Complement activation in patients with sepsis is in part mediated by c-reactive protein

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Complement Activation in Patients with Sepsis Is in Part Mediated by C-Reactive Protein


The involvement of C-reactive protein (CRP) in the activation of complement in patients with sepsis was investigated. In 104 patients with infections of varying severity, circulating levels of CRP-complement complexes, which are specific indicators for CRP-mediated complement activation, were assessed. Complement-CRP complexes were increased in almost all patients and correlated significantly with levels of C3a (r = .59; P < .001) and C-reactive protein (r = .76; P < .001). In addition, they correlated with levels of secretory phospholipase A2 (r = .59; P < .001). Levels of complement-CRP complexes in patients with a pneumococcal type of infection were similar to those in patients with other types of infections. Complement-CRP complexes were significantly higher in patients with shock (P = .01) and in patients who died (P = .03). These results demonstrate that part of the complement activation in patients with sepsis is independent from a direct interaction with microorganisms but rather results from an endogenous mechanism involving CRP.

Excessive activation of inflammatory mediators in patients with sepsis may lead to life-threatening complications such as shock and multiple organ failure. One of the mediator systems activated during sepsis is the complement system. Patients with sepsis have decreased plasma levels of complement proteins and increased levels of complement activation products, such as the anaphylotoxins C3a, C4a, and C5a and the terminal complement complexes [1–3]. Although its precise role in the pathogenesis of sepsis is not clear, activation of complement may contribute to the inflammatory response via the release of cytokines, via activation of clotting and of neutrophils, and via the enhancement of vasopermeability [4].

Many bacteria activate complement in vitro and are therefore considered to be the main activators of complement in patients with sepsis. However, several observations indicate that additional mechanisms may contribute to complement activation in sepsis: Administration of high doses of interleukin-2 (IL-2) to patients with cancer induces a sepsis-like syndrome, which is accompanied by activation of the classical complement pathway [5], demonstrating the existence of a cytokine-driven mechanism of complement activation in vivo. Although the precise molecular mechanism of this activation is unknown, some evidence suggests that the acute-phase reactant C-reactive protein (CRP) may be involved: First, in vitro CRP is able to activate the classical complement pathway on binding to an appropriate ligand [6]; second, we have observed a biphasic activation of complement in a baboon model of septic shock, the later phase coinciding with increases of circulating CRP [7]; and finally, lymphocytes from patients with an IL-2–induced sepsis-like syndrome bind complement fragments as well as CRP [8].

To investigate the role of CRP in complement activation in vivo, we recently developed sensitive assays for complexes of CRP and activated complement proteins (C3b/C3d or C4b/C4d) and showed that circulating levels of these CRP-complement complexes specifically reflect CRP-mediated complement activation [9]. In the study reported here, we used these assays to investigate the role of CRP in the activation of complement in patients with sepsis.

Furthermore, we attempted to determine the possible ligand for CRP by specifically analyzing circulating levels of complement-CRP complexes in patients with pneumococcal infections and by relating levels of complement-CRP complexes with those of secretory type II phospholipase A2 (sPLA2), an enzyme that may generate ligands for CRP by hydrolyzing membrane phospholipids.

Patients and Methods

Patients. All patients included in this study were admitted to the Department of Internal Medicine of the Free University Hospital. Most (88) of them participated in a larger study (300 patients) investigating the release of inflammatory mediators during the development of fever in a general internal medicine ward (fever study) (Bossink et al., unpublished data). The inclusion criterion for the fever study was fever: rectal temperature >38.3°C. The major exclusion criterion was the presence of septic shock. Bacteri
ologic studies were done in each enrolled patient to establish the presence of infection and the type of microorganism involved as well as its sensitivity to antibiotics. On the basis of results of the bacteriologic examinations, patients were classified as having fever only, local infection, or bacteremic infection.

We at random selected 88 patients from the fever study to yield 3 groups of patients with comparable size: 29 patients with fever only, 29 patients who suffered from a local infection, and 30 patients who suffered from a bacteremic infection. In addition to these patients of the fever study, 16 patients admitted to the Medical Intensive Care Unit because of septic shock were included in this study as well. Septic shock criteria used were as described previously [10]. Clinical investigations of all patients included evaluation of the presence of systemic inflammatory response syndrome or septic shock [10, 11]. Survival was determined at 28 days after inclusion in case patients who were still in the hospital. Patients discharged from the hospital during the follow-up period were classified as survivors.

