Role of C5a in Multiorgan Failure During Sepsis


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Role of C5a in Multiorgan Failure During Sepsis

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In humans with sepsis, the onset of multiorgan failure (MOF), especially involving liver, lungs, and kidneys, is a well known complication that is associated with a high mortality rate. Our previous studies with the cecal ligation/puncture (CLP) model of sepsis in rats have revealed a C5a-induced defect in the respiratory burst of neutrophils. In the current CLP studies, MOF occurred during the first 48 h with development of liver dysfunction and pulmonary dysfunction (falling arterial partial pressure of O2, rising partial pressure of CO2). In this model an early respiratory alkalosis developed, followed by a metabolic acidosis with increased levels of blood lactate. During these events, blood neutrophils lost their chemotactic responsiveness both to C5a and to the bacterial chemotaxin, fMLP. Neutrophil dysfunction was associated with virtually complete loss in binding of C5a, but binding of fMLP remained normal. If CLP animals were treated with anti-C5a, indicators of MOF and lactate acidosis were greatly attenuated. Under the same conditions, C5a binding to blood neutrophils remained intact; in tandem, in vitro chemotactic responses to C5a and fMLP were retained. These data suggest that, in the CLP model of sepsis, treatment with anti-C5a prevents development of MOF and the accompanying onset of blood neutrophil dysfunction. This may explain the protective effects of anti-C5a in the CLP model of sepsis. The Journal of Immunology, 2001, 166: 1193–1199.

Sepsis in humans often leads to progressive multiorgan failure (MOF),3 which is still associated with a high mortality rate (1, 2). MOF in either humans or animals appears to emerge as a consequence of progressive development of tissue hypoxia, complement activation, and an unregulated release into the blood of a variety of proinflammatory mediators (ILs, cytokines, chemokines) (3–5). In humans with sepsis, there is well established evidence for the appearance in plasma of complement activation products, especially the anaphylatoxins (C3a, C4a, C5a) (6, 7). Persistent elevation of these anaphylatoxins appears to be correlated with development of MOF and is inversely correlated with survival (7, 8). The complement activation product, C5a, is a potent neutrophil agonist that interacts with a seven-transmembrane spanning receptor (C5aR) (9). Functional responses of neutrophils, such as their release of proteases and generation of oxidants, as well as their enhanced adhesion to endothelial cells, may be associated with development of direct or remote tissue injury that occurs in sepsis and MOF (10). Neutrophils from patients with sepsis-induced MOF exhibit a loss of in vitro chemotactic responsiveness to C5a (10), this functional deficit being correlated with a loss of the ability of C5a to bind to neutrophils (11). Furthermore, LPS- and C5a-induced neutropenia as well as neutrophil migration into the peritoneum in models of acute endotoxemic shock can be inhibited by pretreatment with C5aR-antagonists (12, 13). Our recent studies of sepsis using the cecal ligation/puncture (CLP) model in rats have demonstrated the development of a substantial defect in the ability of blood neutrophils to produce H2O2 when stimulated in vitro with PMA (14). Neutrophil-generated H2O2 is a vital oxygen product required for myeloperoxidase-dependent intracellular killing of bacteria (15). The development of this defect in H2O2 production appears to be reversed by in vivo treatment of CLP animals with anti-C5a (14). Thus, there are several lines of evidence suggesting that sepsis triggers activation of complement and development of abnormalities in neutrophil function. These events may be associated with development of MOF.

In our original study of CLP-induced sepsis, the protective effects of anti-C5a on survival were documented (14), but evidence of MOF and the effects of anti-C5a on MOF were not assessed. In the current studies, we describe the progressive onset of MOF in the CLP model and the multifunctional loss of chemotactic responsiveness of blood neutrophils. We demonstrate the ability of anti-C5a to preserve chemotactic responsiveness of neutrophils and to preserve C5a binding sites on neutrophils, all of which are associated with greatly reduced development of MOF and lactate acidosis following CLP.

