Anti-serum amyloid component P (SAP) antibodies in SLE patients correlate with disease activity

Category-Extended report

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ABSTRACT

Background: Serum amyloid P component (SAP) plays a role in the clearance of apoptotic debris, and hence may be involved in the development of systemic lupus erythematosus (SLE).

Objective: Our primary aim was to determine the presence of elevated titers of anti-SAP antibodies in SLE patients. Our secondary aim, if we were to find elevated titers of anti-SAP antibodies, was to evaluate their correlation with clinical disease by SLEDAI and clinical manifestations.

Methods: 452 samples (328 SLE patients, 124 normal controls) were screened for the elevated anti-SAP antibody titers utilizing the ELISA method. Clinical parameters and SLEDAI scores were independently reviewed from medical records. Twenty-one serial samples from 7 SLE patients were assessed for a change in anti-SAP antibody titers following treatment.

Results: Elevated anti-SAP antibody titers were detected in 44% of 328 SLE samples. In 112 randomly selected samples, 62% of the patients had elevated anti-SAP Ab and anti-dsDNA Ab titers, whereas only 28% had elevated anti-dsDNA antibody titers without elevated anti-SAP antibody titers. The mean titer of anti-SAP antibodies in active patients was higher than in patients with inactive disease and in controls. SLEDAI scores, assessed in 54 patients, were elevated in 84% of patients with anti-SAP antibody titers. A SLEDAI score of at least 8, indicating severe disease was found in 51.6% of patients with elevated anti-SAP antibody titers while only 21.7% of patients without elevated titers of anti-SAP antibodies had a high SLEDAI score. No specific pattern of disease was detected between patients with or without elevated titers of anti-SAP antibodies. Serial sampling from SLE patients with active disease and elevated anti-SAP antibody titers revealed a decrease in anti-SAP antibody titers following treatment that correlated with clinical improvement.

Conclusion: Elevated anti-SAP antibody titers were detected in SLE patients, correlated with disease activity and decreased with improvement of clinical disease. In SLE patients, elevated anti-SAP antibody titers may serve as an additional prognostic marker.

INTRODUCTION

Serum amyloid component P (SAP) is a member of the pentraxin family of proteins [1]. It is a highly conserved plasma protein named for its universal presence in amyloid deposits and a normal component of a number of basement membranes including the glomerular basement membrane [2]. It is the single normal circulating protein with specific calcium-dependent binding to DNA and chromatin in physiological conditions [3]. When bound to ligand, SAP forms a stable decamer with 2 pentameric rings [4]. The SAP-DNA binding interaction involves a decapeptide around Arg 120 [5]. SAP is a constitutive protein (40 microg/ml) synthesized by hepatocytes [6]. It is located on chromosome 1q23, in proximity to one of the putative genes associated with SLE [7].

SAP interacts with nuclear ligands including chromatin and snRNP, is important in the handling of chromatin exposed by cell death and plays a role in the clearance of nuclear ligands from apoptotic and necrotic cells [5,8,9]. SAP binds in vivo both to apoptotic cells, the surface blebs of which bear chromatin fragments, and to nuclear debris released by necrosis [9].

The pentraxin protein family (SAP and CRP) activates complement through the classical pathway and participate in the opsonization of particulate ligands and
bacteria [3]. Both CRP and SAP have nuclear transport signals that facilitate their entry into the nuclei of intact cells [3]. In the mouse, SAP binds to the FcγRI and FcγRIII receptors and therefore can activate the complement system [10]. During the acute phase response, C5a generated as a consequence of complement activation acts in concert with IL-6 and/or IL-1β to promote up-regulation of the SAP gene in a murine model [11]. SAP binds directly to apoptotic cells in the early [12] and late stages [13] of apoptosis. This interaction could mitigate against deposition of these antigens in tissue and autoimmune reactivity.

