The significance of serum matrix metalloproteinase 3 in patients with early rheumatoid arthritis
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Chapter 5

Serum matrix metalloproteinase 3 levels during treatment with sulphasalazine or the combination of methotrexate and sulphasalazine in patients with early rheumatoid arthritis

Marcel D. Posthumus, Pieter C. Limburg, Johanna Westra, Miek A. van Leeuwen, and Martin H. van Rijswijk.

SUMMARY

Objective. To determine the effects of treatment with sulphasalazine (SSZ) or the combination of methotrexate (MTX) and SSZ on serum matrix metalloproteinase 3 (MMP-3) levels in patients with early rheumatoid arthritis (RA).

Methods. Eighty-two patients with early RA (symptoms < 1 year and DMARD-naive at presentation) were selected who had been treated with SSZ (2000 mg/day) or with the combination of MTX (7.5-15 mg/week) and SSZ. Serum MMP-3 levels, C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), swollen joint count (SJC), tender joint count (TJC), Ritchie articular index (RAI), and the disease activity score (DAS) were determined at 4 week intervals during a follow-up of 28 weeks for each treatment group. Response was based on clinical grounds and CRP at 12, 20, and 28 weeks.

Results. SSZ responders (n=52) had lower baseline values of serum MMP-3, CRP, and ESR, compared to partial/non-responders (n=30), but did not differ in joint scores and DAS. In the SSZ responder group all variables decreased. In the SSZ partial/non-responders CRP, ESR, and SJC decreased in contrast to serum MMP-3, TJC, RAI, and DAS-3. After addition of MTX all variables decreased in 24 out of the 30 patients who had shown a partial or no response on SSZ. In the SSZ responders there was a delayed decrease in serum MMP-3 compared to CRP.

Conclusions. Serum MMP-3 levels decrease in early RA patients who respond to SSZ or to the combination of MTX and SSZ. In patients who respond to SSZ the changes in serum MMP-3 levels indicate a delayed response compared to CRP.

Key words: serum matrix metalloproteinase 3, stromelysin 1, early rheumatoid arthritis, disease modifying antirheumatic drugs, sulphasalazine, methotrexate.
INTRODUCTION
In patients with rheumatoid arthritis (RA) matrix metalloproteinase 3 (MMP-3 = stromelysin 1) is of interest because this proteolytic enzyme is thought to play a prominent role in the pathogenesis of matrix degradation \(^1\text{-}^3\) even though it is not the only key enzyme \(^4\). In RA MMP-3 is locally produced and activated within the inflamed joint and released into the peripheral blood. Systemic MMP-3 levels are a reflection of local synthesis. As such, serum MMP-3 can be used as a systemic marker of local joint inflammation \(^5\text{-}^9\) and/or destruction \(^10,11\).

Studies concerning MMPs are not only of importance for the unraveling of the pathogenesis of RA but also for analyzing mechanisms of drug therapy. For example specific MMP-inhibitors may uncouple the relation between surrogate markers of joint inflammation (for example C-reactive protein (CRP)) and joint damage. Radiological progression might be stopped by these new agents without inhibiting inflammation and acute phase response. In that case, especially in early disease, new markers like systemic MMP, including its level of activation, are essential \(^12\).

Sulphasalazine (SSZ) and methotrexate (MTX) are disease modifying antirheumatic drugs (DMARDs), widely used in early RA to suppress disease activity and thereby to prevent or delay joint destruction \(^13,14\). Studies investigating the effects of SSZ or MTX on MMPs, especially on MMP-3, are rare. Therefore we analyzed in a prospective follow-up study of early RA patients (symptoms < 1 year) whether serum matrix metalloproteinase 3 levels are influenced by treatment with SSZ or the combination of MTX and SSZ. In responders to these treatments we also analyzed the influence on serum MMP-3 levels in relation to the influence of treatment on conventional clinical and other biochemical disease activity measures.