Bacteriologic studies. At least 2 blood samples for culture from each patient were obtained by venipuncture at time of inclusion in the study. In addition, local cultures were done when indicated, when clinical signs pointed to a local infection. Supplementary blood cultures were collected at times the treating physician considered necessary.

Blood and local cultures were processed according to standard techniques. Blood cultures containing *Staphylococcus epidermidis* were considered contaminated when only 1 bottle revealed growth and the patient had no indwelling vascular catheters. All other positive blood cultures were considered to indicate bloodstream infection. Local cultures were thought to reflect an infection if the treating physician decided to start or continue antimicrobial therapy on the basis of culture results.

Collection of blood samples. At inclusion, and 24 and 48 h thereafter, blood samples were collected in EDTA-containing Venoject tubes. Plasma was separated by centrifugation within 15 min after venipuncture and frozen in aliquots at −70°C until assayed. From patients with septic shock, blood samples were obtained every day until discharge from the intensive care unit or death.

Assays. C3a levels were determined by an RIA described previously [12] and expressed as nanomolar concentrations. Levels of C3a in healthy persons are <6 nM.

CRP levels were assessed with a sensitive sandwich-type ELISA, in which polyclonal anti-CRP antibodies were used as catching antibodies and a biotinylated monoclonal antibody against CRP as detecting antibody. Results were related to a standard commercially available (Behringwerke, Marburg, Germany) and expressed as milligrams per liter. CRP levels in healthy persons are <3 mg/L.

Complement-CRP complexes were determined by a novel method described in detail elsewhere [9]. In short, complement-CRP complexes as well as noncomplexed CRP were purified from plasma samples by a single-step procedure based on the calcium-dependent affinity of CRP (and its complexes) for phosphorylcholine-Sepharose beads. In control experiments, it was established that this procedure did not induce additional CRP-complement complexes or complement activation. Purified complement complexes were quantified by differential antibody ELISAs. In these ELISAs, four different antibodies, directed against C4c, C4d, C3c, and C3d, respectively, were used to capture complexes. These antibodies, anti–C4-1, anti–C4-4, anti–C3-9, and anti–C3-19, capture C4b/C4bi, C4b/C4bis/C4d, C3b/C3bi, and C3b/C3bis/C3d, respectively, and are described elsewhere [9]. CRP-complement complexes were detected by biotinylated monoclonal antibodies against CRP. Results were referred to an in-house standard containing a known amount of complexes and expressed as picomolar concentrations of complement fixed to CRP. Levels of all four types of complement-CRP complexes in normal healthy volunteers were below the limit of detection (4 pM).

sPLA₂ was determined with a sensitive ELISA as described previously [13]. sPLA₂ levels in healthy volunteers are <5 μg/L.

Generation of CRP-complement complexes in vitro. To illustrate the specificity of complement-CRP complexes for CRP-mediated activation of complement, we incubated 1 vol of recalciﬁed citrated plasma, to which puriﬁed CRP was added to yield a final concentration of 100 μg/mL, with 1 vol of *Escherichia coli* (0.75 × 10¹⁰ bacteria/mL, ﬁnal concentration) [14] or lysophosphatidylcholine (lyso-PC; Sigma, St Louis; 0.5 mg/mL, ﬁnal concentration) in veronal buffer containing 5 mM CaCl₂ and 1 mM MgCl₂ (ﬁnal concentrations). Recalciﬁed plasma was obtained from healthy volunteers by collecting blood at 10 mL citrate (ﬁnal concentration); blood samples were centrifuged for 15 min at 1300 g, after which CaCl₂ was added to yield a ﬁnal concentration of 10 mM. The plasma was then incubated for 15 min at 37°C, whereas the clot was removed by centrifugation at 2000 g for 10 min. The mixture of recalciﬁed plasma and complement activator was incubated for 30 min at 37°C. C4 and C3 activation during this incubation was assessed by measuring the generation of activation products of C4 and C3 (C4b, C4bi, or C4c [C4b/c] and C3b, C3bi, and C3c [C3b/c]) with ELISAs described previously [15]. Furthermore, the formation of complement-CRP complexes in the mixtures was assessed by use of the assays described above.