Mice with targeted deletion of the SAP gene spontaneously develop antinuclear autoimmunity and severe glomerulonephritis, a phenotype resembling systemic lupus erythematosus (SLE) [2,14,15]. The SAP knock-out mice also have enhanced anti-DNA responses to immunization with extrinsic chromatin [2].

SLE is an autoimmune disease whose multi-factorial etiology and diverse mechanisms have yet to be established. One postulated mechanism of dysregulation of the immune system is the abnormal clearance of apoptotic cells [16-18]. The physiological display of nuclear antigens in apoptotic bodies offers a plausible mechanism whereby the autoantigens prominent in SLE are exposed to the immune system, thereby stimulating an autoimmune response [17]. One postulation is that once chromatin is released into the circulation, SAP binds the chromatin and sequesters it from the antigen driven immune response [2].

In humans, SAP is predominantly a physiologic protein whereas in the mouse it is an acute phase reactant protein (as is CRP in humans) [14]. Abnormal levels of SAP in humans are not reported. In addition, no genetic polymorphisms have been detected. SAP levels are not altered when compared to disease activity [19, 20]. However, SAP-DNA complexes are lower in SLE patients when compared to healthy controls [21]. Improper clearance of apoptotic cells may lead to increased levels of apoptotic bodies, stimulating the increase production of autoantibodies during active disease [14]. We speculate that anti-SAP antibody titers may be elevated in SLE patients and hence influence clinical disease. Binding of anti-SAP antibodies to SAP could affect the function of SAP and therefore lead to abnormal clearance of persistent antigens and the propagation of autoimmunity.

In this study, our primary aim was to determine the presence of elevated titers of anti-SAP antibodies in SLE patients. Our secondary aim, if we were to find elevated titers of anti-SAP antibodies, was to evaluate their correlation with clinical disease by SLEDAI and clinical manifestations.

PATIENTS AND METHODS

Patients and controls
Laboratory features of 328 consecutive and unselected patients with SLE were evaluated in a multi-center cross sectional study. All fulfilled 4 or more of the American College of Rheumatology (ACR) revised criteria for the classification of SLE (22). Control samples, sex and age matched (n=124) were obtained from normal blood donors at the Israel National Blood Bank. Clinical findings were assessed in 189 SLE patients.

Serum samples evaluated for the presence of elevated titers of anti-SAP antibodies
The 328 consecutive samples analyzed for the presence of elevated titers of anti-SAP antibodies were from SLE patients attending 4 lupus/rheumatology clinics: 54 from Israel Lupus Clinic (group I), 135 from Germany (group II), 88 from Italy (group III), and 51 from Slovakia (group IV). Serial samples (n=21) measured from 7 SLE
patients attending the Israel Lupus Clinic (group I) were assessed separately for change in anti-SAP antibody titers following treatment.

**Clinical assessment**
Clinical findings were assessed in 189 SLE patients attending 2 outpatient clinics between the years 2000-2001. All patients had documented files and underwent a medical interview and physical examination by a rheumatologist. A serum sample from each patient was collected for anti-SAP antibody testing by ELISA. Disease activity was assessed utilizing the SLEDAI score [23] for 54 SLE patients (group I) and correlation with elevated anti-SAP antibody titers was evaluated. Serial samples from 7 SLE patients with active disease were assessed separately for change in anti-SAP antibody titers before and after treatment and the correlation with clinical improvement. In 135 randomly chosen SLE patients (group II), 31 clinical manifestations (supplement 1) reported in the clinic at anytime from the diagnosis were determined and correlated with elevated anti-SAP antibody titers. Clinical and serological characteristics collected in a protocol form were transferred to a computerized database program (Excel- Microsoft Office). Clinical assessment was not available for 139 SLE patients.

**Laboratory parameters**
Samples were screened for anti-SAP and anti-dsDNA titers utilizing the enzyme linked immunosorbent assay (ELISA) method.

