EXTENDED REPORT

Serum amyloid P component levels are not decreased in patients with systemic lupus erythematosus and do not rise during an acute phase reaction

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Background: Serum amyloid P component (SAP) and acute phase proteins like C-reactive protein contribute to the clearance of apoptotic cells. This response is diminished in systemic lupus erythematosus (SLE).

Objectives: To analyse SAP concentrations in SLE in relation to disease activity, and investigate whether SAP reacts like an acute phase protein.

Methods: SAP was measured in 40 patients with SLE during active and inactive disease and compared with healthy controls and patients with rheumatoid arthritis and Wegener's granulomatosis. Normal SAP values were determined in 120 healthy controls by ELISA. C reactive protein and serum amyloid A (SAA) were measured in all subjects and their levels related to SAP. SAP was also measured serially in 11 patients with breast cancer treated with recombinant human interleukin-6, and in 16 patients with sepsis.

Results: In SLE, SAP was unaltered compared with healthy controls and was not influenced by disease activity, in contrast to C reactive protein and SAA, which increased during active disease. SAP increased in Wegener's granulomatosis but not in rheumatoid arthritis. The rise in C reactive protein and SAA was most pronounced in Wegener's granulomatosis with active disease. SAP did not change significantly during an acute phase response. No correlation was found between SAP and C reactive protein or SAA, but there was a correlation between SAA and C reactive protein ($r = 0.4989$, $p = 0.0492$).

Conclusions: Patients with SLE have normal circulating SAP levels. In contrast to C reactive protein or SAA, SAP does not act as an acute phase protein.

Serum amyloid P component (SAP) is a member of the pentraxin family of proteins. This family consists of several proteins such as C reactive protein and pentraxin-3 (PTX3)[1 2] which are characterised by a cyclic pentameric structure and calcium dependent ligand binding. SAP is the serum precursor of the P component of amyloid. It binds to a broad group of molecules, including autoantigens, through a pattern recognition binding site. The function of SAP is still unknown. It probably serves as an opsonising protein in the clearance of apoptotic cells, because, like the other pentraxins, it can bind to these cells. Chromatin and phosphatidylethanolamine, which are exposed at the outer surface of cells during the process of apoptosis, are supposed to be the major binding sites.[1–8]

Disturbances in the clearance of apoptotic material are associated with the development of autoimmunity. Apoptotic cells have several intracellular (auto)antigens at their outer surface.[9] Normally, such cells are rapidly removed so that exposure of these antigens to the immune system hardly occurs.[10] However, whenever the clearance of apoptotic cells is disturbed, their accumulation will facilitate the development of autoimmunity. Indeed, in mice a deficiency of complement factor C1q, a protein necessary for the opsonisation and phagocytosis of apoptotic cells, results in the production of autoantibodies and subsequently in the development of a glomerulonephritis resembling human SLE.[11] In favour of the above hypothesis is the presence of apoptotic debris in the glomeruli of affected animals. Interestingly, mice with a targeted deletion of SAP also spontaneously produce antinuclear antibodies and develop glomerulonephritis,[12] suggesting that the absence of, or probably even a decrease in, SAP may predispose to autoimmunity.

Following these findings in mice, we speculated that SAP may be deficient in patients with SLE, either qualitatively or quantitatively, compared with healthy controls, and might influence the development and course of the disease. As the structure and function of SAP in SLE patients has been shown to be unaltered,[13] quantitative evaluation of SAP could be important. We propose that the decrease in SAP will be even more pronounced during active disease, thus explaining, in part, the occurrence of apoptotic cells described in these patients.[14 15]

In this study, we measured SAP in patients with SLE during active and inactive disease, and compared these data with levels in healthy and disease controls to investigate the possible role of SAP in the pathogenesis of SLE. We measured SAP, C reactive protein, and serum amyloid A (SAA) levels simultaneously in all the patients and serially in other subjects with an (exogenously induced) acute phase response, to evaluate whether SAP, like C reactive protein, reacts to some extent as an acute phase protein.

