Usefulness of detection of complement activation products in evaluating SLE activity

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Complement activation products, such as C1rs-C1inh, specific for the activation of the classical pathway, C3b(Bb)P, specific for the activation of the alternative pathway and SC5b-9, specific for common terminal pathway of the complement cascade, were measured in healthy donors and in patients with clinically active and inactive systemic lupus erythematosus (SLE). Plasma levels of C3b(Bb)P and SC5b-9 were moderately, those of C1rs-C1inhibitor (C1rs-C1inh) were markedly elevated in patients with clinically inactive SLE, compared with healthy controls. The difference between active and inactive stages of the disease was best reflected by C3b(Bb)P plasma concentration \( P < 0.001 \), which also showed the highest correlation with the SLEDAI \( (R_s = 0.41 \quad P < 0.001) \) and which was the most useful in distinguishing active and inactive sample pairs as well. The difference between SC5b-9 levels in the active and inactive stages was also significant \( (P = 0.007) \), while that of C1rs-C1inh did not differ significantly \( (P = 0.136) \). The correlation of the SLEDAI with SC5b-9 was 0.3 \( (P = 0.015) \), while with C1rs-C1inh it was 0.21 \( (P = 0.089) \). These findings suggest that the measurement of complement activation products, especially that of the alternative pathway, are sensitive markers of the activity of SLE and can be used for clinical purposes.

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Introduction

Systemic lupus erythematosus (SLE) is the prototype of immune complex (IC) diseases, with disease activity varying over time. The determination of disease activity is of great importance, since in the active stage immunosuppressive therapy is usually required, while in the inactive stage therapy should be tapered or stopped. Numerous attempts have been made to find both sensitive and specific laboratory markers for assessing disease activity.

Complement parameters have frequently been studied in order to evaluate SLE activity. Haemolytic activity of complement (CH50), C3 and C4 assays are routine methods for determining disease activity in SLE. However, their sensitivity and specificity are low, because complement activation may be present even when plasma levels of C3, C4 or CH50 are within the normal range, due to increased synthesis of complement proteins. In addition, decreased synthesis can mimic hypocomplementemia.1–4 To overcome the problems mentioned above, complement activation products, split products and activation specific protein-protein complexes have been measured. Split products of the classical and alternative pathways, in particular, Ba, Bb, C4a and C4d have been determined, however, they often have limited value in evaluating SLE activity.5–12 Several reports have analysed activation-specific protein–protein complexes such as SC5b-9 and C1rs-C1inh as potential activation markers in SLE.6,8–22 However, very few data have accumulated on C3b(Bb)P, a specific marker of alternative pathway activation.20,23

The aim of the present study was to analyse complement activation in patients with active and inactive SLE in order to evaluate the diagnostic value of pathway-specific activation products: C1rs-C1inh (classical pathway), C3b(Bb)P (alternative pathway) and SC5b-9 (terminal pathway) in defining SLE activity. Correlation of these parameters to other laboratory markers, such as CH50, C3, C4, circulating immune complexes (IC), anti-dsDNA and anti-C1q antibodies was calculated as well. Among the
parameters tested, C3b(Bb)P was found to be the best tool to distinguish between the active and inactive stages of SLE.

Patients and methods

Patients and samples

Sixty-five (60 female, five male) patients suffering from SLE, fulfilling four or more of the American Rheumatism Association (ARA) criteria for SLE were included. All patients were of Caucasian race. Fifty eight patients had one sample, seven patients had two samples (one from inactive and one from active stage). The samples of the latter patients were used in evaluating active and inactive sample pairs.

Patients with definite diagnosis of a viral or bacterial infection or inflammatory disorder, other than SLE at the time of investigation, were excluded from this study.

Blood and urine samples were obtained at each visit. Venous blood samples were drawn into vacutainer tubes, immediately centrifuged and placed on ice. For anti-dsDNA, CH50, C4, C3, anti-C1q-IgG, anti-C1q-IgA and IC analysis, serum (stored at $\text{-}20 ^\circ\text{C}$) was used. For C3b(Bb)P, C1rs-C1inh and SC5b-9 analysis we used EDTA-plasma (stored at $\text{-}80 ^\circ\text{C}$).