Materials and Methods

Reagents

Unless otherwise specified, chemicals and reagents were purchased from Sigma (St. Louis, MO).

Preparation and characterization of Ab against rat C5a

Rat C5a peptide with the sequence KHRVPKKCYDGARENKYET (corresponding to amino acid residues 17–36) was coupled to keyhole limpet hemocyanin and then used as an Ag to immunize rabbits. After several immunizations, the Ab was affinity purified from serum using the synthetic peptide coupled to beads. The preparation of this Ab was performed by Research Genetics (Huntsville, AL). In vitro, this polyclonal Ab immunoprecipitated from activated rat serum a protein aligning in Western blots with the sequence KHRVPKKCCYDGARENKYET. It precipitated from activated rat serum a protein aligning in Western blots.
with the 14-kDa marker, consistent with the molecular mass of glycosylated C5a (data not shown). This Ab did not interfere with whole hemolytic activity (CH50) in rat serum (data not shown).

**Preparation of rat recombinant C5a (rr C5a)**

A ‘his tag’ based on the TAGZyme system (Unizyme Laboratories, New York, NY) was engineered into the amino terminus of the rat C5a sequence. The presence of the alternating histidine and glutamine residues (in all six, each alternating in tandem) facilitated purification of the recombinant C5a protein over a Ni$^{2+}$ Sepharose column. The his-tagged sequence was cloned into the baculovirus vector pVL1393 (PharMingen, San Diego, CA) and expressed in *Spodoptera frugiperda* (Sf9) cells (PharMingen). Recombinant protein was then purified by lysing the cells in a buffer containing 10 mM Tris pH 7.4, 130 mM NaCl, 1% Triton X-100, 10 mM NaF, 10 mM Na$_2$PO$_4$, 10 mM sodium pyrophosphate, and protease inhibitors (16 µg/ml benzamidine HC1, 10 µg/ml aprotinin, 10 µg/ml leupeptin, and 10 µg/ml pepstatin). After removing the cell debris by centrifugation (10,000 × g for 10 min, then 100,000 × g for 1 h), the lysate was poured over a Ni$^{2+}$ column (Qiagen, Chatsworth, CA). Then, rr C5a was eluted with 10 mM Tris (pH 7.4), 300 mM NaCl, and 0–250 mM imidazole. The eluate was dialyzed extensively with PBS (pH 7.4). This rr C5a was shown to be functionally active in a chemotaxis assay using rat blood neutrophils.

**Experimental sepsis by CLP**

Male Long-Evans specific pathogen-free rats (Harlan Breeders, Indianapolis, IN) weighing 275–300 g were used in all experiments. Anesthesia was induced by i.p. administration of ketamine (20 mg/100 g body weight). After shaving the abdomen and applying a topical disinfectant, a 2-cm midline incision was made, and the cecum was identified and ligated below the ileocecal valve, with care being taken not to occlude the bowel passage. The cecum was then subjected to a single “through and through” perforation with a 21-gauge needle and was gently squeezed to ensure patency of the perforation sites. After repositioning the bowel, the abdominal incision was closed in layers with plain gut surgical suture 4-0 and skin clips (Ethicon, Somerville, NJ). Sham-operated animals underwent the same procedure except for ligation and puncture of the cecum. Animals with CLP treatment were injected i.v. immediately after induction of CLP with 400 µg rabbit IgG against rat C5a (anti-C5a) or with 400 µg preimmune rabbit IgG. Before and after surgery, animals had unrestricted access to food and water. For urine collection and determination of water intake and urinary output, rats were closely followed in metabolic boxes (Nalgene metabolic cage; Nalge Nunc International, Rochester, NY) from time 0–36 h after the surgical procedures, as indicated in the individual experiments. In some animals, a carotid artery catheter (PE-50; Becton Dickinson, Somerville, NJ) was used to monitor systemic arterial pressure and heart rate. Sham-operated animals were not cannulated. Blood was obtained from the inferior vena cava (at 12-h intervals up to 48 h after CLP) for biochemical measurements and for isolation of neutrophils. Organs were processed for morphological studies as described below.