METHODS

Patients

Serum SAP was analysed in 40 patients with SLE (36 female, four male), mean (SD) age 34.2 (12.3) years. All fulfilled at least four of the American College of Rheumatology (ACR) criteria for SLE.[16] SAP values in these patients were compared with those in 120 healthy volunteers, 81 female (47.1 (16.9) years) and 39 male (53.9 (17.4) years). To analyse the influence of disease activity on SAP in patients with SLE, we analysed paired sera collected during active and inactive

Abbreviations: ACR, American College of Rheumatology; SAA, serum amyloid A; SAP, serum amyloid P; SLE, systemic lupus erythematosus

disease phases. Patients with active disease had to fulfil predefined criteria,[17] and were treated with corticosteroids alone or in combination with cyclophosphamide, depending on the severity of the SLE exacerbation and the organ systems involved. More than one organ system could be involved during a relapse of the disease. For the involvement of one or more organ systems during a relapse, the definitions of the SLE disease activity index (SLEDAI) score were used to determine disease activity.[18] Inactive disease was defined as the persistent absence of clinical disease activity for at least four months while patients were on a constant dose of immunomodulating drugs or were not receiving these drugs.

Patients with Wegener's granulomatosis (15 female, 10 male; age 55.8 (8.9) years) and rheumatoid arthritis (27 female, four male; 42.7 (13.9) years) were used as disease controls. The patients fulfilled the respective ACR criteria for these disease,[19 20] and criteria for active and inactive disease were predefined.[21 22] Patients with active rheumatoid arthritis were treated with sulphasalazine and those with active Wegener's granulomatosis with prednisolone and cyclophosphamide, according to a standard protocol.[23] In these patients paired serum samples obtained during active and inactive disease were also analysed, with a time interval of six months. In all the samples obtained from the index patients and disease controls, C reactive protein and SAA were measured in addition to SAP.

To analyse more specifically whether SAP reacts as an acute phase protein, serum was obtained from 11 women with breast cancer (aged 45.3 (10.7) years) and 16 patients (nine male, seven female; age 63.4 (18.3) years) with bacterial sepsis. Women with breast carcinoma had been treated, as previously described,[24] in a dose escalating trial of recombinant human interleukin-6 (rhIL-6) in increasing doses of 0.5 μg/kg/day (n = 1), 1.0 μg/kg/day (n = 2), 2.5 μg/kg/day (n = 2), 5.0 μg/kg/day (n = 2), 10.0 μg/kg/day (n = 3), and 20.0 μg/kg/day (n = 1) for seven days.[24] Serial serum samples from these patients were tested for SAP, C reactive protein, and SAA.

Analyses

Serum SAP, SAA, and C reactive protein concentrations were determined by enzyme linked immunosorbent assay (ELISA). For the detection of SAP, microtitre plates (Costar, Badhoevedorp, Netherlands) were coated in 0.1 M sodium carbonate, pH 9.6, with a monoclonal antibody directed against SAP (Novocastra Laboratories, Newcastle on Tyne, UK; 1:1000). After washing, serum specimens were added starting at a 250-fold dilution, with a further fourfold dilution in 0.05 M Tris, 0.3 M NaCL, 1% bovine serum albumin (BSA), and 0.05% Tween-20; the samples were then allowed to stand for one hour. As a positive control a reference serum was added. Bound SAP was detected with a rabbit polyclonal anti-SAP (Dakopatts, Glostrup, Denmark; 1:1000). After one hour of incubation, plates were washed and HRPO-labelled goat anti-rabbit IgG (Southern Biotechnology Associates, Birmingham, Alabama, USA; 1:4000) was added for 30 minutes in the dark. The SAP concentrations were determined using an SAP standard curve concentration (range 0.1 to 0.78 μg/ml). The intra-assay and interassay variations were 6.2% and 7.4%, respectively.

For the detection of C reactive protein, microtitre plates (Greiner BV, Alfen a/d Rijn, Netherlands) were coated overnight in 0.1 M sodium carbonate, pH 9.6, with a rabbit anti-human C reactive protein (Dakopatts; 1:10 000). After washing, sera were added starting at 250-fold dilution, with a further fourfold dilution in 0.05 M Tris, 0.3 M NaCL, 1% BSA, and 0.05% Tween-20 for one hour. As a positive control a reference serum was added. Rabbit anti-C-reactive protein conjugated with HRPO (Dakopatts; 1:2000) was applied for 30 minutes in the dark. The C reactive protein concentrations were determined using a standard curve (concentration range 0.1 to 0.78 μg/ml).