Routine laboratory tests included a complete blood cell count, serum creatinine, erythrocyte sedimentation rate, urinary sediment and total protein measurement.

Routine immunological tests

Serum anti-(native)dsDNA IgG was determined by ELISA. CH50 was measured in a haemolytic assay. C3, C4 and PEG-precipitable IC were measured by turbidimetry.

The cut-off value of anti-dsDNA level was 7.18 U/ml (mean $\pm$ 2 s.d.), based on the data of 100 healthy controls. The normal range for CH50 was 48–105 U/ml, for C3: 0.7–1.8 g/l, for C4: 0.08–0.3 g/l. The cut-off value of IC level was 97 U/ml (mean $\pm$ 2 s.d.), based on the data of 100 healthy controls.

Measurement of the complement activation products

SC5b-9 was determined by ELISA as described previously. The cut-off value of SC5b-9 level was 661 ng/ml (mean $\pm$ 2 s.d.), based on the results of 40 healthy controls.

Plasma concentrations of C1rs-C1inh and C3b(Bb)P were determined by ELISA, according to Cat et al. In brief: plates (Nunc, Wiesbaden, Germany) were coated overnight at 4$ ^\circ\text{C}$ with anti-human C1inh (Dako, Glostrup, Denmark) or goat anti-human properdin (ATAB, Stillwater, USA). After blocking them with phosphate buffered saline (PBS)-1% bovine serum albumin (BSA) for 60 min at room temperature (RT) diluted plasma samples and standards (C1s-C1inh complex or inulin activated serum for C3b(Bb)P) were incubated in duplicates for 60 min. Goat anti-human C1s (ATAB, Stillwater, USA) or biotin labelled rabbit anti-human C3c (Dako, Glostrup, Denmark) was added as second antibody thereafter for 60 min. Then peroxidase labelled rabbit anti-goat IgG (Dianova, Hamburg, Germany) (60 min) or streptavidine-peroxidase (Amersham, Little Chalfont, England) (30 min) was added. The reaction was visualized by 2,2’-azino-bis (3-ethylbenzthiozoline-6-sulfonic acid (ABTS) (Sigma, St. Louis, Mo, USA)/H$_2$O$_2$ or by o-phenylenediamine (OPD) (Dako, Glostrup, Denmark)/H$_2$O$_2$ and stopped by oxalic acid or H$_2$SO$_4$ (0.5M). The cut-off value of C1rs-C1inh level was 252 U/ml (mean $\pm$ 2 s.d.) and of C3b(Bb)P level was 10.8 U/ml (mean $\pm$ 2 s.d.), based on the results of 40 healthy controls.

Determination of disease activity

Patients were considered to have active lupus if they satisfied two conditions: (a) a score of at least 2

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by the clinical activity scale of modified SLEDAI (mSLEDAI); and (b) the requirement of immediate treatment for control of symptoms. The physician’s global assessment was in all cases in accordance with the diagnosis of activity mentioned above. Clinical assessment was made without knowledge of the laboratory data.

**Statistical analysis**

Differences between the parameters measured in patient groups with active and inactive stages of SLE were tested using the Mann-Whitney’s test. Differences between gender and treatment rates in patient groups with active and inactive stages of SLE were tested using Fisher’s exact test. Analysis of covariance including treatment variables (corticosteroid, immunosuppressive and antimalarial therapy) as covariates was used to estimate the confounding role of treatment on the parameters measured. Correlation of parameters to each other and with mSLEDAI was calculated by Spearman’s rank correlation test. To avoid statistical bias, from patients who had two samples the active one was used for statistical analysis. For evaluating the correlation between clinical activity and measured factors, modified SLEDAI was used because SLEDAI criteria28 contain anti-dsDNA and complement. Modified SLEDAI was calculated by omitting anti-DNA and complement from the SLEDAI.21 Sensitivity, specificity, positive and negative predictive values were calculated using the standard formula. Receiver operator characteristic (ROC) curves10 were used to identify cutpoints for disease activity, measured by C3b(Bb)P, SC5b-9 or anti-dsDNA that provided the greatest sensitivity and specificity in determining lupus disease activity based on the criteria mentioned above.