Statistical methods. The values of the various complement and other parameters measured were abnormally distributed. Therefore, data were expressed as median values of each parameter obtained in the plasma samples collected during the first 3 days following inclusion. The signiﬁcance of differences between groups was calculated with the Mann-Whitney U test. To investigate a relation between parameters, we used Spearman’s rank correlation analysis. Statistical signiﬁcance was designated at the 95% conﬁdence level (two-sided P).

Results

Patients. Sixteen patients fulﬁlling the criteria for septic shock were included. From the patients without septic shock, 29 had negative local or blood cultures and were categorized as the fever only group; 29 other patients had positive local but negative blood cultures and were categorized as the local infection group; and ﬁnally, 30 patients had positive blood cultures without shock and were categorized as the bacteremic infection group. By compiling the patient groups as we did, the fever only, local infection, bacteremic infection, and septic shock groups were considered to represent patients with infections of increasing severity. Indeed the survival rates decreased in the successive groups, from 93% in the fever only group,
Table 1. C3a, CRP, C3d-CRP, and secretory phospholipase A2 (sPLA2) in survivors and nonsurvivors and in patients with or without shock.

<table>
<thead>
<tr>
<th></th>
<th>C3a (nM)</th>
<th>CRP (mg/L)</th>
<th>C3d-CRP (pM)</th>
<th>sPLA2 (μg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Survivors</strong></td>
<td>9 (3–103)</td>
<td>48 (0.3–1310)</td>
<td>99 (5–1505)</td>
<td>164 (2.5–2053)</td>
</tr>
<tr>
<td><strong>Nonsurvivors</strong></td>
<td>18 (7–72)</td>
<td>77 (28–767)</td>
<td>157 (67–979)</td>
<td>456 (60–1257)</td>
</tr>
<tr>
<td><strong>P &lt; .01</strong></td>
<td>51 &lt; .01</td>
<td>51 &lt; .01</td>
<td>51 &lt; .01</td>
<td>51 &lt; .01</td>
</tr>
<tr>
<td><strong>No shock</strong></td>
<td>9 (3–57)</td>
<td>47 (0.3–208)</td>
<td>102 (5–977)</td>
<td>173 (2.5–1250)</td>
</tr>
<tr>
<td><strong>P &lt; .01</strong></td>
<td>51 &lt; .01</td>
<td>51 &lt; .01</td>
<td>51 &lt; .01</td>
<td>51 &lt; .01</td>
</tr>
<tr>
<td><strong>Shock</strong></td>
<td>367 (57–1310)</td>
<td>170 (25–1502)</td>
<td>943 (52–2053)</td>
<td>456 (60–1257)</td>
</tr>
<tr>
<td><strong>P &lt; .01</strong></td>
<td>367 &lt; .01</td>
<td>367 &lt; .01</td>
<td>367 &lt; .01</td>
<td>367 &lt; .01</td>
</tr>
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**NOTE.** Data are median (range). *P* values were calculated by Mann-Whitney *U* test.

80% in the local infection group, and 77% in the bacteremic infection group to 57% in the septic shock group.

**Plasma levels of C3a and C-reactive protein.** C3a levels were increased in 83% of the patients. Levels were significantly (*P* < .01) higher in patients who died than in patients who survived (table 1). Similarly, significantly higher levels were found in patients with shock than in patients without shock (table 1), whereas levels were similar in the other groups. Median (range) C3a levels were 8.5 (3–28) nM in the fever only group; 8.9 (4.4–20) nM in the local infection group; 13.5 (4.8–57) nM in the bacteremic infection group; and 54 (4–104) nM in the septic shock group.

CRP levels were also increased in the majority (98%) of the patients. Patients who died had significantly (*P* < .01) higher levels than patients who survived (table 1); similarly, patients with shock had significantly (*P* < .01) higher levels than patients without shock (table 1). Levels were similar in the other groups. Median (range) CRP levels were 41.6 (0.3–208) mg/L in patients with fever only; 50 (0.7–172) mg/L in patients with local infection; 52 (5.9–205) mg/L in patients with bacteremia; and 367 (57–1310) mg/L in patients with septic shock.

