γ in serum from all patients was determined using cytokine-specific sandwich ELISA. Results of cytokine assessments are shown with medians, 25th and 75th percentiles, and whiskers representing the 10th and 90th percentiles. Serum IFN- γ levels were lower in all RA patients and in groups with particular histological types of synovitis as compared to OA controls. The lowest amount of IFN- γ was found among patients with follicular synovitis differentiating them from those with diffuse disease.

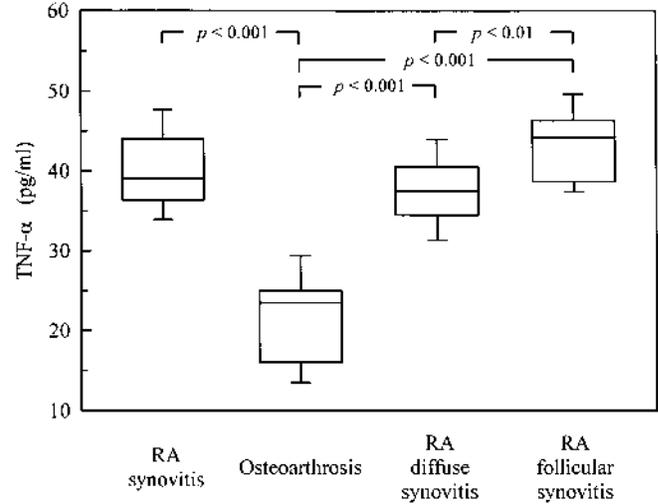


Figure 4. Serum concentration of TNF- α determined as described in Figure 2 legend. Serum TNF- α levels were higher in all patients with RA and among those with both histological forms of synovitis than in patients with OA. TNF- α was a dominant cytokine in follicular synovitis and could be clearly distinguished from the lower serum level among patients with diffuse infiltrates.

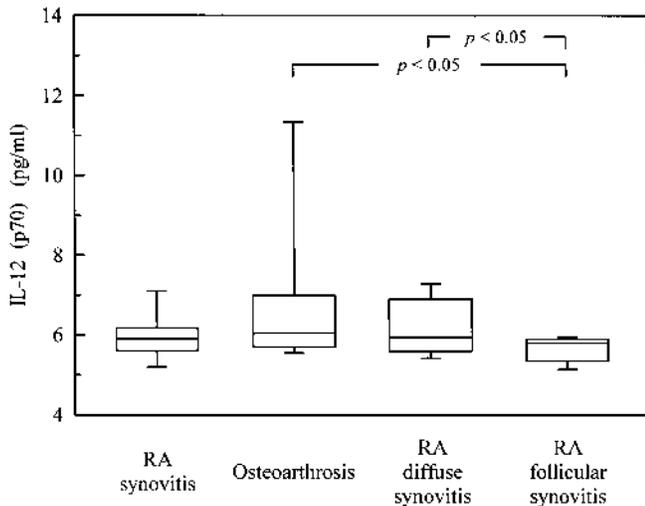


Figure 3. Serum concentrations of IL-12 are presented as described in Figure 2 legend. The level of IL-12 was lower in RA sera compared to OA, reaching statistical significance only in patients with follicular synovitis. The lowest serum concentrations of IL-12 were associated with the follicular disease type.

($p < 0.05$), and number of swollen joints ($p < 0.001$). In the case of TNF- α only a positive correlation with ESR was observed ($p < 0.05$). We did not observe any other significant correlations between measured cytokines in the 25 patients with RA or in both histological subgroups.

As shown in Table 1, 88% of RA patients with diffuse and 75% with follicular synovitis were seropositive. We observed no correlation between RF and any cytokine concentration.

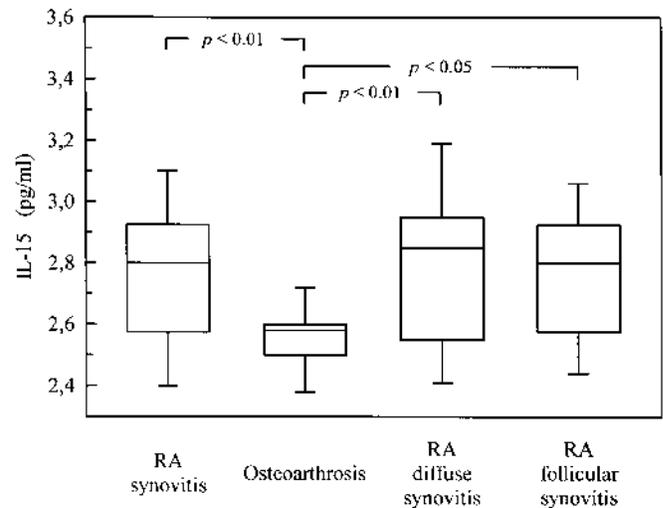


Figure 5. Serum concentration of IL-15 was analyzed and presented as described in Figure 2 legend. IL-15 was found in higher amounts in all patients with RA and in diffuse and follicular microanatomical forms of rheumatoid synovitis as compared to patients with OA. The concentration of this cytokine did not differ between patients with the 2 distinct histological patterns of the disease.

