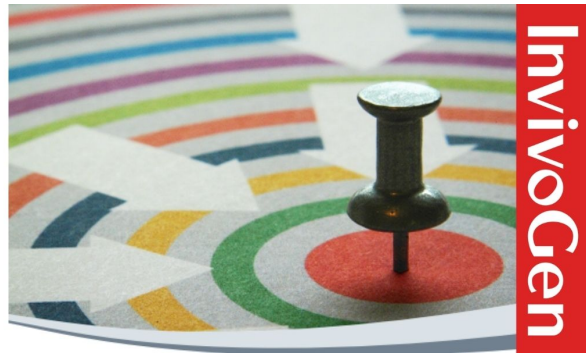


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# Complement-Induced Impairment of Innate Immunity During Sepsis<sup>1</sup>

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**This study defines the molecular basis for defects in innate immunity involving neutrophils during cecal ligation/puncture (CLP)-induced sepsis in rats. Blood neutrophils from CLP rats demonstrated defective phagocytosis and defective assembly of NADPH oxidase, the latter being due to the inability of p47<sup>phox</sup> to translocate from the cytosol to the cell membrane of neutrophils after cell stimulation by phorbol ester (PMA). The appearance of these defects was prevented by in vivo blockade of C5a in CLP rats. In vitro exposure of neutrophils to C5a led to reduced surface expression of C5aR and defective assembly of NADPH oxidase, as defined by failure in phosphorylation of p47<sup>phox</sup> and its translocation to the cell membrane, together with failure in phosphorylation of p42/p44 mitogen-activated protein kinases. These data identify a molecular basis for defective innate immunity involving neutrophils during sepsis. *The Journal of Immunology*, 2002, 169: 3223–3231.**

The innate immune system exists as a protective shield after tissue injury and in the presence of bacterial pathogens. This system can be rapidly activated via cells (the phagocytic cell system) or by plasma components (complement proteins, coagulation factors, etc.) (1–3). It is also recognized that the innate immune system can be perturbed during sepsis, as reflected by extensive activation of the inflammatory, complement, and clotting systems, together with the appearance in plasma of cytokines and chemokines (1, 4). Such perturbations occur in humans and in experimental models of sepsis (5, 6). During sepsis in humans, unregulated activation of the complement system results in excessive generation of anaphylatoxins, especially C3a and C5a (4, 7), and ensuing dysfunction of neutrophils (3, 4, 8, 9). In later stages of sepsis, neutrophils have suppressed chemotactic responsiveness (3, 4, 10), depressed enzyme release (3, 4, 8, 9), alterations of intracellular pH (11), and a defective respiratory burst (diminished production of reactive oxygen species, especially H<sub>2</sub>O<sub>2</sub>) (3, 4, 12, 13), collectively leading to impaired bactericidal activity. Neutrophils from healthy human volunteers incubated with plasma from patients with sepsis or severe trauma have exhibited suppressed superoxide (O<sub>2</sub><sup>-</sup>) production (7, 14, 15), suggesting that a plasma factor may be responsible for suppression of the respiratory burst. During experimental sepsis, we have recently found that blood neutrophils have a greatly diminished ability to bind C5a (16). In addition, these cells demonstrate impaired chemotactic responses to C5a (14) and a loss of H<sub>2</sub>O<sub>2</sub> production (12), all defects being prevented by treatment of cecal ligation/puncture

(CLP)<sup>3</sup> animals with anti-C5a Abs (12, 16). This treatment caused reduced bacteremia and greatly improved survival, suggesting that sepsis induces excessive generation of C5a, which, in turn, leads to serious functional defects in neutrophils.

The oxygen-dependent antimicrobial arsenal in neutrophils is generated by the multicomponent enzyme, NADPH oxidase (17, 18). This oxidase system, which catalyzes the reduction of oxygen to O<sub>2</sub><sup>-</sup>, is dormant in unstimulated cells, but in stimulated neutrophils undergoes activation by phosphorylation and translocation to the cell membrane of a major cytosolic component, p47<sup>phox</sup>, also accompanied by translocation of p67<sup>phox</sup>, p21<sup>rac</sup>, and p40<sup>phox</sup> to the integral membrane protein, flavocytochrome b<sub>558</sub> (19–21). After assembly, electron transfer from NADPH to oxygen occurs, generating O<sub>2</sub><sup>-</sup>, which undergoes dismutation to H<sub>2</sub>O<sub>2</sub>, a key product for phagocyte killing of ingested bacteria (17, 18). Little is known about the status of NADPH oxidase during sepsis. Increased levels of cytosolic p47<sup>phox</sup> in neutrophils have been reported in humans with sepsis occurring after severe trauma (22). In vitro exposure of neutrophils to bacterial LPS does not activate NADPH-oxidase, but primes neutrophils for enhanced assembly of NADPH oxidase when cells are subsequently stimulated with the bacterial chemotactic peptide, fMLP (23). To elucidate the linkage between complement activation and neutrophil dysfunction during sepsis, we evaluated the status of NADPH oxidase in blood neutrophils from CLP rats treated with either preimmune IgG or anti-C5a IgG, and also used in vitro approaches in which neutrophils were incubated with C5a. Treatment of CLP rats with anti-C5a has been shown to be protective against the lethal consequences of sepsis (12). The current studies define the molecular basis for C5a-induced loss of the respiratory burst in neutrophils during sepsis.

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<sup>3</sup> Abbreviations used in this paper: CLP, cecal ligation/puncture; Erk, extracellular signal-regulated kinase; huC5a, human C5a; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein/Erk kinase; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; PMN, polymorphonuclear cell; SOD, superoxide dismutase.

## Materials and Methods

### Reagents

Unless otherwise indicated, all reagents were purchased from Sigma-Aldrich (St. Louis, MO).

### Experimental sepsis induced by CLP

Male Long-Evans-specific pathogen-free rats (275–300 g in body weight; Harlan, Indianapolis, IN) were anesthetized by i.p. administration of ketamine (20 mg/100 g body weight). Via a 2-cm abdominal midline incision, the cecum was tightly ligated below the ileocecal valve, carefully avoiding bowel obstruction. The cecum was then punctured through and through with a 21-gauge needle. After repositioning the bowel, the abdominal incision was closed in layers (4-0 silk suture and skin clips; Ethicon, Somerville, NJ). Immediately after CLP (time 0), animals received either 400  $\mu$ g preimmune rabbit IgG or 400  $\mu$ g rabbit anti-C5a peptide IgG i.v., the latter generated against the middle peptide region of rat C5a (corresponding to amino residues 17–36). This Ab is described elsewhere (10). Before and after surgery, rats had unrestricted access to food and water.

### Neutrophil isolation

Human or rat neutrophils were isolated from whole blood by the traditional techniques of Ficoll-Paque gradient centrifugation (Pharmacia Biotech, Uppsala, Sweden) and dextran sedimentation, followed by hypotonic lysis of residual RBCs. Blood was drawn using 10% anticoagulant citrate dextrose (Baxter, Deerfield, IL).

### Phagocytosis assay

Rat neutrophils (50  $\mu$ l of  $10^6$  cells/ml HBSS; Life Technologies, Bethesda, MD) were layered onto adhesion reaction fields on glass slides (Bio-Rad, Munich, Germany) and allowed to settle down. After washing, the adherent cells were then incubated in absence or presence of different amounts of C5a (0.01–100 nM) for 1 h at 37°C, followed by a 30-min incubation step with IgG-coated zymosan particles (100/polymorphonuclear cells (PMN)). After additional washing, cells were stained with May-Gruenwald stain and trypan blue. Phagocytotic uptake was evaluated by light microscopy by scoring random fields.

