

Interleukin-6 Infusion Blunts Proinflammatory Cytokine Production Without Causing Systematic Toxicity in a Swine Model of Uncontrolled Hemorrhagic Shock

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Background: Serum elevations of interleukin-6 (IL-6) correlate with multiple organ dysfunction syndrome and mortality in critically injured trauma patients. Data from rodent models of controlled hemorrhage suggest that recombinant IL-6 (rIL-6) infusion protects tissue at risk for ischemia-reperfusion injury. Exogenous rIL-6 administered during shock appears to abrogate inflammation, providing a protective rather than a deleterious influence. In an examination of this paradox, the current study aimed to determine whether rIL-6 decreases inflammation in a clinically relevant large animal model of uncontrolled hemorrhagic shock, (UHS), and to investigate the mechanism of protection.

Methods: Swine were randomized to four groups (8 animals in each): (1) sacrifice, (2) sham (splenectomy followed by

hemodilution and cooling to 33°C), (3) rIL-6 infusion (sham plus UHS using grade 5 liver injury with packing and resuscitation plus blinded infusion of rIL-6 [10 mcg/kg]), and (4) placebo (UHS plus blinded vehicle). After 4 hours, blood was sampled, estimated blood loss determined, animals sacrificed, and lung harvested for RNA isolation. Quantitative reverse transcriptase-polymerase chain reaction was used to assess granulocyte colony-stimulating factor (G-CSF), IL-6, and tumor necrosis factor- α (TNF α) messenger ribonucleic acid (mRNA) levels. Serum levels of IL-6 and TNF α were measured by enzyme-linked immunoassay (ELISA).

Results: As compared with placebo, IL-6 infusion in UHS did not increase estimated blood loss or white blood cell counts, nor decrease hematocrit or plate-

let levels. As compared with the sham condition, lung G-CSF mRNA production in UHS plus placebo increased eightfold ($*p < 0.05$). In contrast, rIL-6 infusion plus UHS blunted G-CSF mRNA levels, which were not significantly higher than sham levels ($p = 0.1$). Infusion of rIL-6 did not significantly affect endogenous production of either lung IL-6 or mRNA. As determined by ELISA, rIL-6 infusion did not increase final serum levels of IL-6 or TNF α over those of sham and placebo conditions.

Conclusions: Exogenous rIL-6 blunts lung mRNA levels of the proinflammatory cytokine G-CSF. The administration of rIL-6 does not increase the local expression of IL-6 nor TNF α mRNA in the lung. Additionally, rIL-6 infusion does not appear to cause systemic toxicity.

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Ischemia-reperfusion injury secondary to hemorrhage stimulates inflammation and tissue damage by escalating the endogenous production of proinflammatory cytokines such as interleukin-6 (IL-6), granulocyte colony-stimulating factor (G-CSF), and tumor necrosis factor- α (TNF α). Up-regulation of IL-6 correlates with mortality in numerous clinical studies.¹⁻³ As a result of the significant association be-

tween high circulating serum IL-6 levels, mortality, and multiple organ dysfunction syndrome, IL-6 is widely thought to be detrimental. Conversely, a significant accumulating body of evidence suggests that exogenously administered recombinant IL-6 (rIL-6) has numerous beneficial antiinflammatory effects that may outweigh the harmful influence of endogenously produced IL-6 depending on the experimental or clinical situation. Consequently, an intuitive paradox exists because exogenous intravenous administration of rIL-6 early in the course of resuscitation after hemorrhage appears to abrogate the inflammatory response and provide a protective rather than a deleterious influence.

Data from a controlled hemorrhagic shock rodent model suggest that intravenously administered exogenous rIL-6 benefits tissue at risk for ischemia-reperfusion injury.⁴ This study aimed to examine molecular mechanisms by which rIL-6 protects against organ damage in a clinically relevant large animal model of uncontrolled hemorrhagic shock (UHS). The authors hypothesized that intravenous rIL-6 infusion during resuscitation from UHS would decrease endogenous proinflammatory cytokine messenger ribonucleic acid (mRNA) production, thereby reducing polymor-

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phonuclear neutrophil (PMN) infiltration and dysfunctional inflammation.

MATERIALS AND METHODS

Surgical Protocols

The study protocols were approved by the Animal Protocol Review Committee of Baylor College of Medicine and the Institutional Animal Care and Use Committee serving Stanford University. The protocols were performed according to the provisions of the Animal Welfare Act, PHS Animal Welfare Policy, principles of the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and policies and procedures of Baylor College of Medicine and Stanford University. For the study, 3-month-old Yorkshire crossbred swine weighing approximately 40 kg were used. The animals were allowed access to water and commercial laboratory swine food ad libitum until 24 hours before the procedures.

Study Groups

Swine were randomly subjected to one of four experimental groups of eight animals each: (1) normal control group, (2) sham group, (3) UHS plus placebo infusion protocol group, (4) UHS plus rIL-6 infusion protocol group.

