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EXTENDED REPORT

# Infliximab treatment reduces complement activation in patients with rheumatoid arthritis

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**Background:** Tumour necrosis factor (TNF) blocking agents decrease C reactive protein (CRP) levels in rheumatoid arthritis (RA). It has been shown that CRP may contribute to complement activation in RA.
**Objective:** To assess the effect of intravenous infliximab treatment on complement activation, especially that mediated by CRP, in RA.
**Methods:** 35 patients with active RA (28 joint count Disease Activity Score (DAS28) >4.4) were treated with intravenous injections of infliximab (3 mg/kg, at weeks 0, 2, 6, 14, and 22). Clinical response and plasma levels of complement activation products, of CRP and of CRP-complement complexes, which are specific markers for CRP mediated complement activation, were assessed at the indicated time points up to 22 weeks. The relationship between CRP and CRP-complement complexes was analysed by paired *t* test between two time points and by generalised estimated equation, to test differences of variables over time.
**Results:** At 2 weeks after the first dose, infliximab significantly reduced overall C3 and C4 activation and plasma levels of CRP and CRP-complement complexes were also significantly reduced at this time point. The effects of infliximab on CRP and complement continued throughout the observation period and were more pronounced in patients with a good response to infliximab treatment.
**Conclusion:** Treatment with infliximab decreases plasma levels of CRP and CRP dependent complement activation products and concomitantly may reduce complement activation in RA. Complement activation may be among the effector mechanisms of TNF in RA.

During the past decade, it has become clear that cytokines have a key role in the pathogenesis of rheumatoid arthritis (RA). In particular the cytokine tumour necrosis factor α (TNFα) is a major player in the inflammatory cascade. Expression of TNFα and its receptors has been demonstrated in RA synovial tissue.[1] Furthermore, in vitro experiments with synovial cells[2] and animal studies[3] showed reduction of other inflammatory cytokines upon blockade of TNFα activity. This has led to the concept that TNFα is an essential mediator in inflammation in the rheumatoid joints, and has stimulated the development of drugs that block TNFα as a treatment of RA. Indeed there is now ample evidence that anti-TNF agents improve the clinical course of RA.[4–7]

In addition to the action of cytokines, activation of complement seems to be another important contributor to inflammation in RA. For example, high levels of complement activation products have been demonstrated in the joints and to a lesser extent in the circulation of patients with RA.[8 9] Moreover, intervention studies with complement inhibitors in animal models of arthritis have confirmed that complement has an important role in the pathogenesis of inflammation in the joints.[10 11] Activation of complement induces inflammation by generating several peptides and protein complexes, such as C5a and C5b–C9 complexes, which activate and stimulate endothelial and inflammatory cells.[12–14] Immune complexes are considered to be the main inducers of complement activation in RA.[15 16] However, other compounds can activate complement as well. For example, the acute phase protein, C reactive protein (CRP), a member of the pentraxin family,[17] bound to a ligand can activate the complement system via a classical pathway.[18 19] Recently, we reported increased levels of complexes between the acute phase protein CRP and activated C3 or C4 in the plasma of patients with RA,[20] suggesting that CRP contributes to complement activation in RA.[21] Neutralisation of TNFα decreases plasma levels of CRP in patients with RA.[17] Hence, one may postulate that CRP mediated complement activation may amplify the inflammatory effects of TNFα. However, to our knowledge little, if anything, is known about the effect of anti-TNF drugs on complement activation in patients with RA.

In this study we investigated the effect of treatment with the anti-TNF monoclonal antibody (mAb), infliximab, on overall complement activation, as well as on CRP levels and CRP mediated complement activation in patients with RA.

## PATIENTS AND METHODS

### Study protocol

Thirty five patients with active RA, for whom treatment with standard disease modifying antirheumatic drugs had failed, were selected to enter our study. Each patient fulfilled the American College of Rheumatology criteria for diagnosis of RA and had a 28 joint count Disease Activity Score (DAS28)[22] >4.4, indicating that they had active disease. The study group comprised 29 women and six men with a mean (SD) age of 52 (1) years (range 25–73), and a mean (SD) disease duration of 10 (7) years (range 1–24).