Serum amyloid A (SAA) ELISA was carried out as follows. Microtitre plates (Costar) were coated in 0.01 M PBS with 2 μg/ml of a monoclonal antibody directed against SAA (Rue.86.5; 1:1500) for one hour. After washing, sera were added starting at a 250-fold dilution, with a further fourfold dilution in 0.05 M Tris, 0.3 M NaCL, 1% BSA, and 0.05% Tween-20 for one hour. A reference serum was added as a positive control. Monoclonal antibody conjugated with HRPO directed against human SAA (Rue.86.5; 1:2000) was applied for 30 minutes using an ELISA shaker in the dark.

The substrate used in all ELISA systems was 90.9 μg/ml 3.3′, 5.5′-tetramethyl benzidine (TMB, Roth Chemicals, Karlsruhe, Germany) together with 0.004% H_2O_2. Colour development was stopped using 1N H_2SO_4. The OD values were read at 450–575 nm in an Emax scanner and concentrations were calculated using the standard samples with SOFTmax Pro software (Molecular Devices, Sunnyvale, California, USA).

Statistical analysis

Student's *t* tests or non-parametric Mann–Whitney U tests were used for group comparisons where appropriate. Paired *t* tests were used for differences between inactive and active samples when a normal distribution could be assumed. Otherwise the Wilcoxon signed rank test was used. Differences between multiple groups were calculated by one way analysis of variance (ANOVA) with Bonferroni's multiple comparison test. Correlations were calculated using the Spearman rank correlation coefficient. Reported probability (p) values were two tailed and $p<0.05$ was considered significant.

RESULTS

SAP values in SLE

Mean (SD) serum SAP in SLE patients with inactive disease was 26.4 (11.4) mg/l, which was not statistically different from the values in 120 healthy controls (27.7 (10.6) mg/l). Because the mean SAP tended to be higher in male than in female controls (30.0 (12.7) *v* 26.6 (9.3) mg/l (p = 0.0955)), data from male and female patients were compared separately with their respective controls. The results are shown in fig 1. No significant differences were found. In the control population serum SAP was not related to age (data not shown).

In addition, we analysed SAP values in SLE patients during active disease, assuming that at such times a relative deficiency in SAP might occur. However, disease activity did not influence serum SAP (table 1). Even when we made a subanalysis of SAP values in patients who did develop an

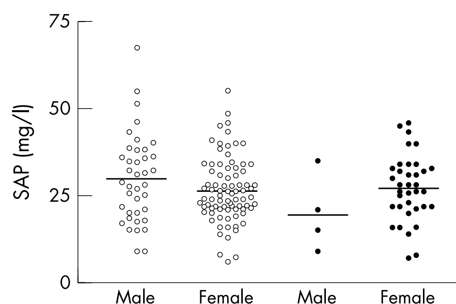

Figure 1 Serum amyloid P component (SAP) in male (n = 39) and female (n = 81) healthy controls (empty circles) and in male (n = 4) and female (n = 36) SLE patients during inactive disease (filled circles). Horizontal bars indicate the mean.

acute phase response during active disease (defined as a C reactive protein concentration of more than 5 mg/l), SAP remained unaltered (data not shown). Furthermore, levels of serum SAP were not associated with specific disease manifestations (fig 2).