Detection of anti-SAP antibody titers- 96 well Nunc plates were coated with SAP (Sigma Chemical, St. Louis, MO, USA), 5 ug/ml in Dulbecco’s PBS with calcium (Biological Industries, Beit Haemek, Israel), overnight at 4°C. The plates were blocked with 3% BSA for 1 hour at 37°C. Serum samples were added at a dilution 1:200, in PBS supplemented with Ca++/1% BSA for 4 hours at room temperature. The binding was probed with anti-human IgG and IgM conjugated to alkaline phosphatase, and incubated for 1 hour at 37°C. An appropriate substrate was added. Washing between steps was performed using PBS with 0.05% Tween-20. The plates were read at 405 nm OD by ELISA Titertek. A value was considered significantly elevated when the OD reading for elevated anti-SAP levels was above 3 standard deviations (3SD) of the mean OD reading for the control group. The specificity of binding was confirmed by inhibition assay.

Detection of anti-dsDNA antibody titers by ELISA- The detection of anti-dsDNA antibodies was previously described by us [24].

Inhibition assay- Total IgG was affinity purified on a protein-G column (Pharmacia, Upsala, Sweden) from 5 SLE patients (4 patients with elevated levels of anti-SAP and one patient which did not have circulating anti-SAP Abs). The affinity purified IgG was further purified on a dsDNA-cellulose column (Pharmacia), and tested for anti-dsDNA activity. Anti-SAP binding of the affinity purified IgG was tested at different concentrations (0.05-50µg/ml) to define the 50% binding to SAP by ELISA. The IgG, at a concentration of 50% binding to SAP, was incubated with different concentrations of SAP (0-50 µg/ml) overnight at 4°C. The mix was centrifuged at 14,000 rpm for 20 minutes. The presence of free anti-SAP Abs in the supernatant was assayed by transferring the supernatants to SAP coated 3% BSA blocked ELISA plates. Following a 4 hour incubation at room temperature, the bound anti-IgG Abs
were probed by anti-human IgG conjugated with alkaline phosphatase, and an appropriate substrate. Between each step, extensive washings with PBS-Tween 0.05% were performed. The percent of inhibition was defined according to: % Inhibition = [(OD of sample with no competitor - OD of sample with competitor)/ OD of sample with no competitor] X 100

**Statistical analysis**
The Pearson linear correlation and the two-tailed p value were utilized to assess the correlation between elevated anti-SAP antibody levels. The ROC curve analysis was performed for the evaluation of elevated anti-SAP antibody levels and the SLEDAI score. Student's t test was utilized for group comparisons when appropriate.

**RESULTS**

**Prevalence of anti-SAP antibodies**
In a multi-center cohort study, 452 samples (328 SLE patients, 124 normal controls) were screened for anti-SAP antibody titers. Elevated anti-SAP antibody titers were found in 44% (range: 22%-69%) of 328 SLE samples, compared to 2% of the control group (p<0.001) (Table 1).

**Table 1-Anti-SAP antibody titers in SLE patients**

<table>
<thead>
<tr>
<th>GROUP</th>
<th># OF PATIENTS WITH ELEVATED ANTI-SAP AB TITERS</th>
<th>TOTAL NUMBER OF PATIENTS</th>
<th>% OF PATIENTS WITH ELEVATED ANTI-SAP AB TITER (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I-ISRAEL*</td>
<td>32</td>
<td>54</td>
<td>59%</td>
</tr>
<tr>
<td>II-GERMANY*</td>
<td>31</td>
<td>135</td>
<td>22%</td>
</tr>
<tr>
<td>III-SLOVAKIA</td>
<td>21</td>
<td>51</td>
<td>41%</td>
</tr>
<tr>
<td>IV-ITALY</td>
<td>61</td>
<td>88</td>
<td>69%</td>
</tr>
<tr>
<td>SLE CLINICS TOTAL</td>
<td>145</td>
<td>328</td>
<td>44%</td>
</tr>
<tr>
<td>CONTROL</td>
<td>3</td>
<td>124</td>
<td>2%</td>
</tr>
</tbody>
</table>

*Serum samples available clinical parameters.