PATIENTS AND METHODS
Patients
Eighty-two patients with early rheumatoid arthritis were selected from a cohort of patients with RA, according to the 1987 ACR criteria \(^15\), with joint symptoms existing less than one year at presentation and who had not previously received disease modifying anti rheumatic drugs (DMARDs). These patients participated in a prospective follow-up study at the department of Rheumatology at the Groningen University Hospital. Clinical and laboratory investigations were performed at monthly intervals during a follow-up of 28 weeks.

Clinical markers of disease activity
Fifty-two peripheral joints were examined for tenderness and soft tissue swelling. The following articular indices were determined: Ritchie articular index (RAI) \(^16\),
tender joint count (TJC), swollen joint count (SJC) and the disease activity score (DAS) according to Van der Heijde with 3 variables 17.

Laboratory analysis
Serum MMP-3 levels were determined by a MMP-3 ELISA developed at our laboratory. In short, 96 well plates were precoated with F(ab)_2 fragment of goat-anti-mouse IgG, 1µg/ml (Jackson Immunoresearch Labs, West Grove, PN, USA). Next a mouse monoclonal antibody against human MMP-3, clone 10D6 (R&D Systems, Abingdon, UK) was coated at 0.1 µg/ml. Serum samples were analyzed in two-fold serial dilutions in high performance ELISA buffer (CLB, Amsterdam, NL) and incubated during 1 hour. After washing bound MMP-3 was detected with a polyclonal rabbit anti-human MMP-3 (AB 810, Chemicon, Temecula, CA, USA), followed by horseradish-peroxidase-labelled F(ab)_2 fragment of goat-anti-rabbit IgG (Zymed, San Francisco, CA, USA). Peroxidase activity was determined using tetramethylbenzidin as substrate. MMP-3 levels were calculated at the linear range of the assay from a standard curve (3-400 ng/ml) using pro-MMP-3, which was purified from serum free supernatant of IL-1β stimulated rheumatoid arthritis synovial fibroblasts 18. The intra-assay coefficient of variation (CV) was 6.8%, the inter-assay CV 8.8%. With an immunoblot we demonstrated that both the monoclonal and the polyclonal antibody reacted with active MMP-3, pro-MMP-3 as well as with MMP-3 bound to tissue inhibitor of matrix metalloproteinases (TIMPs). Furthermore it was demonstrated that rheumatoid factors do not react in this assay and do not interfere with measurement of MMP-3 (data not shown). For normal values of serum MMP-3 the 95 percentile in healthy bloodbank donors (n=80) was used (female < 20 ng/ml, male < 60 ng/ml).

CRP was measured by ELISA 19, ESR according to Westgren. IgM rheumatoid factor (RF) was measured by Dade/Behring BN-2 nephelometer (normal value: < 15 IU/ml).

DMARD treatment and definition of response
During follow-up patients were treated with non-steroidal anti-inflammatory drugs (NSAIDs) as indicated clinically. It is our policy to use an intensive treatment strategy in early RA patients consisting of an immediate start of DMARDs and rapid adjustment of dosage and/or drugs (step up method) in case of an insufficient response 20. DMARD treatment was instituted according to the following guidelines: at study entry all patients with active disease started with SSZ 500 mg/day, increased to 2000 mg/day in weekly increments of 500 mg. In case of an insufficient response at week 12, MTX 7.5 mg/week could be added to SSZ 2000 mg/day. At week 20 the
MTX dose could be increased to 15 mg/week. Corticosteroids were allowed as 
adjuvant therapy.

Decisions about intensifying the treatment with DMARDs, at 12 and 20 weeks 
were discussed by an independent observer and the patients rheumatologist. These 
decisions were based on clinical markers of disease activity in combination with the 
CRP level as effect measures.

Patients with a sufficient response, ≥ 50% reduction in joint scores or CRP (or 
normalization of CRP (< 5 mg/l)) were assumed to be SSZ responders. A SSZ partial 
responder showed some improvement but less than 50% in joint scores or CRP. SSZ 
non-responders did not show any response or had deteriorated.

Statistical analysis
Chi-square and Mann Whitney U were used to detect differences between groups. 
The Friedman test with the Dunnett’s post test were used to analyze differences 
within groups.