Because the acute phase response is relatively inadequate in SLE patients, data were compared with two disease control groups in which an acute phase response, as represented by C reactive protein, is considered representative of disease activity. As in the SLE patients, we analysed blood samples in patients with rheumatoid arthritis and Wegener's granulomatosis for SAP, C reactive protein, and SAA on two separate occasions—that is, during active disease and during inactive disease (table 1). No differences in SAP levels between active and inactive disease were found in either disease group. However, SAP was increased in Wegener's granulomatosis compared with the healthy controls ($p<0.001$), and also compared with the patients with rheumatoid arthritis ($p<0.05$) and SLE ($p<0.001$) during active disease. Even during inactive disease, SAP was increased in the Wegener's group compared both with the healthy controls ($p<0.001$) and with the patients with rheumatoid arthritis ($p<0.05$) and SLE ($p<0.001$) (fig 3). As expected, C reactive protein and SAA concentrations were significantly increased in all patient groups during active as well as inactive disease compared with the controls (data not shown). The rise in C reactive protein during active disease was more pronounced in Wegener's granulomatosis than in either rheumatoid arthritis ($p<0.001$) or SLE ($p<0.001$). During active disease SAA concentrations in Wegener's granulomatosis increased more than in rheumatoid arthritis ($p<0.001$) or SLE ($p<0.001$).

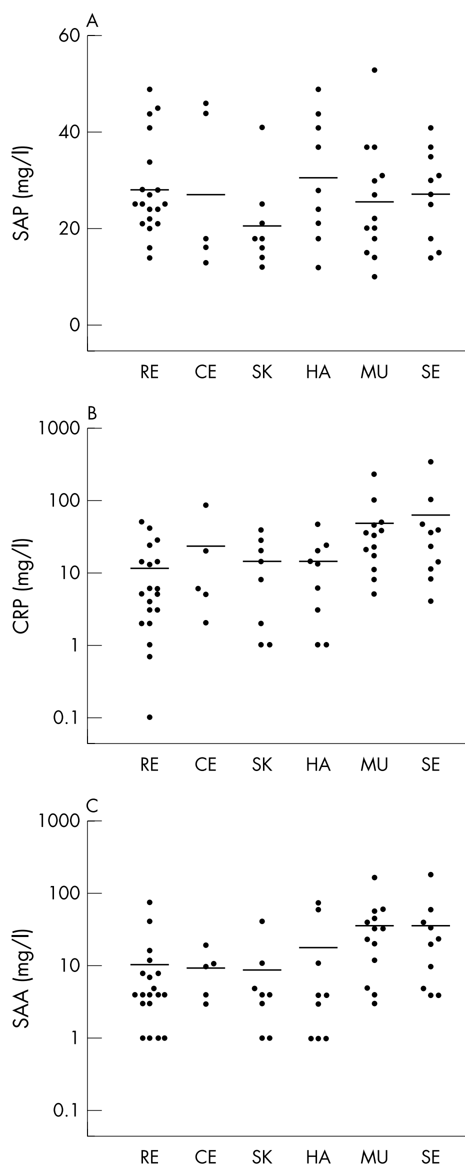

Figure 2 Serum amyloid P component (SAP) (A), C reactive protein (B), and serum amyloid A (SAA) (C) values in 40 patients with SLE during active disease. Disease manifestations were divided into organ manifestations based on the ACR criteria of SLE: RE, renal; CE, cerebral; SK, skin; HA, haematological; MU, musculoskeletal; SE, serositis. More than one organ system could be involved in an individual patient. Horizontal bars indicate the mean.

SAP levels during an acute phase response

To analyse the kinetics of serum SAP and to address the question of whether SAP is an acute phase protein, SAP was also measured in two other clinical situations. In the first model, an acute phase response was initiated by the administration of rhIL-6 to 11 patients with breast carcinoma. After starting rhIL-6, a small decrease in SAP occurred in all patients (fig 4). Values declined from 44.4 (7.5) mg/l at the start of treatment to 36.6 (9.7) mg/l after three days of treatment. No relation between the dose of rhIL-6 used and the level of SAP could be shown. Thereafter an increase in SAP levels was seen, reaching 54.9 (15.4) mg/l by day 10. In contrast to the SAP response, C reactive protein levels increased significantly after the start of treatment, from 18.1 (21.5) mg/l to a maximum of 415.0 (195.1) mg/l on day 3 ($p<0.001$). SAA levels showed a similar pattern, increasing from 13.1 (14.7) to 627.3 (245.9) ($p<0.001$). A representative example of the day by day kinetics of SAP during and after IL-6 administration is given in fig 4. No correlation could be detected between SAP and C reactive protein or between SAP and SAA, while there was a significant correlation between SAA and C reactive protein ($r = 0.4989$, $p = 0.0492$).