Normal Controls

The eight animals in the normal control group were killed without any procedures to obtain constitutive levels of mRNA.

Sham Surgical Protocol

The eight animals in the sham group were given a pre-anesthetic intramuscular injection of glycopyrrolate 0.01 mg/kg and Telazol 4 mg/kg, and a 7-mm-diameter cuffed endotracheal tube was placed after induction of anesthesia. Anesthesia was maintained for the study period with 1% to 3% isoflurane in 50% oxygen. The tidal volume was fixed at 10 mL/kg with a rate of 10 breaths per minute. An electrocardiogram monitor was secured, and an esophageal stethoscope, gastric tube, and thermometer were inserted.

After the animals had been placed in the supine position, the ventral cervical area and abdomen were clipped and surgically prepared with povidone iodine. A central venous internal jugular line was used for fluid resuscitation, and a carotid arterial line was used for continuous blood pressure monitoring and blood sampling. Mean arterial pressure, systolic pressure, diastolic pressure, and heart rate were recorded and averaged. A laparotomy and splenectomy were performed. A splenectomy was necessary because the swine spleen is contractile and contains a variable volume of blood resulting in autoresuscitation during hemorrhage studies leading to a confounding variable. Splenectomy removes this variable.

The spleen was weighed, and lactated Ringer's solution was infused at 50 mL/minute to three times the spleen weight

to replace the removed blood. The animals underwent an isovolemic, exchange transfusion with pharmaceutical grade 5% human albumin (Albumarc; Baxter Healthcare Corporation for the American Red Cross, Westlake Village, CA). Half (50%) of the animals' blood volume was replaced, and the animals were standardized to an esophageal temperature of 33°C by lavage of the abdomen. This rendered the animal hypothermic with a dilutional coagulopathy. The left lateral lobe of the liver was mobilized but not injured. The abdomen was closed, and the animal was maintained under anesthesia for 4 hours, then killed.

UHS Protocol With and Without rIL-6 Infusion

The 16 animals in the UHS group (8 subjected to UHS plus rIL-6 and 8 subjected to UHS plus placebo) underwent the same procedure as the sham-operated animals with the addition of grade 5 liver injury, as described by the American Association for the Surgery of Trauma (AAST) Organ Injury Scaling system. A grade 5 injury is defined as an injury to a central hepatic vein by the AAST Organ Injury Scaling system.⁵ The left lateral lobe of the liver was mobilized, and a grade 5 liver injury was performed using a specially designed clamp. This grade 5 liver injury model has been described previously.⁶

One minute after the grade 5 liver injury was performed, a blinded infusion of either lactated Ringer's solution or rIL-6 (dose, 10 μ g/kg)⁴ was administered. Administration of the blinded infusion was concurrent with initiation of fluid resuscitation. The liver injury was packed with laparotomy sponges simultaneously with administration of the blinded infusion and the fluid resuscitation. The animals were resuscitated to their baseline mean arterial pressure with lactated Ringer's solution at 50 mL/minute. After completion of the 4-hour study, the abdomen was reopened, the blood loss was determined, and the animal was killed.

Exogenous rIL-6

Studies were performed using a rodent model of controlled hemorrhagic shock to determine the appropriate dose of rIL-6. An exogenous dose of 10 μ g/kg rIL-6 given intravenously to rodents undergoing controlled hemorrhage resulted in abrogation of endogenous liver IL-6 levels to control levels (unpublished data from Tweardy et al., 2003). Human rIL-6 was obtained from Novartis Pharma AG (Basel, Switzerland). On the basis of the authors' previous studies using the rodent model, the dose used was 10 μ g/kg, administered intravenously to the swine 1 minute after the grade 5 liver injury.

Blood Collection and Analysis

Baseline and final blood specimens were collected for serum analysis, complete blood counts, and liver enzyme tests.

Enzyme-Linked Immunoassay Analysis of Circulating Protein Levels

Plasma concentrations of the circulating proinflammatory cytokines IL-6 and TNF α were measured using commercially available enzyme-linked immunoassay (ELISA) kits (R & D Systems, Minneapolis, MN).

Tissue Collection and RNA Isolation

After the animals had been killed, a lung sample was collected and flash frozen in liquid nitrogen immediately upon harvest and stored at -80°C for RNA isolation and quantitative reverse transcription-polymerase chain reaction. Total RNA was extracted using the modified method of Chomczynski and Sacchi⁷ with commercially available Trizol reagent (Life Technologies, GibcoBRL).

