Reduced opsonisation of protein A containing *Staphylococcus aureus* in sera with cryoglobulins from patients with active systemic lupus erythematosus

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**SUMMARY** Among a total of 41 patients with systemic lupus erythematosus (SLE) 11 of 14 patients with active disease had reduced capacity (p<0.05) to opsonify *Staphylococcus aureus* in undiluted sera, as compared with nine of 27 patients with inactive disease (p<0.02). The opsonic reduction in the active patients increased with the number of active organ systems (p<0.002). No correlation was found between reduced opsonisation and corticosteroid treatment, or serum concentrations of complement components (C) of the classical pathway, or bacteria-associated activated C3. When the cryoglobulin fraction of immune complexes (IC) was removed, normal opsonic capacity was restored, and the opsonic reduction could be transferred with the cryoglobulins to pooled serum. Increased IC values, as measured by C1q binding assay, were found in conjunction with reduced opsonic capacity (p<0.04). Since opsonisation in SLE sera of a protein A deficient strain of *S. aureus* was normal, reduced *S. aureus* phagocytosis in SLE sera may be explained by IC binding to staphylococcal protein A.

Key words: phagocytosis, granulocytes, monocytes, immune complexes.

In a recent prospective and controlled study we have shown an increase of bacterial infections, especially those caused by *S. aureus*, in an unselected group of SLE patients from a defined population. The infectious episodes occurred predominantly during periods of disease activity.

Phagocytic killing is considered to be the major host defence against bacterial infections. In sera from patients with SLE impaired phagocytosis and microbicidal capacities have been reported for normal phagocytes. Phagocytic cells identify an opsonised prey and attach it to their cell membranes with the aid of receptors for the Fc fragment of immunoglobulin G and the CR1 receptor for activated C3. Defective opsonisation due to reductions of C and correlating with infections in active SLE has been suggested as one possible mechanism behind impaired phagocytosis.

Serum concentration and opsonisation time are of critical importance to results when opsonin functions are studied. In this study we present a modified assay system with short-time opsonisation in undiluted serum. We used as test organism a protein A containing strain of *S. aureus,* which is opsonised by 'natural' antibodies to peptidoglycan in the presence of C of the classical pathway.

With this system reduced opsonisation was found in some SLE sera. The mechanisms behind the reduction were studied, and the results were correlated with the current clinical status of the patients, in conjunction with blood sampling.

**Patients and methods**

Informed consent was obtained from the blood donors and the study was approved by the Ethics Committee of the University of Lund.

**Patients**

From a sequential study of SLE patients a series of 41 consecutive patients were included in the present study. All 41 patients fulfilled at least four of the 1971 American Rheumatism Association (ARA) criteria or four of the 1982 ARA criteria. The
Reduced opsonisation of S. aureus in undiluted SLE sera

PHAGOCYTES

100 ml of blood was drawn from a cubital vein of healthy members of the hospital staff. The blood was immediately defibrinated by gentle rotation for 10 minutes in a flask containing glass beads. A modified Böyum technique was used in the following manner. The defibrinated blood was sedimented at room temperature with an equal volume of 2% dextran T 500 (Pharmacia, Uppsala, Sweden) in isotonic sodium chloride. After 45 minutes the leukocyte-rich supernatant was withdrawn, and 25 ml was layered on top of 15 ml of an Isopaque–Ficoll mixture in each of four 30x115 mm plastic tubes. (Falcon, Oxnard, California, USA). The tubes were centrifuged with a swing out rotor for 15 min at 1350 g. The upper cell layer containing monocytes was pipetted off into two tubes and centrifuged to a pellet. The granulocyte pellets from two tubes were each suspended in 7 ml of the original serum–dextran mixture and gently mixed for 5 min with 35 ml of 0.87% ammonium chloride for erythrolysis. After centrifugation the pellets were washed twice in 40 ml of Parker’s fluid 199 (National Bacteriological Laboratory, Stockholm, Sweden) and suspended in the incubation medium which consisted of 10% heat-inactivated pooled serum in Parker’s fluid 199, or Parker’s fluid 199 only. The cells were counted in a Bürker chamber, and their final concentration was adjusted to approximately 5x10^9 phagocytes/l incubate.

