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C1 Inhibitor-Mediated Protection from Sepsis

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C1 inhibitor (C1INH) protects mice from lethal Gram-negative bacterial LPS-induced endotoxin shock and blocks the binding of LPS to the murine macrophage cell line, RAW 264.7, via an interaction with lipid A. Using the cecal ligation and puncture (CLP) model for sepsis in mice, treatment with C1INH improved survival in comparison with untreated controls. The effect was not solely the result of inhibition of complement and contact system activation because reactive center-cleaved, inactive C1INH (iC1INH) also was effective. In vivo, C1INH and iC1INH both reduced the number of viable bacteria in the blood and peritoneal fluid and accelerated killing of bacteria by blood neutrophils and peritoneal macrophages. In vitro, C1INH bound to bacteria cultured from blood or peritoneal fluid of mice with CLP-induced sepsis, but had no direct effect on bacterial growth. However, both C1INH and iC1INH enhanced the bactericidal activity of blood neutrophils and peritoneal exudate leukocytes. C1INH-deficient mice (C1INH−/− mice) subjected to CLP had a higher mortality than did wild-type littermate mice. Survival of C1INH−/− mice was significantly increased with two doses of C1INH, one given immediately following CLP, and the second at 6 h post-CLP. C1INH may be important in protection from sepsis through enhancement of bacterial uptake by, and/or bactericidal capacity of, phagocytes. Treatment with C1INH may provide a useful additional therapeutic approach in some patients with peritonitis and/or sepsis. The Journal of Immunology, 2007, 179: 3966–3972.

Sepsis is the third leading cause of death in developed societies. In the U.S. alone, it affects as many as 750,000 people and results in >200,000 deaths per year, despite the use of antibiotics and other recent advances in treatment (1–4). Sepsis is characterized by an overwhelming systemic inflammatory response that can lead to lethal multiple organ failure. Gram-negative bacterial LPS and other bacterial components stimulate macrophages to produce proinflammatory cytokines such as TNF-α and IL-1β (5). Excessive production of these cytokines can result in dramatic pathologic sequelae, including a systemic capillary leak syndrome, tissue injury, shock, and fatal organ failure (6–15). Evidence in mice, using the cecal ligation and puncture (CLP)4 model, suggests that neutralization of TNF-α or IL-1β as a therapeutic approach was either ineffective (16, 17) or, in one study, provided a modest benefit that was strain dependent, very sensitive to changes in dose, and effective only when given before CLP (18). In human sepsis, the anticytokine approach also has been ineffective (19–21) or, in some instances, may have provided a slight, usually statistically insignificant, beneficial effect (22–24).

C1 inhibitor (C1INH) is an acute-phase protein with a mean plasma level of ~250 mg/L that can increase up to 2.5-fold during inflammation (25). Via protease inhibition, C1INH regulates both complement and contact system activation. Both of these systems are activated during sepsis and appear to play complex roles in both mediation of, and protection from, endotoxin shock. Treatment with C1INH is effective at improving outcome in a variety of inflammatory disease models (28–38), including endotoxin shock (39–42) and sepsis induced by i.v. infusion of Escherichia coli (43). Therefore, C1INH may have a beneficial effect on the clinical course and outcome of severe sepsis.

Exogenous administration of C1INH protected mice from lethal LPS-induced endotoxin shock via a direct interaction with LPS (39). This C1INH-LPS interaction is dependent both on N-linked glycosylation and on the positively charged residues within the N-terminal domain (39, 44, 45). To further investigate the anti-inflammatory effect of C1INH in sepsis, in this study we used a mouse model of peritonitis/sepsis induced by CLP. CLP is a clinically relevant model that develops slowly and results in a polymicrobial enteric insult similar to that seen in patients with colonic perforations (46). We demonstrated that treatment with C1INH and reactive center-cleaved, inactive C1INH (iC1INH) resulted in improved survival in CLP-induced sepsis. The number of viable bacteria in peritoneal fluid from treated septic mice was reduced, whereas neutrophil phagocytic and bactericidal activities were improved. Furthermore, mortality in C1INH-deficient mice subjected to CLP was increased, and intravascular supplementation with C1INH enhanced survival. These data suggest that C1INH plays an important role in host defense during sepsis.

