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Changes and Regulation of the C5a Receptor on Neutrophils during Septic Shock in Humans

Heike Unnewehr,*† Daniel Rittirsch,*† J. Vidya Sarma,‡,† Firas Zetoune,† Michael A. Flierl,* Mario Perl,* Stephanie Denk,* Manfred Weiss,‡ Marion E. Schneider,‡ Peter N. Monk,§ Thomas Neff,‡ Michael Mihlan,‡ Holger Barth,‡ Florian Gebhard,* Peter A. Ward,‡ and Markus Huber-Lang*

During experimental sepsis, excessive generation of the anaphylatoxin C5a results in reduction of the C5a receptor (C5aR) on neutrophils. These events have been shown to result in impaired innate immunity. However, the regulation and fate of C5aR on neutrophils during sepsis are largely unknown. In contrast to 30 healthy volunteers, 60 patients in septic shock presented evidence of complement activation with significantly increased serum levels of C3a, C5a, and C5b-9. In the septic shock group, the corresponding decrease in complement hemolytic activity distinguished survivors from nonsurvivors. Neutrophils from patients in septic shock exhibited decreased C5aR expression, which inversely correlated with serum concentrations of C-reactive protein (CRP) and clinical outcome. In vitro exposure of normal neutrophils to native pentameric CRP led to a dose- and time-dependent loss of C5aR expression on neutrophils, whereas the monomeric form of CRP, as well as various other inflammatory mediators, failed to significantly alter C5aR levels on neutrophils. A circulating form of C5aR (cC5aR) was detected in serum by immunoblotting and a flow-based capture assay, suggestive of an intact C5aR molecule. Levels of cC5aR were significantly enhanced during septic shock, with serum levels directly correlating with lethality. The data suggest that septic shock in humans is associated with extensive complement activation, CRP-dependent loss of C5aR on neutrophils, and appearance of cC5aR in serum, which correlated with a poor outcome. Therefore, cC5aR may represent a new sepsis marker to be considered in tailoring individualized immune-modulating therapy.

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In critically ill patients, sepsis remains the leading cause of death, with an increasing incidence in both the United States (1, 2) and Europe (3). Despite intensive scientific and clinical efforts, the deleterious pathophysiological consequences of sepsis remain enigmatic (4, 5). Autopsy studies have failed to identify an obvious reason for sepsis-induced death but have revealed an intriguing discordance featuring histological changes and immunohistochemical evidence of immunodeficiency (6). One distinct finding in sepsis is the apoptotic loss of cells of the adaptive immune system (7–9). In contrast, neutrophils and the complement system, both belonging to the innate immune system as the “first line of defense,” were found to demonstrate either excessive function or paralysis of functional responses of phagocytes (10, 11). Overall, the immune response during sepsis seems to be affected by many factors (12), including various pathogen- or danger-associated molecular patterns (4, 13), the respiratory function of cells (14), comorbidity conditions (12), the inflammatory reflex of the nervous system (15), and polymorphism in immune-modulating genes coding for cytokines, coagulation molecules, complement factors, and their corresponding receptors (12, 16). Therefore, measurement of circulating concentrations of inflammatory mediators and complement activation products, as well as assessment of neutrophil function, may be useful in evaluating the immune status of the patient with sepsis and in tailoring individualized immune-modulating therapy. Increasing experimental evidence shows that the complement activation product C5a (17) and its interaction with the C5a receptor (C5aR) (18–21) play key roles in the initiation and progression of the systemic inflammatory response and resulting complications (22), such as septic shock and multiple organ failure. In this regard, the role of C5a in sepsis has been described as “too much of a good thing” (23), referring to excessive generation of C5a during sepsis and subsequent C5a-dependent impairment of innate immunity (17, 24). Furthermore, blockade of either C5a or C5aR greatly improved survival of rodents during sepsis (11, 17, 25). More than three decades ago, receptor assays of dysfunctional neutrophils from four patients with intra-abdominal infection showed a specific loss of cellular C5a binding (26), confirmed by experimental data that revealed a time-dependent loss of C5a binding (22) and C5aR content (25) on neutrophils. In a preliminary study of 12 heterogeneous patients with severe sepsis, reduction of C5aR was found and
negatively correlated with the severity of illness (27). However, little is known about complement function and C5aR status during septic shock in humans. Moreover, the mechanisms of C5aR regulation and its fate during septic shock are still unknown. We demonstrate for the first time, to our knowledge, in a representative cohort of patients in septic shock that C5aR expression is reduced on neutrophils and is predictive of outcome. Loss of C5aR on neutrophils is associated with increased serum levels of C-reactive protein (CRP). C5aR loss could be induced in vitro by use of native CRP concentrations found in serum during sepsis. Furthermore, the data suggest that C5aR is shed during sepsis, resulting in the appearance of a previously undetected circulating C5aR (cC5aR) in the serum of patients that negatively correlates with survival.

Materials and Methods

Selection and demography of patients

The study was approved by the Independent Local Ethics Committee of the University of Ulm, Ulm, Germany (no. 225/05). Written informed consent was obtained from all included consent. However, if the patient was not capable of making decisions because of sedation or altered mental function, in which case the informed consent was obtained after recovery. We recruited 60 consecutive critically ill adults in septic shock according to Bone’s criteria (28). Exclusion criteria were as follows: age < 18 y, pregnancy, infection with HIV, cardiogenic shock as the primary underlying disease, underlying hematologic disease, cytotoxic therapy given within the previous 6 mo, and sepsis of unknown origin or belonging to a rapidly progressing underlying disease anticipated to be fatal within the next 24 h. Serving as the control group were 30 healthy volunteers. Although the volunteers were not individually matched to the patients, their sex and age distributions were similar to those of the patients. All patients were monitored throughout the 30-d study period or until death occurred. Samples of blood were obtained at the time patients required continuous infusion of vasopressors or inotropic agents to maintain blood pressure despite adequate fluid resuscitation. The following items were recorded at enrollment: age distribution (65.9 ± 1.6 y); sex distribution (23 female/37 male; 38% female; 30-d lethality (24 of 60; 40%); Simplified Acute Physiology Score II (score ranging from 0 to 159, with higher scores indicating a higher risk of death) of 50.5 ± 2.9; Sepsis-Related Organ Failure Assessment score (score ranging from 0 to 24) of 8.2 ± 0.6; and leukocyte count of 17.5 ± 1.5 GigaL.