SERUM

Pooled serum was collected from 34 healthy volunteers. Individual sera were collected from 15 healthy members of the hospital staff, seven of the sera being used for determining the variance of single sera from one subject to another. Blood was sampled from the 41 SLE patients according to the SLE control programme of the series of patients under consideration here. Sera collected at 4°C were used for complement analysis, and sera collected at 37°C were used for opsonic studies and cryoglobulin isolation. Fifty-nine sera, usually including the first available sample from each patient during the prospective study, were analysed by the opsonic assay. Serum from one patient in a family with fulminant meningococcal infections and hereditary properdin deficiency, less than 1% of normal was kindly provided by Dr J H Braconier, Department of Infectious Diseases, University Hospital, Lund.

BACTERIA

Staphylococcus aureus (strain 502 A) was used as the test organism. In some experiments separately reported under ‘Results’ tests with S. aureus (strain Wood 46) were run in parallel. Bacteria were cultured overnight in Bacto antibiotic medium 3 (Difco, Detroit, USA), twice washed in isotonic sodium chloride, and then opsonised.

OPSONISATION

The washed bacterial pellet was suspended in 1 ml serum (undiluted) or 0-1 ml serum in 0-9 ml Parker’s fluid 199 (diluted serum) and incubated at 37°C for either 3 or 30 minutes while being gently agitated. Opsonisation was arrested by the addition of 0-01 M ethylenediaminetetra-acetic acid (EDTA). The opsonised bacteria were washed twice in isotonic sodium chloride and adjusted to an optical density of 0-23 at 618 nm. After dilution in Parker’s fluid 199 a final bacterial concentration of approximately 40x10^9 colony forming units/l was achieved.

FIXATION OF C3

Anti-C3d rabbit IgG (antihuman C3d complement Dakopatts a/s, Copenhagen, Denmark) was labelled with 125I (100 mCi/ml, Amersham, Buckinghamshire, England) by the chloramine T method. S. aureus 502A were incubated for 3 min in undiluted patient or control sera in a total volume of 0-5 ml. Opsonisation was stopped by addition of 10 ml isotonic saline at 4°C, and the bacteria were washed twice and adjusted to an optical density of 0-23 at 618 nm. After dilution in Parker’s fluid 199 to a bacterial concentration of 10^9 bacteria/l, 50 μl labelled anti-C3d (250 000 cpm) in Parker’s fluid 199 was added. After 15 min at 37°C with the labelled antibody the bacteria were washed in Parker’s fluid 199. The radioactivity in the bacterial cell pellet was measured (Gamma Sample Counter, LKB, Stockholm, Sweden). The results were expressed as a percentage of the counts per minute in the reference (pooled) sera. In C2-deficient or heat-inactivated sera, the counts per minute were reduced by more than 80%.

BACTERIAL KILLING

A modification of the Maaloe technique was used for bacterial killing. 50 μl of the bacterial suspension was added to 0-2 ml of the cell suspension in 12x75 mm plastic capped tubes (Falcon, Oxnard, Califor-
nia, USA). Incubation was at 37°C, while the tubes were gently agitated in a vertical position. Incubation was stopped after 15 min by adding 2 ml distilled water containing 0.01% bovine serum albumin and 1.5% Triton X 100 (isoctylphenoxypolyethoxyethanol) at 4°C, and the suspension was allowed to stand for 3 min to permit cellular lysis in the tube. 0.2 ml of the suspension was then diluted in 7.8 ml distilled water containing 0.01% bovine serum albumin. Viable bacteria were determined by colony counting by the pour plate method. The process for cellular lysis did not affect bacteria viability.

