Association of Serum C-Reactive Protein Levels With Lupus Disease Activity in the Absence of Measurable Interferon-α and a C-Reactive Protein Gene Variant

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Association of serum C-reactive protein levels with lupus disease activity in the absence of measurable interferon-\(\alpha\) and a C-reactive protein gene variant

Running head: CRP reflects SLE activity in the absence of IFN\(\alpha\) and a CRP gene variant

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ABSTRACT

Objectives: The type I interferon (IFN) system is important in the pathogenesis of systemic lupus erythematosus (SLE). We previously demonstrated an inhibitory effect of IFNα on interleukin 6 (IL-6) induced C-reactive protein (CRP) \textit{in vitro}, hypothetically explaining the poor correlation between disease activity and CRP levels in SLE. Herein we investigated disease activity, IL-6 and CRP in relation to a CRP gene polymorphism and IFNα.

Methods: Sera from 155 SLE patients and 100 controls were analyzed for CRP. Patients were genotyped for a CRP single nucleotide polymorphism (rs1205) associated with low CRP levels. Serum IFNα and IL-6 was quantified by immunoassays. Clinical disease activity was assessed by SLE disease activity index 2000 (SLEDAI-2K).

Results: CRP levels were increased in SLE patients compared to controls, but were not associated with SLEDAI-2K or IL-6 levels. However, exclusion of patients carrying at least one rs1205 minor allele revealed an association between disease activity and CRP levels (p=0.005). We found a strong association between disease activity and CRP levels (p<0.0005) when patients with measurable IFNα as well as the minor allele of rs1205 were excluded from the analysis. Similarly, when patients with raised IFNα and/or the rs1205 polymorphism were excluded, IL-6 associated with CRP levels.

Conclusions: The present study demonstrates that serum IFNα as well as CRP genotype affects the CRP response in SLE patients. Lack of correlation between serum levels of CRP and disease activity could therefore be explained by activation of the type I IFN system and polymorphisms in the CRP gene.
Systemic lupus erythematosus (SLE) is an inflammatory disease characterized by multi-organ involvement, circulating autoantibodies (most notably antinuclear antibodies, ANA), increased levels of interferon α (IFNα) and/or expression of IFNα inducible genes, the “type I IFN signature” [1]. Despite large efforts, we still lack reliable disease activity markers covering all disease phenotypes of SLE. Instead, an array of clinical data and biomarkers are used to evaluate organ involvement and disease activity [2]. The acute phase protein C-reactive protein (CRP) is widely used to monitor inflammation in diseases such as rheumatoid arthritis and bacterial infections, but is not a reliable inflammation marker in SLE as the serum levels often remain low despite high disease activity coinciding with raised levels of interleukin (IL)-6 [3-7]. Hepatocytes are the main producers of human CRP, and the most important inducer of CRP transcription in primary cells is IL-6 [8-11]. Baseline and acute phase levels of CRP are also dependent on polymorphisms in the CRP gene [4].

CRP may contribute to an anti-inflammatory elimination of cell debris due to its opsonizing properties and ability to bind nuclear components [11, 12] and SLE patients would therefore probably benefit from an adequate CRP elevation during disease flares. Apoptotic cells that are not eliminated immediately will expose nuclear components on their surface which in turn can induce immune reactions with autoantibody production and formation of immune complexes that can deposit in tissues and cause further inflammation [12].

As in SLE flares, CRP is often low during viral infections. The difference in CRP response between viral and bacterial infections makes CRP analysis a useful tool to discriminate between these two types of infection. Interestingly, viral infections induce IFNα production in order to fight the viral replication and activate an appropriate immune response [13]. We previously showed that IFNα inhibits IL-6 induced CRP production by human hepatocytes in
vitro [10], and based on this we hypothesized that raised levels of IFNα contribute to an inadequately low CRP response during SLE flares and viral infections, by uncoupling the correlation between IL-6 and CRP. Hence, we now investigated the circulating levels of CRP, IL-6 and IFNα in serum from SLE patients. Since genetic variation of the CRP gene may influence CRP levels, we furthermore analyzed the SNP rs1205 which has been associated with low basal and acute phase levels of CRP [4, 14-20], and to the risk of developing SLE [21, 22].