The mean titer of anti-SAP antibodies in active patients (n=54) was higher than in patients with inactive disease (n=135) and in controls (n=39) (0.34 +/- 0.11 OD at 405nm vs. 0.115 +/- 0.05 OD at 405nm vs. 0.08 +/- 0.04 OD at 405nm) (Figure 1). In a randomly selected group of 112 patients, 69/112 (62%) harbored elevated both anti-SAP antibody and anti-dsDNA antibody titers, whereas only 32/112 (28%) had elevated anti-dsDNA antibody titers without elevated anti-SAP antibody titers.

IgG and IgM isotypes of anti-SAP antibodies were measured in 149 SLE serum samples. The IgG isotype was elevated more frequently than the IgM isotype (88/149 and 61/149 respectively), but was not statistically significant. Only 15 SLE patients harbored both IgG and IgM isotypes.

Competition assay in 5 patients (4 patients with elevated titers of anti-SAP antibodies compared to one patient without elevated titers) revealed that anti-SAP and anti-DNA antibodies are distinct (Figure 2, 3).
Relationship between anti-SAP antibodies and clinical features and disease activity

Clinical assessment of samples from 54 SLE patients was performed utilizing the SLEDAI score. Patient demographics included 87% women, and the mean age was 44.7 years old (range 18-73). The ROC curve analysis was performed for the evaluation of elevated anti-SAP antibody levels and the SLEDAI score. The best value for defining patients with mild-moderate disease compared to severe disease was a SLEDAI score of 7. Patients with SLEDAI score >7 were considered to have severe disease. The SLEDAI score was divided into remission (score=0) mild disease (score 1-4), moderate disease (score 5-7) or severe disease (score 8 or above). Thirty-one patients (57%) harbored elevated anti-SAP antibody titers. SLEDAI scores were elevated (score>0) in 84% of patients with elevated titers of anti-SAP antibodies. Elevated anti-SAP antibody titers correlated with disease activity A SLEDAI score of at least 8, indicating severe disease was found in 51.6% of patients with elevated anti-SAP antibody titers while only 21.7% of patients without elevated titers of anti-SAP antibodies had a high SLEDAI score. The average SLEDAI score in patients with elevated anti-SAP antibody titers was 6.87. Figure 4 represents the percentage of patients with or without elevated anti-SAP antibody titers compared to SLEDAI scores (0-4 indicating remission or mild disease, 5-7 indicating moderate disease, >8 indicating severe disease).

Although elevated anti-SAP antibody titers correlated with severe disease activity (SLEDAI >7), no correlation was detected between elevated anti-SAP antibody titers and specific clinical manifestations described in the SLEDAI score. The positive predictive value was 76.2% for the probability that more severe disease is encountered in the presence of elevated titers of anti-SAP antibody titers. The negative predictive value was 54.5% for the probability that there is mild-moderate disease in the presence of elevated titers of anti-SAP antibody titers. The positive and negative likelihood ratios were 2.37 and 0.62 respectively.

In 135 randomly selected SLE patients, the distribution of 31 clinical manifestations reported in the clinic at anytime from the diagnosis was assessed (supplement 1). All patients were women. The mean age at serum sampling was 46.4 +/- 13.5 years old. Elevated anti-SAP antibody titers in 34 SLE patients (23%) did not reveal a specific pattern when compared to patients without elevated anti-SAP antibody titers. The distribution of clinical manifestations in patients with elevated anti-SAP antibody titers were by prevalence: arthritis (26/34), malar rash (19/34), photosensitivity (18/34), discoid lesions (17/34), myalgia (14/34), neuropathy (14/34), nephritis (7/34), depression (9/34). Other manifestations were less commonly found. Serial sampling (n=21) of 7 SLE patients with clinically active disease by SLEDAI who were not previously treated and harbored initially elevated anti-SAP antibody titers revealed a decrease in anti-SAP antibody titers upon treatment (Figure 5).