When normal pooled sera or SLE were heat inactivated, opsonisation did not result in bacterial killing, either by granulocytes or by monocytes. Nor did opsonisation in sera treated with a chelating agent (EDTA) support bacterial killing. Neither 1 mg/l nor 20 mg/l prednisolone (Percortalon aquosum) affected either granulocyte or monocyte killing. The rate of killing was linear for 60 min when plotted on a linear-log scale. Incubations were always performed in duplicate. Controls without cells were always included, and no deaths occurred.

**COMPLEMENT COMPONENTS**

The complement components (C) C1q, C1s, C3, and C4 were quantified in all sera by electrophoresis and immunofluorescent assay.26 27 All sera were screened by haemolysis in a gel functional complement assay which detected deficiencies in the classical and alternative pathways of complement.28 When pooled sera was incubated at 37°C and C analysed at two-hourly intervals, a reduced lysis of the classical pathway appeared 16 hours after the start of incubation.

**CIRCULATING IMMUNE COMPLEXES**

Circulating immune complexes were measured by a fluid phase Clq binding assay (Clq BA) as described by Zubler et al. The results of Clq BA measurement in nine SLE sera collected both at 4°C and 37°C were comparable for the two temperatures.

**CRYOPRECIPITATION**

Cryoprecipitation was performed according to Svensson et al., with serum collected at 37°C and immediately transferred to a water bath for clotting at 37°C for 2 h. The serum was then stored at 4°C for four days as suggested by Cream.30 The resulting cryoprecipitate was spun down at 4°C, the supernatant was pipetted off and stored, and the pellet was redissolved at 37°C in a corresponding volume of pooled control serum. Native SLE serum, supernatant of SLE serum, and pooled serum with dissolved cryoglobulin, each in a volume of 0.5 ml were collected from nine SLE patients. Five of the patients had reduced opsonic capacity (p<0.05) while four were within the normal range.

**STATISTICS**

When calculating the significance of differences between groups of independent values Student's t test was used for normal distributions. The non-parametric χ² test for two independent samples and Spearman's rank test were used, as appropriate.33

Opsonic bactericidal capacity was rated according to a leucocyte bactericidal index (BI), based on the Student paired t test, after Hoffman and Bullock.34 The following formula was used:

\[
BI = \frac{\text{log} \% \text{ viable control} - \text{log} \% \text{ viable SLE}}{\text{normal 'between-subject' SD}}
\]

The normal 'between-subject' standard deviation (SD) separately determined for monocytes and granulocytes was 0.11 for both phagocytic cell lines.

**Results**

**EVALUATION OF OPSONIC ASSAY**

Opsonisation in undiluted normal pooled serum for 3 min produced significantly higher phagocytic killing of S. aureus than did bacterial opsonisation in 10% serum for 3 or 30 min and was comparable to bacterial opsonisation in undiluted serum for 30 min. The observed killing capacities of granulocytes and monocytes were comparable (Fig. 1).

Bacterial opsonisation for 3 min in undiluted, C2-deficient patient sera is shown in Fig. 2. Opsonic capacities were reduced in sera from two SLE patients with hereditary C2 deficiency, and in sera from one patient with pronounced hypocomplementaemia, probably genetically determined, of C4. The sera from one of the two C2-deficient patients did not have increased concentrations of immune complexes. Opsonisation in sera from a patient with inherited properdin deficiency on the other hand was within the normal range.

**OPSONISATION IN SLE SERA**

Opsonisation was studied in 41 sera, i.e., one sample from each patient included in the sequential study. 14 samples were collected during disease activity and 27 samples during quiescent disease. Opsonic capacity was reduced (p<0.05) in 11 of the 14 'active' sera, as compared with nine of the 27 'quiescent' sera (p<0.02). Table 1 shows the SLE symptoms in the 14 patients with active disease and the granulocyte bactericidal index (GBI). A correlation exists between the number of active organi-The **evaluation of opsonic assay** shows that opsonisation in undiluted normal pooled serum for 3 min produced significantly higher phagocytic killing of S. aureus than did bacterial opsonisation in 10% serum for 3 or 30 min and was comparable to bacterial opsonisation in undiluted serum for 30 min. The observed killing capacities of granulocytes and monocytes were comparable (Fig. 1).