PATIENTS AND METHODS

Patients and controls

A total of 155 SLE patients were included from KLURING (Swedish acronym for “clinical lupus register in northeastern Gothia”) i.e. a prospective follow-up project at the Rheumatology clinic, Linköping university hospital, Sweden [23]. Informed consent was obtained from all subjects and the study protocol was approved by the Regional ethics committee in Linköping (M75-08/2008). Prevalent (92%) as well as incident cases (8%) were recruited consecutively during 2008-2011. All patients met the 1982 American college of rheumatology (ACR) classification criteria [24]. Body mass index (BMI) and SLE International collaborating clinics (SLICC)/ACR damage index [25] was registered at blood sampling. Disease activity was registered according to the SLE disease activity index 2000 (SLEDAI-2K) [26]. Of the 155 patients, 138 (89%) were females and 143 (92%) were Caucasians. The patients were treated as follows: 58 (37%) were prescribed antimalarials (AM) alone, 48 (31%) were medicated with disease-modifying anti-rheumatic drugs (DMARDs) ± AM, and 105 (68%) were treated with prednisolone. Further patient characteristics are shown in Table 1.
One hundred healthy blood donors (50% females) served as control subjects. The mean age was 45.8 years, ranging from 22 to 70 years of age.

**CRP and cytokine analyses**

Venous blood was drawn from each individual and serum was prepared and kept at -70°C until analyzed. High sensitivity CRP (detection limit 0.12 mg/L) was analyzed by turbidimetry at the routine clinical chemistry laboratory at Linköping university hospital. IFNα (detection limit 1 U/mL) was measured in sera from SLE patients by a dissociation-enhanced lanthanide fluorescent immunoassay at Uppsala university, Sweden, as previously described [27]. IL-6 (detection limit 0.64 pg/mL) was analyzed by a high sensitivity multiplex magnetic bead assay (Milliplex, Millipore, Solna, Sweden) according to the manufacturer’s instruction. Non-detectable levels of cytokines or CRP were given half the value of the detection limit.

**Genotyping**

Genomic DNA was obtained from whole blood samples using QIAamp DNA Blood Midi Kit (Qiagen, Hilden, Germany). The SNP rs1205 was genotyped by using the Golden Gate Assay (Illumina Inc. San Diego, CA).
Statistical analyses

Chi-square (Fisher’s exact test) was used when categorical variables were analyzed. Mann-Whitney’s test was used to assess differences in cytokine or CRP levels between patients and controls as well as between patient groups. The associations of CRP and disease activity or IL-6 were evaluated using multiple linear regression models with log10-transformed CRP as the response variable. Residual plots showed that the residuals were randomly distributed around zero suggesting that the multiple linear regression models were appropriate. P-P plots also showed that the residuals were normally distributed (data not shown). Because of age [28, 29] and body mass index (BMI)-dependent variations of CRP levels [30, 31], these were included as independent variables in multiple linear regression analyses. All regression analyses were also performed with adjustment for prednisolone dose but since it did not influence CRP levels significantly, and no other significance arose or disappeared due to prednisolone adjustment we chose not to adjust for this.

Two-tailed p-values of <0.05 were considered significant. Statistical analyses were performed with SPSS Statistics 21 (IBM, Armonk, NY).

RESULTS

Serum CRP levels are elevated in SLE patients, but not related to disease activity

CRP levels were significantly higher in SLE patients (median; mdn=2.11 mg/L) compared to healthy controls (mdn=0.49 mg/L), p<0.0005. There was however no significant difference in CRP levels between patients with high disease activity, defined as SLEDAI ≥ 6 (n=24, mdn=2.00 mg/L) and patients with no or low disease activity (SLEDAI < 6, n=131, mdn=2.52 mg/L) (Figure 1).
Effects of the SNP rs1205 and IFNα on CRP levels

Comparing CRP levels between SLE patients homozygous for the major allele (G) (n=73) with SLE patients heterozygous or homozygous (n=82) for the minor allele (A; associated with lower CRP levels [4]) revealed significantly lower CRP levels among patients with the minor allele (mdn=1.35 mg/L), p=0.045 (Table 2). A significant negative impact of the minor allele of rs1205 on logCRP levels was also found after adjusting for age and BMI in a regression analysis (p=0.046). Genotype frequencies did not deviate from the Hardy-Weinberg equilibrium (p=0.663 by chi-square test). A comparison of CRP levels between patients with (n=36) and without (n=119) measurable levels of IFNα (≥1 U/mL) implied lower median CRP levels among patients with detectable levels of IFNα (1.81 vs. 2.14 mg/L) although this difference was not statistically significant.

Association between serum CRP and SLEDAI-2K or serum IL-6

Based on previous in vitro findings concerning an inhibitory effect of IFNα on IL-6 induced CRP production [10], we performed a multiple linear regression analysis to investigate if CRP is reflected by clinical disease activity (SLEDAI-2K) or IL-6 levels among patients without detectable IFNα levels (<1U/mL) and/or the rs1205 polymorphism (Table 3). Neither SLEDAI-2K, nor IL-6 were associated with logCRP among all patients (n=155), but a significant association between logCRP and IL-6 (p=0.001) was found when excluding patients with IFNα ≥1U/mL. Among patients homozygous for the major allele of rs1205, both SLEDAI-2K (p=0.005) and IL-6 (p=0.001) were associated with logCRP. When limiting the patient group to those homozygous for the major allele of rs1205 and non-measurable IFNα (<1U/mL) (n=57), both SLEDAI-2K (p<0.0005) and IL-6 (p=0.001) were strongly associated with logCRP. The 57 patients without detectable IFNα and CRP-lowering rs1205 variants were not significantly different from the other patients with regard to clinical
features such as SLEDAI-2K, number of fulfilled ACR criteria, specific ACR criteria or SLICC/ACR damage index (not shown).

