

# Cytokinic panel in rheumatoid arthritis and correlation with histological patterns of synovitis – active type of disease

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## Abstract

Rheumatoid arthritis (RA) is a chronically inflammatory disease of the articular synovial, with severe, progressive and irreversible articular destruction. RA pathogeny implies an autoimmune mechanism, the role of cytokines resulting from the exaggerated production of some cytokines that act as pro-inflammatory agents, being directly responsible of certain symptoms and articular destructions, and/or inadequate inhibition of certain cytokines that act as anti-inflammatory agents. *Objective.* We want to determine certain pro-inflammatory cytokines (tumoral- $\alpha$  – TNF- $\alpha$  necrosis factor, interleukine-6 – IL-6, interleukine-8 – IL-8), anti-inflammatory cytokines (interleukine-10 – IL-10) and immunomodulators (interleukin-2 – IL-2) in some RA patients serum at the active stage of the disease and correlation with histological patterns of synovitis – active type of disease. *Material and method.* The 37 patients have been grouped in stage I (9 patients), II (15 patients) and III (13 patients) according to the anatomical staging of the ARA (American Rheumatism Association), the serum levels of the cytokines being determined by ELISA technique. From the 37 patients clinical investigated only 12 were histopathological examined. *Results.* The serum levels of IL-2 have been found low in patients with RA in the active stage of the disease, the lowest values having been determined at the patients in the 3rd stage of the disease, who also have the highest values of pro-inflammatory cytokines. In the case of IL-10 the lowest values have been found at patients in the advanced stages of the disease. In the serum of RA patients with follicular synovitis TNF-alpha was a dominant cytokine compared to patients with diffuse disease, but the greatest serum level was found in patient with granulomatous synovitis. Concentrations of IL-6 and IL-2 were highest in the serum of RA patients with follicular synovitis in comparison to patients with diffuse synovitis and could distinguish RA patients with these two histological variants of the disease. *Conclusion.* In the active stage of the disease the symptoms are a consequence of the interleukin pro and anti-inflammatory game: high serum levels of pro-inflammatory cytokines are accompanied by low serum levels of anti-inflammatory cytokines. Cytokines can be used as monitorization markers of the acutization period in RA, increase in serum levels of pro-inflammatory cytokines showing the progression from the inactive phase to a new period of activity of the disease. The association between distinct histological appearance of rheumatoid synovitis and serum cytokine profile and diverse clinical activity of disease seems to confirm its heterogeneity.

**Keywords:** rheumatoid arthritis, pro-inflammatory, anti-inflammatory and immunomodulators cytokines.

## Introduction

Rheumatoid arthritis (RA) is a chronically inflammatory disease of the articular synovial, with severe articular destruction, progressive and irreversible. Through its systemic and autoimmune character, RA, the most frequent form of inflammatory rheumatism, complete the panorama of the autoimmune systemic diseases. These diseases have, as specific, the presence of dispersed autoantigenes in the entire organism (organs and tissues), autoantigenes that produce the formation of antibodies with different characteristics and extended fixation. The destructive mechanism that appear in these illnesses are, especially, consequences of type III hypersensitivity phenomenon (immune complex illness) [15, 16].

### Morphological alteration in rheumatoid arthritis

The joint inflammation from RA begins in synovium was during the time produce rheumatoid synovitis. In the early stage were observed acute inflammatory changes, rapidly substituted by a chronic inflammation with a proliferative and infiltrative dominant feature

[2, 19, 22]. The main morphological alterations from rheumatoid synovitis are: exudation, inflammatory cell infiltration, cellular proliferation and granulomatous tissue formation (pannus) [2, 19, 20, 22-24].

Lymphocytes are the major cellular population, they representing about 70-80%, from which 92% are CD4<sup>+</sup> helper T cells and 8% CD8<sup>+</sup> suppressor T cells. Initial they have a diffuse or nodular arrangement and later in follicles with germinative centers. Plasma cells with Russel bodies (which contain rheumatoid factors, peculiar to rheumatoid synovitis) appear late and progressive increase to 10-15%. Macrophages that process the antigens and phagocyte the immune complexes represent only 5-10% of inflammatory cells [2, 19, 23, 24].