Consistent with a role of CRP in the activation of complement in the patients, CRP correlated significantly with C3a (*r* = .55, *P* < .001; see figure 1).

**In vitro generation of complement-CRP complexes.** We have previously shown that CRP-mediated activation of complement results in the generation of complement-CRP complexes [9]. To illustrate the specificity of the formation of these complexes for CRP-mediated activation of complement, we incubated recalciﬁed plasma containing CRP at a ﬁnal concentration of 100 mg/L, with *E. coli* or lyso-PC as described above, and assessed the amount of complement-CRP complexes generated in these samples. Notably, we used recalciﬁed plasma rather than serum because on incubation of the latter with CRP in the absence of activators, higher background activation of C4 and C3 was observed. This is probably due to the fact that serum contains higher levels of phospholipids released from cells damaged during the clotting process.

A representative example of these experiments is shown in figure 2. Both *E. coli* and lyso-PC activated complement, as indicated by the almost maximal activation of C4 and C3 (ﬁgure 2, top). In the absence of CRP, the activation of C3 and C4 by lyso-PC was greatly reduced and comparable to that observed with buffer alone (data not shown). In contrast, activation of C4 or C3 by *E. coli* was not inﬂuenced by the presence of CRP. In the same mixtures, we also measured the formation of complement-CRP complexes. Significant amounts of these complexes were observed only when plasma was incubated with lyso-PC in the presence of CRP (see ﬁgure 2, bottom; only C3d-CRP and C4d-CRP complexes are shown). Thus, activation of complement by *E. coli* did not induce the formation of complement-CRP complexes, even in the presence of elevated CRP concentrations. The generation of activated C4 and C3 as well as the formation of complement-CRP complexes was negligible in the mixtures incubated with veronal buffer only. These experiments conﬁrmed our previous observations that complement-CRP complexes are speciﬁc indicators for CRP-mediated complement activation.
The median level of C3d-CRP complexes in the fever only group was 84 pM. The lowest value in this group of patients was found in a patient clinically suspected of diverticulitis (5 pM), and the highest value was in a patient with suspected pneumonia (895 pM). The median C3d-CRP value in the local infection group was 103 pM. The lowest value found in this group occurred in a patient with a Pasteurella urinary tract infection (10 pM) and the highest value (532 pM) in a patient with Salmonella gastroenteritis. The median value in patients with bacteremia was 123 pM. The values in this group of patients ranged from 23 pM in a patient with an S. epidermidis catheter sepsis to 979 pM in a patient with Staphylococcus aureus septic arthritis. The median value of C3d-CRP complexes in the patients with septic shock was 170 pM, ranging from 25 pM in a patient with negative blood cultures to 1502 pM in a patient with Streptococcal pneumoniae sepsis after chemotherapy. The course of complement-CRP complexes, C3a, and CRP levels in this latter patient during the observation

**Figure 2.** CRP-complement complexes specifically reflect CRP-mediated complement activation. Recalculated plasma to which purified CRP was added at 100 mg/L was incubated for 30 min at 37°C with Escherichia coli (7 × 10⁴ bacteria/mL), lyosphosphatidylcholine (lyso-PC; 0.5 mg/mL, final concentration), or veronal buffer (V.B.) only. Top, activation of C4 (open bars) and C3 (solid bars), expressed as percentage of total amount of C4 and C3, respectively. Bottom, C4d-CRP (open bars) and C3d-CRP (solid bars) generated (expressed as nM). C3 and C4 activation by lyso-PC in plasma without added CRP was comparable to that in plasma incubated with buffer, whereas activation by E. coli was not influenced by presence of CRP.

**Figure 3.** Plasma levels of CRP (●) and secretory type II phospholipase A₂ (sPLA₂; □) (top), C3a (○) and C4b-CRP (△) (middle); and C4d-CRP (○) and C3d-CRP (△) (bottom) in 40-year-old male treated with chemotherapy for lymphatic leukemia, who was admitted to intensive care unit because of shock and adult respiratory distress syndrome. Blood cultures revealed Streptococcus pneumoniae. After 7 days of intensive treatment, he was discharged from intensive care unit.
period of 7 days is shown in figure 3. Analyzing the data of all patients together, levels of complement-CRP complexes appeared to correlate significantly with those of C3a (C3d-CRP complexes and C3a: $P < .001$; $r = .59$) and CRP (C3d-CRP complexes and CRP: $P < .001$; $r = .76$).