**Transmission electron microscopy**

Kidney tissues were sectioned into 1-mm cubes, 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% OsO$_4$ 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, infiltrated with increasing mixtures of propylene oxide and epox resin, and embedded in pure Epon. One-micron sections were stained with toluidine blue and evaluated by light microscopy. Thin sections were obtained on an AU Ultracut ultramicrotome, stained with uranyl acetate and lead citrate, and evaluated in a Philips 400 T electron microscope.

**Measurement of biochemical parameters**

Unless otherwise stated, blood was obtained from the inferior vena cava at the time of sacrifice. Creatinine, blood urea nitrogen (BUN), alanine aminotransferase (ALT), aspartate aminotransferase (AST), and lactate dehydrogenase (LDH) were determined in serum samples by standard clinical chemistry techniques. All assays were performed using commercially available reagents (Vitros dry film slides) on a Vitros 950 chemistry analyzer (Ortho-Clinical Diagnostics, Rochester, NY). Electrolytes were determined by conventional flame spectrometry.

Calculation of glomerular filtration rates (GFRs)

The GFR was determined by endogenous creatinine clearance, measured by the urine flow rate (ml/min) over a period of 36 h, and multiplied by the ratio of the creatinine concentration in urine and in serum.

**Urinary protein electrophoresis**

After centrifugation of urine at 14,000 × g for 10 min, a 10-µl aliquot of the supernatant was added to 5 µl of Laemmli’s sample buffer followed by boiling for 5 min. Samples were then electrophoresed on a 10% SDS-PAGE gel and stained with Coomassie blue.

**Isolation of rat blood neutrophils**

Whole blood from rats was drawn into syringes containing the anticoagulant ACD (Baxter Health Care, Mundelein, IL). Neutrophils were isolated using Ficoll-Paque gradient centrifugation (Pharmacia Biotech, Uppsala, Sweden) and dextran sedimentation. After hypotonic lysis of residual RBC, neutrophils were evaluated in chemotaxis assays and in binding studies using rr C5a and fMLP.

**Chemotaxis assay**

Following neutrophil isolation, cells were fluorescein labeled with BCECF (2,7′,bis-[2-carboxyethyl]-5,6-carboxy-fluorescein acetoxymethyl ester) (Molecular Probes, Eugene, OR). Labeled neutrophils (5 × 10$^6$ cells/ml) were then loaded into the upper chambers of 96-well micromich____

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**Statistical analyses**

All values are expressed as mean ± SEM. Data sets were analyzed using a one-way ANOVA. Individual group means were then compared using the Tukey test. Significance was assigned where $p < 0.05$.

**Results**

**Reversal by anti-C5a of early respiratory alkalosis and late metabolic acidosis in CLP animals**

Arterial blood gases (expressed as mmHg) and pH values were determined in normal rats (ctrl) and in CLP rats that were infused i.v. at time 0 (immediately after CLP) with either 400 µg rabbit preimmune IgG or 400 µg anti-rat C5a IgG (anti-C5a). Blood samples were obtained at early (24 h) and late time points (60 h) of CLP. The results are shown in Table I. As expected, at 24 h there was a mild respiratory alkalosis, with the arterial pH rising from 7.47 ± 0.01 to 7.53 ± 0.01 in CLP rats treated with preimmune IgG. In the same group, the blood pCO$_2$ fell from a value of 38.6 ± 0.01 µl/mg benzamidine HCl, 10 µg/ml aprotinin, 10 µg/ml leupeptin, and 10 µg/ml pepstatin. After removing the cell debris by centrifugation (10,000 × g for 10 min, then 100,000 × g for 1 h), the lysate was poured over a Ni$^{2+}$ column (Qiagen, Chatsworth, CA). Then, rr C5a was eluted with 10 mM Tris (pH 7.4), 300 mM NaCl, and 0–250 mM imidazole. The eluate was dialyzed extensively with PBS (pH 7.4). This rr C5a was shown to be functionally active in a chemotaxis assay using rat blood neutrophils.