Experimental sepsis in rodents by cecal ligation and puncture

All procedures were performed in accordance with the guidelines of the local committee on use and care of animals. Specific pathogen-free, 8-wk-old male C57BL/6 wild-type mice (The Jackson Laboratory, Bar Harbor, ME) and male Sprague Dawley rats (The Jackson Laboratory) were used. Intraperitoneal ketamine (100 mg/kg body weight) (Bayer, Shawnee Mission, KS) for sedation. After an abdominal midline incision was made, the cecum was exposed, ligated, and removed. The abdomen was closed in layers, using 6-0 surgical sutures (Ethicon, Somerville, NJ) and metallic clips. Before and after the surgery, animals had unrestricted access to food and water. To induce intermediate-grade cecal ligation and puncture (CLP), 50% of the cecum was ligated. At indicated time points, blood was removed by puncture of the inferior vena cava, followed by euthanization of the animals.

CRP isoforms

For the in vitro CRP stimulation experiments, native pentameric human CRP (Sigma-Aldrich, St. Louis, MO) or recombinant pentameric CRP (Calbiochem, San Diego, CA), and monomeric CRP (mCRP) were used. The mCRP was generated by treating native pentameric CRP with 8 M urea with 10 mM EDTA for 1 h at 37˚C (29). Then mCRP was dialyzed in phosphate buffer (10 mM Na2HPO4, 10 mM NaH2PO4, and 15 mM NaCl, pH 7.4), as described previously (30).

Measurement of the serum concentration of C3a, C5a, and the soluble form of C5b-9

Complement status was assessed by the serum or plasma concentrations of complement activation products C3a, C5a, and the soluble form of the membrane attack complex C5b-9 (sC5b-9), measured by a C3a and sC5b-9 enzyme immunoassay (Quidel, San Diego, CA) and by a C5a ELISA (IBL Immuno-Biological Laboratories, Hamburg, Germany or DRG Diagnostics, Marburg, Germany) according to the manufacturers’ protocols.

Measurement of the serum concentration of CRP

High-sensitivity CRP detection was performed on a routine clinical chemistry analyzer, Dimension RxL (Dade Behring, Newark, DE), by the revised CRP method based on a particle-enhanced turbidimetric immunoassay technique in accordance with the manufacturer’s protocol (Dade Behring). Latex particles coated with Ab for CRP aggregated in the presence of CRP in the sample. The increase in turbidity was determined at 340/700 nm and was proportional to the CRP concentration (sensitivity of CRP, 0.5 mg/l).

Hemolytic complement assay

Serum was serially diluted in TBS (75 g/l NaCl, 177 ml/l 1 N HCl, 28 ml/l triethanolamine, 1.2 ml/l 1.25 M CaCl2, 1.2 ml/l 4.16 M MgCl2) and the dilution causing 50% lysis of sensitized sheep RBCs (Colorado Serum Company, Denver, CO) after incubation at 37˚C for 60 min was determined. The complement reaction was stopped by addition of ice-cold TBS (with 0.05% gelatin) followed by a centrifugation step (2500 rpm, 5 min). Absorbance of the supernatants was measured at (541 nm), and the dilution at which 50% lysis occurs [total complement hemolytic activity (CH50)] was determined.

Neutrophil isolation

Neutrophils were isolated from whole blood by Ficoll-Hypaque gradient centrifugation (Pharmacia Biotech AB, Uppsala, Sweden) and dextran sedimentation, followed by hypotonic lysis of residual RBCs. Evaluation of the viability of neutrophils by the trypan blue exclusion test revealed >98% vital cells. For ex vivo stimulation of neutrophils (<4 h), human recombinant C5a (100 ng/ml; Sigma-Aldrich, Munich, Germany), native CRP (0–250 µg/ml), mCRP (250 µg/ml), or other indicated stimuli were used and subsequently analyzed for C5aR expression by flow cytometry or Western blotting.

Oxidative burst of neutrophils

Isolated neutrophils (2 × 106 cells per milliliter) were incubated in the presence or absence of PMA (25 ng/ml) for 10 min, and generation of hydrogen peroxide (H2O2) was determined (in the presence of 1 mM sodium azide). The reaction was stopped by addition of 0.1 ml 50% trichloroacetic acid. Then, 1.5 mM ferrous ammonium sulfate and 0.25 M potassium thiocholate were added to the supernatant fluid. The absorbance of the ferrithiocyanate complex was measured at 480 nm and compared with a standard curve generated from dilutions of reference solutions of H2O2.

Immunoblotting for C5aR

After isolation, neutrophils were lysed with a hypotonic buffer containing a protease-inhibitory mixture (Roche Diagnostics, Rotkreuz, Switzerland). Equal amounts of protein, as determined by protein assay (Pierce, Rockford, IL) from lysates, were electrophoretically separated using reducing conditions in 10% SDS-PAGE and transferred onto a polyvinylidene fluoride membrane. The membrane was blocked in 5% milk for 1 h and then incubated with mouse mAb against human C5aR (clone 5S/1; Serotec, Düsseldorf, Germany) or polyclonal rabbit anti-human C5L2 (HyCult Biotechnology, Uden, the Netherlands), using a concentration of 1 µg/ml. As a secondary Ab, alkaline phosphatase (AP)–conjugated goat anti-mouse or anti-rabbit IgG (1:5000; Jackson ImmunoResearch, West Grove, PA) was added and the blot was developed using an AP color development system (Bio-Rad Laboratories, Hercules, CA). For coexpression experiments, sera from mice with sepsis were incubated overnight with pre-blocked protein A and G beads (Santa Cruz Biotechnology, Santa Cruz, CA) in the presence of polyclonal goat anti-mouse CD88 (clone P1H, Santa Cruz Biotechnology), mapping within the extracellular domain. After centrifugation, pellets were resuspended in Laemmli sample buffer (Bio-Rad), followed by boiling of the samples. After a final centrifugation step, supernatant fluids were analyzed by Western blotting using polyclonal rabbit anti-mouse CD88 (Santa Cruz Biotechnology) or polyclonal rabbit anti-mouse CD66e (Abcam, Cambridge, U.K.), respectively, as a primary Ab. As a secondary Ab, AP-conjugated goat anti-rabbit IgG (1:5000) was applied before AP development.