RESULTS
All patients started with SSZ. After 12 weeks of treatment there were 2 groups: SSZ 
responders (n=52), and SSZ partial/non-responders (n=30). Of these 30 SSZ 
partial/non responders 24 patients had a sufficient response on subsequent 
combination therapy with MTX/SSZ (MTX/SSZ responders (n=24)). The 6 
MTX/SSZ non responders were subsequently treated with the combination of 
MTX/cyclosporine.

SSZ responders
After 12 weeks of SSZ treatment the response was determined as described in the 
method section. Sixteen patients (31%) had ≥ 50% reduction in CRP, 13 patients 
(25%) ≥ 50% reduction in joint scores and 23 patients (44%) ≥ 50% in both 
variables.

In table 1 the baseline characteristics of the SSZ responders (n=52) and the SSZ 
partial/non responders (n=30) are shown. The SSZ responders had lower baseline 
values of serum MMP-3, CRP, and ESR levels but did not differ in joint scores or 
DAS.
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**TABLE 1.** Baseline characteristics of 82 patients with early rheumatoid arthritis; sulfasalazine (SSZ) responders (n=52) and SSZ partial/non-responders (n=30).

<table>
<thead>
<tr>
<th></th>
<th>SSZ responders (N=52)</th>
<th>SSZ partial/non responders (N=30)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>49.8 (20.3-78.6)</td>
<td>51.4 (21.5-66.2)</td>
</tr>
<tr>
<td>Gender, female/male</td>
<td>37/15 (71)</td>
<td>16/14 (53)</td>
</tr>
<tr>
<td>IgM-RF positive (%)</td>
<td>45 (87)</td>
<td>26 (87)</td>
</tr>
<tr>
<td>Tender joint count</td>
<td>12 (0-35)</td>
<td>12 (0-38)</td>
</tr>
<tr>
<td>Swollen joint count</td>
<td>11 (3-29)</td>
<td>12 (4-28)</td>
</tr>
<tr>
<td>Ritchie articular index</td>
<td>6 (0-34)</td>
<td>6 (0-24)</td>
</tr>
<tr>
<td>Disease activity score</td>
<td>3.52 (1.73-5.69)</td>
<td>3.76 (1.88-5.55)</td>
</tr>
<tr>
<td>Serum MMP-3 (ng/ml.)</td>
<td>64 (14-495)</td>
<td>112 (17-1290)</td>
</tr>
<tr>
<td>CRP (mg/l.)</td>
<td>14.5 (2-110)</td>
<td>33 (2-139)</td>
</tr>
<tr>
<td>ESR (mm/h.)</td>
<td>27 (5-114)</td>
<td>40 (9-114)</td>
</tr>
<tr>
<td>Sharp &gt; 0 (%)</td>
<td>32 (63)</td>
<td>16 (53)</td>
</tr>
</tbody>
</table>

Values are the median and range. Chi-square for gender, IgM-RF positivity and Sharp > 0. Mann Whitney U for the other variables.

¶: SSZ-responders vs. SSZ-partial/non-responders p < 0.01.

After 12 weeks of SSZ all variables were significantly decreased in the SSZ responders (table 2). In the SSZ partial/non responders CRP, ESR, and SJC decreased in contrast to serum MMP-3, TJC, RAI and DAS-3 (table 3).