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of an additional respiratory acidosis with a mild hypercapnia were unchanged in these two sets of animals at this early period of time. In contrast, in CLP rats treated with anti-C5a, the blood pH and pCO2 values at 24 h were the same as those values obtained in normal, unmanipulated rats.

When similar groups of rats were examined at 60 h, the CLP group treated with preimmune IgG showed evidence of metabolic acidosis, with the blood pH values falling from 7.48 ± 0.003 to 7.34 ± 0.008, and blood HCO3− (mmol/L) falling from 26.6 ± 0.47 to 20.8 ± 1.86 (Table I). At the same time point, first signs of an additional respiratory acidosis with a mild hypercapnia were found (increase in blood pCO2 from 36.6 ± 1.29 to 44.5 ± 3.32) and there was clear evidence of hypoxia, with a fall in the pO2 value from 105 ± 0.88 to 69.8 ± 9.79. Remarkably, in the CLP group treated with anti-C5a, all values remained in the normal range at 60 h (Table I).

Parameters of MOF and effects of anti-C5a treatment

The parameters of MOF are described in Table II as well as the protective effects of anti-C5a. There was clear evidence of MOF developing in CLP rats and the reversal of these parameters by treatment of CLP rats with anti-C5a (400 μg given i.v. at the time of CLP) but not when preimmune IgG was used. Blood lactate levels rose 2.64-fold during CLP. This rise was prevented in anti-C5a-treated rats. Evidence of liver dysfunction was indicated by elevations in serum bilirubin, ALT, and AST (2.5-, 5-, and 7-fold, respectively). In animals treated with anti-C5a, most of these parameters remained in the normal range, whereas the rise in LDH levels was attenuated. With regard to kidney function, in unprotected CLP animals, serum creatinine, BUN, and urine protein levels rose by 67, 280, and 150%, respectively, whereas in CLP rats treated with anti-C5a, the levels were not statistically different from those levels in sham animals. Electrophoretic analysis of urine (SDS-PAGE, followed by Coomassie blue staining) revealed faint amounts of albumin in urine from shams and greatly increased urinary albumin in CLP rats treated with preimmune IgG involving lower and higher m.w. proteins relative to urinary albumin. In CLP rats treated with anti-C5a, albumin was somewhat increased above that found in sham rats, whereas the higher and lower m.w. bands were largely abolished (data not shown). These data suggest defects both in glomerular filtration as well as in tubular absorption. Urine output and GFR values in the unprotected CLP animals fell by 80 and 75%, respectively; these changes were greatly reduced in the animals receiving anti-C5a. Thus, manifestations of MOF in CLP animals are greatly attenuated in animals receiving anti-C5a.

Transmission electron microscopy of kidneys from CLP animals

Given the changes in proteinuria, urine output, and GFR (see above) we investigated presence of morphological changes in kidneys by transmission electron microscopy. Tissue samples were obtained 36 h after CLP-induced sepsis in rats treated with either preimmune IgG or anti-C5a. As shown in Fig. 1, in CLP animals treated with preimmune IgG, it was evident that there was extensive fusion of foot processes of podocytes in glomeruli (A, arrowheads). In proximal convoluted tubules, the cells had lost their cell membranes and exhibited mitochondrial swelling together with intracellular edema (B). In CLP animals that had been treated with anti-C5a, renal glomeruli appear normal with prominent podocytes (C), and epithelial cells of proximal convoluted tubules were intact and had normal structures (D).

Electrophoretic analysis of rr C5a

The relative purity of recombinantly produced rr C5a was determined by electrophoretic analysis. Coomassie staining revealed a single predominant band in the expected m.w. position for a non-glycosylated product (data not shown) This C5a preparation was used both for functional (chemotaxis) assessment of rat blood neutrophils and binding of 125I-rr C5a to rat blood neutrophils.