**Results**

We examined 16 (22.2%) samples from patients with active SLE and 56 (77.7%) samples from patients with inactive disease. Of the 16 active samples, 6 (37.5%) were from patients with disease activity in more than one organ system and 10 (62.5%) were from patients with one affected organ system (musculoskeletal system (4), nervous system (3), renal system (2) and mucocutaneous system (1)).

Demographic and treatment variables are presented in Table 1. No significant differences were found in gender, age and disease duration between active and inactive patients.

### Table 1 Patients’ characteristics (in parentheses: 95% confidence intervals)

<table>
<thead>
<tr>
<th></th>
<th>Active</th>
<th>Inactive</th>
<th>Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients</td>
<td>16</td>
<td>49</td>
<td></td>
</tr>
<tr>
<td>Male/female</td>
<td>0/16</td>
<td>5/44</td>
<td>n.s.</td>
</tr>
<tr>
<td>Age (y)</td>
<td>39.5±11.8</td>
<td>38.8±11.7</td>
<td>n.s.</td>
</tr>
<tr>
<td>Disease duration (y)</td>
<td>11±?</td>
<td>10.7±8</td>
<td>n.s.</td>
</tr>
<tr>
<td>SLEDAI</td>
<td>9.31±2.93</td>
<td>1.61±1.35</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>Treatment:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corticosteroid</td>
<td>16</td>
<td>22</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>Immunosuppressive</td>
<td>6</td>
<td>9</td>
<td>n.s.</td>
</tr>
<tr>
<td>Antimalarial</td>
<td>4</td>
<td>7</td>
<td>n.s.</td>
</tr>
<tr>
<td>No medication</td>
<td>0</td>
<td>20</td>
<td>P = 0.001</td>
</tr>
</tbody>
</table>

n.s.: not significant.

Analysis of covariance revealed that treatment does not have significant effect on the parameters measured (data not shown).

The normal ranges of healthy donors, the mean values and standard deviations in samples from patients with inactive and active disease, differences between active and inactive patients in C3b(Bb)P, SC5b-9, anti-dsDNA, anti-C1q IgG, anti-C1q IgA, CH50, C1rs-C1inh, C4, C3 and IC levels are shown in Table 2. The greatest difference between patients in the active and inactive stages was found in the values of C3b(Bb)P (P < 0.001). Lower but still highly significant differences were found both in the SC5b-9 values (P = 0.007) and anti-dsDNA levels (P = 0.01). For the anti-C1q IgG and CH50 values

### Table 2 Normal ranges (values of healthy controls), mean ± s.d. values of complement parameters, IC, anti-dsDNA, anti-C1q IgG and anti-C1q IgA found in the inactive and active SLE patient groups (in parentheses: 95% confidence intervals)

<table>
<thead>
<tr>
<th>Test</th>
<th>Normal values n = 49</th>
<th>Inactive n = 16 vs Active n = 16</th>
</tr>
</thead>
<tbody>
<tr>
<td>C3b(Bb)P</td>
<td>1.9–10.8 U/ml</td>
<td>7.97±2.65 (7.21–8.73)</td>
</tr>
<tr>
<td>SC5b-9</td>
<td>120–661 ng/ml</td>
<td>414±257 (340–488)</td>
</tr>
<tr>
<td>Anti-dsDNA</td>
<td>&lt; 7.18 U/ml</td>
<td>15.5±28.1 (5.48–21.7)</td>
</tr>
<tr>
<td>Anti-C1q IgG</td>
<td>&lt; 0.575 (OD)</td>
<td>0.42±0.46 (0.28–0.55)</td>
</tr>
<tr>
<td>CH50</td>
<td>48–105 U/ml</td>
<td>82.8±32.2 (73.6–92)</td>
</tr>
<tr>
<td>C1rs-C1inh</td>
<td>40–252 U/ml</td>
<td>338±103 (408–368)</td>
</tr>
<tr>
<td>C4</td>
<td>0.08–0.3 g/l</td>
<td>0.19±0.14 (0.153–0.237)</td>
</tr>
<tr>
<td>C3</td>
<td>0.7–1.8 g/l</td>
<td>1.31±0.44 (1.18–1.44)</td>
</tr>
<tr>
<td>IC</td>
<td>&lt; 97 U/ml</td>
<td>126.3±17.3 (106–147)</td>
</tr>
<tr>
<td>Anti-C1q IgA</td>
<td>&lt; 0.612 (OD)</td>
<td>0.37±0.43 (0.24–0.494)</td>
</tr>
</tbody>
</table>
the difference between active and inactive patients was also significant ($P = 0.012$ and $P = 0.015$, respectively). However, no significant differences were observed between the active and inactive patients in the C1rs-C1inh, C4, C3, IC and anti-C1q IgA levels.