**Complement-CRP complexes in patients with pneumococcal infections.** As CRP is known to bind to C-polysaccharide of pneumococci, we studied the course of CRP and complement parameters in detail in the patients infected by these bacteria. In 5 patients, a pneumococcal infection was documented. Complement-CRP complex levels of these patients did not significantly differ from those of the other patients, ranging from 5 to 57 pM for C4b-CRP, 83 to 672 pM for C4d-CRP, and 110 to 522 pM for C3d-CRP complexes.

**Relation of C3a, CRP, and CRP-complement complexes to sPLA₂.** sPLA₂ levels were elevated in 98% of the patients. Levels of sPLA₂ in patients who died were higher than those in patients who survived. Moreover, the levels in patients with septic shock were significantly ($P < .01$) higher than in patients without shock (table 1). The median (range) sPLA₂ levels in patients with fever only were 77 (3–1250) μg/L, in patients with local infection were 193 (4–1250) μg/L, and in patients with bacteremic infection were 309 (15–1250) μg/L. In patients with septic shock, the median value was 943 (4–1250) μg/L, and in patients with local infection were 193 (4–1250) μg/L, and in patients with bacteremic infection were 309 (15–1250) μg/L. In patients with septic shock, the median value was 943 (4–1250) μg/L, and in patients with local infection were 193 (4–1250) μg/L, and in patients with bacteremic infection were 309 (15–1250) μg/L. In patients with septic shock, the median value was 943 (4–1250) μg/L, and in patients with local infection were 193 (4–1250) μg/L, and in patients with bacteremic infection were 309 (15–1250) μg/L. In patients with septic shock, the median value was 943 (4–1250) μg/L, and in patients with local infection were 193 (4–1250) μg/L, and in patients with bacteremic infection were 309 (15–1250) μg/L.

**Discussion**

The release of cytokines, such as IL-1, tumor necrosis factor, and IL-6, during an inflammatory response induces changes in the plasma concentrations of acute-phase proteins. The prototypical acute-phase reactant is C-reactive protein, whose levels may increase up to 1000-fold. In vitro, a number of functional properties have been ascribed to CRP, including its capacity to activate the classical complement pathway [16, 17]. In the present study, we show that levels of C3a significantly correlate with levels of CRP in patients with sepsis. Moreover, we report the presence of increased plasma levels of complement-CRP complexes in these patients, thereby providing evidence that in clinical sepsis, CRP mediates activation of complement.

Activation of complement via the classical pathway results in the exposure of an internal thioester in C4 and C3 [18]. These thioesters have the capacity to react with any nearby hydroxyl or amino group. The rapid inactivation of the exposed thioester by the large excess of water molecules present in plasma and the interstitial fluid considerably limits the fixation of C4 and C3 to proteins other than the activator. Demonstration of complement-CRP complexes in biologic fluids could therefore provide a means to assess CRP-mediated activation of complement in vivo. Indeed, complement-CRP complexes are generated during CRP-mediated complement activation in vitro [19, 20].

It has been shown that C3 may fix to IgG during activation induced by non-immunoglobulin activators [21]. It was, therefore, essential to demonstrate specificity of complement-CRP complexes for CRP-mediated activation of complement. Using recently developed assays, we studied the formation of CRP-complement complexes under a number of conditions and found that, in vitro, ~1%–5% of activated C4 and C3 will fix to CRP during CRP-mediated complement activation. CRP-complement complexes were not generated when complement was activated by aggregated human immunoglobulin, even in the presence of acute-phase concentrations of CRP [9]. Similarly, these complexes were not formed during activation of complement by *E. coli*, even in the presence of elevated CRP concentrations (see figure 2). Furthermore, increased plasma levels of complement-CRP occurred in patients 3 days after the implantation of a renal allograft, but these complexes did not increase during classical pathway activation induced by a bolus injection of monoclonal antibody OKT3 (used to prevent transplant rejection), even though these patients had increased CRP levels [9]. Taken together, these data indicate that circulating levels of complement-CRP complexes are specific indicators for CRP-mediated complement activation and do not increase specifically following complement activation by activators other than CRP.