**TABLE 2.** Median values of clinical and biochemical variables in SSZ responders (n=52) during 28 weeks of follow-up.

<table>
<thead>
<tr>
<th>Week</th>
<th>MMP-3 (ng/ml)</th>
<th>CRP (mg/l)</th>
<th>ESR (mm/h)</th>
<th>SJC</th>
<th>TJC</th>
<th>RAI</th>
<th>DAS-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>64</td>
<td>14</td>
<td>27</td>
<td>11</td>
<td>12</td>
<td>6</td>
<td>3.52</td>
</tr>
<tr>
<td>4</td>
<td>68</td>
<td>13</td>
<td>22</td>
<td>9</td>
<td>7</td>
<td>5</td>
<td>2.97</td>
</tr>
<tr>
<td>8</td>
<td>56</td>
<td>9</td>
<td>15</td>
<td>7</td>
<td>6</td>
<td>4</td>
<td>2.54</td>
</tr>
<tr>
<td>12</td>
<td>¶</td>
<td>55</td>
<td>8</td>
<td>13</td>
<td>5</td>
<td>4</td>
<td>2.32</td>
</tr>
<tr>
<td>16</td>
<td>44</td>
<td>6</td>
<td>10</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>1.89</td>
</tr>
<tr>
<td>20</td>
<td>38</td>
<td>4</td>
<td>8</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>1.96</td>
</tr>
<tr>
<td>24</td>
<td>39</td>
<td>4</td>
<td>8</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1.72</td>
</tr>
<tr>
<td>28</td>
<td>¶</td>
<td>38</td>
<td>4</td>
<td>9</td>
<td>2</td>
<td>1</td>
<td>1.56</td>
</tr>
</tbody>
</table>

¶: after 12 weeks of sulphasalazine the response was determined. In the SSZ responders all variables were significantly decreased at week 12 and at the end of the follow-up, at 28 weeks (P < 0.05, Friedman test).


TABLE 3. Median values of clinical and biochemical variables in SSZ partial/non responders (n=30) at study entry and at 4, 8 and 12 weeks.

<table>
<thead>
<tr>
<th>Week</th>
<th>MMP-3 (ng/ml)</th>
<th>CRP (mg/l)</th>
<th>ESR (mm/h)</th>
<th>SJC</th>
<th>TJC</th>
<th>RAI</th>
<th>DAS-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>112</td>
<td>33</td>
<td>40</td>
<td>12</td>
<td>12</td>
<td>6</td>
<td>3.76</td>
</tr>
<tr>
<td>4</td>
<td>136</td>
<td>35</td>
<td>36</td>
<td>13</td>
<td>14</td>
<td>7</td>
<td>3.67</td>
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<td>8</td>
<td>121</td>
<td>30</td>
<td>35</td>
<td>12</td>
<td>9</td>
<td>7</td>
<td>3.40</td>
</tr>
<tr>
<td>12 †</td>
<td>132</td>
<td>17</td>
<td>30</td>
<td>9</td>
<td>10</td>
<td>6</td>
<td>3.27</td>
</tr>
</tbody>
</table>

P ns < 0.01 < 0.01 0.03 ns ns ns

†: after 12 weeks of sulphasalazine the response was determined. In the SSZ partial/non responders CRP, ESR and swollen joint count were significantly decreased (P < 0.05, Friedman test) in contrast to serum MMP-3, TJC, RAI and DAS-3.

**Effects of MTX in SSZ partial/non responders**

Study variables at the moment of the addition of MTX were taken as a new baseline (week 0 MTX). In most patients MTX was added at week 12 or 16. Due to this variation the values at week 12 in SSZ partial/non responders (table 3) were not exactly the same as the values at week 0 MTX (table 4).

Ten patients (42%) had a ≥ 50% reduction in CRP, 4 patients (16%) a ≥ 50% reduction in joint scores and 10 patients (42%) a ≥ 50% in both variables. Because of the small size of the non responder group (n=6) only the responders on MTX/SSZ (n=24) were evaluated.

The effects of adding MTX to SSZ partial/non-responders during a follow-up of 28 weeks is shown in table 4. All variables decreased significantly.

TABLE 4. Median values of clinical and biochemical variables in MTX/SSZ responders (n=24) during 28 weeks after the addition of MTX.