The mSLEDAI displayed the highest correlation with C3b(Bb)P ($R = 0.41$ p < 0.001 Table 3). mSLEDAI also positively correlated with anti-dsDNA ($P = 0.011$) and SC5b-9 ($P = 0.015$) values, while a significant negative correlation between mSLEDAI and CH50 values was observed ($P = 0.018$). By contrast mSLEDAI did not significantly correlate with C1rs-C1inh, C4, C3, anti-C1q IgG, anti-C1q IgA and IC levels. C1rs-C1inh showed significant correlation with SC5b-9 ($p < 0.001$), IC ($p < 0.001$), anti-dsDNA ($P = 0.004$) and anti-C1q IgG ($P = 0.05$) pointing to an antibody mediated classical pathway activation. CH50, C3, C4 and anti-dsDNA correlated significantly with one another.

Among the complement activation products tested, C3b(Bb)P correlated only with C3 concentration and a correlation of marginal significance between C3b(Bb)P and SC5b-9 was also observed.

Table 4 shows the sensitivity, specificity, positive and negative predictive values of each parameter at one representative cutpoint. C3b(Bb)P measurement had the highest sensitivity, specificity, positive and negative predictive values.

Seven patients had a flare-up during the study period (Figure 1). C3b(Bb)P changes with the disease activity in 7/7, SC5b-9 in 6/7, and C1rs-C1inh in 4/7 cases.

The three parameters showing the greatest correlation with the mSLEDAI and the highest difference between the active and inactive stages—C3b(Bb)P, SC5b-9 and anti-dsDNA—were used to construct receiver operator characteristic (ROC) curves (Figure 2). Based on the analysis of the ROC curves, the greatest sensitivity and specificity was found to be 93–71% respectively, at the cutpoint 9.2 U/ml for C3b(Bb)P. SC5b-9 had 75% sensitivity and 71%
specificity at the cutpoint 460 ng/ml, while the best sensitivity and specificity was found to be 62.5–63% at the cutpoint 8.8 U/ml for anti-dsDNA. The ROC curves of the other parameters measured reflected lower clinical utility than that of C3b(Bb)P and SC5b-9 (data not shown).

There was no significant correlation between serum creatinine and any measured parameters (data not shown).

No significant differences were observed between plasma C3, C4, CH50, C3b(Bb)P, SC5b-9 and anti-C1q IgA levels in patients with different patterns of organ involvement. C1rs-C1inh, IC, anti-dsDNA and anti-C1q IgG levels were more frequently elevated in patients with renal involvement.

Discussion

Our findings confirm and extend others’ observations regarding the value of assessing the activation of the alternative pathway of complement system in estimating the activity of SLE.5–10,13,21,29 We found that C3b(Bb)P, a protein-protein complex formed during the activation of the alternative pathway is a valuable tool for this purpose. C3b(Bb)P showed the highest difference between active and inactive stages of the disease and the best correlation with mSLEDAI among the parameters studied and it had the highest sensitivity, specificity, positive and negative predictive values as well. Moreover, C3b(Bb)P was the only
parameter, which changes with the disease activity in all 7 examined patients. In our hands, C3b(Bb)P was more useful in evaluating SLE activity than C3, C4, CH50, SC5b-9, or anti-dsDNA, known markers of SLE activity.

Activation of the alternative pathway in SLE was investigated by the measurement of B, Ba, Bb, factor D, properdin, AH50 (alternative haemolytic assay) and IC solubilization during long incubation. Complement split products, such as Ba, Bb were found to be more useful in evaluating SLE activity than native complement components. C3b(Bb)P, a highly specific marker of alternative complement activation, was first measured in SLE patients by Auda et al. It was found to be higher in SLE patients than in controls, but no further analysis was performed. Chiu et al. — in contrast with our observations — found no significant difference in C3b(Bb)P concentrations between inactive and active subgroups of SLE patients. The difference between the results of the two studies can be explained probably by the differences between the two patient groups.