### Measurement of respiratory burst

$O_2^-$  generation was measured by the superoxide dismutase (SOD)-inhibitable reduction of ferricytochrome *c*. Neutrophils ( $5 \times 10^6$  cells/ml) were preincubated with ferricytochrome *c* (80  $\mu$ M)  $\pm$  SOD (24  $\mu$ g/ml) in the presence or absence of human C5a (huC5a) for 60 min at 37°C. As indicated, cells were subsequently stimulated by PMA (100 ng/ml for 10 min). Then absorbance of the supernatant fluids was measured at 550 nm, and the amount of  $O_2^-$  calculated was based on the amount of reduced ferricytochrome *c* in the presence or absence of SOD. Hydrogen peroxide ( $H_2O_2$ ) generation was determined in the presence of 1 mM sodium azide. As indicated, neutrophils ( $2 \times 10^6$  cells/ml) were pretreated with various concentrations of huC5a (1.0–100  $\mu$ M) for 60 min at 37°C. To stimulate neutrophils, cells were incubated with various concentrations of PMA (10–1000 ng/ml) for additional 10 min. The reaction was stopped by addition of TCA (50% v/v). Then, ferrous ammonium sulfate (1.5 mM) and potassium thiocyanate (0.25 M) 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  $H_2O_2$ .

### Translocation of p47<sup>phox</sup> and immunoblotting

After stimulation, neutrophil lysates were centrifuged (400  $\times$  g, 5 min) to remove nuclei and unbroken cells. The resulting supernatant fluids were further centrifuged (15,000  $\times$  g, 45 min) to isolate the crude cytosol-derived fraction (supernatant) and the crude membrane fraction (pellet). Equal amounts of protein of the crude subcellular fractions were separated under reducing conditions by 10% SDS-PAGE and transferred onto polyvinylidene fluoride membrane. The membrane was blocked in 5% milk (in PBS) for 1 h and then incubated with rabbit anti-p47<sup>phox</sup> IgG Ab at a concentration of 1  $\mu$ g/ml (Genentech, San Francisco, CA). As a secondary Ab, alkaline phosphatase-conjugated goat anti-rabbit IgG (1:1000; Jackson ImmunoResearch Laboratories, West Grove, PA) was added, and the blot was developed using alkaline phosphatase color development system (Bio-Rad, Hercules, CA).

For determination of the phosphorylation state of p47<sup>phox</sup>, anti-p47<sup>phox</sup> mAb was used to immunoprecipitate from equal amount of cell lysates (100  $\mu$ g). After Western blotting, anti-phosphoserine Ab (Zymed Labora-

tories, San Francisco, CA) was used for probing the membranes, followed by ECL (Amersham, Piscataway, NJ).

### Mitogen-activated protein kinase (MAPK) activity

MAPK activity (p42/p44 MAPK or extracellular signal-regulated kinase (Erk) 1/2) of human neutrophils was determined by Western blot analysis, as per manufacturer's instructions, for p42/p44 protein and phospho-p42/p44 protein (NEB, Beverly, MA). Image analysis of the signal on the films obtained by the chemiluminescence procedure was performed using Adobe System software (San Jose, CA).

### Analysis of C5aR on neutrophils

Flow cytometric analysis was conducted immediately after blood collection. A total of 10  $\mu$ l anti-C5aR serum (Research Diagnostics, Flanders, NJ) in 100  $\mu$ l staining buffer (PBS with 0.1% sodium azide and 1% FBS) was incubated with 100  $\mu$ l human whole blood for 30 min. After washing, the cells were suspended in FITC-conjugated rat anti-rabbit IgG (Biosource International, Thousand Oaks, CA) diluted 1/50 in staining buffer. Erythrocytes were lysed for 10 min by addition of 1 $\times$  FACS lysing solution (BD PharMingen, San Diego, CA). After washing, the leukocytes were reconstituted in a fixing solution of 1% paraformaldehyde prepared in PBS plus 0.1% sodium azide and analyzed on a flow cytometer (Coulter, Miami, FL). Granulocytes were gated by the typical forward and side light scatter profiles. The same gated population in rat blood cells has been confirmed to be granulocytes by staining of whole blood with a rat granulocyte marker, HIS48 (BD PharMingen). More than 90% of gated cells were found to be granulocytes.

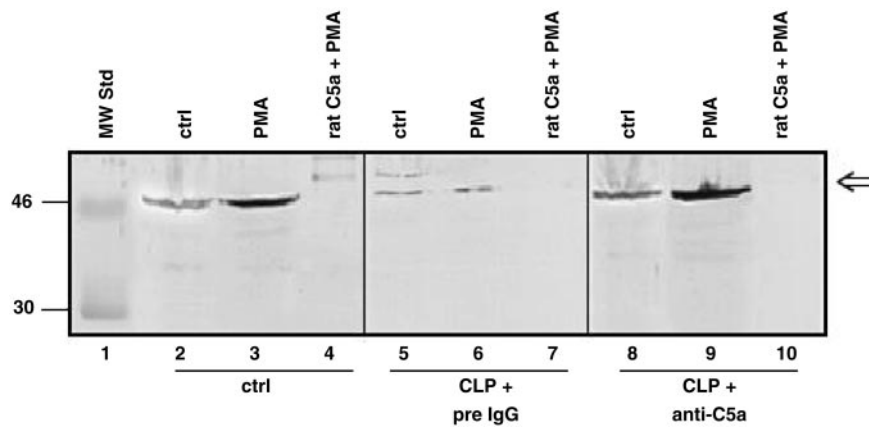
### Statistical analysis

All values were expressed as mean  $\pm$  SEM. Data sets were analyzed with one-way ANOVA; differences in the mean values among experimental groups were then compared using the Tukey multiple comparison test. Results were considered statistically significant where  $p < 0.05$ .

## Results

### Treatment with anti-C5a during CLP sepsis reverses the respiratory burst defect in neutrophils

During sepsis, plasma levels of complement-derived anaphylatoxins are increased (see above) and alteration of the respiratory burst (production of  $O_2^-$  and  $H_2O_2$ ) occurs, resulting in impaired bacterial killing (9–14). In CLP-induced experimental sepsis, we have recently shown the  $H_2O_2$  response of neutrophils to be defective in a manner that is C5a dependent (12). We have now evaluated in blood neutrophils (from normal rats or from rats 24 h after CLP) the status of the assembly (activation) of NADPH oxidase, which requires translocation of cytosolic p47<sup>phox</sup> to the cell membrane after stimulation with PMA. CLP animals were treated i.v. at time 0 with 400  $\mu$ g preimmune IgG or affinity-purified anti-rat C5a. The ability of p47<sup>phox</sup> to translocate to the cell membrane of neutrophils stimulated with PMA was evaluated in blood neutrophils obtained 24 h after CLP. As shown in Fig. 1, Western blot analysis of neutrophil crude membrane fractions from control rats without sepsis revealed some cell membrane-associated p47<sup>phox</sup> (arrow) in nonstimulated (ctrl) neutrophils (*lane 2*) and a more intense band in the membrane fraction of PMA-stimulated neutrophils (*lane 3*). p47<sup>phox</sup> was not detectable in membrane fractions if these neutrophils had been first exposed to C5a (10 nM), followed by stimulation with PMA (100 ng/ml) (Fig. 1, *lane 4*). Blood neutrophils from CLP rats (treated with preimmune IgG) had only faint evidence of p47<sup>phox</sup> in the cell membrane fraction (Fig. 1, *lane 5*). After stimulation of these cells with PMA, no change in cell membrane-associated p47<sup>phox</sup> band was found (Fig. 1, *lane 6*). If blood neutrophils from these animals were first exposed in vitro to rat C5a (10 nM) followed by PMA, the membrane-associated band vanished altogether (Fig. 1, *lane 7*). In CLP rats treated with anti-C5a, blood neutrophils that were not otherwise treated in vitro showed a cell membrane-associated p47<sup>phox</sup> band in the expected position in the gel (Fig. 1, *lane 8*), and, as in control cells stimulated with PMA (*lane 3*), this band was much more intense if the