DISCUSSION

Genetic, clinical, and biological heterogeneity of RA is unquestioned^{2,11-15}. Also, microscopic evaluation of rheumatoid synovium by conventional histology has shown the existence of at least 3 distinct histological patterns¹⁸. We were able to distinguish only 2 of them. Two-thirds of our RA synovium samples were characterized by diffuse lymphocyte infiltration with no additional microanatomical organization. In the

Table 2. Correlation between cytokine concentrations and clinical variables in all 25 patients with RA.

Correlation between	r	p
IFN- γ		
ESR	-0.516	< 0.05
Morning stiffness	-0.413	< 0.07
IL-12		
TNF- α	-0.384	< 0.07
ESR	-0.509	< 0.01
Morning stiffness	-0.594	< 0.01
Ritchie index	-0.488	< 0.05
No. swollen joints	-0.662	< 0.001
TNF- α		
ESR	0.432	< 0.05
Morning stiffness	0.406	< 0.06
IL-15		
Platelet count	0.460	< 0.05
ESR		
Morning stiffness	0.846	< 0.001
Ritchie index	0.448	< 0.05
No. swollen joints	0.501	< 0.05
Morning stiffness		
No. swollen joints	0.642	< 0.001
Platelet count	0.565	< 0.01
Ritchie index		
No swollen joints	0.678	< 0.001

remaining one-third of the specimens, lymphocytic nodular aggregates with germinal center-like structure formation typical of RA were found. The formation of clear necrobiotic granulomas was not observed. However, in the previous study they were also very rare¹⁸. The report mentioned above showed that each of the histological patterns of rheumatoid synovitis might be characterized by the unique cytokine mRNA expression in the synovium¹⁸. In our study the profile of serum cytokines is associated with different morphological forms of synovitis.

As the concentration of T cell derived cytokines in peripheral blood of patients with RA is low²¹⁻²³, several methods are used to activate T cells to evaluate the capacity of peripheral T cells to produce cytokines^{22,24,25}. In such experiments IFN- γ production by stimulated T cells was shown to be lower and production of IL-4 higher than in patients with OA and/or healthy controls. However, a significantly higher percentage of IFN- γ -producing CD4+ (Th1) cells was found among stimulated peripheral blood mononuclear cells (PBMC) of RA patients in comparison with healthy subjects²⁶. Only patients in the initial stages of RA exhibited a reduced percentage of such Th1 cells in stimulated peripheral blood, compared to controls²⁷. In studies using quantitative reverse transcriptase polymerase chain reaction (RT-PCR) T cell production of cytokine mRNA by unstimulated PBMC was measured. Spontaneous IFN- γ mRNA expression was similar in active RA and healthy controls, but decreased in RA PBMC after their *in vitro* stimulation²⁸. But analyses of stimulated PBMC would not necessarily reflect spontaneous cytokine production by T cells in peripheral blood.

Thus we tried to use cytokine-specific ELISA to determine low amounts of serum IFN- γ in RA and OA. We investigated whether the concentration of this cytokine differs between patients with distinct histological types of synovitis. We found IFN- γ concentration in RA patient serum was lower compared to the OA patient controls. Numerous studies have shown that IFN- γ in RA is a dominant cytokine in synovial fluid²³ and synovium^{18,23} compared to IL-4. Furthermore, Bucht, *et al* observed increased IFN- γ mRNA expression in RA synovial fluid mononuclear cells (SFMC) compared with PBMC²⁹. In view of the predominance of articular T helper type 1 cells, the low spontaneous serum IFN- γ level in RA patients that we observed might be explained by selective Th1 cell migration into the joint or peripheral suppression of Th1 cell activity^{24,26,27}. Moreover, IFN- γ concentration was lowest among patients with follicular synovitis. The decrease in IFN- γ production in this and other studies was associated with laboratory and clinical disease activity variables^{22,24}. This would suggest that the follicular histological form of RA could be considered more severe. These results support the hypothesis of selective migration of Th1 cells, mainly in the case of follicular synovitis, into the joint, where they play the crucial role in rheumatoid synovitis⁷⁻¹⁰.