Analysis of C5aR expression on neutrophils

To determine C5aR expression on neutrophils, flow cytometric analyses were conducted after whole-blood collection in an EDTA-containing syringe. A total of 10 µl FITC-labeled anti-CD88 (clone P1H/2; Serotec) was
incubated with 100 μl human whole blood for 20 min at room temperature. For control purposes, FITC-labeled isotype-matched IgG (Serotec) was added to control samples in equal amounts and with equal incubation time. Erythrocytes were lysed by the addition of 2 ml 1× FACS lysing solution (BD Pharmingen, San Diego, CA) for 10 min. After washing twice, the leukocytes were resuspended in a fixating solution (1% paraformaldehyde plus 0.1% sodium azide) and analyzed on a flow cytometer (FACSCanto II; BD Pharmingen). The granulocytes and monocytes were separated by the typical forward and side light scatter profiles and additional staining with anti-CD45/anti-CD14 (Leukogate; Beckman Coulter, Krefeld, Germany). The number of apoptotic neutrophils as assessed by TUNEL staining was < 1% in the sepsis group in comparison with 6% in the healthy control group (data not shown).

**Human proteome profiler array**

For simultaneous analysis of various potential signaling proteins involved in the CRP-induced downregulation of C5aR on polymorphonuclear neutrophils (PMNs), a proteome profiler array (protein Stress Array Kit, R&D Systems, Abingdon, UK.) was undertaken of PMN lysates from healthy volunteers after exposure to CRP (100 μg/ml) in the absence or presence of anti-FcRy1 (monoclonal anti-human CD64) and anti-FcRy1 (monoclonal anti-human CD32 Fab), both from Ancell Immunology Research, Bayport, MN. The assay was conducted according to the manufacturer’s protocol.

**Detection of cC5aR in human serum and rat plasma**

For detection of circulating C5aR in serum, both immunoblotting of serum (see above) and a flow cytometric capture analysis were used. For the latter method, polyclonal goat anti-human C5aR (Santa Cruz Biotechnology) was bound to Cytoplex microspheres (1.4 × 10^8 particles per milliliter) with a size of 4 μm (Duke Scientific Corporation, Palo Alto, CA), in accordance with the manufacturer’s protocol. After washing, 100 μl either serum or saline (as a negative control) was incubated with 2 μl anti-C5aR microspheres on a rotator for 90 min at 25°C. After washing, microspheres were then resuspended in 100 μl PBS. One part of these microspheres was further incubated with 10 μl FITC-labeled monoclonal mouse anti-human C5aR (clone P12/1; Serotec) and another part with FITC-labeled isotype-matched IgG (Serotec) for 20 min at room temperature. After subsequent washing, microspheres were gated and analyzed on a flow cytometer (BD Pharmingen) for their C5aR content.

A similar flow-based protocol was used to detect cC5aR in the plasma of rats. For immuno-capturing, a custom-made polyclonal rabbit anti-rat C5aR targeting the N terminus of rat C5aR aa 1–38 (sequence: mdpisdsceytdsgtppndmpagvypikmpged; Invitrogen, Carlsbad, CA) was applied; and for detection, a monoclonal anti-rat CD88 FITC (clone R63; Serotec, Germany) raised against the N-terminal sequence was applied.

**FITC labeling of human C5a and binding assay**

For FITC labeling of recombinant human C5a (Sigma-Aldrich), the carboxylic acid–sucinimidyld ester labeling kit, MFP 488 (Mobitec, Rastatt, Germany), was used strictly according to the manufacturer’s instructions. After labeling, the FITC-C5a still exhibited chemotactic activity for human neutrophils (data not shown). In subsequent in vitro experiments, isolated human blood neutrophils from healthy volunteers were exposed to either HBSS as a control or C5a (10 nM) for 3 h at 37°C. After separation of the cellular fraction, supernatant fluids were ultracentrifuged (100,000 × g for 60 min) to pellet the remaining membrane fragments or microparticles. This fraction was then washed with FITC-C5a (10 μg/ml) or nonlabeled C5a for 1 h on ice. After washing with cold PBS and another ultracentrifugation step, the residual pellet was analyzed by flow cytometry.

**Transmission electron microscopy of neutrophils**

After exposure of neutrophils to HBSS, C5a (100 ng/ml), or CRP (150 μg/ml), neutrophils were fixed in 4% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3) at room temperature overnight and washed in two changes of 0.1 M cacodylate buffer (pH 7.3). Samples were postfixed in 2% OsO4 in 0.1 M cacodylate buffer (pH 7.3) for 1 h at 25°C, dehydrated in a graded series of alcohols for final dehydration in propylene oxide, and then infiltrated with increasing mixtures of propylene oxide and Epon resin, and embedded in pure Epon. Sections of 1 μm stained with toluidine blue were evaluated by light microscopy. Thin sections were obtained with an AG Ultracut Ultramicrotome, stained with uranyl acetate and lead citrate, and evaluated with a Phillips 400 T Electron Microscope.

**Statistical analysis**

All values were expressed as mean ± SEM. Datasets were analyzed by Kruskal–Wallis one-way ANOVA on ranks; differences in the mean values among experimental groups were then compared using a multiple comparison procedure (Dunn’s method). The unpaired t test was used for comparison of the serum or plasma anaphylatoxins obtained from patients with sepsis and from healthy volunteers. Receiver-operating-characteristic (ROC) curves were constructed to analyze different cutoff levels of C5aR expression. The Spearman rank-order correlation was performed to investigate correlation of the C5aR expression level on neutrophils with CRP concentrations in sera. Results were considered statistically significant when p < 0.05.