All patients received 3 mg/kg of infliximab (Centocor, Malvern, Pa) at weeks 0, 2, 6, 14, and 22. Each patient was clinically examined for assessment of response to treatment before each anti-TNF injection. Clinical response was defined as improvement of the DAS28 score according to criteria established by the EULAR.[23] Briefly, a good responder was defined as a patient in whom the DAS28 had improved by >1.2 and who had a DAS28 <3.2 at the time of evaluation. A

**Abbreviations:** CRP, C reactive protein; DAS28, 28 joint count Disease Activity Score; ELISA, enzyme linked immunosorbent assay; GEE, generalised estimating equation; IL, interleukin; mAb, monoclonal antibody; RA, rheumatoid arthritis; RF, rheumatoid factor; TNFα, tumour necrosis factor α

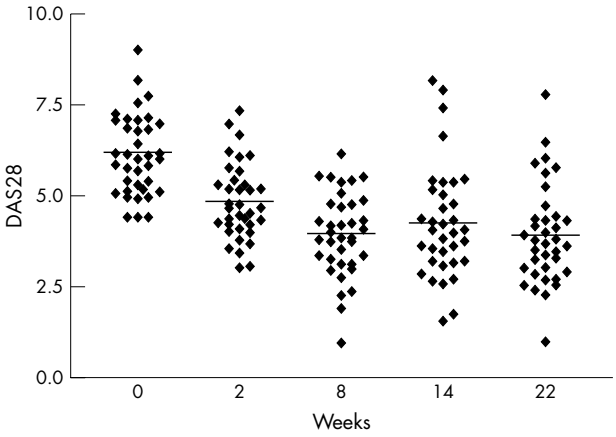


**Figure 1** Effect of infliximab (anti-TNF) treatment on Disease Activity Score 28 (DAS28): DAS28 was assessed in each patient before each injection of infliximab. Note that at 2 weeks, in all patients DAS28 had decreased, but that in time in some patients this decrease became less or even reversed (p value for repeated measurement of analysis of variance <0.0001).

moderate responder was defined as a patient who had either an improvement of DAS28 of 0.6–1.2 and who had a DAS28 <3.2, or an improvement of DAS28 >0.6 and a current DAS of 3.2–5.1, or who had an improvement of DAS28 >1.2 and a current DAS28 >5.1. Patients who did not fulfil these criteria were considered to be non-responders.

The study protocol was approved by the medical ethical committee of the VU University Medical Centre. Each patient had given informed consent to participate in the study.

## Blood samples collection

Before every infliximab infusion, blood was collected from each patient in citrate or EDTA containing tubes . Plasma was obtained by centrifugation at 1300 *g* for 10 minutes at 4°C. EDTA or citrate plasma was immediately frozen and kept at −70°C until tests were performed.

To establish normal values for complement, plasma samples were also obtained from 33 healthy people, and processed in a similar way to the samples from the patients.

## Laboratory measurements

### Activation of complement

Activation of complement system was assessed by specific enzyme linked immunosorbent assays (ELISAs) for measurement of plasma levels of activated C4 and C3.[19] We used specific mAbs against neo-epitopes exposed on activated fragments as capture antibodies, and biotinylated polyclonal sheep antibodies against C4 and C3, respectively, to detect bound complement fragments. As these assays do not discriminate between C4b, C4bi, or C4c, or between C3b, C3bi, or C3c, the activation products detected in the assays are further referred to as C4b/c and C3b/c, respectively. Results were expressed as nmol/l C4b/c or C3b/c, referring to an in house standard with known levels of activation products.

### CRP and CRP mediated complement activation

CRP was measured with a specific ELISA as described previously.[24] CRP mediated complement activation was evaluated using specific ELISAs for covalently bound complexes between CRP and activated C4 or C3.[19] Briefly, CRP and CRP complexes were isolated from plasma through calcium dependent affinity for phosphorylcholine coupled to Sepharose beads. After extensive washing of the beads, CRP and complexes were eluted with EDTA containing buffer, and applied to ELISA plates coated with appropriate anticomplement component mAbs as catching antibodies. Bound CRP-C4d and CRP-C3d complexes were detected with biotin conjugated mAb against CRP. The affinity based separation of CRP and CRP complexes was necessary to reduce interference in the ELISA by the excess of complement components present in the sample to be tested, and was performed in the presence of 0.5 M NaCl to prevent in vitro complement activation. Levels of CRP complexes in samples were expressed as mU/ml.

