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Molecular Intercommunication between the Complement and Coagulation Systems

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The complement system as well as the coagulation system has fundamental clinical implications in the context of life-threatening tissue injury and inflammation. Associations between both cascades have been proposed, but the precise molecular mechanisms remain unknown. The current study reports multiple links for various factors of the coagulation and fibrinolysis cascades with the central complement components C3 and C5 in vitro and ex vivo. Thrombin, human coagulation factors (F) XIa, Xa, and IXa, and plasmin were all found to effectively cleave C3 and C5. Mass spectrometric analyses identified the cleavage products as C3a and C5a, displaying identical molecular weights as the native anaphylatoxins C3a and C5a. Cleavage products also exhibited robust chemoattraction of human mast cells and neutrophils, respectively. Enzymatic activity for C3 cleavage by the investigated clotting and fibrinolysis factors is defined in the following order: FXa > plasmin > thrombin > FIIa > FIXa > control. Furthermore, FXa-induced cleavage of C3 was significantly suppressed in the presence of the selective FXa inhibitors fondaparinux and enoxaparin in a concentration-dependent manner. Addition of FXa to human serum or plasma activated complement ex vivo, represented by the generation of C3a, C5a, and the terminal complement complex, and decreased complement hemolytic serum activity that defines exact serum concentration that results in complement-mediated lysis of 50% of sensitized sheep erythrocytes. Furthermore, in plasma from patients with multiple injuries (n = 12), a very early appearance and correlation of coagulation (thrombin–antithrombin complexes) and the complement activation product C5a was found. The present data suggest that coagulation/fibrinolysis proteases may act as natural C3 and C5 convertases, generating biologically active anaphylatoxins, linking both cascades via multiple direct interactions in terms of a complex serine protease system. *The Journal of Immunology, 2010, 185: 000–000.

Traditionally, the complement and coagulation systems are described as separate cascades. As descendants of a common ancestral pathway, both proteolytic cascades are composed of serine proteases with common structural characteristics, such as highly conserved catalytic sites of serine, histidine, and aspartate (1, 2). Furthermore, both systems belong to a complex inflammatory network (3) and exhibit some similar characteristics regarding the specialized functions of their activators and inhibitors. In particular, the clotting factor [human coagulation factor (F) XIIa] can activate the complement factor C1r and thereby initiate the classical pathway of complement activation. In turn, the C1 esterase inhibitor suppresses not only all three established complement pathways (classical, lectin, and alternative, but also the intrinsic coagulation cascade (kallikrein, FIIa) (4, 5). Recently, it has been shown that thrombin is capable of generating the complement activation product C5a in the absence of C3 (6). In another study, Clark et al. (7) suggested that thrombin and plasmin may contribute to nontraditional complement activation during liver regeneration even in the absence of C4 and during inhibition of factor B. Thrombin may also act as a physiological agonist of the protein kinase C-dependent regulation of the complement decay-accelerating factor and thereby may provide a negative-feedback loop helping to prevent thrombosis during inflammation (8). In the setting of systemic inflammation, activation of the coagulation cascade is accompanied by a profound activation of the complement system, resulting in the generation of the anaphylatoxins C3a and C5a (9, 10). According to a previous report, C5a induces tissue factor (TF) activity in human endothelial cells (11) and may therefore be involved in the activation of the extrinsic coagulation pathway. Furthermore, C5a has been shown to stimulate the expression of TF on neutrophils via the C5aR, which was associated with a higher procoagulant activity (12). Additional evidence of procoagulant effects by complement components has been provided by a recent report demonstrating in vitro that mannan-binding lectin-associated serine protease 2 of the lectin pathway was capable of promoting fibrinogen turnover by cleaving prothrombin into thrombin (13).
In 1986, Sims et al. (14) showed that the terminal complement complex (TCC, C5b-9) can catalyze prothrombin cleavage to thrombin even in the absence of FV and thereby specifically increase platelet prothrombinase activity. In contrast, C5a has been described as having fibrinolytic effects by upregulating the plasminogen activator inhibitor I expression in the human mast cell line HMC-1 (15).