**Results**

**Sepsis-induced complement activation and impairment of complement function**

Whereas an enhanced C3a/C3 ratio in the serum of patients who developed sepsis has been reported (31), changes in C5a levels during sepsis, especially during the shock phase of human sepsis, have been little studied (32, 33). As shown in Table I, there was a 3.4-fold increase in C3a levels during septic shock, an almost 5-fold increase in serum for C5a, and a modest but significant increase in the formation of the nonlytic sC5b-9. These results were reflected by significantly reduced serum hemolytic activity in patients in septic shock (Table II). In comparison with patients who survived the lethal consequences of septic shock, nonsurvivors retained significantly higher CH50 values. The data indicate complement activation and consumption during septic shock. In a second cohort of healthy volunteers and patients in septic shock (n = 8), different sampling methods were applied in regard to measured levels of C5a, revealing differences in plasma versus serum, as shown in Table I.

**Impairment of neutrophil function during septic shock**

Recently, we have described a C5a-induced loss of the oxidative burst response of neutrophils during experimental sepsis (11). To evaluate neutrophil function in humans during septic shock, in vitro generation of H2O2 was measured. As shown in Table II, neutrophils isolated from patients in septic shock and stimulated in vitro by PMA (25 ng/ml) exhibited a significant reduction (nearly 66%, p < 0.01) in H2O2 production, in comparison with neutrophils obtained from healthy volunteers. Neutrophils from patients with a lethal outcome showed a further reduction (nearly 75%) in H2O2 production, although this difference between survivors and nonsurvivors missed statistical significance (p = 0.08). In accordance with our previous report from a rodent model of experimental sepsis, these data confirm acquired dysfunction of neutrophils during septic shock in humans.

**Loss of C5aR on neutrophils correlates with outcome of patients in septic shock**

Numerous reports have suggested that neutrophil C5aR expression is essential for effective innate immune defense (22, 25). Therefore, using flow cytometric analyses, we determined whether the C5aR status on blood neutrophils was altered during septic shock in humans (n = 60). As expected, all neutrophils obtained from healthy volunteers (n = 30) expressed C5aR on their surfaces, as determined by a significant right-shift (original measurement, Fig. 1A, left panel) in comparison with the isotype-matched control (inset). In contrast, C5aR expression was significantly reduced on neutrophils from patients during septic shock (Fig. 1A, middle and right panels). As shown in Fig. 1A (right panels) and as summarized in Fig. 1B, a significant further decrease in expression to nearly background levels occurred in neutrophils from patients who did not survive septic shock, indicating nearly total loss of C5aR on the neutrophil surface. The C5aR loss on neutrophils was independent of the blood sampling method applied (EDTA, lepirudin, or citrate plasma; n = 9–10) (Supplemental Fig. 1). The
capacity of C5aR on neutrophils to predict the outcome of patients in septic shock was assessed by an ROC curve analysis (Fig. 1C). The area under the ROC curve was 0.98 (95% confidence interval = 0.97–0.99) when C5aR status was used to differentiate between healthy status and septic shock. A C5aR cutoff value of 0.97–0.99, the area under the outcome curve was 86%. For differentiation between survival and nonsurvival of patients in septic shock was assessed by an ROC curve analysis (Fig. 1C).

The area under the ROC curve was 0.98 (95% confidence interval = 0.97–0.99), indicating that differences in C5aR expression of neutrophils correlate with clinical outcome.

Reduction of C5aR content in blood neutrophil lysates after in vitro exposure to C5a or during septic shock

To further investigate C5aR regulation, neutrophils from healthy donors were exposed for increasing time periods (1–4 h) to C5a concentrations found during sepsis (34), followed by the measurement of C5aR content in cell lysates. As shown in Fig. 2A, neutrophil lysates from healthy volunteers (control [ctrl]) revealed a single band detected by anti-C5aR Ab aligning with the 45-kDa marker (arrow). When neutrophils were incubated with 10 nM of C5a for up to 1 h, the intensity of the C5aR band (arrow) was slightly increased but became much fainter at 2 h and was barely detectable at 4 h. Cell viability at the end of the incubation period was consistently > 98% in all samples (as determined by the trypan blue exclusion test; data not shown). In contrast, when neutrophils were incubated with 1 μM of MLLF (formyl-Met-Leu-Phe) for 1–4 h, no change in the intensity of the C5aR band was noted (data not shown). Exposure of neutrophils to a range of concentrations of C5a (0.1–1000 nM) caused a decrease in the intensity of the C5aR band at C5a concentrations > 10 nM (Fig. 2B). These data indicate that in vitro exposure of neutrophils to C5a at concentrations found during sepsis causes a reduction of cellular C5aR content. In translating these findings to patients in septic shock, cell lysates of neutrophils were analyzed by immunoblotting (Fig. 2C, exposition of three representative Western blots). Neutrophil lysates from 14 of 15 patients who survived the septic shock period exhibited in comparison with healthy probands a similar intensity of the C5aR band. This finding stood in striking contrast to neutrophil lysates from patients who succumbed to septic shock, which showed in 12 of 15 cases a considerable reduction of detectable C5aR.