Rheumatoid factor (RF) levels were assessed with an ELISA, in which human IgG was used as coating and peroxidase conjugated mouse antihuman IgM as detecting antibody. Circulating immune complexes were measured with the $^{125}$I-C1q binding test.[25]

## Analysis of data

The GraphPad InStat program, version 3.0 (GraphPad Software, San Diego, CA), was used to analyse the data. Data are given as mean and standard deviation in cases of normal distribution, and median and range in cases of non-normal distribution. The difference between variables in patients and those in healthy people was analysed with Mann-Whitney tests. The effect of anti-TNF on levels of a given variable was evaluated by analysing levels before and at 2 weeks after the first dose of anti-TNF using Wilcoxon matched pairs signed ranks tests. Measures at various time points after treatment were compared with baseline using analysis of variance for repeated measurements. The relation of complement parameters to clinical response was analysed by generalised estimating equations (GEEs). A GEE is a regression technique for studying intervariable relationships in longitudinal studies. This technique corrects for the dependency of observations within the patients.[26] When this technique is used, time as well as time independent and time dependent covariates are taken into account. In this set of data we analysed the relation between CRP, CRP complexes, and complement with the DAS28 response from 2, 6, 14 and 22 weeks (outcome variable). In all GEE analyses a correction for the baseline DAS28 score was performed and GEE analyses were performed with STATA (version 7).

A two sided value of $p<0.05$ was considered to reflect a significant difference.

**Table 1** Plasma levels of the complement activation products C3b/c and C4b/c in the patients with RA before the start of infliximab treatment in comparison with those in healthy volunteers

| | Healthy volunteers (n = 33) | | | Baseline values in patients with RA (n = 35) | | |
|---|---|---|---|---|---|---|
| | Maximum | Minimum | Mean (SD) | Maximum | Minimum | Mean (SD) |
| C3b/c | 28 | 8.3 | 15 (4.7)* | 53 | 5 | 27.3 (10.8)* |
| C4b/c | 20 | 1.7 | 6.9 (4.7)* | 152 | 5 | 42.6 (34.9)* |

*p<0.0001 for the difference between healthy controls and patients (Mann-Whitney test).

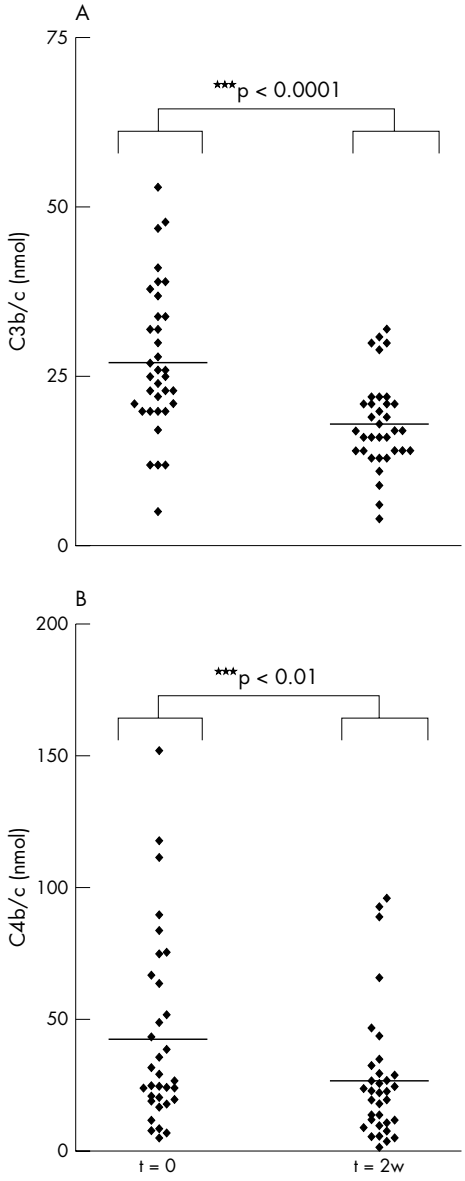


**Figure 2** Activation of complement before treatment and at 2 weeks after the first injection of infliximab. Data represent plasma levels of C3b/c (A) and C4b/c (B). Note that both measures had significantly decreased after treatment with infliximab.