Thus, it is now becoming clear that both cascades may interact on a much larger scale than previously anticipated (16, 17). However, many of the underlying molecular mechanisms remain poorly understood. As indicated above, several factors of the coagulation/fibrinolysis cascade and components of the complement system display similar serine protease properties. In the current study, we hypothesized that various serine protease components of the clotting/fibrinolytic cascade directly cleave complement proteins, challenging the classic dogma that the two systems are separate cascades, and propose a model of a complex serine protease network.

Materials and Methods
Reagents

Unless stated otherwise, reagents were purchased from Sigma-Aldrich (Taufkirchen, Germany). Purified human C3 and C5 were obtained from Quidel (San Diego, CA). Human coagulation factors (F) VII, VIIa, IXa, Xa, XIa, and (activated) protein C were purchased from Calbiochem (Darmstadt, Germany). FIIa, FXIIa, and (activated) protein C were purchased from Calbiochem (Darmstadt, Germany). rFX, rFXI, and rTF were acquired from R&D Systems (Wiesbaden, Germany). Sodium enoxaparin and sodium fondaparinux were obtained from Sanofi-Aventis (Frankfurt, Germany).

In vitro cleavage of C3 and C5

In vitro experiments were performed by incubating native C3 (100 μg/ml) or C5 (100 μg/ml) in Dulbecco’s PBS (DPBS) in the absence or presence of various coagulation factors [FIIa, FIIa, FXa, FXa, FXa, FVIIa, FVIII, TF, protein C, activated protein C, thrombin (FIIa), plasmin and plasminogen] at 37°C in a dose- and time-dependent manner, followed by ELISA and Western blot analyses. Experiments were repeated in the absence or presence of selective FXa inhibitors (sodium enoxaparin and sodium fondaparinux). Human serum and plasma was incubated for 90 min at 37°C with FXa (ranging from 0–100 ng/ml) and assessed for C3a and C5a production as well as the assembly of TCC using Western blot and ELISA analysis.

Western blot analysis for C3a and C5a

Samples and controls were separated by SDS-PAGE under reducing conditions and transferred onto a polyvinylidene fluoride membrane (Schleicher & Schuell, Keene, NH). The blots were incubated overnight at 4°C with 1:1000 polyclonal antibody against human C3a IgG (Calbiochem) or 1:1000 polyclonal rabbit anti-human C3a IgG (Calbiochem). Hemolytic activity of human serum in the absence and presence of FXa was assessed as previously described (19, 20). Briefly, sheep erythrocytes (Oxoid, Wesel, Germany) were sensitized with hemolysin (Colorado Serum Company, Denver, CO) and exposed to dilutions of serum samples in TBS (pH 7.35, 37°C, 60 min). The hemolytic activity was stopped by the addition of ice-cold TBS followed by a centrifugation step (700 × g, 5 min). Absorption values of the supernatant fluids were determined by spectrophotometry at 541 nm. The complement hemolytic serum activity (CH50) defines the exact serum concentration that results in complement-mediated lysis of 50% of sensitized sheep erythrocytes.

Complement hemolytic serum activity

Hemolytic activity of human serum in the absence and presence of FXa (100 μg/ml) was assessed as previously described (19, 20). Briefly, sheep erythrocytes (Oxoid, Wesel, Germany) were sensitized with hemolysin (Colorado Serum Company, Denver, CO) and exposed to dilutions of serum samples in TBS (pH 7.35, 37°C, 60 min). The hemolytic activity was stopped by the addition of ice-cold TBS followed by a centrifugation step (700 × g, 5 min). Absorption values of the supernatant fluids were determined by spectrophotometry at 541 nm. The complement hemolytic serum activity (CH50) defines the exact serum concentration that results in complement-mediated lysis of 50% of sensitized sheep erythrocytes.

Measurement of thrombin–antithrombin complexes and C5a early after multiple injury in humans

Twelve patients after multiple injury (10 males, 2 females, median age: 38 y, range: 19–74 y) with a median injury severity score of 48 (range: 25–60) as defined by the Consensus Criteria were enrolled in the study in accordance with the Independent Local Ethics Committee of the University of Ulm (approval number 44/06). For all patients, informed consent was obtained. If the patient was incapable of making decisions because of sedation or altered mental status, informed consent was obtained postrecovery. Blood was drawn within 1 h postinjury on admission to the emergency room at the University Hospital Ulm, and the thrombin–antithrombin complex (TAT) as well as the anaphylatoxin C5a concentrations were determined (see above). Plasma levels of TAT were measured using a sandwich ELISA. TAT was captured in wells coated with anti-human thrombin, and HRP-coupled anti-human antithrombin Ab was used for detection (both Abs from Enzyme Research Laboratories, South Bend, IN). A standard prepared by diluting pooled human serum in normal citrate-phosphate-dextrose plasma was used. The standard was calibrated using TAT complexes produced from purified thrombin and antithrombin. Values were expressed as milligrams per liter.