**CRP-induced loss of C5aR**

Apart from a possible C5a-induced downregulation of C5aR (see Fig. 2), no other mechanisms for the reduction of C5aR on neutrophils during experimental and clinical sepsis have been described so far. Therefore, we examined various serum factors known to be elevated during septic shock in humans. In vitro incubation of LPS (1 ng/ml–100 μg/ml) as well as a variety of cytokines (IL-6, IL-10, IL-13, all at 1 ng/ml–10 μg/ml) and ATP (1 nM–100 μM) failed to significantly alter C5aR status on neutrophils (data not shown). To rule out the possibility that endogenously produced or infused catecholamines (the clinical need for which defines septic shock) might alter C5aR expression, nor-epinephrine, epinephrine (both at 0.02 ng/ml–100 μg/ml), and hydrocortisone (0.1–1000 μg/ml) in concentrations typically found (or administered) during septic shock were incubated with neutrophils from healthy volunteers in vitro, but again failed to significantly alter C5aR expression (data not shown). This observation was in striking contrast to the effect of the proinflammatory agent, the native pentameric form of CRP, which induced a dose- and time-dependent reduction of C5aR expression on neutrophils (Fig. 3A, 3B). When neutrophils were incubated with either the monomeric form of CRP (mCRP, 250 μg/ml) or fMLP (0.1 μM), no measurable decrease in C5aR detection was found. Remarkably, the reduction in C5aR expression became significant when native

<table>
<thead>
<tr>
<th>Function of Complement</th>
<th>Healthy Donors</th>
<th>Septic Shock Patients</th>
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</thead>
<tbody>
<tr>
<td>CH 50&lt;sup&gt;a&lt;/sup&gt;</td>
<td>157 ± 9</td>
<td>130 ± 14</td>
</tr>
<tr>
<td>Neutrophil H&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt; generation&lt;sup&gt;b&lt;/sup&gt;</td>
<td>18 ± 6</td>
<td>10 ± 1</td>
</tr>
<tr>
<td>Unstimulated</td>
<td></td>
<td>8 ± 1</td>
</tr>
<tr>
<td>PMA stimulated</td>
<td>116 ± 12</td>
<td>44 ± 10</td>
</tr>
<tr>
<td>Acute phase reaction CRP&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2 ± 0.2</td>
<td>151 ± 15</td>
</tr>
</tbody>
</table>

<sup>a</sup>Complement activity (× CH<sub>50</sub>, U/ml) to obtain 50% hemolysis of opsonized sheep RBCs.
<sup>b</sup>nmol/2 × 10<sup>6</sup> neutrophils stimulated in vitro with 25 ng/ml PMA.
<sup>c</sup>Serum concentration in mg/l.
<sup>d</sup>Survivor versus nonsurvivor.
CRP was used at concentrations found during systemic inflammatory processes. As shown in Table II, in nonsurviving patients, serum CRP concentrations of $208 \pm 6$ mg/l were measured versus $151 \pm 15$ mg/l in survivors ($p < 0.03$). In vitro incubation of human neutrophils with native human CRP resulted in an almost complete loss of C5aR expression in vitro (Fig. 3A). Similar results occurred when neutrophils were exposed to recombinant CRP (data not displayed). To rule out a direct effect of CRP on C5aR detection, CRP concentrations ranging from 0.1 to 1000 mg/ml were incubated with neutrophils at 5°C for 60 min and the effects on C5aR levels, as determined by flow cytometry, were evaluated. Under these conditions, CRP failed to reduce C5aR detection (data not shown). In the in vivo setting, performance of a Spearman correlation analysis of CRP values found in patients with sepsis (and healthy volunteers) with C5aR expression on neutrophils revealed a significant inverse correlation ($rs = -0.43$, $p < 0.01$, $n = 71$). These in vitro and in vivo data suggest that pentameric CRP may play a role in the loss of C5aR expression on neutrophils during septic shock. To determine whether the CRP effects were a consequence of C5aR internalization, we incubated isolated human neutrophils with either buffer or CRP (100 µg/ml) in the presence or absence of the Clostridium botulinum C2 toxin components C2IIa (800 ng/ml) or C2I (400 ng/ml). In this context, C2IIa serves as a binding/translocation factor, whereas C2I displays the enzymatic component leading to depolymerization of the intracellular actin cytoskeleton, consequently inhibiting receptor internalization. As depicted in Supplemental Fig. 2, despite blockage of receptor internalization, CRP led to significant loss of C5aR.

Inhibition of various potential cross-reacting signaling pathways, using staurosporine (100 nM), wortmannin (100 nM), BAPTA (10 µM), the broad-spectrum matrix metalloproteinase inhibitor GM6001 (10–25 µM), anti-CD14 (10 µg/ml), the MAPK inhibitor SB203580 (1–10 µM), the sodium–proton exchanger (NHE1) inhibitor guanidine derivative (5 µM), and monensin (20 µM), failed to significantly modulate the CRP-induced loss of C5aR on neutrophils (data not shown). Thus, a proteome profiler array (using a stress protein array kit) was performed and identified a CRP-induced FcγR-dependent downregulation of heat shock protein 70 (HSP70) (Supplemental Fig. 3). Mortalin (Mkt077) as an inhibitor of the HSP70 family dose-dependently reduced C5aR on neutrophils, as determined by flow cytometry (Supplemental Fig. 4). These findings are supported by data obtained from the clinical setting of sepsis, as neutrophil lysates from patients in septic shock contained significantly lower HSP70 concentrations (203 ng/mg protein $\pm 25$) than did those from healthy volunteers (821 ng/mg protein $\pm 175$) (data not shown).

**Detection of circulating C5aR in serum of patients in septic shock; correlation with outcome**

The reduction of C5aR expression (in lysates and on neutrophil surfaces) during septic shock raised the question about C5aR in septic patients. Therefore, we examined sera from healthy volunteers and patients in septic shock for the presence of C5aR, using

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**FIGURE 1.** Flow-based analysis of C5aR on human neutrophils isolated from blood. (A) Typical patterns of C5aR from healthy donors (left), survivors of sepsis (middle), and nonsurviving patients with sepsis (right). The insets of each of the three panels show results with an isotype normal IgG control. (B) Comparisons of C5aR expression on blood neutrophils from the three groups described in (A). (C) Statistical evaluation of the various groups involving C5aR content on blood neutrophils, as analyzed by ROC curve analysis.
two independent methods (Western blots and a flow cytometry-based system). As shown in Fig. 4A, healthy volunteers exhibited barely detectable C5aR levels in serum, with a band aligning with the 45-kDa marker and reacting with anti-C5aR (arrow). This finding was in striking contrast to sera obtained from patients in septic shock, which showed strong bands reacting with anti-C5aR. A clear difference in the intensity of C5aR bands was observed in relation to survival or death of patients. In 12 of 15 patients with a lethal outcome, a more intense C5aR band was seen in comparison with the survivor group, which showed in 13 of 15 a less intense band at 45 kDa (Fig. 4A).