Nearly all patients had increased plasma levels of complement-CRP complexes. In all cases, both C3d-CRP and C4d-CRP complexes were increased, as was expected considering that CRP activates the classical complement pathway. In addition, most patients also had similar plasma levels of C3b-CRP. However, C4b-CRP complexes were ~10-fold lower than C4d-CRP complexes. In vitro, we have observed rapid cleavage of C4b-CRP complexes into C4c and C4d-CRP, whereas a similar
breakdown of C3b-CRP complexes into C3c and C3d-CRP did not occur (although our assays do not discriminate between C3b and C3bi), indicating differential regulation of C4b and C3b bound to CRP [9]. Thus, the observed increases of the various types of CRP-complement complexes in the patients with sepsis indicate that this differential regulation of C3b and C4b bound to CRP also occurs in vivo.

CRP activates the complement system on binding to an appropriate ligand. Determination of the ligands for CRP in patients with sepsis will be a critical step in understanding the physiologic role of CRP-mediated activation of complement. Our study does not allow definite conclusions regarding the nature of this ligand but may provide some clues. CRP in vitro has been shown to bind to the C-polysaccharide of the pneumococcal cell wall and to products derived from damaged tissue [22]. The interaction between CRP and pneumococci is likely relevant in vivo, since mice transgenic for human CRP are protected against infections with these microorganisms [23]. We included 5 patients with pneumococcal infections. These patients had levels of complement-CRP complexes similar to those in patients with other types of infections. This does not exclude a role of CRP-mediated complement activation in the defense against pneumococci but rather indicates that the majority of CRP binds to a ligand other than C-polysaccharide in septic patients.

In clinical studies, CRP has been found to correlate with the total infarct size in acute myocardial infarction and with the prognosis in unstable angina [24, 25]. The mechanisms that relate the level of CRP to myocardial infarction are unclear. Interestingly, in experimental models, CRP was found to be able to bind to inflamed and necrotic tissues [26]. In a recent study, we showed that CRP may colocalize with complement in infarcted myocardial tissue [27]. This suggests that CRP at a tissue level may amplify inflammation by activating complement. Whether such a mechanism explains the increase of CRP-complement complexes in the patients described in this study needs to be established in future studies.

In this study, we found significantly higher levels in patients with shock or patients who died in the other patients; that is, levels were higher in patients with presumably more tissue damage. The products derived from damaged tissue that bind CRP include polycations, small nuclear ribonucleoprotein, and phospholipids [28–30]. The latter are the major constituent of the normal cell wall. Volanakis and Wirtz [29] demonstrated that CRP binds to vesicles of phospholipids containing a certain amount of lysophospholipids. Lysophospholipids are generated by hydrolysis of membrane lipids by PLA2 enzymes. One of the PLA2 enzymes characterized in humans, sPLA2, is an acute-phase protein that shares regulatory elements with CRP and circulates at high levels in sepsis [31]. In the patients described here, levels of sPLA2 strongly correlated with levels of CRP and also with levels of C3a and with complement-CRP complexes. Although these correlations do not provide conclusive evidence for a role of sPLA2 in CRP-mediated activation of complement, we suggest that this enzyme is involved in generating binding sites for CRP in damaged tissues.

Studies in animal models have yielded conflicting results regarding the role of complement in the pathogenesis of sepsis. Dogs with a genetic C3 deficiency have an impaired clearance of endotoxin and are more susceptible to endotoxin than are healthy animals [32], suggesting complement to be important for the defense against the infecting microorganisms. On the other hand, inhibition of the biologic effects of C5a in baboons with lethal sepsis attenuates the lethal complications [33], illustrating that the proinflammatory effects of complement activation may contribute to the complications of sepsis. Our results indicate that at least part of the complement activation in patients with sepsis is not due to direct interaction with bacteria but rather involves CRP and correlates with morbidity and mortality of the patients. Future studies should reveal the role of this CRP-mediated complement activation in the pathogenesis of sepsis, that is, whether this route is merely a marker of infection-associated tissue damage or contributes by itself to morbidity and mortality.

References