Statistical analysis

All values are expressed as mean ± SD. Data sets of ELISA and chemotaxis assays were analyzed by Kruskal-Wallis ANOVA on ranks;
of glycosylated C3a was found (Fig. 1A). Abs to human C3a, a single band at the predicted molecular mass of 8.6 kDa, was found (Fig. 1A). Identical results were obtained when mAb to human C3a was employed (data not shown). The data were confirmed by parallel ELISA analysis of the samples (Fig. 1B). To determine the time dependency of thrombin-mediated cleavage, 5 μg/ml thrombin was incubated with 100 μg/ml C3 as a function of time (0–480 min). C3 cleavage appeared to reach a plateau after ~60 min of incubation (Fig. 1C). HMC-1 cells were used to assess biological (chemotactic) activity of the thrombin-induced cleavage product, which positively correlated with the amount of C3a generated (data not shown). To compare the thrombin-induced C3 cleavage with that of C5, cleavage of C5 was assessed using the same in vitro conditions, which in agreement with previous findings (6) resulted in significant generation of C5a as detected by ELISA (Fig. 1D).

In vitro cleavage of C3 and C5 by human FXa

FXa, the junction of the extrinsic and intrinsic paths of the coagulation system, was evaluated for its capability to cleave C3. When human C3 and human FXa were incubated at 37°C for 90 min and subjected to Western blot analysis for C3a, a single band at 9 kDa was found. The intensity of the band initially correlated with the amount of FXa added, peaking at 2 μg/ml FXa (Fig. 2A). When supraphysiological concentrations of FXa were used (20–100 μg/ml), the bands appeared at slightly lower molecular levels, suggesting further cleavage most likely at the C-terminal site of C3a. When the polyclonal Ab used for detection of C3a was preblocked with recombinant human C3a, the C3a bands were significantly fainter, confirming the specificity of C3a detection by the Western technique (data not shown). A similar in vitro cleavage pattern was also indicated by the ELISA data, in which the Abs employed might not detect further C3 cleavage products at lower molecular weights than the native C3a (Fig. 2B). Reflecting these results, FXa-generated C3a induced chemotaxis of HMC-1 cells in a concentration-dependent fashion, peaking at 2 μg/ml FXa and declining at higher concentrations (Fig. 2C).

Subsequently, the capability of human FXa to cleave human C5 (5 μg; concentration: 100 μg/ml) in vitro was evaluated. Samples were incubated at 37°C for 90 min and then subjected to Western blot and ELISA analysis for C5a. A C5 cleavage product was present at ~14 kDa that was reactive with anti-human C5a IgG (Fig. 2D). The band intensity was dependent on the concentration of FXa added. Confirmation of these results was obtained using a monoclonal anti-C5a IgG (data not shown). No evidence of autocleavage existed in the absence of human FXa. To further specify the cleavage product obtained, the anti-C5a detection Ab was preblocked with recombinant human C5a, which resulted in faint bands for the expected cleavage product (data not shown). The amount of C5a generated in the presence of increasing concentrations of FXa was confirmed using C5a ELISA analysis (Fig. 2E). Finally, functional analysis of the C5 cleavage product following FXa exposure was performed. The cleavage product

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**FIGURE 1.** Thrombin-induced cleavage of C3 and C5. Human C3 (5 μg; concentration 100 μg/ml) was incubated in DPBS in the absence or presence of increasing concentrations of human thrombin for 90 min. A. Western blot analysis of the C3 cleavage product in the presence of thrombin using polyclonal anti-C3a IgG as detection Ab. Equal protein loading was ensured for all samples. Depicted blot is representative of n = 3 experiments. B. ELISA assessment for C3a from samples coincubated with thrombin and C3 in DPBS (n = 3). C. C3 (100 μg/ml) and thrombin (5 μg/ml) were incubated as a function of time with subsequent evaluation for C3a by ELISA. D. Western blot analysis of C5 cleavage in the presence of thrombin. *p < 0.05 versus control.
showed clear evidence of chemotactic attraction for human blood neutrophils (Fig. 2F).