These data suggest for the first time, to our knowledge, the presence of circulating C5aR in serum during sepsis. A second independent method was developed to detect circulating C5aR, using a minimal amount of serum. We employed a bead capture flow cytometry-based method (as described in Materials and Methods). As shown in Fig. 4B (left panel), microspheres coated with anti-C5aR Ab and incubated with serum from healthy volunteers revealed a slight increase in fluorescence, when compared with an isotype-matched normal IgG (inset), indicating that a few microspheres had bound C5aR and were reactive with a secondary FITC-labeled anti-C5aR Ab. In contrast, almost all microspheres showed a pronounced increase in fluorescence when incubated with serum of surviving patients who were in septic shock (Fig. 4B, middle panel). Moreover, an additional increase in the fluorescence intensity was associated with microspheres exposed to serum from patients who did not survive septic shock (right panel). As summarized in Fig. 4C, the existence in serum of cC5aR was found, which was significantly increased during septic shock, especially in those patients with lethal outcome (Fig. 4C). The 45-kDa molecular position of cC5aR (Fig. 4A) suggests that this is an intact form of C5aR.

Similar findings were also obtained in the experimental setting of rodent sepsis. Levels of circulating C5aR in individual animals (detected by flow cytometry-based assay) were plotted against cumulative C5a plasma concentrations of rats with sepsis as a function of time (Fig. 4D). During sepsis, plasma C5a levels were significantly raised as a function of time after CLP (Fig. 4D, dotted line). In contrast, levels of cC5aR began to increase immediately after sepsis induction, peaking after 12 h. Between 12 and 24 h, the C5aR concentration rapidly declined (Fig. 4D, straight line).

To assess the role of C5L2, as well, human PMNs from healthy volunteers were incubated with recombinant C5a (10 nM) up to 4 h. As depicted in Fig. 4E, the circulating form of C5aR was detectable in supernatant fluids as a function of time after exposure to C5a, whereas a circulatory form of C5L2 could not be detected in supernatant fluids of PMNs under the conditions maintained.

FIGURE 2. Western blots of C5aR of lysates from human neutrophils. (A) Lysates from cells incubated with buffer or 10 nM human C5a at the times indicated. (B) Dose-response loss of C5aR in neutrophil lysates as a function of the concentration of C5a used. Cells were incubated for 4 h at 37°C. (C) Characteristic findings in neutrophil lysates from a healthy survivor, and from surviving and nonsurviving patients with sepsis.

FIGURE 3. The effects of native pentameric (CRP) and monomeric (mCRP) forms of CRP on C5aR expression on blood neutrophils. (A) Dose-response effects of CRP on C5aR expression, with incubation of cells at 37°C for 1 h. *p < 0.03. (B) Effects of exposure time on C5aR expression on blood neutrophils incubated with CRP. *p < 0.05. (C) Spearman rank-order correlation analysis of the C5aR expression on neutrophils versus CRP concentration in serum (n = 71).
Appearance of cC5aR in supernatant fluids from neutrophils exposed to C5a

To determine if neutrophils are a potential source of cC5aR, neutrophils from healthy volunteers were exposed in vitro to 10 nM C5a for 1 h at 37˚C, and after separation of the cellular fraction, supernatant fluids were further examined by immunoblotting. As shown in Fig. 5A, a band was detected by C5aR Abs aligning with the 45-kDa marker (left panel, left band). After incubation with C5a, this band in the supernatant fluid was more intense (right panel, left band), suggesting an increase in cC5aR presence. However, when these supernatant fluids were ultracentrifuged (100,000 g for 60 min), this band disappeared (left and right panels, middle lane). This observation contrasted with the pellet analysis after ultracentrifugation, which revealed a single band at the 45-kDa position (left and right panels, right lane). Again, the band from C5a-treated neutrophils was more intense than the non–C5a-treated cells. The data suggest that cC5aR can be generated from neutrophils and is associated with the pellet fraction of the cellular supernatant fluids, likely in the form of microparticles.

Morphological changes of neutrophils during C5a and CRP exposure

Thin sections of plastic-embedded neutrophils were analyzed for qualitative changes in neutrophil morphology by cell incubation with C5a (10 nM) or with native CRP (150 µg/ml). Fig. 5B exhibits in the top row (a–c) otherwise nontreated neutrophils after a 3-h incubation period at 37°C. The middle row shows representative neutrophils after a similar incubation period with C5a. Almost all neutrophils revealed changes in the cell surface, with the appearance of blebs (or microparticles), some of which had been shed from cells. A remarkable reduction in the amount of cytoplasm can also be seen. Similar changes were found when cells were exposed to native CRP (Fig. 5B, lower row). This transmission electron microscopy analysis was devoid of the typical signs of apoptosis, a finding also confirmed by parallel TUNEL assays that revealed apoptotic cell rates between 6.5 and 9% in both control group and treatment groups, independent of the incubation period (1–3 h) and stimulation (10 nM C5a or 150 µg/ml CRP, respectively, n = 5; data not shown).

Microparticles or membrane fragments obtained from nontreated or C5a-treated neutrophils revealed some evidence of FITC-C5a binding, as MFI baseline levels rose from 9 to 60 MFI or from 8 to 107 MFI, respectively (data not shown). To further support the cC5aR-associated membrane fragment/microparticle theory, coimmunoprecipitation experiments using anti-CD66e as a marker for microparticles originating from granulocytes were performed. In experimental mouse CLP sepsis, cC5aR was found to be coexpressed with CD66e (Fig. 5D), but not with anti-CD14 (data not shown), supporting the idea that cC5aR is associated with PMN-derived membrane fragments or microparticles.