Materials and Methods

CLP-induced sepsis in mice

C57BL/6J mice (6–8 wk old, 18–22 g; Charles River Laboratories), C1INH-deficient mice (C1INH−/− mice), and their wild-type littermates were...
used for these studies. The genetic background of the C1INH−/− mice was mixed and consisted of 129/Sv, C57BL/6 albino, and C57BL/6. Controls in these experiments were always wild-type littermates. CLP was performed, as described (47). Mice were anesthetized with avertin (0.2 ml of 2.5% solution per 10 g body weight), and a 1-cm midline incision was made in the peritoneum. The distal 25% of the cecum was ligated with a silk suture, punctured once with an 18-gauge needle, and then gently squeezed to ensure release of the cecal contents. Preliminary experiments determined that ligation of 25% of the cecum resulted in 80–100% mortality (data not shown). The cecum was returned to the peritoneal cavity and the abdomen was closed. Mice were injected with C1INH (600 μg; Berinert; ZLB Behring) through the tail vein. Control-untreated mice were subjected to CLP and injected with BSA (600 μg/100 μl/mouse), also through the tail vein. In the sham control, mice were subjected to laparotomy and the cecum was manipulated, but was not ligated or punctured. All experiments were performed in compliance with relevant laws and institutional guidelines, and were approved by the CBR Institute for Biomedical Research Animal Care and Use Committee. In some experiments, to provide additional evidence for a protective role for C1INH in sepsis, C1INH−/− mice were used (48). Preliminary experiments determined that ligation of ~10% of the cecum resulted in minimal, but detectable, mortality of wild-type mice (data not shown). Therefore, to determine whether the absence of C1INH resulted in increased susceptibility to sepsis, 10% of the cecum, rather than 25%, was ligated before puncture.

Collection of leukocytes from blood and peritoneal fluid

Blood neutrophils and peritoneal exudate cells were isolated by modifications of previously described procedures (49, 50). Mouse blood collected by intracardiac puncture was added to acid citrate dextrose (ACD) (ACD: blood = 1:10). Dextran (6%)/NaCl (0.9%) was added to the ACD/blood mixture to a final concentration of 2% and inverted 18–20 times to ensure adequate mixing. The mixture was then subjected to centrifugation at 1150 rpm for 12 min at 4°C, and the supernatant was removed. The cells were mixed with RBC lysing buffer (Sigma-Aldrich), incubated at room temperature for 30 min, and subjected to centrifugation at 2000 rpm for 10 min at 4°C. The cell pellet was resuspended in PBS and layered over Ficoll-Hypaque (Sigma-Aldrich). After centrifugation at 1550 rpm for 30 min at 4°C, the cell pellet was resuspended in RPMI 1640, and the cell number was quantitated using a hemocytometer. The cells were washed twice in RPMI 1640 and cultured for 2–3 h, following which nonadherent cells were removed by washing with RPMI 1640. The purity of the neutrophils was consistently greater than 99%. To isolate peritoneal leukocytes, sterile PBS (10 ml) was injected into the peritoneal cavity, followed by gently massaging the abdomen. The peritoneal fluid was then collected through an incision in the abdominal wall. The resulting fluid was subjected to centrifugation at 400 × g for 10 min at 4°C, and the supernatant was removed. The cell pellet was cultured in RPMI 1640 for 2–4 h, following which the nonadherent cells were removed by washing with RPMI 1640. The cell number was determined by counting in a hemocytometer.

Detection of bacterial CFUs

Peripheral blood and peritoneal lavage fluid were collected from mice following CLP. The plasma and peritoneal fluid were incubated overnight on 5% FBS-agar base plates (Sigma-Aldrich) at 37°C, and CFUs were counted. In other experiments, leukocytes isolated as described above were treated with lysis buffer and incubated overnight on 5% FBS-agar base plates (Sigma-Aldrich) at 37°C, and CFUs were counted.

Analysis of H2O2 generation

Peripheral blood neutrophils and peritoneal exudate cells were isolated at 4 and 24 h after CLP. These cells were stimulated with PMA (100 ng/ml; Sigma-Aldrich) for 1 h at 37°C, then incubated in fresh RPMI 1640 (supplemented with 5% FBS). H2O2 was measured in the supernatants with an H2O2 assay kit, as described by the manufacturer (Assay Designs).

Detection of TNF-α

TNF-α was measured using a commercially available TNF-α ELISA kit (Assay Designs). Serum and peritoneal fluid were incubated overnight at 4°C, and the detecting Ab (2.1 μg/ml) was added. The samples were used at a dilution of 1/2 and measured twice. The assay was performed, as described by the manufacturer, with the capture Ab (3 μg/ml) coated on the plates.