Identification of FXa-induced cleavage products as C3a and C5a

To identify the FXa-induced cleavage products on a molecular level, MALDI-TOF MS analyses were performed. C3 (100 μg/ml) or C5 (100 μg/ml) were incubated with FXa (10 μg/ml). The molecular mass of the C3-cleavage product generated by FXa exactly matched the predicted molecular mass for native C3a of 9090 Da (Fig. 3A). As an internal control, C3a expressed with a histidine tag (MRGSHHHHHHGS) from Escherichia coli revealed an identical molecular mass of 9090 Da after subtracting the mass of the histidine tag (data not shown).

Due to the N-linked glycosylation of C5a, deglycosylation of C5a was necessary prior to MS analysis. Purified C5a from human plasma (kindly provided by Complement Tech, Tyler, TX) was used as a standard. The molecular mass of the deglycosylated C5a standard (Fig. 3B, upper panel) was identical to the FXa-induced cleavage product C5a (Fig. 3B, bottom panel).

C3 and C5 cleavage by human plasmin

As the central factor of the fibrinolysis cascade, plasmin not only cleaves fibrin, but also fibronectin, thrombospondin, laminin, and von Willebrand factor (23). To determine whether the fibrinolysis system also interacts with the complement cascade, human plasmin was incubated with human C3 or C5 as a function of concentration. Plasmin was found to cleave C3 into C3a fragments, as detected by Western blot analysis using anti-C3a-IgG (Fig. 4A) and quantitative ELISA measurements (Fig. 4B). Plasmin-induced

![FIGURE 2](http://www.jimmunol.org/)

**FIGURE 2.** FXa-mediated C3 and C5 cleavage with subsequent generation of C3a and C5a. Human C3 or C5 (5 μg; concentration 100 μg/ml) were incubated in DPBS in the absence or presence of increasing concentrations of FXa for 90 min at 37˚C. A, Western blot detection of C3a following exposure of C3 to FXa. Equal protein loading was ensured for all samples. Blots are representative of at least three independent experiments. B, ELISA evaluation for C3a following coincubation of FXa and native C3. *p < 0.05 versus control (n = 3). C, Chemotaxis assay using HMC-1 cells to determine biological activity of C3-cleavage product postexposure to FXa (n = 6). Recombinant human C3a (100 ng/ml) served as a positive control. D, Western blot analysis of samples following exposure of C5 to FXa using anti-C5a IgG. Nonglycosylated, rC5a served as a positive control. Blot is representative of three independent experiments. E, ELISA assessment of the preparations described in D. *p < 0.05 versus control. F, Evaluation of C5 + FXa preparations for chemotactic activity using human neutrophils. Recombinant human C5a (100 ng/ml) served as a positive control. For each experimental condition, n = 6.

![FIGURE 3](http://www.jimmunol.org/)

**FIGURE 3.** MS analysis of FXa-induced cleavage products of C3 and C5. MALDI-TOF MS analysis of the C3 and C5 cleavage products following FXa incubation. A, Mass sizes of C3a produced by FXa-induced C3 cleavage. B, Mass sizes of deglycosylated, recombinant human C5a (upper panel) and C5a generated by incubation of C5 with FXa (lower panel).
generation of C3a peaked at 20 μg/ml plasmin and decreased at higher concentrations (Fig. 4A, 4B). In addition, plasmin-derived C3a dose-dependently induced chemotactic activity of HMC-1 cells (Fig. 4C), suggesting biological activity for the C3 cleavage product obtained.

When human plasmin was incubated with human C5, a concentration-dependent cleavage of C5 was found, as assessed by Western blotting and ELISA (Fig. 4D, 4E). Maximal plasmin-induced C5a-generation was found at plasmin concentrations above 2 μg/ml. Again, generated C5a was biologically active, as determined by neutrophil chemotaxis assays (Fig. 4F).