CRP-induced increase of cC5aR

To further investigate the induction of cC5aR, neutrophils from healthy volunteers were exposed to various native CRP concentrations, ranging from 0 to 250 µg/ml for 1 h, and supernatant fluids were then incubated with cC5aR-detecting microspheres.
(n = 7). With increasing concentrations of CRP, there was an increasing appearance of cC5aR in supernatant fluids (Fig. 5C). This finding is indicative of the generation of cC5aR by CRP-stimulated neutrophils, which is in accordance with the CRP-induced loss of cC5aR generation from neutrophils by CRP incubation, as detected by the flow-based capture method (n = 7, *p < 0.05). (D) Coexpression of cC5aR and CD66e in serum from mice with sepsis, determined by coimmunoprecipitation experiments.

Discussion

Nearly 40 y ago, complement activation (as assessed by consumption of C3) was described in human Gram-negative bacteremia and was considered a prognostic factor (34). It is somewhat surprising that only a few studies were conducted since then to more precisely define the role and fate of complement during septic shock. A common finding of these studies was an enhanced C3a/C3 ratio during sepsis, which seemed to correlate with sepsis severity and outcome (31). However, contradictory claims have arisen concerning hemolytic serum activity (CH50) and the concentration of C5a in serum obtained from patients with sepsis (32, 35). In the current study, which included 60 patients in septic shock, the evidence was dramatic for consumption of complement, as reflected by significant reduction in CH50 values in those patients who failed to survive when compared with survivors of sepsis. The latter group revealed a much more modest consumption of complement (Table II). This finding is in accord with a previous report on 11 patients in septic shock, in whom lower CH50 values were observed and were associated with a higher organ failure score (36). For the anaphylatoxin C5a, previous studies either found C5a below the detection limit in serum (33) or suggested that most C5a generated was bound to circulating leukocytes in plasma (31) and therefore not detectable. C5a concentrations rarely exceed 10 nM in plasma from patients with sepsis and is highly reactive with C5a receptors (C5aR, C5L2) on phagocytic cells at very low concentrations (1–10 nM) (37). In the septic sera from our study, significantly enhanced levels (n = 60) were consistently detected in the septic sera, although the levels failed to distinguish between survivors and nonsurvivors of septic shock. However, care has to be taken to interpret the measured complement activation products on the basis of some evidence that “diseased” blood has been shown to have a higher exogenous activation capacity than does “healthy” blood (38) and that the coagulation process in serum samples might additionally activate complement, which can mainly be excluded using EDTA-plasma samples. In support of the clinical data, plasma samples taken during experimental rodent sepsis also revealed a time-dependent increase in C5a generation (Fig. 4D). Experimentally, similar concentrations of C5a were associated with hemodynamic depression equivalent to that found during septic shock (39) and were also linked to sepsis-induced immunodeficiency and organ dysfunction (22). Neutrophils underwent a state of innate signaling paralysis after exposure to C5a, with downregulation of surface C5aR, defects in cellular signaling, and impaired cellular function (11, 17, 22, 25). In the current study, a significant reduction in the oxidative burst response during septic shock indicated neutrophil dysfunction (although differentiation between survivors and nonsurvivors was not possible) (Table II). During experimental sepsis in rodents, dysfunctional blood neutrophils exhibited an almost complete loss of C5a binding (22). Accordingly, a reduced expression of the classical C5aR (25), as well as of the C5a-like receptor (C5L2), was detected on neutrophils in rats with sepsis, being associated with a poor clinical outcome (40). It has been suggested that C5L2 acts as a high-affinity scavenger receptor for C5a and C5adesArg during sepsis (41), thereby effectively clearing or limiting the inflammatory response. However, recent studies revealed negligible C5L2 surface expression on human PMNs and indicate that C5L2 is predominantly intracellular (42). This finding might explain the absence of a circulating form of the “decoy” C5a receptor C5L2. With respect to classical C5aR, we found a remarkable reduction in C5aR content both on the surface and in the cell lysates of neutrophils from patients in septic shock, when compared with normal PMNs (Figs. 1C, 2). Furthermore, the C5aR status on neutrophils could reliably distinguish between those patients who would succumb to septic shock and those who would survive. This observation suggests that C5a–C5aR interaction may play a crucial role during septic shock in humans. Little is known about the fate of C5aR on neutrophils during sepsis. After binding of C5a, C5aR on neutrophils undergoes phosphorylation of serine/threonine residues in the C-terminal cytosolic region, followed by rapid internalization of the ligand–receptor complex into endosomes within 10–15 min.

FIGURE 5. (A) Circulating C5aR detection in supernatant fluids from neutrophils incubated with either control medium (ctrl) or C5a and analysis of the ultracentrifugate of supernatant fluid or pellet areas. (B) Transmission electron microscopy: morphological changes of neutrophils during C5a (10 nM) or native CRP (150 µg/ml) incubation for 3 h (representative figure of n = 3). (C) cC5aR generation from neutrophils by CRP incubation, as detected by the flow-based capture method (n = 7, *p < 0.05). (D) Coexpression of cC5aR and CD66e in serum from mice with sepsis, determined by coimmunoprecipitation experiments.
(43). Thereafter, the intracellular trafficking of C5aR appears to depend on the cell type (44). In neutrophils, following prolonged exposure to C5a (which may closely reflect reality in the blood of patients with sepsis), C5aR has been shown to mainly colocalize with the late endosomal/lysosomal marker LAMP2 (lysosome-associated membrane glycoprotein-2), suggesting routing to the degradation pathway (45). This finding agrees with our results of C5aR loss in cell lysates after incubation with C5a (up to 4 h). Furthermore, a diminished cellular C5aR content in neutrophils from patients in septic shock was detected. It is remarkable that the cellular C5aR content in neutrophils from survivors of septic shock was higher than the C5aR content in those from non-survivors. Other reports suggest that a fraction of the total amount of C5aR recycles to the plasma membrane (43). In blood leukocytes of 26 patients with severe sepsis, compared with healthy volunteers, real-time RT-PCR revealed a 20-fold increase in C5aR levels, suggesting sepsis-induced de novo C5aR synthesis (46). Thus, cellular loss of C5aR could be due to increased degradation rates of C5aR and/or the shedding of C5aR. Circulating and soluble forms of receptors released by blood monocytes or neutrophils, such as sIL-1RII (47, 48) or sTNF-αR (49), were elevated during systemic inflammatory responses and may represent a strategy to regulate inflammatory processes.