### Table I. Arterial blood gas analyses

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Early Phase (24 h)</th>
<th>Late Phase (60 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>CLP + preimmune IgG</td>
</tr>
<tr>
<td>pH</td>
<td>7.47 ± 0.01</td>
<td>7.53 ± 0.01*</td>
</tr>
<tr>
<td>pCO2 (mmHg)</td>
<td>38.6 ± 0.89</td>
<td>34.2 ± 0.91*</td>
</tr>
<tr>
<td>HCO3− (mmol/L)</td>
<td>26.4 ± 1.14</td>
<td>26.9 ± 0.50</td>
</tr>
<tr>
<td>pO2 (mmHg)</td>
<td>106 ± 0.88</td>
<td>106.4 ± 7.86</td>
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</table>

* Significant difference (p < 0.05) vs effects of anti-C5a treatment.

### Table II. Effects of anti-C5a on parameters of MOF

<table>
<thead>
<tr>
<th>Organ</th>
<th>Parameter</th>
<th>Sham + preimmune IgG</th>
<th>CLP + preimmune IgG</th>
<th>CLP + anti-C5a</th>
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</thead>
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<tr>
<td>Blood</td>
<td>Lactate (mmol/L)</td>
<td>2.87 ± 0.40</td>
<td>7.42 ± 1.31</td>
<td>3.54 ± 0.30</td>
</tr>
<tr>
<td></td>
<td>Bilirubin (mg/dl)</td>
<td>0.30 ± 0.01</td>
<td>0.53 ± 0.08</td>
<td>0.23 ± 0.03</td>
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<tr>
<td></td>
<td>ALT (U/L)</td>
<td>46.6 ± 1.8</td>
<td>255.0 ± 65.2</td>
<td>63.6 ± 2.4</td>
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<tr>
<td></td>
<td>AST (U/L)</td>
<td>74.0 ± 2.0</td>
<td>386.6 ± 71.6</td>
<td>128.0 ± 8.5</td>
</tr>
<tr>
<td>Kidney</td>
<td>LDH (U/L)</td>
<td>288.3 ± 85.8</td>
<td>926.0 ± 47.0</td>
<td>628.0 ± 11.5</td>
</tr>
<tr>
<td></td>
<td>Creatinine (mg/dl)</td>
<td>0.43 ± 0.03</td>
<td>0.70 ± 0.05</td>
<td>0.43 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>BUN (mg/dl)</td>
<td>11.6 ± 1.4</td>
<td>44.5 ± 12.2</td>
<td>17.6 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>Urine protein (mg/dl)</td>
<td>1.30 ± 0.04</td>
<td>3.27 ± 0.29</td>
<td>1.57 ± 0.19</td>
</tr>
<tr>
<td></td>
<td>Urine output (ml/36 h)</td>
<td>26.2 ± 0.8</td>
<td>6.6 ± 1.0</td>
<td>19.0 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>GFR (ml/min)</td>
<td>1.89 ± 0.03</td>
<td>0.49 ± 0.07</td>
<td>1.90 ± 0.24</td>
</tr>
</tbody>
</table>

The Journal of Immunology
Chemotactic dysfunction of blood neutrophils from CLP animals

The ability of blood neutrophils to respond in vitro to recombinant rat C5a and to the structurally unrelated chemotactic peptide, fMLP, both of which are known to be powerful chemotactic factors for neutrophils, was assessed. Blood neutrophils were obtained from each of the three groups of rats (control and 36 h after CLP from rats treated with either preimmune IgG or anti-C5a) as described above. The isolated cells were labeled with a fluorescent probe and evaluated by cytofluorometry for chemotactic responses in vitro as described in Materials and Methods. The results are shown in Fig. 2. When blood neutrophils were obtained from normal rats (ctrl), chemotactic responses to rr C5a showed the expected bell-shaped dose-response profile, starting at 0.1 nM and peaking between 1 and 100 nM (Fig. 2A). Blood neutrophils from CLP rats treated with either preimmune IgG or anti-C5a, as described above, showed responses that were not statistically different from those obtained from blood of normal animals. Thus, blood neutrophils from CLP rats acquired a chemotactic defect that was nonspecific, involving impaired responses to both C5a and fMLP. In vivo blockade of C5a in CLP rats resulted in retention of normal chemotactic function in response to both C5a and fMLP.