DISCUSSION

In this study, we approached the concept that SLE patients may harbor elevated titers of SAP antibodies and these autoantibodies may influence disease activity. We are the first multi-center group to assess the levels of anti-SAP antibodies in SLE patients. We found that elevated anti-SAP antibody titers were significantly higher in a large number of SLE active patients (defined by SLEDAI) when compared to non-active patients and healthy controls. The different subgroups were valuable in comparing the frequency of anti-SAP antibody titers in different patient populations. The main aim of this study was to investigate the presence of elevated titers to anti-SAP antibodies
in SLE when compared to a control group. For this reason, we chose to evaluate a large number of samples taken from SLE patients of European and Israeli populations. Upon finding an increased frequency of these antibodies in SLE patients, we chose then to compare the presence of anti-SAP antibody titers to (a) disease activity utilizing SLEDAI (group I) and (b) to specific clinical manifestations (group II). The clinical information was available in two groups only. Group I for the most part had active disease and group II for the most part had inactive disease. The difference in the nature of the clinics cannot be fully explained, only that the clinic from group I was a new clinic and hence had more newly diagnosed patients that had not received therapy.

In group I, 57% of patients had elevated anti-SAP antibody titers. Elevated anti-SAP antibody titers scores correlated with elevated SLEDAI scores. In another group of 135 patients, where 31 clinical manifestations were assessed, most patients were in remission that may explain a relatively low frequency (22%) of elevated titers of anti-SAP antibody titers was encountered. SLE patients may have non-active clinical disease but still harbor elevated titers of autoantibodies. This is supported by a prospective cohort study where patients who were serologically active but clinically quiescent (SACQ) in 3 consecutive clinic visits were analyzed for the development of a clinical flare over the subsequent year and were evaluated for predictive factors for flare before and during their SACQ period. Forty-six episodes of SACQ went on to clinical flare within one year while 60 did not. No predictive factors for flare were found either during or before the SACQ period. Hence, a significant population of patients with SLE are SACQ and must be followed over time and treated only on the basis of clinical criteria [25]. Conversely, patients may be clinically active and serologically quiescent. In another study, 514 patients followed for 4 years in a single clinic who on at least 3 consecutive visits had clinical activity in the absence of a low complement and elevated DNA binding. Demographics, disease characteristics, and therapy for the CASQ periods, as well as prior and subsequent disease course were analyzed. Sixty-two patients had at least one episode of CASQ lasting a 9.8 +/- 6.4 months. During these periods, patients showed evidence of clinical disease activity with a high SLEDAI score. Major organ involvement occurred in 43 patients. Of the 58 patients who had follow-up after their last CASQ defining visit, 9 remained CASQ for 39 +/- 23 months. Of the remaining 49 patients, 23 became inactive, 21 became clinically and serologically active, and 5 were serologically active but clinically quiescent (SACQ). This study supports the idea that clinical laboratory correlation in SLE is a heterogeneous relationship. The majority of patients have clinical-serological concordance. However, a minority of patients has discordance between clinical and serological status, and are either SACQ or CASQ. Therefore, monitoring both clinical and serological features in patients with SLE is important. [26].

Elevation of anti-SAP antibody titers correlated with disease activity by SLEDAI, making them potentially a good marker for active disease.