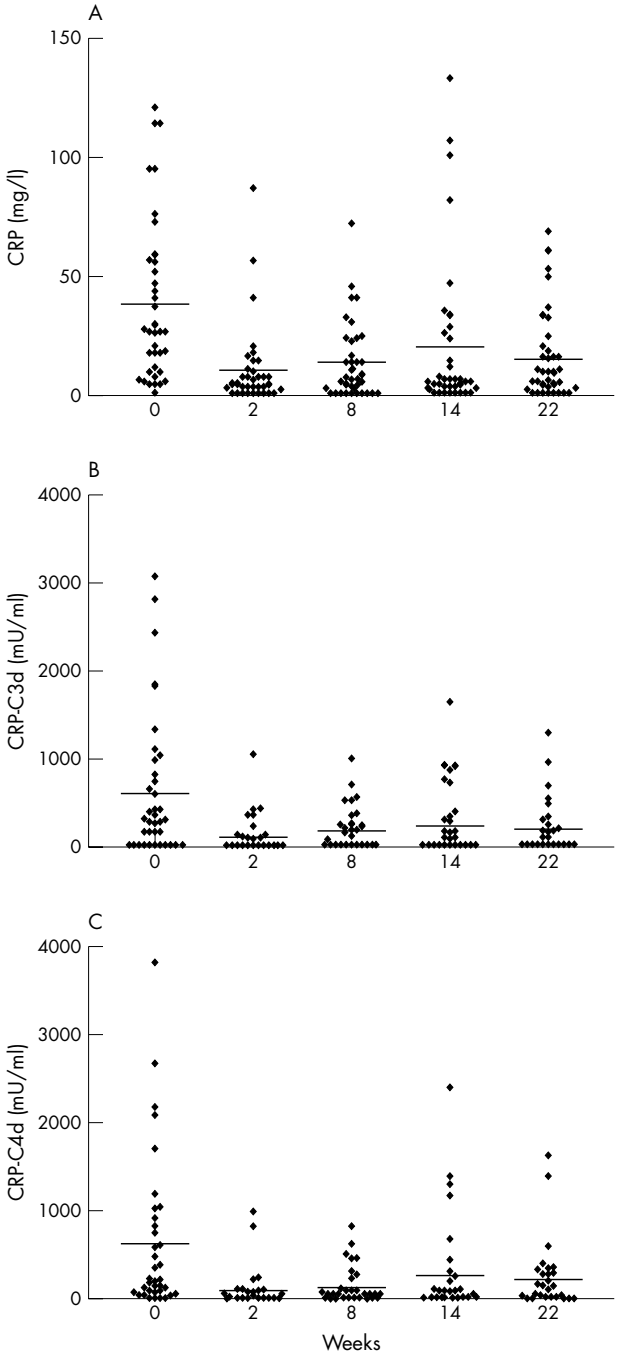


**Figure 3** Effect of infliximab treatment on plasma levels of (A) CRP and (B, C) CRP-complement complexes: (B) CRP-C3d; (C) CRP-C4d, which reflects CRP mediated complement activation. Note that both CRP and CRP-complement complexes significantly decreased upon treatment (p values for all panels are <0.001).

## RESULTS

### Clinical course of the patients upon infliximab treatment

Thirty five patients were included in the study. The DAS28 was assessed in every patient before the start of infliximab treatment and before each subsequent injection to establish clinical responses to treatment. Figure 1 shows the DAS28 of the whole group at different time points. At the first evaluation point—that is, at 2 weeks after the first injection, all the patients showed clinical improvement, as reflected by a decreased DAS28 compared with the score at baseline. At the later time points, some patients no longer responded to infliximab according to the criteria described in ''Patients and methods''. For example, at the end of the observation period—that is, at 22 weeks after the first injection, 10 showed a good response, 20 had a moderate response, and 5 had no response to the treatment, according to the criteria described in ''Patients and methods''.

### Effect of infliximab on complement activation

Plasma levels of C3b/c and C4b/c, reflecting activation of C3 and C4, respectively, in the patients before the start of infliximab treatment were indeed significantly higher than levels in healthy volunteers (table 1), 85 and 88% of the patients with RA having raised C3b/c and C4b/c, respectively, as compared with normal values ($p<0.0001$ in both cases for the difference between patients and healthy controls, Mann-Whitney test).

At 2 weeks after the start of infliximab treatment all patients were responders according to change of the DAS28.