Characterization of the C3- and C5-proteolytic activity of coagulation factors

To determine whether the above-described clotting and fibrinolysis factors exhibit different degrees of catalytic activities, analysis of enzymatic activity was performed. Accordingly, increasing concentrations of C3 or C5 were used as a substrate, whereas a constant concentration of 80 nM each factor was added as the enzyme. Various coagulation factors (TF, FVIIa, FVII, plasminogen, and activated protein C) failed to generate C3 or C5 cleavage activity (data not shown). In contrast, FXa and plasmin most effectively cleaved both C3 and C5 (Fig. 5A, 5B).

The enzymatic activity analyses (n = 5 for each) revealed the following order: FXa > plasmin > thrombin > FIIXa > FXIa > control for C3 and plasmin > FXa > FIIXa = FXIa > thrombin > control for C5.

Serum complement activation by FXa: inhibition by anticoagulants

To determine whether the results obtained in vitro can be transferred into a more complex biological system, the ability of FXa to activate the complement system in serum was investigated ex vivo. We also sought to determine whether the in vitro cleavage process is capable of assembling the TCC ex vivo, which was assessed by the CH50. Human serum from healthy volunteers (50 ml) was incubated in the absence or presence of increasing concentrations of FXa (0–100 μg/ml) and analyzed by ELISA for generated C3a and C5a. Serum levels of C3a and C5a were both found to be increased in a concentration-dependent manner on incubation with FXa (Fig. 6A, 6B). Similarly, serum incubation with FXa (100 μg/ml) resulted in significant generation of TCC (384 ± 24 ng/ml TCC), reflected functionally by a small but significant decrease in serum hemolytic activity (Fig. 6C).

The specificity of FXa-dependent anaphylatoxin generation in serum was further evaluated using fondaparinux and enoxaparin, both selective inhibitors of FXa. Serum incubation with FXa in the copresence of increasing concentrations of fondaparinux led to a significantly suppressed production of C5a (Fig. 6D). Similar results were found when a different inhibitor of FXa protease
activity, enoxaparin, was used (Fig. 6E). Additional experiments substituting human plasma for human serum demonstrated similar patterns of C3a and C5a generation in the presence of FXa (data not shown). Collectively, these data suggest functional ex vivo cleavage of C3 and C5 by FXa.

For a first transfer of the reported ex vivo findings to a relevant clinical setting, plasma from 12 polytrauma patients within the first hour after trauma was analyzed for activation of the coagulation and complement systems (for detailed patients’ demography, see Ref. 24). In accordance with a previous study in multiple injured patients (25), very early activation (and dysfunction) of the coagulation system was found with significant formation of TAT complexes. A subsequent analysis determined whether these changes in TAT complexes were associated with complement activation. Early posttrauma (within 1 h postinjury), there was a positive correlation between the generation of the TAT complexes and the anaphylatoxins help in activating cellular immune responses (30).

FIGURE 5. C3-and C5-proteolytic activity of various coagulation factors (FXa, FIXa, FXIa, plasmin, thrombin). A and B, C3- and C5-proteolytic activity of various coagulation/fibrinolysis factors (each at 80 nM) were assessed by ELISA measurements of C3a and C5a generated within 90 min in the presence of increasing concentrations of the substrate (10, 54, 108, 270, 540, 810, and 1080 nM C3 or C5). Every proteolytic activity value represents the average of duplicate measurements based on five independent experiments.

Discussion

In the current study, we investigated the interaction between the coagulation/fibrinolysis cascades and the complement system in vitro and ex vivo. Exposure of C3 to thrombin resulted in time- and concentration-dependent generation of C3a in vitro. C5 was also cleaved by thrombin to produce C5a. In parallel experiments, incubation of C3 and C5 with either FXa or plasmin resulted in generation of C3a and C5a. The resulting cleavage products exhibited intact chemotactic activity and were indistinguishable from native C3a or C5a when assessed by MS. Incubation of serum or plasma with FXa resulted in a concentration-dependent generation of C3a and C5a, which, in turn act as chemoattractants for phagocytic cells to the site of inflammation, where these cells release their major arsenals of tissue-damaging proteases, reactive oxygen species, and cytokines/chemokines (15, 18, 33, 34). An important role for C5a/C5aR signaling has also been postulated in the fibrinolysis system (35). Finally, many proinflammatory cytokines can cause decreased levels of several anticoagulant proteins including thrombomodulin, the endothelial cell protein C receptor, and protein S (36), resulting in an inflammatory, procoagulant state. Mannan-binding lectin-associated serine protease 2, a protease that is characteristic of the lectin pathway of complement activation, can trigger coagulation by cleaving prothrombin into active thrombin (13).