FIGURE 1. C1INH improves survival in CLP-induced peritonitis/sepsis. CLP was performed in C57BL/6J mice, as described in Materials and Methods. Mice were either untreated, treated with C1INH, or treated with iC1INH. A, ●, Untreated CLP mice (n = 55); ○, treatment with C1INH (600 μg/100 μl, i.v.) immediately after CLP (n = 35); ▲, treatment with C1INH (600 μg/100 μl, i.v.) at 0, 12, and 24 h (n = 20); ▽, treatment with iC1INH (600 μg/100 μl, i.v.) immediately after CLP (n = 15). B, ●, Untreated CLP mice, as in A (n = 35); ○, treatment with C1INH (600 μg/100 μl, i.v.) at 6 h after CLP (n = 15); ▲, treatment with C1INH (600 μg/100 μl, i.v.) at 3 and 9 h after CLP (n = 12).

Detection of C1INH binding to bacteria

Plasma and peritoneal fluid were incubated overnight on 5% FBS-agar base plates at 37°C, and CFUs were counted. Nitrocellulose membranes (Invitrogen Life Technologies) were placed on the plates for 1 h at 37°C and then incubated with C1INH for 1 h at room temperature. Nitrocellulose membranes (Invitrogen Life Technologies) were placed on the plates for 1 h at 37°C, then removed and incubated with C1INH (200 and 600 μg) for 1 h at room temperature. After washing with 1× PBS, the membranes then were incubated with rabbit anti-human C1INH Ab (1:1000) (DakoCytomation) for 2 h and then with Immunoprobe goat anti-rabbit IgG (H + L) conjugated with HRP (1:10,000) (Pierce Biotechnology) for 2 h at room temperature. Development was performed using a SuperSignal Chemiluminescent Substrate kit (Pierce Biotechnology).

The effect of C1INH on bacterial growth in vitro

Viable bacteria from blood and peritoneal fluid in mice subjected to CLP were incubated with C1INH or iC1INH (0, 200, and 600 μg/ml) for 1 h at 37°C and then plated on 5% FBS-agar base plates at 37°C, and CFUs were counted. In some experiments, to directly determine the total number of bacteria in the cells and medium, the lysis buffer was added to the cells without separation from the supernatant; these then were plated and incubated, as above.
Survival analysis was performed using GraphPad Prism 4.00 (GraphPad). Data were compared using the log-rank test and Tukey’s multiple comparison test. All the experimental data were presented as mean ± SD. A p value of <0.05 was considered significant.

Results

C1INH protects against CLP-induced sepsis

CLP was performed in C57BL/6J mice. A single dose of C1INH (600 μg) given immediately after CLP increased survival from 14 to 43% (p < 0.05) (Fig. 1A), whereas administration of the same dose at 0, 12, and 24 h after CLP increased survival to 68% (p < 0.001). iC1INH was produced by cleavage of active C1INH at its Arg-Thr reactive center peptide bond with insoluble trypsin. This reactive center-cleaved inhibitor was the appropriate size on SDS-PAGE, and it had lost all serine proteinase inhibitor activity (data not shown) (39, 44). A single dose of iC1INH (600 μg) at the time of CLP was nearly as effective as the three doses of intact C1INH (p < 0.01). We next examined whether delayed treatment with C1INH still provided protection. A single dose of C1INH (600 μg) at 6 h after CLP did not improve mortality in comparison with untreated control mice (p = 0.05) (Fig. 1B). However, survival improved to 58% when treatment with C1INH (600 μg) was given at 3 and 9 h after CLP (p < 0.01). These data demonstrate that C1INH protects mice from CLP-induced sepsis. Furthermore, the effect is not simply a result of inhibition of complement and contact system activation because iC1INH is also effective (Fig. 1A).