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Reduced opsonisation of S. aureus in undiluted SLE sera

**MECHANISM OF REDUCED OPSONISATION IN SLE SERA**

Reduced opsonisation (p<0.05) with the present method was found in 20 of the 41 SLE patients. Ten of the patients with reduced opsonisation were receiving corticosteroid treatment (mean dose 10 mg/day), compared with 12 of the patients with normal opsonisation (mean dose 12.5 mg/day). These results do not indicate an influence of corticosteroid treatment on the opsonisation results. Fig. 3 shows the simultaneous granulocyte and monocyte killing of S. aureus 502 A resulting from 22 of the experiments. Granulocyte and monocyte killing of bacteria opsonised in the same SLE sera were similarly reduced.

Concentrations of C determined from sera with reduced opsonic capacity and from sera with normal opsonic capacity showed no significant differences (Table 2). However, increased concentrations of systems and the reduction of opsonic capacity (p<0.002, Spearman's rank test). Six of the 11 patients with reduced in-vitro opsonisation had moderately reduced (p<0.025) C concentrations (Clq,C3, or C4), and eight had increased (p<0.05) IC concentrations (as measured by Clq BA).

In eight cases patient sera were studied both when SLE was clinically active and when it was quiescent. In all patients (one patient being C2 deficient) the opsonic capacity of their sera was reduced in active disease, as compared with that in quiescent disease (p<0.05).

Opsonic capacity was reduced in four out of five sera sampled within the two-month period preceding bacterial infection, as compared with nine out of the remaining 36 sera with no infection within two months (p<0.05). Two of the four predicted infections were deep major infections caused by S. aureus, and one was a septicemia with S. albus. In six patients sera were collected during infection but before antibiotic treatment was started, and opsonic capacity of sera was within the normal range in all six sera.

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![Fig. 1 Phagocytic killing of S. aureus by monocytes and granulocytes.](image1)

![Fig. 2 Reduced phagocytic killing by granulocytes of S. aureus opsonised for 3 min in undiluted sera from two SLE patients deficient in complement component 2 (▲, p<0.001), one SLE patient deficient in complement component 4 (△, p<0.05), one healthy individual with properdin deficiency (○, not significant) compared with a normal pool (□). The results are means of duplicate experiments and bars indicate ‘between-subject’ SD.](image2)
Table 1  SLE symptoms and opsonic capacity (GBI)

<table>
<thead>
<tr>
<th>Patient</th>
<th>SLE symptoms</th>
<th>GBI</th>
</tr>
</thead>
<tbody>
<tr>
<td>1*</td>
<td>Pancreatitis, leucopenia, thrombocytopenia, fever</td>
<td>-12.3†</td>
</tr>
<tr>
<td>2</td>
<td>Pericarditis, malar rash, cytoid bodies, fever</td>
<td>-5.5†</td>
</tr>
<tr>
<td>3</td>
<td>Lupus nephritis, exanthema, fever</td>
<td>-4.8†</td>
</tr>
<tr>
<td>4</td>
<td>Pleuritis, malar rash, fever</td>
<td>-3.4‡</td>
</tr>
<tr>
<td>5</td>
<td>Arthritis</td>
<td>-3.2‡</td>
</tr>
<tr>
<td>6</td>
<td>Malar rash, arthritis</td>
<td>-2.9§</td>
</tr>
<tr>
<td>7</td>
<td>Lupus nephritis</td>
<td>-2.9§</td>
</tr>
<tr>
<td>8</td>
<td>Lupus nephritis, CNS symptoms, cardiomyopathy, malar rash</td>
<td>-2.7§</td>
</tr>
<tr>
<td>9</td>
<td>Arthritis</td>
<td>-2.5§</td>
</tr>
<tr>
<td>10</td>
<td>Myelitis, fever</td>
<td>-2.4§</td>
</tr>
<tr>
<td>11</td>
<td>Myelitis</td>
<td>-2.4§</td>
</tr>
<tr>
<td>12</td>
<td>Carpal tunnel syndrome</td>
<td>-1.6</td>
</tr>
<tr>
<td>13</td>
<td>Myocardial infarction</td>
<td>-1.6</td>
</tr>
<tr>
<td>14</td>
<td>Arthritis</td>
<td>0.6</td>
</tr>
</tbody>
</table>