The rs1205 polymorphism, CRP and IFNα in relation to disease phenotypes

Significantly higher levels of CRP were found in patients meeting the ACR criteria of serositis (mdn=2.98 mg/L) compared to patients not fulfilling this criteria (mdn=1.37 mg/L), p=0.007. Patients fulfilling the photosensitivity criterion had significantly lower levels of CRP (mdn=1.49 mg/L) than patients without this criterion (mdn=2.70 mg/L), p=0.015. IFNα was significantly higher in patients fulfilling the criteria of malar rash (p=0.023), renal disorder (p=0.006), hematologic disorder (p=0.008), immunologic disorder (p=0.027) and neurologic disorder (p<0.0005). Mdn values of IFNα were 0.5 U/mL regardless of fulfilling the above mentioned criteria, except for neurologic disorder were the mdn value was 4.22 U/mL for patients fulfilling this criterion. There was no significant association between rs1205 genotypes and ACR criteria.

DISCUSSION

In the present study we found an association between SLE disease activity and CRP levels in patients without detectable IFNα levels in serum, and without a genetic variant associated with low CRP levels. We also found an association between IL-6 and CRP in these patients. These observations, together with the previously reported mechanistic evidence [10], indicate an interfering effect of IFNα on IL-6-induced CRP production and pronounced effects of the CRP lowering genetic variant of rs1205 on CRP levels. The absence of correlation between IL-6 and CRP [3, 32-34] and the poor performance of CRP as an inflammation marker in SLE [5, 7] could therefore be due to a heterogeneous SLE population with regard to IFNα and CRP genetics.
We found elevated levels of CRP in patients with ongoing and/or a history of serositis (ACR criterion 6) and higher levels of IFNα in patients with renal, neurologic and hematologic involvement (ACR criteria 7, 8 and 9), essentially consistent with previous studies [35-38]. Interestingly, CRP and IFNα levels, respectively, were thus raised in patients with disparate disease phenotypes.

CRP, an opsonin with affinity for phosphocholine (PC) exposed on apoptotic cells, can activate the classical complement pathway. This results in further opsonization, but typically not to membrane attack complex formation. Thereby, CRP contributes to a non-inflammatory clearance of dying cells [12, 39]. In addition, CRP can bind to several nuclear components such as small nuclear ribonucleoproteins (snRNP), dsDNA and histones [11], all of which are known targets for autoantibodies in SLE. It is therefore tempting to speculate that CRP could protect from SLE by interrupting the “vicious circle” of autoantibody production, immune complex formation and tissue inflammation. In support of these assumptions, studies show beneficial effects of CRP-treatment in murine SLE models [40, 41] and delay of SLE onset in lupus-prone mice expressing a human CRP transgene [42], although conflicting results have been published [43]. Further, CRP was recently shown to inhibit immune complex induced IFNα-production by plasmacytoid dendritic cells [44], and certain CRP-lowering genetic variants and haplotypes of the CRP gene are more frequent among SLE patients [22, 45, 46]. Given these observations, it is of great importance to understand the regulation of CRP production. This is also important in relation to the emerging anti-IFNα therapy, which could hypothetically lead to a normalized and beneficial CRP response.
An association between genetic variations in the CRP gene and serum CRP levels was first described in 2002 by Szalai et al [47], and a large number of SNPs have now been described, but only a few have consistently been related to CRP levels [4]. In the present study we evaluated rs1205, a SNP located in the 3’ untranslated region of the CRP gene. This SNP has been associated with low CRP in numerous studies [4, 14-20] and is associated with SLE susceptibility and ANA levels [22]. Further, rs1205 was the SNP with strongest independent association to low CRP levels in a Bayesian meta-analysis [48]. Despite a relatively small number of patients, we found significantly lower levels of CRP in patients with one or two rare alleles of rs1205. Excluding these patients from the regression analysis revealed a highly significant association of IL-6 or SLEDAI with CRP levels, indicating that gene polymorphisms can be a major reason for a modest CRP response during SLE flares. Yet, SLE patients mount an apparently normal CRP response during bacterial infections [6, 35, 49] which could suggest a multifaceted rather than a single explanation [4].