Blood and peritoneal fluid were obtained from both untreated C57BL/6J mice and mice treated with C1INH or iC1INH (600 μg) at 8 h after CLP. The numbers of bacteria from the blood of mice treated with both C1INH and iC1INH were significantly decreased (p < 0.0001 in both instances) in comparison with the untreated mice (Fig. 2A). With this single dose of C1INH or iC1INH, the peritoneal fluid bacterial counts did not change significantly (p = 0.0540 and 0.0804, respectively) (Fig. 2A). However, when mice subjected to CLP were given two doses of C1INH or iC1INH (600 μg)
g) at 0 and 4 h, the number of bacteria in both blood and peritoneal fluid was significantly decreased ($p < 0.0001$ in each instance) (Fig. 2A). In addition, fewer viable bacteria were recovered from both blood neutrophils and peritoneal exudate cells in mice treated with C1INH or iC1INH in comparison with untreated control mice ($p < 0.0001$ in each instance) (Fig. 2B).

The effect of C1INH on H$_2$O$_2$ generation and TNF-α production in CLP-induced sepsis

Neutrophils derived from mice subjected to CLP are damaged in their ability to produce H$_2$O$_2$ in vitro, suggesting that CLP suppresses neutrophil function (51). We examined whether C1INH has any effect on CLP-induced neutrophil inactivation and bactericidal activity. After blood neutrophils and peritoneal exudate cells were stimulated with 100 nM PMA in vitro, H$_2$O$_2$ production was tested. Treatment with both C1INH and iC1INH resulted in an increase in CLP-induced H$_2$O$_2$ production from blood neutrophils ($p < 0.0001$) at both 4 and 24 h. H$_2$O$_2$ also increased in peritoneal exudate cells at both 4 h ($p = 0.005$ with C1INH and 0.0158 with iC1INH) and 24 h ($p < 0.0001$ with both) after CLP, respectively (Fig. 3A). As observed with other effective therapeutic interventions in this model (52–56), C1INH and iC1INH each significantly suppressed CLP-induced TNF-α levels at 4 and 8 h in both blood ($p < 0.0001$) and peritoneal fluid ($p < 0.0001$) (Fig. 3B). The mechanism responsible for the suppression of TNF-α production is unknown. However, we have previously shown a similar suppression in Gram-negative endotoxin shock (39). In vitro, suppression of TNF-α production by LPS-treated macrophages was caused by a direct interaction of C1INH (or iC1INH) with the lipid A moiety of LPS, which prevents LPS binding to the macrophage (39).

C1INH does not interfere with bacterial growth or viability in vitro

The interaction of C1INH with LPS is dependent on both N-linked glycosylation and the positively charged residues within the N-terminal domain of C1INH (44, 45). C1INH clearly bound to the cultured bacteria in both the blood (Fig. 4A) and peritoneal fluid (Fig. 4B) from mice subjected to CLP. However, neither C1INH nor iC1INH at doses up to 600 µg had any effect on bacterial growth in vitro ($p > 0.05$) (Fig. 4C).

C1INH enhances the bactericidal activity of neutrophils and macrophages in vitro

In preliminary experiments, we determined that incubation of C1INH with bacteria before the addition of cells was more effective than initial incubation of C1INH with the cells (data not suppressed CLP-induced TNF-α levels at 4 and 8 h in both blood ($p < 0.0001$) and peritoneal fluid ($p < 0.0001$) (Fig. 3B). The mechanism responsible for the suppression of TNF-α production is unknown. However, we have previously shown a similar suppression in Gram-negative endotoxin shock (39). In vitro, suppression of TNF-α production by LPS-treated macrophages was caused by a direct interaction of C1INH (or iC1INH) with the lipid A moiety of LPS, which prevents LPS binding to the macrophage (39).

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C1INH and iC1INH (150 µg/ml) enhanced phagocytosis by both cell types in comparison with untreated controls (Fig. 5). These results suggest that C1INH suppresses the number of viable bacteria in the neutrophils or peritoneal exudate cells, the number of bacteria in the supernatant medium, and the total number of bacteria in the cells and medium, as described in Materials and Methods. The data suggest that both C1INH and iC1INH (150 µg/ml) enhanced phagocytosis by both cell types in comparison with untreated controls (Fig. 5). These results suggest that C1INH suppresses the number of viable bacteria in blood or peritoneal fluid via an increase in the phagocytosis and/or the bactericidal activity of neutrophils and macrophages.