The granulomatous tissue formation (pannus) consists in: extensive neoangiogenesis, stromal fibroblastic proliferation, inflammatory infiltrate. This stage is crucial for disease progression, because is the cause of adjacent structures erosions which finally produce the joint destruction [2, 19, 22].

RP, like any other inflammatory process, means aside from activation of cells with phagocytic

functions and the production of mediation products of inflammation and the secretion of a complex cytokine network with role in “mediating and modulating” the inflammatory answer [3].

These cytokines amplify the cellular answer and stimulates, uncharacteristically, polyclonal, B cells in inflammatory focus, with IgG secretion altered with unprecise specificity, against which an anti-IgG antibody will appear (rheumatic factor-RF). IgG immune complex RF-altered determine phagocytosis, liberation of lizozomal enzymes and oxygen active species with activation of the complement in synovial liquid that determines growth in liberating mediators of the inflammation and chemotactic factors [16].

In the case of RP, cytokines control not only the proliferation, the activation, the traffic and the homing of immunocompetent cells in articulations, also the function of synovial cells from cartilages, bone, endothelia.

Cytokine rolls in the pathogenesis of this disease can be a consequence of the over-production of cytokines (proinflammatory agents that respond directly to same symptoms and to articular destruction) and/or inadequate inhibition of certain cytokines (anti-inflammatory agents), the balance between them having a decisive effect over the evolution of the disease. Also, some of their cytokines or receivers can act as markers of disease activity or of immune activation and could be used in monitoring treatment, while other cytokines or anti-cytokines were already used in treatment RP [12-14].

### Objective

RA, the most frequent form of inflammatory rheumatism, it is a systemic disease witch involves systemic humoral and cellular autoimmune mechanisms with important changes of cytokines' network.

*Starting from these literature data we want to make a study that has as objective determining serum levels of pro-inflammatory, anti-inflammatory and immunomodulating cytokines, in a group of patients suffering from RA in the active stage of the disease and correlation with histopathological alteration of synovitis.*

### Material and methods

The group of study was composed of 37 patients diagnosed with RA active form of the disease, coming from medical clinics of the Emergency County Hospital of Craiova, C.F. Hospital, or from the ambulatory.

The subjects are in the I stage-early stage – 9 patients (X-ray show lack of erosive lesions; with or without osteoporosis), II stage-moderate – 15 patients (X-ray show visible osteoporosis; no articular deformities but with atrophy the muscles next to the articulation; with or without nodules and tenosinovites) and III stage-severe – 13 patients (osteoporosis, damage to the bone and to cartilage showed on X-ray; articular deformation, but without fibrosis or bone ankylosis; marked and extended muscle atrophic) according to anatomical standardization ARA (American

Rheumatism Association) [1].

The criteria used to define the active stage of the disease were clinical (waking up during the night at least once because of pain; morning pain for minimum 30 min; at least 3 joints tumefied) and biological criteria (VSH over 50 mm / 1 h; C reactive protein-CRP over 30 ng/ml; circular immune complex - CIC over 100 mg%).

From the 37 patients clinical investigated only 12 were histopathological exanimate. From this patients were obtained by blind needle biopsy of clinically involved knee joint synovial tissue biopsies. These were fixed formalin (10%) and embedded in paraffin (Giemsa, haematoxylin and eosin staining) to be used for diagnosis and scoring of the degree of inflammation.

Before beginning immunosuppressive treatment, we determined through the immunoenzymatic technique ELISA, serum levels of certain proinflammatory cytokines (tumor necrosis factor- $\alpha$  – TNF- $\alpha$ , IL-6, IL-8), anti-inflammatory cytokines (IL-10) and immunomodulators (IL-2). We used the next kits for human interleukins: for IL-2 h-Interleukin-2 Roche Diagnostics GmbH; Pelikine™ human ELISA kit Nederlands for IL-6 and TNF- $\alpha$ ; Milenia Biotec GmbH for IL-8 and IL-10.