**Table 2** Plasma levels of CRP, CRP-complement complexes, and complement activation products in patients with RA treated with infliximab, classified according to their clinical response (good, moderate, or no response) at 14 weeks after the start of treatment

| | Good (n = 9) | | | Moderate (n = 17) | | | No response (n = 9) | | |
|---|---|---|---|---|---|---|---|---|---|
| Plasma levels | Before treatment | After treatment | p Value | Before treatment | After ?treatment | p Value | Before treatment | After treatment | p Value |
| CRP | 33 (24.8) | 2.2 (1.8) | <0.01 | 31.6 (25.7) | 15.2 (26.2) | <0.001 | 57.2 (48.6) | 45.6 (44.4) | 0.160 |
| C3b/c | 33.3 (11.8) | 22.8 (6.9) | 0.054 | 24.6 (10.9) | 17.5 (6.4) | <0.01 | 27.4 (9.1) | 34.1 (25.2) | 0.547 |
| C4b/c | 60.1 (49.8) | 58 (45.6) | 0.91 | 37.4 (31.8) | 22.9 (13.4) | 0.120 | 39 (25.5) | 23.4 (6.6) | 0.431 |
| CRP-C3d | 406 (417.5) | 62 (65.2) | <0.05 | 434 (502.4) | 145 (276.6) | <0.001 | 1043 (1249) | 535 (507) | 0.250 |
| CRP-C4d | 632 (686.6) | 83 (142.6) | <0.05 | 370 (379.7) | 148 (334) | <0.01 | 1071 (1403) | 598 (806) | 0.312 |

Data are shown as mean (SD) before and after 14 weeks after treatment. p Values show the differences between baseline and the follow up in every group (Wilcoxon matched pairs test).

Hence, we evaluated the effect of infliximab on complement activation by comparing levels of C3b/c and C4b/c at 2 weeks after treatment with those at baseline, because at that time all patients had responded to the treatment and levels of complement activation parameters, C3b/c and C4b/c, were both significantly lower than levels before treatment (fig 2).

RF can form immune complexes with autologous IgG and hence may be involved in complement activation in RA. To analyse the effect of infliximab on circulating levels of RF, we measured the plasma levels of IgM RF before and after the first injection—that is, at 2 weeks. Levels at 2 weeks were comparable with baseline levels (data not shown). Also, levels of circulating immune complexes, as assessed with the $^{125}$I-C1q binding assay, had not changed at 2 weeks after the start of treatment as compared with baseline levels (data not shown).

### Plasma levels of CRP and CRP mediated complement activation

Using specific ELISAs, we measured plasma levels of CRP and of CRP-C3d and CRP-C4d complexes in the patients before and after start of the infliximab treatment—that is, at 2, 6, 14, and 22 weeks. Before treatment 29/35 (83%) patients had raised CRP, and 24 (69%) and 31 (89%) had increased levels of CRP-C3d and CRP-C4d complexes, respectively, as compared with normal levels. Plasma levels of CRP significantly decreased in the patients during treatment (fig 3A), as did those of both CRP-complement complexes (figs 3B and C).

### CRP and complement in relation to clinical response

The relationship between variations in complement or CRP parameters and clinical response to infliximab was assessed before and after 14 weeks of treatment. Based on the clinical response to treatment at this time point, patients were divided into three different subgroups—that is, patients with a good, moderate, or no response, as defined in ''Patients and methods''. The decrease of CRP and C3b/c levels was more pronounced in the patients with a good response. C4b/c levels, however, did not show this relationship (table 2). Plasma levels of CRP-complement complexes decreased six- to sevenfold in the group of responders, which was significant, whereas they dropped non-significantly about twofold in the group of non-responders. Analysis of the data at other time points also pointed to more pronounced decreases of these variables in patients who showed a good response to infliximab, in comparison with those who did not respond.

The relationship between complement or CRP parameters and clinical responses upon treatment with infliximab were analysed with GEEs. These analyses showed a significant association between a decrease of CRP and a good response ($p<0.01$). Significant associations of CRP-C3d and CRP-C4d with a good response were also seen ($p<0.01$). However, these relationships were less strong when a correction was made for CRP (the corresponding p values for CRP-C3d and CRP-C4d were 0.34 and 0.06, respectively). No significant associations between C3b/c and C4b/c and clinical response were found ($p = 0.56$ and $p = 0.84$. respectively).