In this study the serum IL-12 profile was similar to that observed in the case of IFN- γ . Its level was also lower in RA compared to OA, but reached statistical significance only in serum of patients with follicular synovitis. In contrast to serum IL-12 concentrations, RA synovial tissues express IL-12 mRNA more strongly than those from patients with OA³⁰. Further, IL-12 mRNA expression was shown to be increased in RA SFMC compared with PBMC²⁹. The similarity of serum profiles of both cytokines in our study is compatible with a regulatory role of IL-12 in IFN- γ production. IL-12 is a macrophage derived cytokine promoting the selective expansion of Th1 cells and Th1-like cytokine response^{31,32}. Thus low levels of serum IL-12 in RA might be another explanation for decreased IFN- γ concentrations in RA sera. Another possibility is that both IL-12 and IFN- γ production by PBMC are inhibited by possible circulating serum factors²⁵. The lowest serum concentrations of IL-12 were associated with the presence of follicular synovitis, where the level of this cytokine was lower than in diffuse synovitis and could differentiate between these 2 variants of rheumatoid synovitis. Despite the similarities of serum level patterns of IL-12 and IFN- γ , we did not observe significant correlation between these 2 cytokines ($r = 0.0364$, $p = 0.872$; data not shown).

In contrast to IFN- γ or IL-12, the TNF- α level in serum was higher in RA and in both histological types of synovitis compared to OA. Van Roon, *et al* observed a similar association between serum concentration of TNF- α and IFN- γ production²⁴. In their report the decrease in IFN- γ production by stimulated RA PBMC was associated with the increase in serum TNF- α ²⁴. TNF- α is known to play a pivotal role in RA pathogenesis^{6,33,34}. 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 E₂, and platelet activating factor. These secondary mediators can amplify the inflammatory reaction, as well^{6,34,35}. In other reports TNF- α concentration was also higher in serum and synovial fluid of patients with RA than in those with OA or healthy subjects^{23,34-36}. Abundance of TNF- α was associated with increased ESR and serum α_2 macroglobulin, but with decreased hemoglobin and serum iron concentrations³⁴. As well in our study TNF- α concentration was correlated with ESR. Furthermore, in patients with follicular synovitis TNF- α was a dominant cytokine and could be differentiated from the lower serum level among those with diffuse infiltrates. For a TNF- α level ≥ 44 pg/ml, 5 (56%) of 9 patients with follicular RA had such elevated values vs one (< 10%) of 16 patients with diffuse disease ($p < 0.02$). This could suggest that follicular synovitis may reflect greater severity of RA and the serum concentration of TNF- α may predict disease activity. However, in some reports stimulated PBMC of patients with active RA produced less TNF- α compared to controls²¹. A decreased synthesis of TNF- α by stimulated RA PBMC is another reason why PBMC stimulation cannot reflect spontaneous cytokine production, although in our study its serum concentration in patients with RA was higher than in those with OA.

IL-15 produced by macrophages and other cells shares biological activity with IL-2. It is important for the growth and differentiation of T and B lymphocytes, natural killer cells, macrophages, and monocytes. IL-15 can promote TNF- α production through T cell activation. T lymphocytes activated by this cytokine induce TNF- α production by macrophages via a cell-contact-dependent mechanism. IL-15 may also induce direct TNF- α production by T cells³⁷⁻³⁹. This cytokine was found in high amounts in RA synovial fluids and was strongly expressed in RA synovium^{37,39,40}. All these observations suggest that IL-15 may play an important role in protective immune responses, but also in the pathogenesis of various chronic immuno-inflammatory diseases. In our study IL-15 concentrations in RA serum were similar to those of TNF- α . They were also significantly higher in all patients with RA and in groups with different histological types of synovitis. Similarity in serum profiles of both cytokines is probably related to the regulatory role of IL-15 in TNF- α production. IL-15 concentration was not different in the 2 distinct morphological forms of synovitis. Some similarities of serum level patterns of TNF- α and IL-15 were observed; however, there was no significant correlation between these 2 cytokines ($r = 0.193$, $p = 0.374$; data not shown).

We found that follicular and diffuse synovitis are characterized by different levels of serum cytokines. Further, associations of serum cytokine concentrations with laboratory and clinical disease activity variables were noted. Therefore the serum levels of these cytokines seem not only to be associat-

ed with the morphological appearance of the disease but also might determine its severity. However, predictive studies will require multiple samplings over a longer period, especially in patients with early diagnosed RA.

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