<table>
<thead>
<tr>
<th>Weeks</th>
<th>MMP-3 (ng/ml)</th>
<th>CRP (mg/l)</th>
<th>ESR (mm/h)</th>
<th>SJC</th>
<th>TJC</th>
<th>RAI</th>
<th>DAS-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>145</td>
<td>29</td>
<td>26</td>
<td>11</td>
<td>11</td>
<td>6</td>
<td>3.31</td>
</tr>
<tr>
<td>4</td>
<td>102</td>
<td>15</td>
<td>18</td>
<td>7</td>
<td>6</td>
<td>5</td>
<td>2.72</td>
</tr>
<tr>
<td>8</td>
<td>79</td>
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<td>7</td>
<td>3</td>
<td>3</td>
<td>2.61</td>
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<td>5</td>
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<td>16</td>
<td>64</td>
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<td>2.16</td>
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<td>7</td>
<td>10</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>1.89</td>
</tr>
<tr>
<td>24</td>
<td>45</td>
<td>6</td>
<td>7</td>
<td>3</td>
<td>2</td>
<td>2</td>
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<td>6</td>
<td>10</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1.68</td>
</tr>
</tbody>
</table>

P †: During follow-up all variables decreased significantly (P < 0.05, Friedman test)

†: During follow-up all variables decreased significantly (P < 0.05, Friedman test)
Serum MMP-3 levels compared to other disease activity variables
SSZ responders (n = 52) and MTX/SSZ responders (n = 24) were eventually followed for 28 weeks. In both groups serum MMP-3 and CRP decreased during follow-up (tables 2 and 4).

To evaluate differences between serum MMP-3 and CRP, changes of each variable were expressed as a percentage of the initial level. Serum MMP-3 was significantly decreased after 16 weeks in both groups (figure 1). A significant reduction in CRP was reached after 8 weeks of treatment in both groups.

In both responder groups there were some patients with serum MMP-3 levels in the normal range (female < 20 ng/ml, male < 60 ng/ml). To analyze the influence of these "normal levels" on the overall results we separately evaluated the patients with an elevated serum MMP-3 level at study entry. The results were the same in the SSZ responders (n=42 out of 52). In the MTX/SSZ responders (n=22 out of 24) serum MMP-3 and CRP were both significantly decreased after 12 weeks (data not shown).

We evaluated differences between serum MMP-3 and CRP by changes expressed as a percentage of the initial level. Small variations in or close by the normal range could result in exceptional relative (in terms of percentage) elevations. In some instances serum MMP-3 and CRP were, for example > 400% from baseline (see figure 1). Further evaluation of these data showed that these exceptional, relative elevations were indeed mainly caused by variations within or close by the normal range.
FIGURE 1. Serum MMP-3 and CRP as a % of the initial level in SSZ responders (A & B (n=52)) and MTX/SSZ responders (C & D (n=24)).
All variables decreased during a follow-up of 28 weeks. P < 0.05 was reached after 16 weeks for serum MMP-3 and after 8 weeks for CRP (Friedman with Dunnett's post test on the original crude data).
Inter-individual differences in the course of serum MMP-3 and CRP levels

Data from 4 randomly selected patients are shown in figure 2. These data are illustrative for the wide variations in absolute values of serum MMP-3 and CRP, for the close relation between MMP-3 and CRP, and for the individual response on treatment.

**FIGURE 2.** Individual serum MMP-3 (●) and CRP (○) levels in SSZ-responders (patient A and B) and MTX/SSZ responders (patient C and D). Broken line represents the normal value for serum MMP-3: for women < 20 (patients A, B, C) and for men < 60 ng/ml (patient D).

**DISCUSSION**

Our data show that serum MMP-3 levels decrease in early RA patients who respond to SSZ or to the combination of MTX and SSZ. Actually all variables, including CRP, ESR, and clinical variables decreased, confirming the close relation between serum MMP-3 and markers of disease activity.

The MMPs are thought to play an important role in the pathogenesis of RA based on their capacity to degrade many matrix components, their local expression in synovial tissue, and their increased levels in synovial fluid and serum. Inhibition of the production and/or activation of these MMPs could be an explanation for the
restraining effects of DMARDs on radiological progression. Therefore it is of interest to investigate the influence of DMARDs like SSZ and MTX on MMPs.