Serial sampling of patients provided important information regarding the change in anti-SAP antibody titers correlating to clinical improvement and treatment. Interestingly, two representative patients suffered from SLE and secondary APS, presented with organic brain syndrome manifested as cognitive dysfunction, revealed initially elevated titers of anti-SAP antibody titers prior to treatment and a decrease in anti-SAP antibody titers following treatment with prednisone and intravenous immunoglobulin (IVIg) or IVIG alone [27,28]. Both patients did not have elevated titers of anti-dsDNA, but ANA was positive, and complement levels were low. The decrease in anti-SAP antibody titers correlated to an increase in complement levels.
and to clinical improvement. We suggest that anti-SAP antibody titers may be an ancillary test to that of anti-dsDNA antibody titers for detection of disease activity. However due to the small patient sample, further evaluation would be necessary. The clinical association to elevated anti-SAP antibody titers suggests that these antibodies may play a role in the aberrant clearance of nuclear debris during apoptosis. The SAP-anti-SAP antibody complex may explain the normal levels of SAP detected in the serum, and could suggest an abnormal function of the protein [20].

We established an ELISA for anti-SAP antibody detection in the sera. No differences in the data were obtained in the presence or absence of Ca\(^{+2}\) ions, perhaps due to sera dilutions. Purified IgG bound SAP with no dependency on Ca\(^{+2}\) ions. SAP molecules in the samples did not interfere with anti-SAP binding.

There are two populations of anti-SAP antibodies. One population is cross reactive with dsDNA, and the second population does not react with dsDNA. Figure 2. represents the population which is not dependent on dsDNA, exhibiting the dose dependent specific binding of anti-SAP antibodies to SAP.

In conclusion, we were able to show that elevated titers of anti-SAP antibodies are present in SLE patients, and hence may play a role by binding to SAP and altering its function. Elevated anti-SAP antibody titers were detected in 44% of SLE patients. There was a significant correlation between elevated anti-SAP and anti-dsDNA titers. Elevated anti-SAP antibody titers correlated with disease activity by SLEDAI. Anti-SAP antibody titers decreased upon improvement of clinical disease. We propose that in SLE patients, elevated anti-SAP antibody titers may serve as an additional prognostic marker.

**Legends**

**Figure 1.** Titers of anti-SAP antibodies in 189 SLE patients; 54 SLE patients with clinically active disease (Group I-Israel), 135 SLE patients with clinically inactive disease (Group II-Germany), and in 54 healthy controls.

**Figure 2.** SAP dose dependent binding of protein-G and dsDNA-cellulose affinity purified total IgG from 4 lupus patients positive for SAP (patients 1-4), one lupus patient negative for SAP recognition (patient 5). The binding was assayed by ELISA. Data presented as OD at 405 nm, mean of 3 experiments ±SD.

**Figure 3.** Inhibition of binding of affinity purified IgG from lupus patients to SAP by SAP. Total IgG affinity purified from 5 lupus patients were incubated with different concentrations of SAP. Patient 1-4 harbored elevated titers of anti-SAP antibodies. Patient 5 did not have elevated titers of anti-SAP antibodies. The inhibition of the binding was calculated as the percent of inhibition of total IgG to SAP by SAP. The data are presented as mean +/-SD of 3 experiments.

**Figure 4.** SLEDAI scores for 54 SLE patients, comparing the percentage of patients with elevated anti-SAP antibody titers vs. those without elevated titers. Remission and mild disease: SLEDAI: 0-4; Moderate disease-SLEDAI: 5-7; Severe disease-SLEDAI: 8 and above.

**Figure 5.** The serial sampling of anti-SAP antibody titers in 7 patients on 2-4 occasions. All patients were recently diagnosed with SLE or had a flare. The first samples were taken before therapy. The OD for 3 SD of the mean controls was 0.154.
REFERENCES
Figure 1.
Figure 2.

![Graph showing OD 405nm against SAP (µg/ml) for different individuals (ptn.1 to ptn.5).]
Figure 3.
Fig. 4

<table>
<thead>
<tr>
<th>SLEDAI</th>
<th>Mild</th>
<th>Moderate</th>
<th>Severe</th>
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<tbody>
<tr>
<td>anti-SAP+</td>
<td>35%</td>
<td>13%</td>
<td>52%</td>
</tr>
<tr>
<td>anti-SAP-</td>
<td>57%</td>
<td>22%</td>
<td>22%</td>
</tr>
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Figure 5.