**FIGURE 1.** Defective translocation of p47<sup>phox</sup> in neutrophils obtained from animals with CLP-induced sepsis. Neutrophils were isolated from control rats (ctrl) (lanes 2–4) or from CLP rats at 24 h (lanes 5–10). Animals were infused i.v. at time 0 with 400  $\mu$ g preimmune IgG (pre IgG) (lanes 5–7) or with 400  $\mu$ g affinity-purified anti-rat C5a (lanes 8–10). Neutrophils from each of the three groups were then pre-exposed to buffer only (ctrl) (lanes 2, 5, and 8), pre-exposed to buffer with subsequent stimulation by PMA (100 ng/ml) (lanes 3, 6, and 9), or pre-exposed to rat C5a (10 nM), followed by addition of PMA (lanes 4, 7, and 10). Crude cell membrane fractions were then prepared, as described in *Materials and Methods*, and solubilized, and equal amount of protein (50  $\mu$ g/lane) was immunoblotted using anti-p47<sup>phox</sup> IgG Ab. Data are representative of two independent and separate experiments. A minimum of six animals was used for each group.

cells were stimulated *in vitro* with PMA (lane 9). When these neutrophils were first exposed *in vitro* to C5a, followed by addition of PMA, the p47<sup>phox</sup> band in the cell membrane fraction was undetectable (Fig. 1, lane 10). These data indicate that PMA-induced translocation of p47<sup>phox</sup> is defective in neutrophils from CLP rats, that the *in vivo* acquisition of this defect is C5a dependent, and that this defect can be reproduced by *in vitro* exposure of normal blood neutrophils to C5a.

#### *Exposure of rat neutrophils in vivo or in vitro to C5a induces a defect in phagocytic function*

To evaluate the status of phagocytic function in neutrophils, two separate experiments were conducted. In the first, blood neutrophils were obtained from normal rats (ctrl) or from CLP rats (at 24 h) that received at time 0 either 400  $\mu$ g preimmune IgG or 400  $\mu$ g affinity-purified anti-rat C5a IgG. The results are shown in Fig. 2A. Neutrophils were incubated with IgG-opsonized zymosan particles (100 particles/PMN, 30 min at 37°C), and the number of intracellular particles in neutrophils was determined after this period of incubation. Neutrophils from normal rats contained  $3.0 \pm 0.30$  particles/cell, while neutrophils from CLP rats pretreated with preimmune IgG showed a significantly lower level of particle ingestion ( $1.2 \pm 0.31$  particles/cell), indicating sepsis-induced dysfunction. In CLP rats treated with anti-C5a, uptake of particles was the same as in neutrophils from normal rats (Fig. 2A). These results indicate that the phagocytic defect acquired by neutrophils during CLP-induced sepsis is C5a related. In companion experiments (B), *in vitro* exposure of blood neutrophils from normal rats to increasing amounts (0.01–100 nM) of rat C5a (at 37°C for 60 min), followed by incubation of cells with particles (for 30 min at 37°C), led to a progressive reduction in numbers of zymosan particles taken up into neutrophils as a function of the concentration of C5a. The IC<sub>50</sub> for C5a was  $\sim 0.1$  nM C5a.

#### *In vitro exposure of neutrophils reduces detection of C5aR*

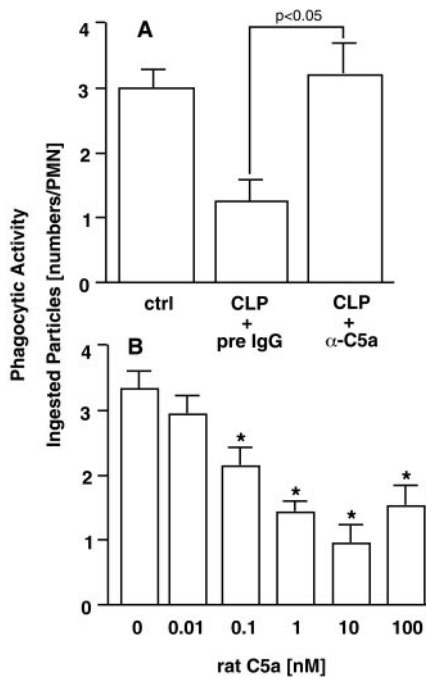
For these and all subsequent experiments, human neutrophils were exposed to human rC5a, and the effects on C5aR and on signaling pathways were evaluated. For the first series of experiments, human neutrophils were exposed to 100 nM C5a at different periods of time and at different temperatures, and then the cells were eval-

uated for C5aR content by flow cytometry. As shown in Fig. 3A, there was a measurable reduction in detectable C5aR content on neutrophils exposed to C5a at 25°C for 30 min. In Fig. 3B, as can be seen in the *upper panel*, there was virtually no difference in C5aR content (mean channel fluorescence) over a 1-h period when neutrophils were or were not exposed to 100 nM C5a, indicating that presence of C5a does not affect detection of C5aR at 4°C. In the *lower panel*, there was a striking reduction (nearly 40%) in C5aR content on neutrophils exposed to C5a at either 25 or 37°C. By 15 min, the reduction in detectable C5aR was complete, at least over a 1-h period. These data are consistent with other studies that suggest the C5a/C5aR complex is partially internalized.

#### *In vitro exposure of neutrophils to C5a suppresses the assembly of NADPH oxidase and the respiratory burst in PMA-stimulated neutrophils*

As shown in Fig. 4, H<sub>2</sub>O<sub>2</sub> production was used as an indicator for activation of NADPH oxidase. In A, human blood neutrophils were incubated with 0.01–100 nM huC5a for 60 min at 37°C and the amount of H<sub>2</sub>O<sub>2</sub> generated was determined. At doses of 1.0, 10, and 100 nM C5a, small but statistically significant increases in H<sub>2</sub>O<sub>2</sub> were noted. In B, a dose response for PMA-induced generation of H<sub>2</sub>O<sub>2</sub> revealed sharp increases between 10 and 1000 ng PMA/ml, with no further increase at 1.0  $\mu$ g/ml. In C, neutrophils were exposed to C5a (0.01–100 nM) for 60 min at 37°C, and the subsequent amount of H<sub>2</sub>O<sub>2</sub> generated after addition of PMA (100 ng/ml, for 10 min) was determined. When exposed to 0.1–100 nM C5a (60 min at 37°C), neutrophils showed a progressive loss in production of H<sub>2</sub>O<sub>2</sub> generation. Finally, exposure of neutrophils to 10 nM C5a for 0–60 min revealed a time-dependent loss in H<sub>2</sub>O<sub>2</sub> production by these neutrophils after addition of PMA (Fig. 4D).