In this article, we present for the first time, to our knowledge, data suggesting the in vitro shedding of C5aR, but not of the "decoy" receptor C5L2, from neutrophils and the appearance in serum of C5aR as cC5aR (Figs. 4, 5). Because C5aR is embedded in the cell membrane, the fact that the Western blots of cC5aR indicate a molecular mass of 45 kDa is evidence that intact C5aR is present together with the associated lipid bilayer of the cell membrane. This evidence would be consistent with C5aR being shed in the form of microparticles from PMNs. In support, we found that circulating C5aR in rodent sepsis was coexpressed with CD66e, a marker for microparticles originating from granulocytes. With the use of both Western blot analysis and a flow-based assay to detect C5aR in serum, evidence for C5aR in serum from healthy donors was limited. In contrast, enhanced levels of cC5aR were detected in serum from humans in septic shock. Strikingly, much more cC5aR was found in sera from nonsurvivors than in sera from survivors of septic shock. These data correlated with the loss of neutrophil-associated C5aR from patients with sepsis (Fig. 1B). Furthermore, Western blot analyses of ultracentrifuged supernatant fluids of neutrophils revealed that cC5aR was associated with the pellet fraction, which is known to contain membrane fractions, microvesicles, and blebs. This association with the pellet fraction may represent cell membrane blebs containing intact C5aR that have been released into serum from neutrophils. Shedding of blebs (microparticles) from neutrophils released by inflammatory stimuli (such as fMLP), by cell-derived ATP (50), or during apoptotic processes has been recently described (51). In our study, we found C5a or CRP induction of microparticles from neutrophils (Fig. 5B), whereas the apoptotic rate of neutrophils was unaltered, as determined by TUNEL assays. The latter is in accord with the observation that apoptotic rates in neutrophils are reduced during sepsis (5, 52). The rapid detection of cC5aR in serum by new flow-based capture assay (as described in the current article) may represent a biomarker for the diagnosis of sepsis, but this remains to be proven.

It is still unclear which factors are involved in the regulation of C5aR on neutrophils during inflammatory processes. Theoretically, the loss of C5aR on neutrophils during sepsis could be induced by various stress or inflammatory mediators present during septic shock. However, these factors, including epinephrine, norepinephrine, hydrocortisone, ATP, ILs (IL-1,-6,-10), insulin, and metalloproteinase inhibitors, have all failed to alter in vitro C5aR expression and cellular content of C5aR. Because CRP is considered one of the molecular links between the early systemic response and subsequent specific immunity (53) and also regulates various signaling pathways in phagocytic leukocytes (54), the effects of native pentameric CRP on C5aR regulation were investigated. In addition, we analyzed the role of mCRP, which is to some extent expressed at inflammatory sites and was shown to affect various biological functions in human neutrophils (55). Whereas mCRP did not interfere with C5a receptor binding, native CRP was found to effectively reduce the expression of C5aR on neutrophils (Fig. 3), especially when CRP concentrations were used at levels found in the serum of patients in septic shock (>100 μg/ml). In accordance with other reports (56), CRP levels in patients with a lethal outcome were significantly enhanced in comparison with the survivor group and reversely correlated with C5aR expression. Complement activation in patients with sepsis has been reported to be mediated in part by CRP, with increasing levels of complement-CRP complexes in patients with sepsis and in nonsurviving patients (57). Evidence is also present that C5a acts in concert with IL-6 and IL-1β to promote upregulation of the CRP gene (58) and that CRP (at 50 μg/ml) activates shedding of IL-6R from human neutrophils (59). By our flow-based capture assay, we also found evidence for CRP-induced shedding of C5aR in vitro (Fig. 5C). Furthermore, in vivo, an inverse correlation between C5aR expression on neutrophils and CRP serum concentration was found (Fig. 3C). A competitive interference of CRP on C5aR binding could be excluded by real-time flow-based binding assays (data not shown). CRP could possibly indirectly downregulate C5aR by C5a generation via activation of the classical complement pathway. However, this is rather unlikely because the enhanced C5a levels during sepsis failed to discriminate between survivors and nonsurvivors (which might be caused by the vast binding capacity of the almost ubiquitously expressed C5aR). In the current study, some evidence is provided that HSP70 is involved in CRP-induced loss of C5aR, supported by reduced HSP70 protein levels in neutrophils during septic shock. However, additional studies are required to further define a crosstalk between HSPs and complement. Another factor that may alter C5aR expression could be bacterial LPS, as recently proposed for LPS-stimulated neutrophils, which exhibited a slightly reduced C5aR expression (~12%) in contrast to a greatly increased CR-1 and CR-3 expression (60). Of interest, a negative regulation by C5a-C5aR interaction with TLR-4–induced immune response in macrophages has been reported (61). In the current report, we could not detect a significant effect of LPS or anti-CD14 treatment on C5aR content in neutrophils.

Overall, the cellular and immune dysfunction found in advancing sepsis has been explained by both “cellular hibernation” (5) and impairment of the innate and adaptive immune responses (4). The data from dysfunctional neutrophils from patients in septic shock suggest that C5a and the CRP-induced loss of cellular C5aR and the appearance of circulating C5aR in serum or plasma may play a crucial role in the pathophysiology of sepsis and its outcome. The C5aR status on neutrophils and the presence of cC5aR in serum, as detected by flow-based methods, may both be important markers defining the immune status of the patient. Such findings might be key for effective patient monitoring and patient-tailored treatment.

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