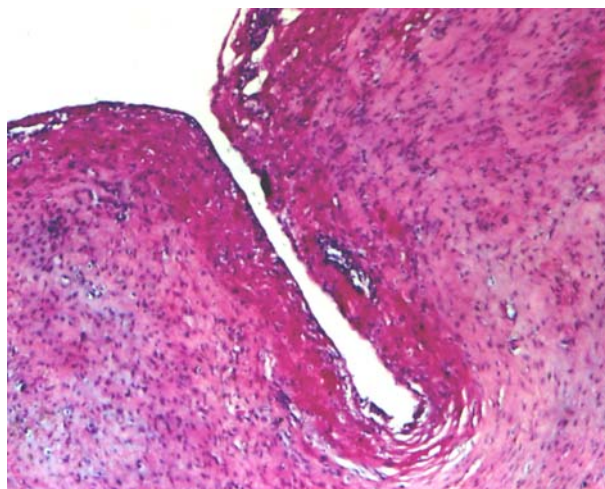
The results obtained were compared with the ones from a control group, composed of 25 healthy subjects. IL-2 serum values in these healthy individuals were between 5.4–50 pg/ml, TNF- $\alpha$  was under 10 pg/ml, IL-6 under 11 pg/ml, IL-8 under 31 pg/ml, IL-10 between 2–24 pg/ml.

## Results

### 1. Histopathological alteration

After histological analysis RA synovial biopsies were divided into 3 distinct types.

Three samples were characterized by diffuse lymphocyte infiltrates with no additional microanatomical organization (Figures 1 and 2), five specimens had lymphocytic aggregates with germinal center-like structures and four were found with granulomatous inflammatory reaction.



**Figure 1 – Rheumatoid synovitis. Synovial exulceration and diffuse chronic inflammatory infiltrate (H.E. staining,  $\times 40$ )**

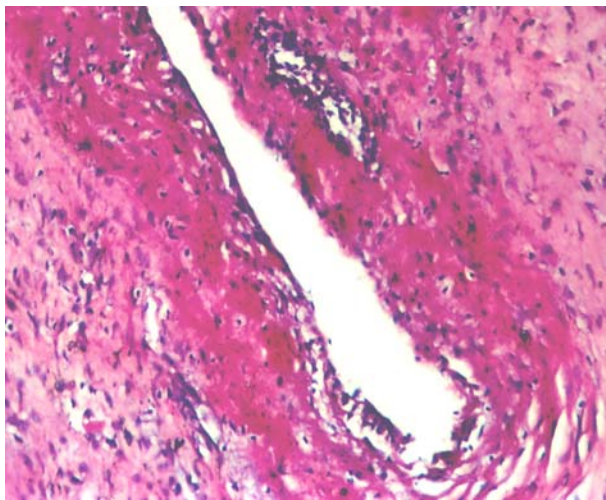


Figure 2 – Rheumatoid synovitis, detail (H.E. staining,  $\times 100$ )

## 2. Cytokines

### a. Cytokines immunomodulators

IL-2 serum concentrations of IL-2 RA patients, comparatively to the healthy group, showed low average values.

In the active stage of the disease average concentrations are much lower; their lowest values being determined in stage III (Figure 3).

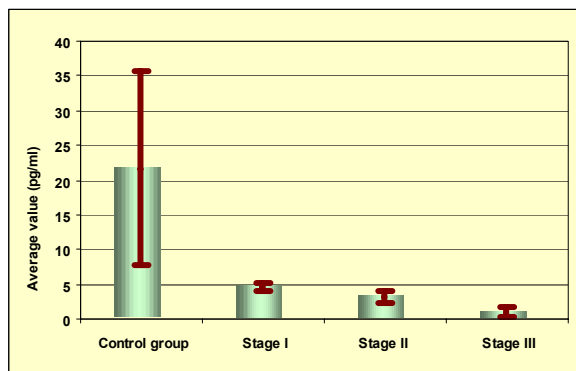


Figure 3 – Average values and standard IL-2 deviation in the healthy group and in study groups

### b. Pro-inflammatory cytokines

For all three cytokines of this study group - *TNF-alfa*, *IL-6*, *IL-8* - high serum values were determined at patients with RA active phase of the disease, higher serum levels being shown in the third stage of the disease (Figures 4-6).

### c. Anti-inflammatory cytokines

*IL-10* serum values in the studied group were different not only when compared to the healthy group, but also depending on the stage of the disease.

So, at all RA patients in the active stage, we found low *IL-2* levels (stages I, II) and even very low (stage III) (Figure 7).