Complement activation in SLE is generally thought to occur via the classical pathway. Specific markers, such as C4, C4a, C4d and C1rs-C1inh have been measured, however, these studies showed limited usefulness of measuring these factors in evaluating SLE activity. The C1rs-C1inh level did not differ significantly between our patients with active and inactive disease. The correlation with the mSLEDAI also failed to be significant. C1rs-C1inh, which was markedly elevated even in inactive patients, changed with the disease activity only in four of seven cases.

Other reports emphasize the value of SC5b-9 as a disease activity marker in SLE. We also found marked differences in SC5b-9 plasma concentrations between active and inactive patients, they correlated well with disease activity and they change with the disease activity in six/seven cases. The overall sensitivity and specificity of the mSLEDAI also failed to be significant. C1rs-C1inh, which was markedly elevated even in inactive patients, changed with the disease activity only in four of seven cases.

Anti-dsDNA differed significantly between the active and inactive stages and correlated with the mSLEDAI. However the ROC curve of anti-dsDNA mirrored lower clinical utility than that of C3b(Bb)P or SC5b-9.

Anti-C1q IgG antibodies also showed significant difference between the active and inactive stages, but failed to correlate significantly with mSLEDAI. However, anti-C1q IgG autoantibodies showed significant correlation with C3, C4, CH50, anti-dsDNA and with SC5b-9 level, confirming previous observations. Moreover, anti-dsDNA IgG and anti-C1q IgG correlated significantly with C1rs-C1inh, suggesting their role in activating the classical pathway of the complement system.

A significant positive correlation was found between C3b(Bb)P and C3. Seemingly it is in contrast with the finding that C3b(Bb)P level increased when the disease activity was higher and C3 decreased at the same time. However, the above mentioned correlation can be explained as follows: C3 is an acute phase protein (C3 synthesis increases with the activation of disease which may obscure the increased consumption) and C3 is required for the formation of C3b(Bb)P.

In our study, IC level did not show a significant difference between active and inactive SLE patients and did not correlate significantly with mSLEDAI. IC levels showed highly significant correlation with C1rs-C1inh and with SC5b-9 levels, reflecting that IC primarily activates the classical pathway of complement system, as previously described. This finding is consistent with others’ observations, indicating that IC solubilization ability inversely correlates with C1rs-C1inh level.

Based on previous findings and on our present observations, we assume the following mechanism for complement activation in SLE: in the inactive stage the permanently high IC level — probably in part due to defective clearance mechanisms — causes a continuous classical pathway activation, with a limited amplification via the alternative pathway and relatively low SC5b-9 levels. With the activation of the disease, the levels of specific autoantibodies, such as anti-dsDNA, anti-C1q and possibly others increase, enhancing classical pathway activation and resulting in increased generation of C3b. At this point complement activation is amplified by the positive feedback between C3b and C3b(Bb)P. Due to this positive feedback minor changes in classical pathway activation can be amplified to major changes in the alternative pathway activation. However, some other factors such as the half-life of the markers examined and the length of time and kinetics of their level-changes may all influence the diagnostic value of these markers. The chemotactic peptides produced during complement activation, such as C3a, C5a and the formation of C5b-9 lead to inflammation and tissue injury.

According to our results, the most important demographic and treatment variables does not have confounding role on the parameters measured. However, longitudinal studies are needed to confirm this latter finding. Longitudinal studies can also help to avoid spectrum bias and provide information on the ability of the laboratory test to change over time.
Our patient cohort contained mostly patients with moderate disease activity, and only few patients with severe disease activity. Thus, it remains possible that in more severe cases traditional measures (C3, C4, CH50) are more informative than in our study, although they have been reported to have little clinical value even in severe cases.\textsuperscript{3,4}

In conclusion, C3b(Bb)P is a highly specific and sensitive indicator of SLE activity showing the highest difference between the active and inactive stages and the greatest correlation with the mSLEDAI among the parameters examined. It changes with the disease activity in all examined patients. However repeated longitudinal studies, with a higher number of patients are needed to confirm our present findings.

Acknowledgements

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