In the second model, SAP was measured in patients admitted to the intensive care unit (ICU) with severe sepsis. In these patients a similar pattern could be recognised. In the initial phase of the acute phase response, on admission to the ICU, SAP concentrations tended to be decreased in 16 patients with severe sepsis (23.1 (13.4) mg/l) compared with controls ($p = 0.06$). However, in the same patients, C reactive protein and SAA were increased to 183.2 (63.4) mg/l and 261.6 (175.4) mg/l, respectively, on admission to the ICU.

DISCUSSION

In this study we addressed the question of whether levels of serum amyloid P component in patients with SLE are decreased compared with healthy controls and whether the levels are related to disease activity.

In contrast to our expectations based on data obtained in the SAP knock-out mice,[12] we did not find a decrease in serum SAP in the patients with SLE, even in those with active disease. The question whether SAP levels differ in SLE patients compared with controls has been addressed before.[25–27] However, in all these studies the numbers of patients were small (ranging from eight to 16) and the extent of the disease was not taken into account.

It has been proposed that the spontaneous development of autoimmune features in the SAP knock-out mice may result from a disturbed clearance of apoptotic cells. Indeed, the persistence and accumulation of apoptotic cells can induce the production of autoantibodies and even overt autoimmune disease.[28 29] Our results show that the disease expression of human SLE cannot be explained by a deficiency of SAP. However, a contributing role of SAP in the elimination of

Table 1 Serum amyloid P component, C reactive protein, and serum amyloid A in patients with systemic lupus erythematosus, rheumatoid arthritis, and Wegener's granulomatosis

	SLE (n = 40)		Rheumatoid arthritis (n = 31)		Wegener's granulomatosis (n = 25)	
	Active	Inactive	Active	Inactive	Active	Inactive
SAP	27.8 (11.3)	26.4 (11.4)	32.3 (12.3)	30.3 (19.3)	40.4 (13.9)	39.3 (12.3)
C reactive protein	32.6 (64.5)***	9.3 (15.9)	22.7 (23.1)*	14.3 (12.6)	131.3 (132.6)***	16.5 (19.4)
SAA	23.4 (40.1)***	9.7 (24.3)	40.9 (49.5)	26.4 (32.0)	339.3 (349.1)***	35.5 (78.1)

Paired analysis of SAP, C reactive protein, and SAA detected in patients with SLE, rheumatoid arthritis, and Wegener's granulomatosis patients during active and inactive disease. Values are mean (SD), mg/dl.
**$p<0.01$, ***$p<0.001$, paired *t* test.
SAA, serum amyloid A; SAP, serum amyloid P; SLE, systemic lupus erythematosus.

apoptotic cells cannot be excluded. SAP has been found to bind to late apoptotic cells.[8] Moreover, we have recently shown that the binding of SAP to these cells has functional consequences for their elimination, SAP depletion resulting in a 50% decrease in the uptake of late apoptotic cells by monocyte derived macrophages.[30] From the concept that pentraxins have a function in the opsonisation and clearance of apoptotic cells,[1 2 8] a particular role for these proteins can be proposed during inflammation, when large numbers of cells are dying and have to be cleared rapidly to prevent their accumulation. Indeed, both C reactive protein and PTX3 levels rise rapidly during inflammation. C reactive protein is produced by the liver after IL-6 induction. PTX3 is produced locally on the site of inflammation by macrophages and endothelial cells. However, levels of both C reactive protein and PTX3 are low in active SLE.[31–34] Complement proteins, which opsonise apoptotic cells, are also low during active disease. Therefore, hampered opsonisation of apoptotic cells in SLE in the face of increased production will contribute to their persistence. It is conceivable that SAP acts as a rescue protein in the clearance of late apoptotic cells. For a role as rescue protein the amount of SAP that is constitutively present will be a sufficient backup if opsonisation and clearance of apoptotic cells fails and late apoptotic cells persist.[30]

We cannot rule out the possibility that SAP levels in SLE are higher than we have measured. In the first place, SAP–DNA complexes have been described in the sera of SLE patients.[6] These complexes might have interfered with our assay. Second, it has been shown recently that the majority of SLE patients have antibodies to SAP, which in theory might also reduce the free SAP levels measured.[6 35] Evaluation of this potential source of interference is currently under way.