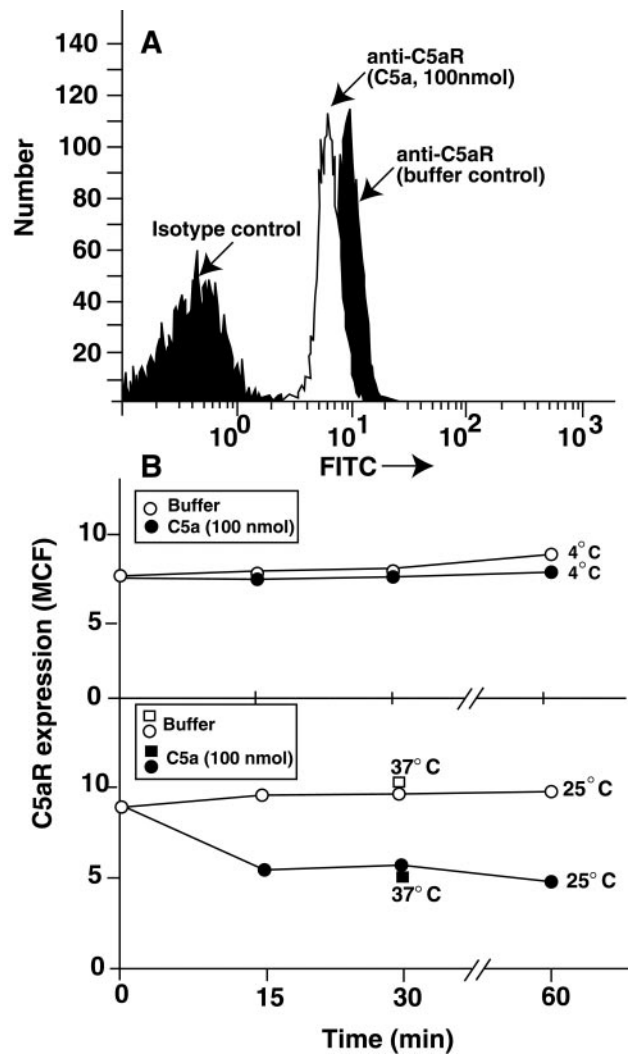
In Fig. 5, neutrophils were exposed *in vitro* to buffer alone or 10 nM C5a for 60 min at 37°C, followed by addition (where indicated) of PMA (100 ng/ml, for 10 min at 37°C). As shown in Fig. 5A, C5a induced a modest, but significant increase in O<sub>2</sub><sup>-</sup> production in neutrophils that were not otherwise treated. Addition of PMA to neutrophils not exposed to C5a caused the expected robust increase in O<sub>2</sub><sup>-</sup>. In C5a-exposed neutrophils that were then stimulated with PMA, the production of H<sub>2</sub>O<sub>2</sub> was reduced by nearly 60%. When companion studies were done with the same batch of



**FIGURE 2.** Inhibitory effects of C5a on the phagocytic response of neutrophils. **A**, Phagocytic activity of neutrophils (number of ingested IgG-opsonized zymosan particles/PMN/30 min), obtained from untreated control rats (ctrl) or from rats with CLP-induced sepsis (at 24 h). CLP rats were injected i.v. at time 0 with either 400  $\mu$ g affinity-purified anti-C5a IgG Ab (anti-C5a) or preimmune IgG (pre IgG). **B**, Phagocytic activity of normal rat neutrophils preincubated in vitro with rat C5a. The cells were exposed to increasing doses of recombinant rat C5a (rat C5a) before addition of opsonized zymosan particles. Data are expressed as mean  $\pm$  SEM; asterisks indicate  $p < 0.05$  vs control (absence of exposure to C5a). Data are representative of two independent and separate experiments. Eight animals were used for each group.

neutrophils, effects of the same manipulations on  $H_2O_2$  generation were similar (Fig. 5B), with pre-exposure of neutrophils to C5a (10 nM) causing an 81% reduction in  $H_2O_2$  after stimulation by PMA. Thus, the effect of C5a exposure to neutrophils causes similar inhibitory effects on  $O_2^-$  and  $H_2O_2$  generation following cell stimulation with PMA.

As indicated above, assembly (activation) of NADPH oxidase requires translocation of cytosolic  $p47^{phox}$  to the cell membrane. Human neutrophils were untreated or exposed to 10 nM huC5a for 60 min at 37°C. Where indicated, cells were then exposed to PMA (100 ng/ml) for 10 min at 37°C. Some neutrophils not otherwise treated were incubated with PMA under conditions described above. Then crude cytosolic and crude membrane fractions were obtained, and equal amounts of protein from each fraction were evaluated in Western blots for  $p47^{phox}$ . The results are shown in Fig. 6. In normal (ctrl) neutrophils, a strong cytosolic band for  $p47^{phox}$  was found, together with a faint band in the cell membrane fraction (lower panel, lane 2). When normal neutrophils were exposed to PMA, cytosolic  $p47^{phox}$  was detected, but there was also a strong band detected in the membrane fraction (lane 5). When neutrophils were exposed only to C5a, the cytosolic band for  $p47^{phox}$  remained, but no membrane band was detected (lane 3). In neutrophils exposed to C5a followed by PMA, the intensity of the cytosolic band for  $p47^{phox}$  diminished in intensity and no translocation to the cell membrane could be detected (lane 4). As shown in this and in other blots, fragmentation products of  $p47^{phox}$  were not detected, and supernatant fluids failed to contain detectable  $p47^{phox}$  (data not shown). The defect in translocation of  $p47^{phox}$



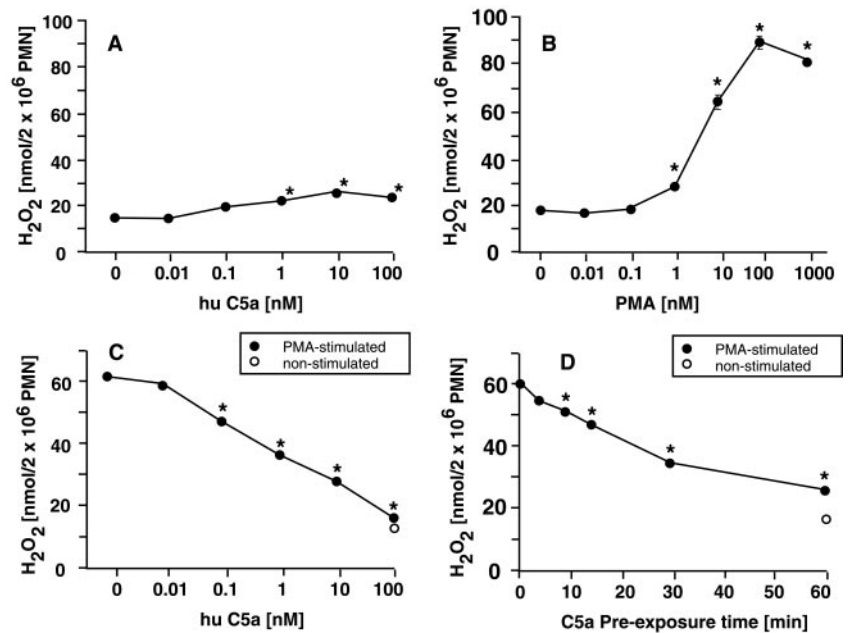
**FIGURE 3.** Changes in C5aR content in human neutrophils after exposure to C5a. Whole human blood was exposed to buffer alone or C5a (100 nM) for 30 min at 25°C (as described in *Materials and Methods*). C5aR was detected by flow cytometry using Ab to huC5aR. **A**, Results using anti-C5aR or isotype-matched control. **B**, C5aR content (expressed as mean channel fluorescence (MCF)), after exposure to 100 nM C5a at various temperatures and for various periods of time. Data are representative of three independent experiments.

after PMA stimulation could be detected after an exposure time of cells to C5a of only 10 min (data not shown). Thus, in vitro exposure of neutrophils to C5a under the conditions described induces a defect in the ability of  $p47^{phox}$  to translocate to the cell membrane after addition of PMA.