Binding of 125I- rr C5a to normal rat blood neutrophils

For binding studies, neutrophils were obtained as described above. As shown in Fig. 3A, over a range of 0.6–39.2 nM 125I-rr C5a, there was progressively increased binding to neutrophils with a plateau in the binding occurring between 19.6 and 31.4 nM 125I-rr C5a. When unlabeled rr C5a was mixed together with 125I-rr C5a in a ratio of 100:1, the binding of the latter was almost totally suppressed (Fig. 3A, lower line of graph). The computed $K_d$ value was 20 nM. For subsequent studies, the dose of 31.4 nM 125I-rr C5a was used to determine whether there was defective binding when blood neutrophils from CLP rats were used.

**FIGURE 1.** Transmission electron micrographs of renal glomeruli and proximal tubular epithelium 36 h after onset of CLP in animals treated (at time 0) with 400 µg preimmune IgG (A and B) or anti-C5a (anti-C5a) (C and D). In A, arrowheads indicate extensive fusion of foot processes of podocytes in glomeruli, which did not occur in anti-C5a treated animals (C). Proximal tubular epithelial cells have intact nuclei but show intracellular edema, loss of the cell membrane, and mitochondrial swelling (B), features of which were not seen in anti-C5a-treated animals (D). A and C, ×4600; B and D, ×3500.
Defective binding of rat C5a to blood neutrophils from animals with CLP

These studies were undertaken to assess the status of binding of \(^{125}\)I-rr C5a to blood neutrophils as a function of time after onset of CLP. Neutrophils were isolated from blood at 0, 12, 24, 36, and 48 h after onset of CLP in animals that had been treated i.v. at time 0 with 400 \(\mu\)g preimmune IgG or anti-C5a. The results of these binding studies are shown in Fig. 3B. Binding values of \(30,000\) cpm with a nonspecific binding fraction of \(5000\) cpm (Fig. 3B) were found in blood neutrophils obtained from normal rats (time 0). When CLP rats were pretreated with 400 \(\mu\)g preimmune IgG and blood neutrophils were harvested at 12, 24, 36, and 48 h after CLP, the specific binding values for rr C5a relative to the 0 time values had fallen to 3.96, 4.35, 33.2, and 40.25\%, respectively. In CLP animals that had been infused with anti-C5a, the results for 12, 24, 36, and 48 h were significantly different from that of preimmune IgG-treated rats. Specific C5a binding values at these time points were 40.7, 61.2, 76.7, and 92.3\%, respectively. Thus, it appears that in CLP rats, treatment with anti-C5a caused retention of the binding sites in blood neutrophils. Whether this was due to occupancy by C5a of C5aR or due to internalized C5a-C5aR complexes remains to be determined.

Binding of \(^{3}\)HjMLP to rat blood neutrophils

Binding of \(^{3}\)HjMLP to rat neutrophils isolated from normal rat blood was determined as described above. As seen in Fig. 4, there was a progressive increase in binding over a range of 0.1–100 nM fMLP, reaching a plateau at a concentration of 50 nM or higher (\(K_d = 35\) nM). Nonspecific binding was determined by incubating neutrophils with a 100:1 ratio of nonlabeled fMLP to \(^{3}\)HjMLP. Neutrophils obtained from normal rats or from CLP rats (24 h after CLP) treated with either 400 \(\mu\)g preimmune IgG or 400 \(\mu\)g anti-rat C5a (anti-C5a) did not show statistically significant differences in binding of