Intravascular supplementation with C1INH enhanced survival of C1INH-deficient mice subjected to CLP

To further investigate the role of C1INH in sepsis, we analyzed the mortality of C1INH-deficient mice compared with that of wild-type littermates following CLP. Ligation of 10% of the cecum followed by puncture resulted in a mortality of 54% of C1INH-deficient mice, whereas wild-type littermate mice had a mortality of 5% (p < 0.001) (Fig. 6). A single dose of C1INH (600 µg) given immediately following CLP increased the survival of C1INH-deficient mice from 46 to 64%. This, however, was not a statistically significantly increase (p > 0.05). Treatment of C1INH-deficient mice with two doses of C1INH (600 µg each) at 0 and 6 h increased survival to 80%, which was significant (p < 0.05).

Discussion

The complement and contact systems each appear to have multiple effects in sepsis. The complement system, in particular, appears to be important both in protection (via generation of C5a) and in the mediation of damage (via generation of C5a). C1INH suppresses excessive complement activation, which would limit the amount of C5a produced, but, if too effective, might have the opposite effect via excessive suppression of C3 activation (57). In addition, inhibition of contact system activation would limit the amount of activated plasma kallikrein, factor XIIa, and bradykinin generated (58). C1INH prevented endotoxin shock via a direct interaction with LPS (39, 44). The binding of C1INH to LPS inhibits the interaction of LPS with LPS-binding protein, which in turn prevents the delivery of LPS to cells that express the LPS receptor complex. This binding is mediated by the lipid A moiety of LPS, and does not require an intact C1INH-reactive center loop (and therefore is not dependent on protease inhibitory activity), but does require intact N-linked carbohydrate, the C1INH N-terminal serpin domain, and the positively charged residues within this domain (45).

Administration of C1INH effectively decreased mortality in the CLP model. This model is very similar in its pathogenesis to that of human sepsis. Treatment with a single dose of C1INH after CLP significantly increased survival, which was further increased by the administration of multiple doses (Fig. 1). However, the survival rate was not improved with a single dose of C1INH at 6 h rather than immediately after puncture (Fig. 1B), but was improved with two doses (at 3 and 9 h after CLP) (Fig. 1B). The observation that iC1INH also provided protection indicates that a portion of the effectiveness of C1INH in sepsis is related to its ability to enhance phagocytosis and killing of Gram-negative bacteria rather than via complement or contact system inhibition (Fig. 1A).

A secondary deficiency in innate immunity, which may include dysfunction of neutrophils or monocytes, contributes to sepsis-induced mortality. Both C1INH and iC1INH reversed the defect in H₂O₂ production (Fig. 3A), augmented the in vivo clearance of bacteria (Fig. 2), and directly enhanced the in vitro bactericidal activity of blood neutrophils and peritoneal exudate cells (Fig. 5). Neither C1INH nor iC1INH had a direct effect on bacterial growth (Fig. 4). The finding that C1INH enhances neutrophil bactericidal activity has important implications. In another study using the CLP model in rodents, evidence was obtained for excessive activation of the complement system, which led to a severe defect in innate immune protective functions of neutrophils (59). These were associated with defects in chemotaxis, respiratory burst (H₂O₂ production), and phagocytosis. The molecular mechanisms that result in these defects may be linked to the complement activation product C5a. Treatment of CLP in rats and mice with anti-C5a, anti-IL-6, or anti-C5aR dramatically improved survival rates, which indicates a linkage between C5a and the C5a receptor in the harmful outcome of sepsis in rodents (60). The similar enhancement of survival from CLP with both C1INH and iC1INH, and their effectiveness in vitro, in the absence of complement, indicate that the beneficial effect is not mediated solely via inhibition of complement activation.

The susceptibility of C1INH-deficient mice to CLP-induced sepsis together with improvement in survival following supplementation with C1INH suggests that C1INH may play a normal biologic role in protection from sepsis. Other observations are consistent with this suggestion. First, C1INH is an acute-phase protein that increases up to 2.5-fold during inflammation (25). Secondly, administration of C1INH improves survival of mice both in mixed bacterial peritonitis/sepsis, as shown in this study, and in Gram-negative endotoxin shock (39, 44). Combined with our previous observations in LPS-induced endotoxin shock and in TNF-α-induced leukocyte rolling (39, 44, 61, 62), and other published studies (26, 27), the studies described in this work demonstrate that C1INH exerts its antibacterial and anti-inflammatory functions via several different mechanisms. These include, in addition to complement and contact system inhibition, the direct interaction of C1INH with endotoxin LPS (and with LPS-containing bacteria) and the inhibition of selectin-mediated leukocyte rolling (61, 62).

Disclosures

The authors have no financial conflict of interest.
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