#### Naturally occurring mediators suppress $H_2O_2$ responses of neutrophils

Studies were conducted using naturally occurring mediators to determine whether the effects of C5a were mediator specific. The mediators used and effects on  $H_2O_2$  responses are shown in Table I. The concentrations of various mediators used were based on preliminary data in which a dose of each mediator that elicited a robust  $H_2O_2$  response in neutrophils was determined (data not shown). In experiment A, the suppressive effects of C5a on the PMA-induced  $H_2O_2$  response of human neutrophil were confirmed (79% suppression). It was also shown that the fMLP-induced  $H_2O_2$  response could be reduced by 97% in cells that had been pre-exposed to C5a. In experiment B, exposure of neutrophils to C5a

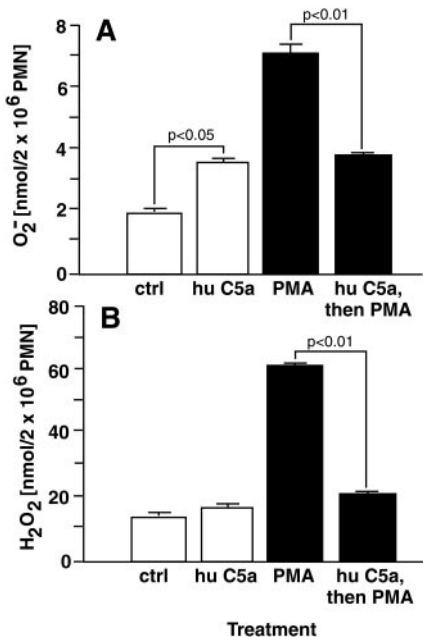
**FIGURE 4.** C5a-induced inhibition of the respiratory burst in neutrophils. *A*, huC5a induction of H<sub>2</sub>O<sub>2</sub> in otherwise unmanipulated human neutrophils. *B*, H<sub>2</sub>O<sub>2</sub> generation in neutrophils as a function of dose of PMA. *C*, Inhibitory effects of C5a on H<sub>2</sub>O<sub>2</sub> generation in PMA (100 ng/ml)-stimulated neutrophils. *D*, Inhibitory effects of C5a (10 nM), as a function of time of exposure of cells, on H<sub>2</sub>O<sub>2</sub> production triggered by PMA (100 ng/ml). Asterisks represent *p* values < 0.05 when compared with control (0 values on horizontal axis). Data are representative of six or more independent experiments.



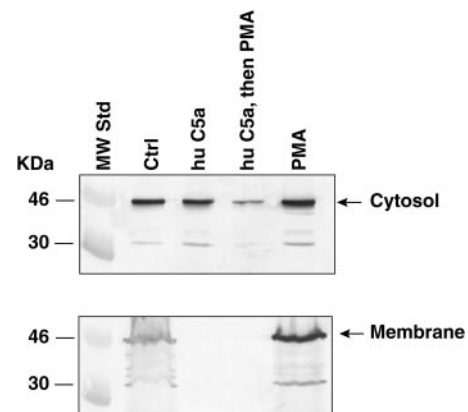
suppressed the TNF- $\alpha$ -induced response by 88%. Prior cell exposure to platelet-activating factor resulted in a 92% reduction in the PMA-induced H<sub>2</sub>O<sub>2</sub> response. Finally, prior cell exposure to platelet-activating factor reduced the H<sub>2</sub>O<sub>2</sub> response to fMLP by 93%. These data indicate that, when neutrophils are exposed to a variety of naturally occurring mediators, the H<sub>2</sub>O<sub>2</sub> responses induced by other naturally occurring mediators are also suppressed. The importance of the potent ability of C5a to suppress the respiratory burst in neutrophils may relate to the fact that high concentrations (10 nM) of C5a have been found in sera of humans with sepsis (4, 7).

*C5a causes inhibition in PMA-induced phosphorylation of p47<sup>phox</sup> in stimulated neutrophils*

To determine the possible basis for the failure of p47<sup>phox</sup> to translocate in stimulated neutrophils exposed to C5a, experiments were conducted to assess by Western blots the status of phosphorylation of p47<sup>phox</sup>, using Ab to phosphoserine after immunoprecipitation using mAb to p47<sup>phox</sup>. Immunoprecipitation was performed using equivalent amounts of cell lysates (100  $\mu$ g protein). The results are shown in Fig. 7. Faint evidence for phosphorylation of p47<sup>phox</sup> was found in unstimulated (ctrl) neutrophils (lane 1). When neutrophils were exposed to 10 nM C5a for 60 min at 37°C, no increase in phosphorylation of p47<sup>phox</sup> occurred (lane 2), in striking contrast to p47<sup>phox</sup> that was immunoprecipitated from PMA (100 ng/ml, 37°C, 10 min)-stimulated neutrophils, in which strong evidence of phosphorylation was found (lane 3). When neutrophils were first exposed to C5a, followed by stimulation with PMA, there was no evidence of increased phosphorylation of p47<sup>phox</sup> (lane 4). Thus,



**FIGURE 5.** C5a-induced depression in generation of O<sub>2</sub><sup>-</sup> and H<sub>2</sub>O<sub>2</sub> in neutrophils. O<sub>2</sub><sup>-</sup> (*A*) and H<sub>2</sub>O<sub>2</sub> (*B*) generation from human neutrophils after exposure to rhuC5a. A total of 2 × 10<sup>6</sup>/ml neutrophils was incubated for 60 min at 37°C in the presence or absence of C5a (10 nM) and then stimulated with (filled bars) or without (open bars) PMA (100 ng/ml) for additional 10 min. Results are expressed as mean  $\pm$  SEM from four separate experiments.



**FIGURE 6.** Blocking effects of C5a on PMA-induced translocation of p47<sup>phox</sup>. Western blot analysis of crude cytosol and crude membrane fractions isolated from human neutrophils that had been preincubated with either buffer (ctrl) or 10 nM huC5a and subsequently stimulated in the presence or absence of PMA (100 ng/ml). The Western blots were probed with rabbit anti-p47<sup>phox</sup> polyclonal IgG Ab. The results shown are representative of three separate and independent experiments.