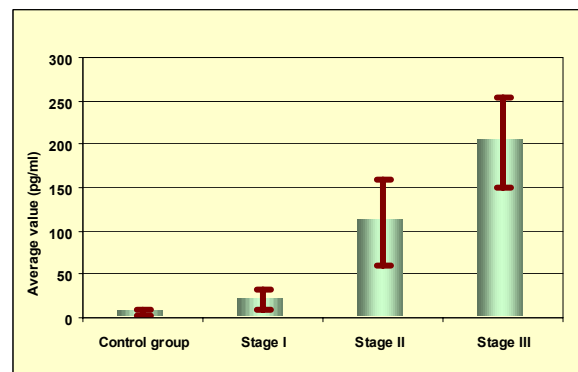


Figure 4 – Average values and standard TNF-alfa deviation in the healthy group and in study groups

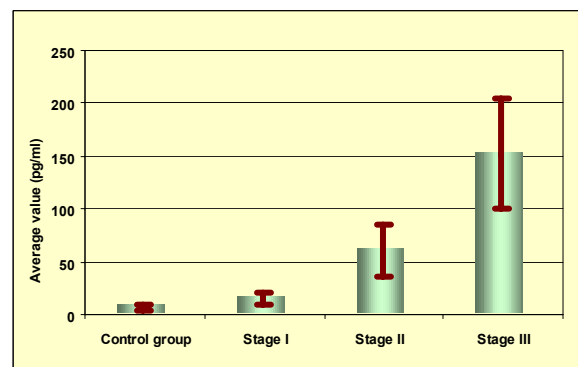


Figure 5 – Average values and standard IL-6 deviation in the healthy group and in study groups

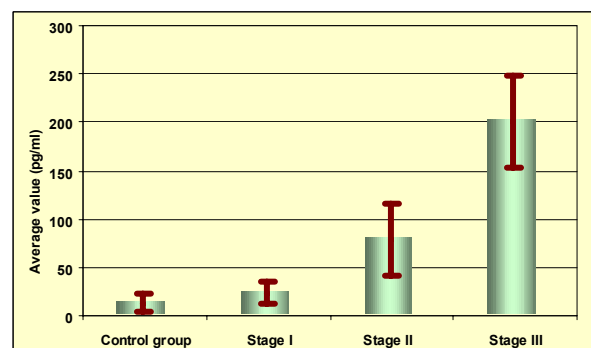


Figure 6 – Average values and standard IL-8 deviation in the healthy group and in study groups

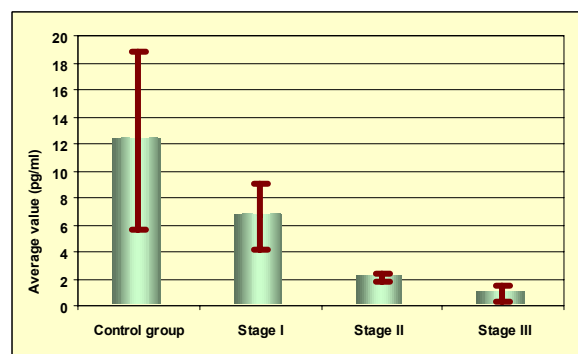


Figure 7 – Average values and standard deviations of the *IL-10* in the healthy group and in three groups of patients suffering from rheumatoid arthritis

In Table 1 we can observe a powerful growth tendency in serum levels of pro-inflammatory cytokines (TNF-alfa, IL-6 and IL-8) at the same time with the developmental stage of the disease, meanwhile the values of IL-2 and IL-10 cytokines decreased.

We determined for TNF-alfa, IL-6 and IL-8 high serum values, the highest being in stage III of the disease. At some patients, serum levels of IL-2 and IL-10 are low in stages I and II and very low in stage III.

**Table 1 – Average values and standard deviation in the free cytokines depending on the evolution stage of RA**

		IL-2	IL-10	TNF-alfa	IL-6	IL-8
Control group	No of subjects	25	25	25	25	25
	Average value	21.70	12.27	5.80	7.02	13.22
	Standard deviation	14.05	6.59	2.92	2.92	8.97
Stage I	No of subjects	9	9	9	9	9
	Average value	4.48	6.64	20.38	15.59	23.48
	Standard deviation	0.58	2.46	10.85	6.12	11.48
Stage II	No of subjects	15	15	15	15	15
	Average value	3.13	2.10	110.23	61.01	78.54
	Standard deviation	0.77	0.30	49.08	24.85	36.82
Stage III	No of subjects	13	13	13	13	13
	Average value	0.98	0.89	202.78	152.30	200.07
	Standard deviation	0.70	0.60	52.22	52.45	47.32
Total	No of subjects	62	62	62	62	62
	Average value	10.36	6.61	74.48	51.79	69.69
	Standard deviation	12.94	6.56	85.41	62.29	77.87

Now we present for your consideration the values obtained from statistics testing that show the significance of this increase (Table 2).