## DISCUSSION

It has been suggested that TNFα blockade exerts its anti-inflammatory effects by interfering with the recruitment of leucocytes into the joints, and by attenuating the synthesis of several cytokines such as interleukin (IL) 1 and IL6, and other mediators such as matrix metalloproteinases-1 and -3, and vascular endothelial growth factor. Here we show that infliximab treatment reduces plasma levels of activated C4 and C3 of the complement system, and of CRP-complement complexes in patients with RA.

Various studies point to activation of the complement system as a crucial event in the inflammatory cascade in rheumatoid joints RA.[27 28] Raised levels of C3a and C5a indeed have been noted in the synovial fluid and plasma of patients with RA,[29–31] and these potent inflammatory peptides may play a part in inflammatory cell recruitment to the synovial fluid and tissue. Also, in this study we found increased plasma levels of C3b/c and C4b/c in the majority of patients with RA before the start of infliximab treatment. We did not investigate the origin of these activation products of the complement system. Considering that several studies have shown increased levels of complement activation products in the joints, in particular, we assume that the increased plasma levels were due to spill over from the joints, although extra-articular activation—for example in patients with subclinical vasculitis,[9] might also have contributed to the activation.

As all patients showed clinical responses upon administration of infliximab at 2 weeks after the start of the treatment, we decided to analyse the effect of anti-TNF on complement activation itself by comparing levels at 2 weeks with those at baseline. In this way, the results were not blurred by analysing non-responding patients as well. A significant decrease of complement activation at 2 weeks was seen. Immune complexes involving RFs are frequently considered as a major cause of complement activation in RA.[15 16] Hence, an obvious explanation for the effect of infliximab on complement activation would be that this treatment affected levels of RFs or immune complexes. However, we found no clear differences in these measures at 2 weeks as compared with levels at baseline. These findings argue against an effect of infliximab on immune complexes or RFs as an explanation for the observed effects of anti-TNF on complement.

A decrease in plasma levels of CRP, a member of the pentraxin family, which acts as an acute phase protein in

humans, as a consequence of TNF blockade has been reported before by Elliott *et al*.[32] Human CRP can activate the complement system via a classical pathway and we have shown previously that activation of the complement system in RA is partly mediated through this pentraxin.[20] Hence, the effect of infliximab on complement may well be related to the effect of the antibody on CRP levels. Indeed we observed a significant decrease of CRP-complement complexes, which specifically reflect complement activation through CRP,[19] upon infliximab treatment. However, a significant number of patients still had increased levels of complement activation products despite effective anti-TNF treatment and lower CRP levels, which suggests that part of the complement activation in RA is independent of CRP, and results, for example, from interaction of complement with immune complexes.

Intervention studies in animal models for arthritis indicate a pivotal role for complement activation in the pathogenesis of arthritis.[10 33 34] Considering the observed effects of infliximab, one may postulate that the clinical responses to infliximab in RA are in part due to its effects on complement. To substantiate this further we analysed the effect of infliximab on complement in patients who responded well to the treatment in comparison with patients who did less well. When analysing the relationship between clinical responses and complement at one time point, we observed a somewhat more pronounced effect of infliximab on complement in patients who responded well. However, the differences were not marked. One might argue that these effects of infliximab on complement and on the clinical course may not occur at the same time. Hence, we also analysed by GEE the effect of infliximab on complement and clinical response by considering the changes at all time points after the start of the treatment. In this way a somewhat more pronounced effect of infliximab was seen in patients with a good response, but again the differences between poor and good responders were not very strong. Among the explanations for this moderate difference between good and poor responders are the somewhat small numbers of patients analysed, as well as the variability of responses in time in individual patients.

Several studies have shown that recombinant cytokines such as IL2 and TNF may induce complement activation when given at high doses to patients.[35 36] In this study we show that blockade of an endogenous cytokine such as TNF in RA attenuates complement activation in vivo. Blockade of the activity of another proinflammatory cytokine, IL1, with IL1 receptor antagonist, significantly improves RA.[37] Data on the effect of a IL1 receptor antagonist on complement activation in RA are not yet available. However, we have shown that the effect of IL1 receptor antagonist treatment on C3a levels is significant in septic patients.[38] Taking these data together, one may postulate that cytokines released during inflammation trigger complement activation, presumably through mechanisms involving CRP. We suggest that reduction of this activation contributes to the anti-inflammatory effects of anti-cytokine reagents in humans.

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