In RA MMP-3 is locally produced and activated in the inflamed joints and systemic levels are a direct reflection of this local synthesis. This is in contrast to CRP which is an indicator of inflammation in general that may be influenced by other stimuli of the acute phase response, like infections. Especially in early disease the use of markers of joint inflammation and destruction are of importance for prognostic and therapeutical reasons. Therefore serum MMP-3 is an interesting marker to investigate the influence of SSZ or the combination of MTX/SSZ on MMP production.

Studies concerning effects of DMARDs, such as sulfasalazine and methotrexate on MMPs are scarce. There is growing evidence that Nuclear Factor $\kappa B$ (NF-$\kappa B$) is involved in MMP induction. NF-$\kappa B$ is an important transcription factor for inflammatory cytokine genes such as TNF-$\alpha$, IL-1 and IL-6 as well as MMPs such as MMP-3.

SSZ is a potent and specific inhibitor of NF-$\kappa B$ in in-vitro cell cultures by interfering with I$\kappa B\alpha$ phosphorylation. By this path the effects of SSZ on MMP-3 and thereby on serum MMP-3 levels could be explained.

MTX in combination with steroids was reported to be effective in reducing neutral protease activity in RA synovium and cartilage. In a more recent study MTX therapy decreased collagenase (MMP-1) but not stromelysin-1 (MMP-3) gene expression in synovium of RA patients. More detailed studies concerning the effects of MTX on for example transcription factors have, to our knowledge, not been published. Nevertheless, MTX interferes with pro-inflammatory cytokines like IL-1 which is a potent inducer of MMPs by activation of transcription factors such as activator protein 1 (AP-1) and NF-$\kappa B$.

With regard to serum MMP-3 levels, treatment with anti-TNF-$\alpha$ (chimeric monoclonal antibody) resulted in a significant and rapid fall of serum MMP-3. Studies concerning corticosteroids showed conflicting results: intra-articular steroids resulted in a decrease but systemic corticosteroids resulted in an increase in serum MMP-3. A prospective open label trial concerning MTX and Tenidap showed close correlations between the DAS, ESR, and CRP, and serum MMP-3 but effects on the absolute values were not given.

In our study all variables decreased in the SSZ and MTX/SSZ responders. This was to be expected considering the clinical definition of response and the close relation between serum MMP-3 and markers of disease activity like swollen joint count, CRP, and ESR. In the SSZ partial/non responders ESR, CRP, and SJC decreased, but obviously not sufficient to the opinion of the patients.
rheumatologist (table 3). After the addition of MTX 24 out of 30 patients showed a sufficient response and in these patients all variables decreased significantly (table 4).

The serum MMP-3 levels decreased in both responder groups; however, there were differences in comparison with CRP. Especially in the SSZ responders serum MMP-3 showed a delayed response in comparison with CRP (figure 1). This difference did not change when only patients with an initially elevated serum MMP-3 (n=42) were evaluated. In the MTX/SSZ responders serum MMP-3 showed only a delayed response when the complete group was evaluated. Analysis of only the patients with an initially elevated serum MMP-3 (n=22) showed a significant response of serum MMP-3 comparable to the CRP response.

This delayed response of serum MMP-3 in comparison with CRP could be explained by differences in metabolism of MMP-3 but, as far as we know there are no data concerning the half-life and clearance of MMP-3 or MMP-3/TIMP complexes. Furthermore there is the possibility of an uncoupled relationship between joint inflammation and joint damage. The differences between CRP and serum MMP-3 may indicate that inhibition of the inflammatory response, resulting in decreased production of IL-6 and subsequent decline in serum CRP does not immediately result in decline in MMP production. This may implicate a temporary cytokine independent production (”autonomous production”) of MMP by synovial cells reflecting continuing matrix degradation.

Data from individual patients are shown in figure 2. Although there was a wide inter-individual variation in absolute values, intra-individually there was a close relation between serum MMP-3 and CRP, consistent with our previous study 11.

In conclusion serum MMP-3 levels decrease in early RA patients who respond to sulphasalazine or to the combination of methotrexate and sulphasalazine. In patients who respond to sulphasalazine the changes in serum MMP-3 levels indicate a delayed response compared to CRP.

ACKNOWLEDGEMENTS
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