**Table 2 – Values of the statistic Kruskal-Wallis test for IL-2, IL-10, TNF-alfa, IL-6 and IL-8**

IL-2	Control group	Stage II	Stage III
Stage I	0.00000259		
Stage II	0.00000881	0.00008223	
Stage III	0.00000014	0.00000000	0.00000003
IL-10	Control group	Stage II	Stage III
Stage I	0.00099261		
Stage II	0.00000006	0.00056892	
Stage III	0.00000001	0.00007357	0.00000423
TNF-alfa	Control group	Stage II	Stage III
Stage I	0.00405831		
Stage II	0.00000098	0.00000413	
Stage III	0.00000001	0.00000002	0.00006106
IL-6	Control group	Stage II	Stage III
Stage I	0.00294954		
Stage II	0.00000079	0.00000342	
Stage III	0.00000037	0.00000077	0.00002392
IL-8	Control group	Stage II	Stage III
Stage I	0.03197250		
Stage II	0.00000651	0.00004172	
Stage III	0.00000001	0.00000000	0.00000013

Charts with  $p$  result values of the *statistic Kruskal-Wallis test*, at each element (IL-2, IL-10, TNF-alfa, IL-6 and IL-8), comparing the healthy group to each of the three stages (on the „witness” column of each chart) as between the three stages as well (on two columns of each chart).  $p$  values will be explained as follows:

- if  $p > 0.05$ , the difference between the average values of the 2 compared groups, is insignificant;
- if  $p < 0.05$  the difference between the average values of the 2 compared groups, is significant;

- if  $p < 0.01$  the difference between the average values of the 2 compared groups, is highly significant;
- if  $p < 0.001$  the difference between the average values of the two compared groups, is extremely significant.

## Discussions

The association between distinct histological appearance of rheumatoid synovitis and serum cytokine profile and diverse clinical activity of disease seems to confirm its heterogeneity.

RA indicates not only the activation of certain phagocytic function cells and producing inflammation mediators but also over-production and/or inadequate cytokine inhibition.

Some cytokines, such as TNF, IL-6, IL-8 have proinflammatory effects and others such as IL-10, have anti-inflammatory effects. Within the inflammatory answer a true hierarchy can be established between cytokines participating in producing and intensifying biological effects, cytokines production being able to stimulate or on the contrary, block the production of other cytokines [5, 8-10].

Cytokines that stimulate the inflammation recognize as “target” cell a heterogeneous cellular population represented by neutrophiles, macrophages, endothelial cells, hepatocytes, etc.

These cytokines act over neutrophiles through chemotactic stimulation and phagocytosis stimulation, and over endothelial cells through increase in adhesion towards white blood cells and stimulation of production of inflammation mediators and procoagulation factors. TNF stimulates the IL-6, IL-8 production from originating cells.

Aside from IL-6, TNF act on the hepatocyte stimulating the acute phase protein production and over the central nervous system giving fever, anorexia and

sleepiness. IL-6 has, on its turn proinflammatory effects synergic to TNF, assuring the amplification of this action, IL-8 is the most important chemotactical factor for neutrophils and induces liberation of proteolytic enzymes responsible for local tissular “injury” [5, 6, 8-11, 15].

On the other hand, IL-10, act as anti-inflammatory factor through inhibiting inflammatory cytokine synthesis [7]. To this category of factors we add solvent receptors of TNF that through fixating circular cytokines, stop this action on membrane receptors. There are, inhibitors or natural opposites of proinflammatory cytokines (natural antagonist of TNF- $\alpha$ ) [5, 6, 8-10].