Table I. *Suppression of H<sub>2</sub>O<sub>2</sub> responses of human neutrophils<sup>a</sup>*

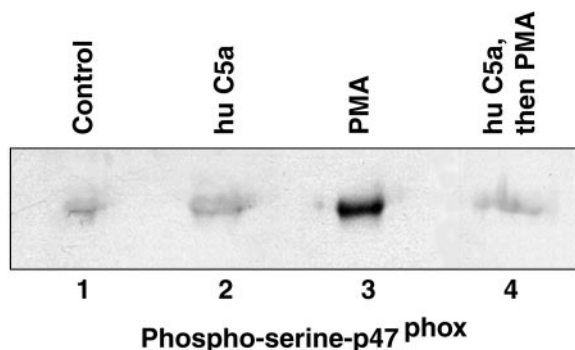
Expt.	First Exposure	Second Exposure	H <sub>2</sub> O <sub>2</sub> Response (OD <sub>480</sub> )	Inhibition (%)
A	None	None	0.199 ± 0.008	
	PMA	None	1.35 ± 0.025	
	C5a	None	0.255 ± 0.011	
	C5a	PMA	0.438 ± 0.020	79
	fMLP	None	0.920 ± 0.027	
B	C5a	fMLP	0.223 ± 0.011	97
	None	None	0.187 ± 0.001	
	PMA	None	1.124 ± 0.002	
	TNF- $\alpha$	None	0.804 ± 0.027	
	C5a	TNF- $\alpha$	0.258 ± 0.011	88
	PAF	None	1.12 ± 0.003	
	PAF	PMA	0.212 ± 0.001	92
	fMLP	None	0.564 ± 0.011	
	PAF	fMLP	0.252 ± 0.015	93

<sup>a</sup> Concentrations of agonists: PMA, 100 ng/ml; C5a, 10 nM; fMLP, 10<sup>-6</sup> M; platelet-activating factor, 10<sup>-5</sup> M; TNF- $\alpha$ , 1 nM. Neutrophils were exposed to the first agonist for 60 min at 37°C (with the exception of PMA, TNF, and fMLP, which were for 10 min), then to the second agonist for 10 min at 37°C. Conditions were similar to those described in Fig. 4. The data are representative of three or more separate and independent experiments performed in triplicate for each condition.

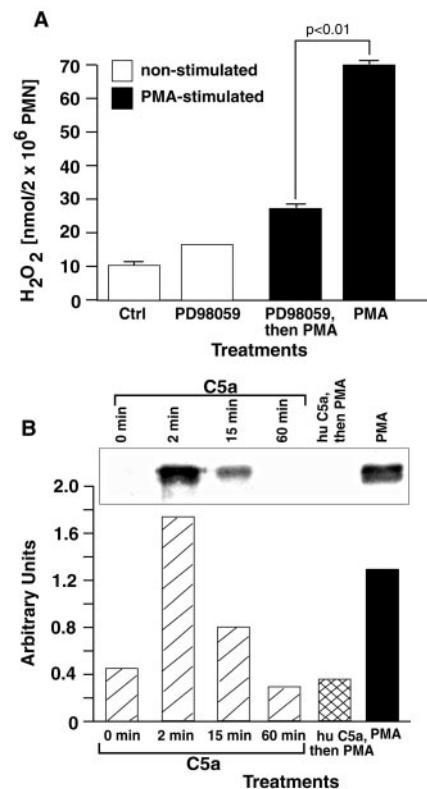
C5a blocks both phosphorylation of p47<sup>phox</sup> as well as its translocation (Fig. 6) to the cell membrane in PMA-stimulated neutrophils.

*Inhibition of mitogen-activated protein/Erk kinase-1 (MEK-1) reduces the respiratory burst; C5a inhibits phosphorylation of p42/p44 MAPK (Erk 1/2)*

Recently, extracellular signal-regulated kinase 1/2 (p42/p44 MAPK, Erk 1/2) has been shown to be required for phosphorylation of p47<sup>phox</sup> in neutrophils exposed to the bacterial chemotactic peptide, fMLP (24). In addition, PD98059 has been described to be a selective inhibitor of MEK-1 (25), which immediately precedes Erk 1/2 in the signaling pathways of neutrophils. Therefore, two separate experiments were conducted. In the first, neutrophils were exposed to PD98059 (50  $\mu$ M for 1 h at 37°C); these cells were then assessed for H<sub>2</sub>O<sub>2</sub> production in the presence or absence of PMA (100 ng/ml). The results are shown in Fig. 8A. Cells exposed to PD98059 alone showed a small increase in H<sub>2</sub>O<sub>2</sub> when compared with otherwise untreated cells (ctrl). When PMA was used as a stimulus, the prior exposure of cells to PD98059 greatly suppressed (by 72%) H<sub>2</sub>O<sub>2</sub> production, when compared with cells that



**FIGURE 7.** Detection of phosphoserine p47<sup>phox</sup> in neutrophils. Cells were untreated (ctrl); exposed to C5a (10 nM) for 60 min at 37°C; stimulated with PMA (100 ng/ml) for 10 min at 37°C; or exposed first to C5a, followed by stimulation with PMA. p47<sup>phox</sup> was first immunoprecipitated with a mAb, Western blotted, and probed with anti-phosphoserine Ab. Data are representative of at least three independent experiments.



**FIGURE 8.** Requirement of MEK-1 for generation of H<sub>2</sub>O<sub>2</sub>; loss of phosphorylation of MAPK in neutrophils exposed to C5a. *A*, H<sub>2</sub>O<sub>2</sub> generation from unstimulated (open bars) or PMA-stimulated (filled bars) neutrophils in presence or absence of the MEK-1 inhibitor PD98059 (50  $\mu$ M for 1 h at 37°C). *B*, Cells were stimulated with huC5a (10  $\mu$ M) for the indicated lengths of time at 37°C or with PMA (100 ng/ml) alone (10 min). Cells were also pre-exposed to C5a for 60 min, followed by PMA for 10 min. Equal amounts (100  $\mu$ g) of cell lysates were immunoblotted with both anti-p42/44 MAPK Ab as well as anti-phospho-p42/p44 MAPK Ab. Signal obtained for the anti-phospho-Ab was normalized to the signal obtained for the nonphospho-Ab by image analysis, and the ratio obtained was graphed. The Western blot and the graph are representative of at least three separate and independent experiments.

were not exposed to PD98059, suggesting that MEK-1 activation is required for PMA-induced generation of H<sub>2</sub>O<sub>2</sub> in neutrophils.

The effect of neutrophil exposure to C5a was assessed on the basis of phosphorylation of p42/44 MAPK (Erk). Equal amount of cell lysates (100  $\mu$ g) from cells exposed to C5a for various lengths of time and from cells exposed to C5a for 60 min and then PMA for 10 min or with PMA alone for 10 min was electrophoresed on SDS-PAGE. After Western blotting, blots were probed with Abs to phosphorylated and nonphosphorylated p42/44 MAPK, as per the manufacturer's instructions. The signal obtained from the blots probed with the phosphorylated p42/44 MAPK Ab was normalized to the signal obtained when probed with the nonphosphorylated Ab and the ratio obtained graphed (Fig. 8B). As shown, when anti-phospho-Ab was used, neutrophils at 0 time point showed little evidence of phosphorylation of p42/44 MAPK (Fig. 8B, upper panel). However, when cells were exposed to C5a, there was an increase in phosphorylation by 2 min, with a decline by 15 min and no signal by 60 min. Similarly, an increase in phosphorylation was observed when cells were treated with PMA. But, when neutrophils were first exposed to 10 nM C5a, followed by PMA, all evidence of phospho-p42/44 disappears (Fig. 8B, upper panel), indicating that neutrophil contact with C5a inhibits phosphorylation of Erk (p42/44 MAPK) or triggers dephosphorylation of Erk.