*Homozygous C2 deficiency. GBI during inactive SLE = −5.5. †p < 0.001. ‡p < 0.01. §p < 0.05.

Circulating IC were found in conjunction with reduced opsonic capacity (Table 2).

Labelled antibodies to C3d showed that the bacteria-associated C3d was comparable in nine sera with reduced opsonic capacity (median 68%, mean 82%, SD±23.6%), to that in five sera with normal opsonisation (median 83%, mean 82%, SD±13.2%). The C3d concentrations observed showed no correlation with opsonisation as determined by granulocyte killing of bacteria (Spearman’s rank test, r = −0.04).

The opsonic capacities of nine SLE sera from which cryoglobulins had been eliminated were compared with those of the nine corresponding native SLE sera. When the cryoglobulin fraction of IC was eliminated opsonic capacity was restored to normal. When bacterial opsonisation was simultaneously performed in nine pool sera, to each of which had been added one separated SLE cryoglobulin, and the results were compared with those of native pool sera, it was found that the opsonic reduction, or increase, could be transferred with the cryoglobulins to pooled sera. The relationships between the two differences calculated from each cryoglobulin elimination are shown in Fig. 4.

The possible role of staphylococcal protein A in opsonisation was studied with the protein A deficient strain S. aureus Wood 46, which was opsonised in parallel with S. aureus 502 A in six SLE sera. Five sera showed reduced opsonic capacities for S. aureus.
Table 2  Complement components (C) and circulating immune complexes (IC) in sera from SLE patients with normal or reduced opsonic capacity

<table>
<thead>
<tr>
<th>Complement Component</th>
<th>Normal (n=21)</th>
<th>Reduced (n=20)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1q</td>
<td>2</td>
<td>6</td>
<td>NS</td>
</tr>
<tr>
<td>C1s</td>
<td>1</td>
<td>2</td>
<td>NS</td>
</tr>
<tr>
<td>C4</td>
<td>3</td>
<td>8</td>
<td>NS</td>
</tr>
<tr>
<td>C3</td>
<td>2</td>
<td>6</td>
<td>NS</td>
</tr>
<tr>
<td>IC</td>
<td>10</td>
<td>17</td>
<td>&lt;0.04</td>
</tr>
</tbody>
</table>

NS = not significant.

502 A, whereas opsonisation of S. aureus Wood 46 was within the normal range in all cases (data not shown).

Discussion

We have described a modified method for determining heat-labile opsonin capacity in non-diluted serum and its application to SLE sera.

Most earlier methods are based on the use of diluted sera for opsonisation.8 32-34 Dilution might introduce pitfalls, for example, the alternative complement pathway might be more sensitive to dilution than the classical complement pathway.35 Furthermore dilution affects both time-dependent and concentration-dependent kinetics.9 With our method sources of error due to serum dilutions or to time factors were minimised.

Our finding of an almost identical response to opsonisation of S. aureus in both monocytes and granulocytes is in agreement with a recent study on the opsonisation of S. pneumoniae.36 Earlier findings of a slower rate of monocyte ingestion37 could not be reproduced with our assay technique.