IL-2, one of the main cytokines involved in mediation and immune response modulation, is the main factor that amplifies T cell helper response cytotoxic cell response and stimulates antibodies production. In RA patient lymphocytes not only produce imprecisely IL-2 but they are also hyporesponsive to IL-2 explaining according to some authors, the proliferative response low in mitogens and hypersensitivity delayed reactions reduced at patients with RA.

In RA, like in other autoimmune disease, data in literature signal high concentrations of its solvent receptor (sIL-2R) [12, 13, 15, 16].

Concentrations of IL-2 were highest in the serum of RA patients with follicular synovitis in comparison to patients with diffuse synovitis and could distinguish RA patients with these two histological variants of the disease.

*TNF- $\alpha$* . It is a pro-inflammatory cytokines with beneficial role in the physiological immune response, but its inadequate production determines inflammation tissular destruction and organ affection. It is one of the cytokines secreted during RA, orchestrating the entire articular inflammatory process.

It is the main starter, being responsible for most RA symptoms and signs and for the appearance and maintenance of the unspecific inflammatory biological syndrome with increase in proteins in the acute phase [4, 8, 12, 13].

In the active stage of the disease, patients in any of the three stages showed high and very high average values of TNF- $\alpha$  as the superior stage of the disease. This observation comes as one more argument regarding the pro-inflammatory effect of this cytokine, TNF- $\alpha$  being used as an evolution marker of RP as well.

In the serum of RA patients with follicular synovitis TNF- $\alpha$  was a dominant cytokine compared to patients with diffuse disease, but the greatest serum level was found in patient with granulomatous synovitis.

IL-6 is a multifunctional cytokine that act on the numerous target cells, being involved in the immune response, in the inflammatory response and in hematopoiesis. It stimulates the protein synthesis in the acute phase differentiating B and T active lymphocytes, differentiating cytotoxic T lymphocytes and the cytotoxic function with the perforine formation [3, 11, 17, 18, 21].

IL-6 serum levels at patients suffering from RA comparatively to the healthy group, showed high levels

of the average. IL-6 variations were shown depending on the evolving stage of the disease: with almost normal values in the stage I and slowly high in the II stage, and with the highest values in stage III.

Concentrations of IL-6 were highest in the serum of RA patients with follicular synovitis in comparison to patients with diffuse synovitis and could distinguish RA patients with these two histological variants of the disease.

IL-8 is the most powerful chemotactical factor for neutrophils and on a lower scale for eosinophils, basophils and T lymphocytes.

It activates neutrophils and induces degranulation with lactoferrin liberation. Its production is stimulated by IL-1, TNF- $\alpha$ , IL-6.

In the case of IL-8 we have also determined high serum values at patients with rheumatoid polyarthritis – active phase of the disease, the highest serum levels being registered in the III stage.

IL-10, cytokines known under the name „inhibition factor of cytokine secretion”, is an endogenous major anti-inflammatory mediator through inhibiting pro-inflammatory production (IL-6, TNF- $\alpha$ , IL-8).

It has an immunosuppression effect by inhibiting the macrophages function of antigen presentation cell (reduces the expression of the HLA molecules class II, adhesion molecules ICAM-1), and through blocking the activation capacity of Th lymphocytes (CD4+), so suppressing the specific immune response. At the same time, some cytokine has immunostimulating effect over the population of T cytotoxic lymphocytes and B-lymphocytes [17, 18].

## ☒ Conclusions

The symptoms in the active stage are a consequence of the interleukin pro and anti-inflammatory game.

Cytokines can be used as monitoring markers of the acutization period in RA increase in serum levels of pro-inflammatory cytokines announcing the evolution from inactivity to a new period of activity of the disease.

The tendency of backward proportions: high serum levels of pro-inflammatory cytokine are accompanied by low serum levels in anti-inflammatory cytokines (IL-10) and immunomodulating ones (IL-2), can be used in medical practice.

Thus, determining only one of the 2 cytokines pro and anti-inflammatory indirectly suggests the evolution tendency of another parameter in the some group, just like the evolution of the parameters in the other group.

Between studied parameters, TNF- $\alpha$  is more useful in diagnosing in the active stage of the disease. It can be a marker in disease evolution, its determination being made in the early stages. At only RP patients increase in TNF- $\alpha$  values predict an acutization of the disease.

The association between distinct histological appearance of rheumatoid synovitis and serum cytokine profile and diverse clinical activity of disease seems to confirm its heterogeneity.

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