Finally, we evaluated whether SAP reacts as an acute phase protein. Although others have reported that SAP levels remain relatively stable in different diseases, including malignancy and infections,[25–27] no studies on the kinetics of SAP have been carried out before. To evaluate this, we used two different models. We could show that, compared with C reactive protein or SAA, SAP does not have the kinetics of an acute phase protein. SAP tended to decrease in the earliest stage of the acute phase reaction and showed some increase in a case of sustained inflammation. It is conceivable that during chronic inflammation SAP levels are slightly raised, and we propose that this explains the increase found in patients with Wegener's granulomatosis.

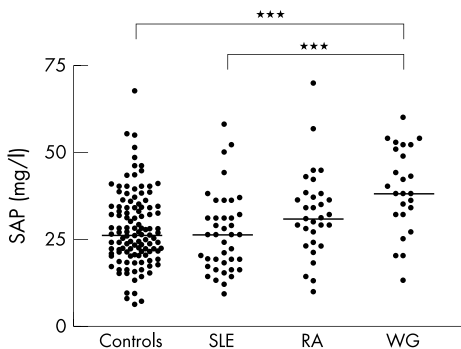

Figure 3 Serum amyloid P component (SAP) in healthy controls (n = 120), SLE (n = 40), rheumatoid arthritis (n = 31), and Wegener's granulomatosis (n = 25). SAP in the patients was measured during inactive disease. Horizontal bars indicate the mean. ***$p<0.001$ by one way analysis of variance with Bonferroni's multiple comparison test.

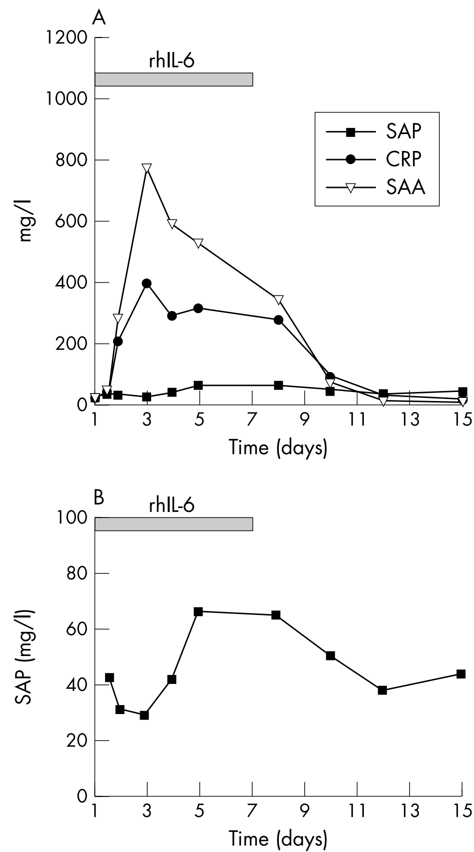

Figure 4 Serum amyloid P component (SAP) does not behave as an acute phase protein. (A) SAP, C reactive protein, and serum amyloid A (SAA) responses in a patient with breast cancer treated for seven days with recombinant interleukin 6 (rhIL-6) at a dose of 20 μg/kg/day. Even though a significant increase in C reactive protein (from18 to 395 mg/l on day 3) and an even more pronounced increase in SAA (from 11 to 770 mg/l on day 3) occurred, SAP levels hardly changed. (B) SAP levels of the same patient in more detail, showing a decline in SAP up to day 3, at the maximum of the acute phase response, and a slight increase thereafter.

Conclusions

We have shown that SAP concentrations are not decreased in patients with SLE and are not related to disease activity. Compared with C reactive protein and SAA, SAP does not react as an acute phase reactant. Serum SAP even decreased slightly in the initial stage of an acute phase reaction. However, these findings do not exclude a role for SAP in the pathogenesis of SLE, as a relative deficiency of this protein during acute inflammation may contribute to the persistence of (late) apoptotic cells. This should be addressed in further studies.

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