The procoagulant activities of complement are increased when anticoagulant mechanisms are inhibited; for example, the formation of a complex between C4b-binding protein and protein S results in a decrease in the availability of protein S to act as a cofactor for the anticoagulant protein C pathway (37). In addition, the thrombin-prothrombin complex activates carboxypeptidase B, which, in turn, blocks C5a to counteract the inflammatory mediators generated at the site of vascular injury (38). Profound effects of the complement system on the coagulation system and vice versa have also been found for some innate regulators, such as the C1-inhibitor or the thrombin activatable fibrinolysis inhibitor. The latter is a potent and broadly reactive carboxypeptidase (39), which is generated by the thrombomodulin–thrombin complex and reacts in an anticoagulant manner (40). It not only moderates fibrinolysis but also has some anti-inflammatory effects due to its ability to inactivate C3a and C5a by removing carboxy-terminal arginine residues from these components (41). Thus, we are now beginning to understand that, rather than acting as separate, independent cascades, the coagulation/fibrinolysis system and the complement cascade cross talk extensively with each other and mutually fine-tune their activation status (16, 17).

Previously, the presence of C3 was thought to be indispensable for the assembly of the C5-convertase to generate C5a. Surprisingly, the presence of C5a was recently discovered in activated C3-deficient serum, where enhanced levels of thrombin, a primary component of the activated coagulation system, induced C5 conversion to C5a (6). In the current study, we found evidence of thrombin-mediated cleavage of C3 into C3a in a dose- and time-dependent manner in complement-sufficient human serum. In line with these findings, Kalowsky et al. (42) reported in 1975 that thrombin and thromboplastin injected into rabbits somehow led to
activation of complement, which appeared to be partially dependent on platelets. Similar to thrombin, kallikrein and plasmin have been described to directly cleave C3 (43, 44).

Serine proteases are characterized by a serine residue at the active center of these proteases (45), which participates in the catalytic mechanism during peptide bond hydrolysis (46). Under physiological conditions, complement C3 and C5 are cleaved by the C3-convertase and C5-convertase at a single arginine-serine peptide bond at position 77 and 75 in the α-chain of C3 and C5 to release C3a and C5a, respectively (47, 48). Similarly, the coagulation factors FXa, FXIa, FIXa, plasmin, and thrombin are known to be potent serine proteases (49) with specific affinity for the arginyl-X peptide bonds of their natural substrates (50), which led us to the above-proposed hypothesis. In the current study, our hypothesis was supported by the identification of thrombin-, FXa-FXIa-, FIXa-, and plasmin-mediated cleavage products of C3 and C5. The proteolytic effectiveness of these serine proteases to cleave C3 and C5 was further compared using a proteolytic activity analysis, identifying coagulation factor FXa as the most potent one. Interestingly, maximal C3 cleavage via FXa or plasmin was achieved at lower concentrations (2 μg/ml and 20 μg/ml, respectively) than the peak of C5 cleavage (20–100 μg/ml). In fact, FXa- and plasmin-mediated cleavage of C3 drastically declined at higher concentrations of these serine proteases. In this

FIGURE 6. FXa-induced serum complement activation. Human serum was incubated in the presence or absence of increasing concentrations of FXa for 90 min at 37˚C. Evaluation of serum samples for C3a (A) and C5a (B) concentration following FXa-incubation using ELISA analysis. *p < 0.05 versus control (n = 3). C, CH50 was assessed in human serum in the presence or absence of 100 μg/ml FXa. Data are representative of 12 separate and independent experiments. *p < 0.05 versus control. ELISA analysis of serum samples for C5a postincubation with FXa (40 μg/ml) in the copresence of increasing amounts of the FXa-selective inhibitors sodium fondaparinux (D) or sodium enoxaparin (E). *p < 0.05 versus control; n = 6. F, Correlation analysis between generated TAT complexes and complement activation product C5a in the plasma of patients early (<1 h) after multiple injury.
The authors have no financial conflicts of interest.
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