The need for activation of the classical complement pathway to opsonise S. aureus 502 A13 is in agreement with our findings of highly reduced opsonisation in sera from patients with hereditary C2 deficiency and of chiefly normal opsonic capacity in serum from a patient with a hereditary properdin deficiency.

The finding of reduced opsonisation in clinically active SLE is in keeping with our recent report of increased frequency of bacterial infections in patients with active disease. Furthermore the correlation, found in this study, of opsonic reduction with the number of active organ systems parallels our clinical finding that bacterial infections are more common during peak activity of lupus flares.1a

Fig. 4 Changes of granulocyte bactericidal index (GBI) between opsonisation in native SLE sera and in supernatant (with the cryoprecipitate eliminated) were comparable with changes of GBI between opsonisation in pool sera and in pool sera in which the corresponding SLE cryoprecipitate had been dissolved (n=9, r=0.91), p<0.01 Spearman's rank test. Opsonisation time was 3 min and the sera were undiluted.
Although based on limited material, the clinical importance of reduced opsonic capacity is suggested, since bacterial infections are more common in the two months after reduction of opsonisation than when opsonic capacity was normal. Our observation of normal opsonic capacity during infection is in contrast to earlier findings, probably due to the fact that the few days interval between onset of infection and blood sampling in our study allowed activation of host defence mechanisms.

Jasin showed that reductions of complement components correlated both with reduced opsonic capacities and with infections in active SLE. Almost all of Jasin’s patients (27/30) had reduced complement concentrations, whereas half of ours did not—a discrepancy presumably reflecting the different populations under study, our patient group probably having less renal disease, as hypocomplementaemia is predominantly seen in active SLE with renal involvement. Nevertheless in this series the proportion of patients with reduced opsonisation during active disease is higher than the corresponding value (12/30) reported by Jasin. We found that heat-labile dependent opsonic capacity was reduced even when complement concentrations were not, this may be due to the use of undiluted sera in our assay technique, or to our blood sampling at 37°C. When radiolabelled antibodies to C3d were added the amounts of bacteria-associated activated C3 after opsonisation in SLE sera were comparable to those of the controls, indicating that defective opsonisation in SLE sera cannot be explained by reduced bacterial C fixation.

Our results thus indicate the possibility of some factor in SLE sera inhibiting either Fc or CR1 receptor contact on the phagocyte. SLE is the prototypic IC disease in man, and IC might be a blocking factor in our assay by binding to protein A of the bacterial cell walls through the Fc portion of its IgG. Protein A has actually been used to isolate IC and to assay IC in SLE sera, with results paralleling those of Clq BA.

When an IC fraction was eliminated from our SLE sera by cryoprecipitation, opsonisation was restored, thus indicating the involvement of IC in the reduced opsonic capacity of SLE sera; and the finding that reduced opsonic capacity could be transferred to normal pooled sera by the cryoprecipitate provides further support for a hypothesis of unspecific IC-mediated reduction of opsonisation. The correlation between increased concentrations of IC as measured by Clq BA, and reduced opsonisation is additional, indirect evidence for IC involvement. Our results with normal opsonisation of the S. aureus Wood 46 strain, which has been shown to be protein A deficient, in SLE sera with low opsonic capacity for S. aureus 502A, also indicate the involvement of IC interaction with protein A in phagocytosis.

Thus we have found that reduced in-vitro opsonisation of S. aureus in undiluted SLE sera is correlated with a clinically active lupus is related to the cryoglobulinuria fraction of IC and might increase the risk of bacterial infection, especially deep infections caused by S. aureus, within the next two months. Circulating IC, which block contact between phagocytic receptors and opsonised bacteria, seem to be of importance, since they reduce host defence against protein A bearing bacteria; this is quite independent of the many other factors that have been considered as being more or less conducive to infection proneness in SLE patients. In fact infections in SLE patients are most frequently caused by S. aureus.

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Reduced opsonisation of S. aureus in undiluted SLE sera 259

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