### Proposed pathways leading to neutrophil dysfunction during sepsis

Fig. 9 depicts proposed mechanisms of C5a-induced neutrophil dysfunction during sepsis, focusing on intracellular signal transduction pathways involved in NADPH oxidase activation. In non-stimulated (resting) neutrophils, the multicomponent NADPH oxidase is dormant in its unassembled state and consists of cytoplasmic components (p21<sup>rac</sup>, p40<sup>phox</sup>, and p47<sup>phox</sup>), or cytoskeletal component (p67<sup>phox</sup>), and the integral membrane protein, flavocytochrome *b*<sub>558</sub> (Fig. 9A). The response of neutrophils to C5a (and to other agonists such as fMLP or TNF- $\alpha$ ) involves interaction with transmembrane receptors. Separate intracellular signaling pathways (especially involving protein kinase C (PKC) and MAPK) are known to be activated, leading within minutes to phosphorylation and activation of numerous target proteins. To simulate cell activation in vitro, PMA was used as an in vitro stimulus of PKC and MAPK. Whereas phosphorylation and translocation of p47<sup>phox</sup> are considered key for assembly of NADPH oxidase, generation of arachidonic acid also appears to play a key role in activation events, which mainly involve the PKC and MAPK pathways (Fig. 9B). In an attempt to simulate conditions of sepsis, exposure of neutrophils to amounts of C5a (10 nM) that have been found in serum during sepsis (3, 4, 7) paralyzed the process, leading to assembly (activation) of NADPH oxidase. Using PMA-stimulated neutrophils, C5a-induced defects were associated with the appearance of at least three different events: greatly reduced phosphorylation of Erk 1/2, reduced phosphorylation of p47<sup>phox</sup>, and failure of p47<sup>phox</sup> to translocate to the cell membrane. It is also possible that C5a-induced dysfunction in neutrophils might be linked to production of cAMP, which could activate protein kinase A (PKA). In turn, PKA would inhibit both Raf-1 and B-Raf (and reduce intracellular levels of extracellular Ca<sup>2+</sup>). Under these conditions, it would also be predicted that downstream activation of Erk 1/2 (p42/p44 MAPK) and phosphorylation and translocation of p47<sup>phox</sup> would be suppressed. It is also possible that the generation of phospholipase A<sub>2</sub>-dependent arachidonic acid in neutrophils exposed to C5a was inhibited by neutrophil exposure to C5a, resulting in suppressed activation of NADPH oxidase. Collectively, these events would lead to defective bacterial killing. To what extent the suppressive effects of C5a are tied to the cAMP and PKA pathways or have direct effects on the Raf-1/B-Raf pathway remains to be determined. Such an outcome could occur by generation of cAMP and PKA-induced inhibition of phospholipase C (PLC), PKC, and Raf-1/B-Raf; inhibition of p42/p44 MAPK (Erk 1/2) phosphorylation; blockade of the p47<sup>phox</sup> phosphorylation and translocation; and, possibly, inhibition of phospholipase A<sub>2</sub>-dependent arachidonic acid, finally resulting in the inability to assemble NADPH oxidase and generate an effective respiratory burst, resulting in impaired bacterial killing (Fig. 9C).

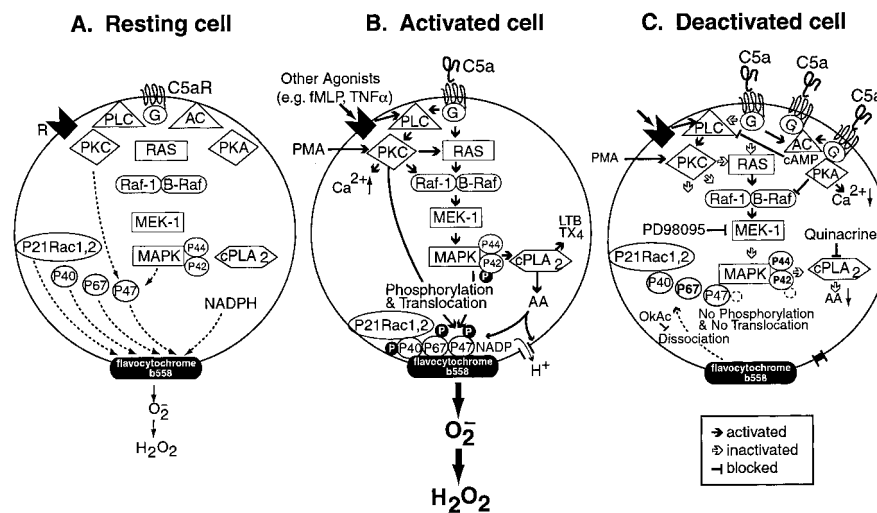
### Discussion

In the early period (<24 hour) following trauma, burn injury, or sepsis, neutrophils have been reported to be "primed" and hyperactive (on the basis of in vitro stimulation) potentially putting patients at increased risk of neutrophil-mediated tissue injury (3, 4, 8). At later time points (48 h), neutrophils are unresponsive to activation and exhibit a variety of depressed functions: chemotactic responses (3, 4, 10, 16), enzyme release (3, 4), microtubular reorientation (26), phagolysosomal acidification (11), and respiratory burst activity (3, 4, 8, 9, 13–15). Collectively, this compromise of innate immunity leads to a loss of oxygen-dependent bacterial killing, causing these individuals to be at high risk for

bacterial infections (3, 4, 8). The mechanisms involved in sepsis-induced neutrophil dysfunction are poorly understood, precluding definitive specific therapeutic interventions.

In the present study, experimental sepsis was induced by CLP. This model, which closely simulates events in human sepsis (5, 6), was used to investigate mechanisms of neutrophil dysfunction during sepsis. Two aspects of innate immunity were found to be seriously compromised in blood neutrophils: phagocytosis and the ability to mount a respiratory burst. We have recently demonstrated that in the CLP model of sepsis, a third component of innate immunity is defective in neutrophils: chemotactic responsiveness (16). All three acquired defects have now been shown to be C5a dependent. Phagocytotic activity (ingestion of IgG-opsonized zymosan particles) was suppressed in neutrophils from CLP animals, but this response was completely restored when complement activation product, C5a, was blocked in vivo by anti-C5a Abs (Fig. 2). When normal neutrophils were exposed in vitro to C5a at levels found during sepsis, there was a dose-dependent impairment of in vitro phagocytosis (Fig. 2). Recently, it has been shown during phagocytosis that the onset of the respiratory burst (featuring generation of reactive oxygen species O<sub>2</sub><sup>-</sup>, H<sub>2</sub>O<sub>2</sub>) is linked to phosphorylation of the NADPH oxidase cytosolic subunit, p47<sup>phox</sup>, which is then translocated to the cell membrane of the phagosome (27). A dysfunction of the NADPH-dependent respiratory burst during sepsis has been described in clinical (8, 9, 13, 14, 22) and in experimental settings (12, 16). To elucidate the mechanisms involved, neutrophils were activated in vitro by PMA, which bypasses conventional membrane receptors often defective during sepsis (3, 16) and which leads to direct activation of intracellular PKC. As expected, activation of normal neutrophils by PMA induced robust O<sub>2</sub><sup>-</sup> and H<sub>2</sub>O<sub>2</sub> production. Exposure of neutrophils to C5a induced the expected reduction in C5aR content (Fig. 3), presumably due to partial internalization of C5a/C5aR complexes. Our recent report of greatly reduced binding of rat C5a to blood neutrophils obtained from CLP rats (16) appears to be due to both receptor occupancy as well as loss of receptors due to internalization. After in vitro exposure of neutrophils to C5a, O<sub>2</sub><sup>-</sup> and H<sub>2</sub>O<sub>2</sub> production in PMA-activated cells was greatly inhibited in a dose- and time-dependent manner, indicating that exposure to C5a in vitro or in vivo can result in impairment of the respiratory burst (Fig. 4). Exposure of neutrophils in vitro to C5a alone caused very modest production of O<sub>2</sub><sup>-</sup> and H<sub>2</sub>O<sub>2</sub>. These responses are known to be greatly magnified in the presence of cytochalasin B, which amplifies the oxidative response by inhibiting internalization of NADPH oxidase subunits (28). Similar to our demonstration of a C5a-induced defect in the PMA-induced respiratory burst in neutrophils, a recent study has demonstrated that C-reactive protein, which activates the classical complement cascade, inhibits chemotaxis and the PMA-induced respiratory burst by affecting PKC-dependent translocation and phosphorylation of p47<sup>phox</sup> (29). Phosphorylation of at least two serine residues on p47<sup>phox</sup> and translocation of this molecule from the cytosol to the membrane (which takes place after phosphorylation) are required to initiate assembly and activation of NADPH oxidase (21, 30). The proline-rich region of cytochrome *b*<sub>558</sub> is considered to interact with the Src homology 3 domains of p47<sup>phox</sup> and p67<sup>phox</sup>, enabling the docking process (19, 20). The subsequent electron transfer from NADPH to O<sub>2</sub> generates O<sub>2</sub><sup>-</sup> and is charge compensated by the opening of a proton channel in the cell membrane of the neutrophil (19, 31). In the current study, the presence of small amounts of p47<sup>phox</sup> in the membrane fraction of nonstimulated neutrophils suggests the presence of basal oxidase activity, in accordance with earlier reports (19, 20, 24). This could represent an artifact of neutrophil isolation. After PMA stimulation, enhanced p47<sup>phox</sup>