It has been hypothesized that CRP, like complement, is consumed by the increased apoptosis rate, but the consumption of CRP appears to be normal in SLE patients [50]. Further, the anti-CRP antibodies found in SLE patients do not correlate with CRP levels and these autoantibodies are directed against monomeric CRP, but not against the pentameric circulating form [51]. Hence, it is likely that the inappropriately low serum CRP during SLE flares is due to a decreased production, rather than increased clearance.

To conclude, the interaction between different pro-inflammatory molecules as well as the patients’ genetic setup needs to be considered when evaluating biomarkers in lupus. The findings in this study indicate that CRP genetic variants and raised serum IFNα at least partly underlie the poor performance of CRP as a disease activity marker in SLE patients.
ACKNOWLEDGEMENTS

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REFERENCES


Table 1. Baseline characteristics of the SLE patients (n=155)

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Mean (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACR criteria fulfilled (n)</td>
<td>5.1 (4-9)</td>
</tr>
<tr>
<td>SLICC/ACR damage index (score)</td>
<td>1.2 (0-9)</td>
</tr>
<tr>
<td>SLEDAI-2K (score)</td>
<td>2.5 (0-16)</td>
</tr>
<tr>
<td>Disease duration (years)</td>
<td>11.5 (0-45)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>49.9 (18-88)</td>
</tr>
<tr>
<td>Body mass index</td>
<td>26.1 (17.5-48.7)</td>
</tr>
<tr>
<td>Prednisolone dose (mg/day)</td>
<td>5.1 (0-60)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>ACR criteria</th>
<th>n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Malar rash</td>
<td>79 (51.0)</td>
</tr>
<tr>
<td>2. Discoid rash</td>
<td>31 (20.0)</td>
</tr>
<tr>
<td>3. Photosensitivity</td>
<td>93 (60.0)</td>
</tr>
<tr>
<td>4. Oral ulcers</td>
<td>17 (11.0)</td>
</tr>
<tr>
<td>5. Arthritis</td>
<td>120 (77.4)</td>
</tr>
<tr>
<td>6. Serositis</td>
<td>62 (40.0)</td>
</tr>
<tr>
<td>7. Renal disorder</td>
<td>41 (26.5)</td>
</tr>
<tr>
<td>8. Neurologic disorder</td>
<td>10 (6.5)</td>
</tr>
<tr>
<td>9. Hematologic disorder</td>
<td>97 (62.6)</td>
</tr>
<tr>
<td>10. Immunologic disorder</td>
<td>81 (52.3)</td>
</tr>
<tr>
<td>11. Antinuclear antibody*</td>
<td>152 (98.1)</td>
</tr>
</tbody>
</table>

Table 2. Association of CRP polymorphism rs1205 with CRP levels

<table>
<thead>
<tr>
<th>rs1205 Genotype</th>
<th>Patients (n)</th>
<th>Median CRP (mg/L)</th>
<th>p-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>GG</td>
<td>73</td>
<td>2.63</td>
<td>0.045</td>
</tr>
<tr>
<td>GA</td>
<td>65</td>
<td>2.14</td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>17</td>
<td>1.13</td>
<td></td>
</tr>
</tbody>
</table>

*Mann-Whitney (comparing GG versus GA and AA). CRP = C-reactive protein.

Table 3. Association of SLEDAI-2K and IL-6 with logCRP

<table>
<thead>
<tr>
<th>Patient selection</th>
<th>n</th>
<th>SLEDAI-2K p-value</th>
<th>Beta</th>
<th>IL-6 p-value</th>
<th>Beta</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. All patients</td>
<td>155</td>
<td>0.2</td>
<td></td>
<td>0.9</td>
<td></td>
</tr>
<tr>
<td>2. IFNα&lt;1U/mL</td>
<td>119</td>
<td>0.1</td>
<td></td>
<td>0.001</td>
<td>0.29</td>
</tr>
<tr>
<td>3. rs1205 major alleles*</td>
<td>73</td>
<td>0.005</td>
<td>0.33</td>
<td>0.001</td>
<td>0.37</td>
</tr>
<tr>
<td>4. Combination of 2 and 3</td>
<td>57</td>
<td>&lt;0.0005</td>
<td>0.52</td>
<td>0.001</td>
<td>0.41</td>
</tr>
</tbody>
</table>

*Homozygous for the major allele (G). P-values and standardized beta coefficient (Beta) are from a multiple linear regression analysis adjusting for age and body mass index. CRP = C-reactive protein, SLEDAI-2K = Systemic Lupus Erythematosus Disease Activity Index 2000, IFN = Interferon, IL = Interleukin.
**Figure 1.** Serum levels of C-reactive protein (CRP) in healthy controls and systemic lupus erythematosus (SLE) patients. (A) SLE patients had significantly higher levels of CRP compared to healthy controls, (B) but there was no significant difference in CRP comparing patients with active disease (SLE disease activity index, SLEDAI ≥ 6), with patients with low/no disease activity (SLEDAI < 6). Bars represent median values.