\[\text{FIGURE 2.} \quad \text{Chemotactic responses of blood neutrophils obtained from normal rats (ctrl) or from CLP rats (at 36 h) treated with 400 \(\mu\)g preimmune IgG or 400 \(\mu\)g anti-rat C5a (anti-C5a) at time 0. In vitro chemotactic responses to rr C5a (A) and fMLP (B) were measured by cytofluorometry. For each data point, } n = 4. \text{ Asterisks represent statistical differences} (p < 0.05) \text{ between neutrophil responses obtained in cells from CLP animals treated with anti-C5a and CLP animals treated with preimmune IgG.}\]

\[\text{FIGURE 3.} \quad \text{Binding of }^{125}\text{I-rr C5a to neutrophils obtained from normal rat blood (A). Details are described in Materials and Methods. The calculated } K_a \text{ value was 20 nM. The ability of unlabeled rr C5a (in a ratio of 100:1) to compete with binding of }^{125}\text{I-rr C5a is also shown. In B, blood neutrophils were obtained at the times indicated (after onset of CLP), and binding of }^{125}\text{I-rr C5a was determined. Neutrophils were obtained from CLP rats treated with 400 \(\mu\)g preimmune IgG or anti-rat C5a (anti-C5a).}\]

\[\text{FIGURE 4.} \quad \text{Binding of }^{3}\text{HjMLP to normal rat neutrophils (○) with a calculated } K_a \text{ value of 35 nM and binding to neutrophils obtained 24 h after CLP from rats injected with 400 \(\mu\)g preimmune IgG (■) or anti-rat C5a (anti-C5a) (▲). Nonspecific binding was determined by binding in the presence of 100-fold excess of cold fMLP (⊙).}\]
[\textsuperscript{3}H]fMLP (50 nM). Thus, CLP does not reduce fMLP binding in blood neutrophils, in striking contrast to the situation with C5a binding (Fig. 3), even though the chemotactic responses of fMLP are greatly suppressed (Fig. 2B).

Discussion
The role of complement activation products in sepsis and in complications such as MOF is a debatable issue. There is fairly good evidence to suggest that, in the absence of complement such as in complement-depleted animals (14) or in C3 or C4 mutant mice lacking this critical complement component (18, 19), animals are highly susceptible to the early and lethal effects of experimental sepsis. These observations reinforce the long-standing concept that the complement system functions as a critical protective pathway via products such as C3b and iC3b. Evidence also exists that, during sepsis, extensive activation of the complement system occurs resulting in loss of homeostasis, which may in some manner compromise survival. Intravenous infusion of C5a into dogs generated a shock syndrome characterized by portal blood pooling and an associated decrease in venous return, cardiac output, and arterial pressure (20). In experimental sepsis caused by massive i.v. infusion of live, Gram-negative bacteria into pigs or monkeys, development of biochemical and hemodynamic abnormalities, and early death have been described within 4–6 h. Some of these events were partly attenuated if the pigs were first treated with a mAb to the carboxyl-terminal region of porcine C5a or monkeys treated with a polyclonal Ab to whole human C5a (21–23). Our own studies have indicated that lethality associated with CLP-induced sepsis in rats can be significantly diminished by treatment of animals with anti-C5a (14). This treatment significantly preserved the respiratory burst of blood neutrophils, thus maintaining the \( \text{H}_2 \text{O}_2 \) response (14), which is vital for the effective myeloperoxidase-dependent pathway of bactericidal activity of neutrophils (15). Collectively, these studies suggest that sepsis may be associated with excessive activation of the complement system, resulting in defects in bactericidal function of neutrophils.