**FIGURE 9.** Model for C5a-induced dysfunction of NADPH oxidase. *A*, In resting neutrophils, the multicomponent NADPH oxidase is dormant and consists of subunits located in the cytoplasm and in the membrane. *B*, Cell activation via surface receptors by various inflammatory mediators (C5a, fMLP, TNF- $\alpha$ ) involves several signaling pathways (e.g., PKC, MAPK, cAMP, etc.), leading to phosphorylation of numerous target proteins. Phosphorylation and translocation of p47<sup>phox</sup> are key for assembly of NADPH oxidase on the cell membrane. Generation of arachidonic acid (AA) is also important for optimal activation of NADPH oxidase. These events are regulated by the PKC and MAPK pathways. *C*, Exposure of neutrophils to C5a (e.g., during sepsis) blocks activation of NADPH oxidase by ultimately interfering with phosphorylation of p42 MAPK, thus preventing phosphorylation and translocation of p47<sup>phox</sup> to the cell membrane. Under these conditions, cell exposure to C5a may also produce excessive cAMP, which could interfere with activation of PLC, PKC, etc.

translocation was observed, which was completely abolished in neutrophils that had been pre-exposed to C5a (Fig. 6), indicating a C5a-induced defect in NADPH oxidase activation via blockade of p47<sup>phox</sup> translocation. LPS, which is often present during sepsis, primes neutrophils for an enhanced respiratory burst after subsequent stimulation of these cells (27). The MAPK pathway has recently been shown in neutrophils to be involved in the fMLP-induced phosphorylation of p47<sup>phox</sup> (24). Because both phosphorylation and translocation of p47<sup>phox</sup> alone are not sufficient enough to fully activate NADPH oxidase (32–34), it was of interest to determine whether the MAPK pathway was also involved in activation of NADPH oxidase. Using the potent and selective MEK-1 inhibitor, PD 98059 (35), the H<sub>2</sub>O<sub>2</sub> response of neutrophils after PMA stimulation was significantly suppressed, indicating that MEK-1 in the MAPK pathway is involved in activation of NADPH oxidase (Fig. 8). Some studies have suggested that fMLP and C5a may activate the MAPK pathway via tyrosine phosphorylation of p42/p44 MAPK (35–37), which has been shown to occur in human astrocytes incubated with C5a. This process is time dependent, with a peak at 15 min (37). Exposure of neutrophils to C5a virtually abrogated all signs of PMA-induced p47<sup>phox</sup> phosphorylation as well as p42/p44 MAPK (Erk 1/2) phosphorylation (Figs. 7 and 8). Whereas transient stimulation of neutrophils with C5a may activate the MAPK pathway, the present data suggest that a persistent presence of C5a or the presence of relatively high concentrations (10 nM or higher) of C5a results in an inhibition of the MAPK pathway. This is consistent with other findings that C5a causes the activation of Raf-1 and B-Raf, which are upstream stimulators of MEK-1 in human neutrophils, with a peak of activation at 5 min, followed by suppressed activation thereafter (38). Activated p42/p44 MAPK (Erk 1/2) is known to phosphorylate p47<sup>phox</sup> (24) and, thereby, to initiate NADPH oxidase assembly in a PKC-independent way (34). When assembled, p47<sup>phox</sup> undergoes a continuous cycle of phosphorylation and dephosphorylation throughout the period of O<sub>2</sub><sup>-</sup> release, with the phosphorylation reaction predominating (20). Termination of oxidase activity correlates with dissociation of p47<sup>phox</sup>/p67<sup>phox</sup> from flavocytochrome b<sub>558</sub> (20, 27). It is possible that events that occur during internalization

of C5a/C5aR and receptor recycling perturb the MAPK pathway, interfering with the ability of PMA to induce generation of H<sub>2</sub>O<sub>2</sub>.

Whereas many *in vitro* studies of NADPH oxidase exist, little is known about alterations of NADPH oxidase occurring during sepsis. In the present study, we demonstrated in later stages (12 h) of CLP-induced sepsis nearly complete loss of p47<sup>phox</sup> translocation after activation of neutrophils by PMA (Fig. 1). The loss of the respiratory burst, which is essential for bacterial killing, could be reversed in animals that had been treated with an anti-C5a Ab preparation. In our recent studies, such treatment has greatly improved survival rates during CLP-induced sepsis and has reduced the accompanying multiorgan failure (12, 16). These *in vivo* data strongly suggest that in the sepsis model, C5a is a major contributor to NADPH oxidase dysfunction, and that this defect can be reversed by treatment of animals with a blocking Ab to C5a. The current studies establish that C5a blocks three critical steps in the pathway leading to activation of NADPH oxidase: phosphorylation of the p42 MAPK (Erk 2); phosphorylation of p47<sup>phox</sup>; and translocation of p47<sup>phox</sup> to the cell membrane.

Possible pathways involved in C5a-induced neutrophil dysfunction are demonstrated in a hypothetical model (Fig. 9). Despite the persistent presence of complement activation products (7) and inflammatory mediators during sepsis (3, 4), the intracellular signaling events are often transient (Ras, Raf-1, B-Raf, Erk-1, PLC, etc.), with activation of these factors occurring within minutes (37–40). The brief duration of these events may result from the loss of receptors through their phosphorylation and rapid internalization (41). Lack of phosphorylation of C5aR (42) was found to result not only in sustained Ca<sup>2+</sup> mobilization and MAPK activity, but also in enhancement of the respiratory burst (43, 44), suggesting that phosphorylation of C5aR may be a regulatory mechanism for NADPH oxidase activity. Additionally, C5a has been shown to activate the adenylate cyclase-cAMP pathway in neutrophils (40, 45). Stimulation of adenylate cyclase by forskolin (1 h, 100 nM) reduced the respiratory response of PMA-stimulated cells by >50% (data not shown), suggesting involvement of cAMP and PKA in regulation of the respiratory burst. Sustained elevation of cAMP in neutrophils has been reported to suppress the O<sub>2</sub><sup>-</sup> release

(40, 43, 46). If cAMP levels were elevated, the MAPK response was also inhibited (47–49), which may represent an additional mechanism of C5a-induced neutrophil dysfunction.

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