In this study, CLP in rats caused a time-dependent pattern of MOF, with a late onset of hypoxia (60 h) in CLP rats treated with preimmune IgG but not in CLP rats after blockade of C5a. In a short-term sepsis model (4–6 h), pigs or monkeys challenged with very high doses of live \textit{Escherichia coli} and treated with an Ab to porcine or human C5a showed (in comparison to otherwise untreated animals with sepsis) a partial improvement in some functional parameters (such as oxygenation, lung edema, and oxygen uptake) and an improvement in the oxygen extraction ratio accompanied by an attenuated increase in lactate levels (21–23). Hyperlactatemia is known to be a reliable indicator of advancing MOF in humans (24). In CLP animals treated with preimmune IgG, blood lactate levels were very high at 48 h. This elevation was abrogated in CLP rats treated with anti-C5a. Data in this report clearly indicate that CLP animals develop evidence of progressive lung, kidney, and liver failure that is C5a dependent. All of these outcomes may be linked to excessive C5a generation during sepsis, resulting in a compromised respiratory burst of neutrophils and their oxygen-dependent bactericidal pathways.

Our data also document in the CLP model in rats that blood neutrophils acquire a significant defect in chemotactic responsiveness both to C5a as well as to fMLP. Because these two chemotactic peptides are structurally totally distinct and use different receptors on neutrophils (25, 26), our observations may imply a postchemotactic factor receptor-acquired functional defect. This may be the result of excessive in vivo stimulation by C5a, resulting in defects in signal transduction that extend to agonists beyond C5a and impair chemotactic responsiveness to C5a as well as to other chemotactic factors. Our studies document development of chemotactic defects in blood neutrophils from CLP rats involving responses to both C5a and to fMLP. These defects are associated with loss of binding of rat \textsuperscript{125}I-rr C5a to neutrophils but normal binding of \textsuperscript{[3}H]fMLP. fMLP is known to be the major neutrophil chemotactic factor in culture filtrates of \textit{E. coli} (27). These data would support the possibility that in vivo contact of neutrophils with C5a induces a downstream defect in signal transduction beyond a point at which C5aR and fMLP-receptor (fMLPR) pathways merge. In other words, although blood neutrophils from CLP rats appear to demonstrate normal binding of fMLP, this binding is unable to effectively engage a post receptor signaling pathway that is common to both C5aR and fMLPR. This is consistent with earlier studies in which, when neutrophils were exposed to low levels of C5a (present in activated serum), the cells acquired a chemotactic defect specific to C5a. When neutrophils were exposed to higher concentrations of C5a, they then acquired a broad defect in chemotactic function characterized by loss of responsiveness to C5a and to bacterial chemotactic peptides (11). In vivo, the chemotactic responses of neutrophils obtained from patients with sepsis or multisystem trauma with adult respiratory distress syndrome were found to be specifically deactivated to C5a or deactivated to both C5a and fMLP depending on clinical severity and time after trauma or onset of sepsis. In contrast, no alteration in neutrophil function was found in patients who did not develop adult respiratory distress syndrome (28). Our recent studies in the CLP model indicate that the \( \text{H}_2 \text{O}_2 \) response of neutrophils to PMA is impaired (14), suggesting a broad defect in postreceptor signaling pathways.

Perhaps relevant to the chemotactic dysfunction of blood neutrophils described in this report is the finding that binding sites for rat C5a (but not for fMLP) on neutrophils have been virtually lost (<5% of binding in control neutrophils) during the first 24 h of sepsis. By 48 h, neutrophils still demonstrated <50% of C5a binding, in striking contrast to nearly normal binding values of C5a for neutrophils obtained from CLP rats treated with anti-C5a (Fig. 3). Under these latter conditions, chemotactic responsiveness to C5a and to fMLP was totally preserved. These data are in accord with findings of specific loss of C5a binding (but intact fMLP binding) to neutrophils in patients with sepsis (11). It seems possible that the mechanism by which anti-C5a may protect against development of MOF during sepsis is its ability to cause preservation in the chemotactic and respiratory burst function of blood neutrophils by intercepting C5a before it has a chance to bind to C5aR on neutrophils. Retention of these functional responses would facilitate effective in vivo recruitment of neutrophils and maintenance of bactericidal function in these cells. Understanding the relationship between C5a and the induction of host defense dysfunction in blood neutrophils is the topic of current focus.

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References