Quantitative Reverse Transcription-Polymerase Chain Reaction

Total RNA (5 ng for 18S and 500 ng for the gene of interest) was added to a reverse transcriptase, first-strand cDNA synthesis reaction. A 20- μL reaction volume was subjected to reverse-transcriptase first strand cDNA synthesis using the commercially available Taqman Multiscribe Reverse Transcriptase Kit (Applied Biosystems, Branchburg, NJ) under the following conditions: 10 minutes at 25°C , then 30 minutes at 48°C , followed by inactivation of the enzyme at 95°C for 5 minutes (ABI Prism 7700 Thermocycler; Applied Biosystems). Polymerase chain reaction then was performed, and conditions were as follows: stage 1: 2 minutes at 50°C , stage 2: 10 minutes at 95°C , and stage 3: 40 cycles of 15 seconds of melting at 95°C followed by DNA synthesis for 1 minute at 60°C . The ABI Prism PE7700 was used as well as Taqman Universal Master Mix (Applied Biosystems).

Polymerase chain reaction primers and probes for swine G-CSF, TNF α , and IL-6 were designed using the computer program Primer Express (Applied Biosystems) based on the published swine sequences of these cytokines.⁸⁻¹⁰ The forward primer sequence for swine G-CSF was 5'-CCTGCCCCAGCTTCCT-3', whereas the reverse primer for G-CSF was 5'-AGCTCGGCGCCATCAG-3'. The probe for G-CSF, located two nucleotides downstream of the 3' end of the forward primer, also was end-labeled with 6-FAM and TAMRA: 6-FAM-CTGGATTTTCCTCACTTGCTCTAAGCACTTTGA-TAMRA. The forward primer sequence for swine TNF α was 5'-GGCCCAAGGACTCAGATCATC-3', whereas the reverse primer was 5'-CGGCTTTGACATTGCTACAA-3' and the TNF α probe sequence was 6-FAM-AACCTCAGATAAGCCCGTCGCCCA-TAMRA. The IL-6 forward primer sequence was 5'-GCTGCTTCTGGTGATGCTACT-3', whereas the IL-6 reverse primer was 5'-GGCATCACCTTTGGCATCTT-3' and the IL-6 probe sequence was 6-FAM-CCTTCCCTACCCCGGAACGCCT-TAMRA.

A negative control condition for each set of polymerase chain reactions contained sterile water instead of cDNA template. The cycle threshold of each triplicate determination was normalized by subtraction of the cycle threshold (CT) for its corresponding 18S cycle threshold ΔCT . Each ΔCT then was calibrated by subtracting the mean normalized cycle threshold ΔCT of the eight normal control lungs ($\Delta\Delta\text{CT}$). The control animals killed without operation were considered to have "normal" or constitutive levels of cytokine production. The level of mRNA in the group of normal animals was arbitrarily assigned the value 1, and the fold change in cytokine mRNA levels in the sham, UHS protocol + rIL-6, and UHS protocol plus placebo groups, as compared with normal control subjects, was calculated using $2^{-\Delta\Delta\text{CT}}$, as described in User Bulletin 2 (Perkin-Elmer Applied Biosystems).

Statistical Analysis

Groups were analyzed using one-way analysis of variance (ANOVA). Statistical analysis of differences between groups was determined by post hoc Student Newman Keuls test. Comparisons between final and initial laboratory values were analyzed using the paired Student's *t* test. Data are presented as means \pm standard error of the mean (SEM). Statistical significance was defined as a *p* value less than 0.05. Statistical analysis was performed using commercially available software from Primer (McGraw-Hill, Inc).

RESULTS

Mortality of Model

In this study, 19 animals were subjected to the UHS protocol. Of these 19 animals, 3 died of exsanguination before the 4-hour study period predetermined for killing and harvesting of tissues. Data from the three animals that died were not included in the analysis because they did not survive to the 4-hour goal. No tissues were harvested from these animals. Only the 16 animals that lasted the full 4-hour resuscitation period underwent tissue analysis. The lethality of the UHS model was 16% secondary to the three deaths.

Hematologic and Physiology Data

As compared with placebo, rIL-6 infusion in UHS did not increase estimated blood loss or white blood cell counts, nor decrease hematocrit or platelet levels (Table 1). When the average mean arterial pressures were compared, there were no significant differences between UHS plus rIL-6 and UHS plus placebo (Fig. 1).

Quantitative Reverse Transcription-Polymerase Chain Reaction Data G-CSF mRNA Levels

As compared with the sham group, lung G-CSF mRNA production in the UHS plus placebo group increased eightfold ($*p < 0.05$). In contrast, rIL-6 infusion plus UHS blunted G-CSF mRNA levels to 1.9-fold above sham conditions, which was not statistically significant ($p = 0.1$). As compared with the control group, the sham group showed an 11-fold

Table 1 Final Laboratory Parameters Of Swine Randomized To UHS + rIL-6 vs. UHS + Placebo

	EBL (ml)	WBC ($\times 10^6/ml$)	Hct	Plts ($\times 10^6/ml$)	AST
UHS + rIL-6	997 (31)	11,170 (2,640)	10.74 (0.94)	157 (22)	352 (120)
UHS + Placebo	1211 (167)	8,000 (1,000)	12.80 (1.18)	175 (14)	103 (23)
p-value	0.23	0.35	0.06	0.5	0.07

Mean (+/-SEM).

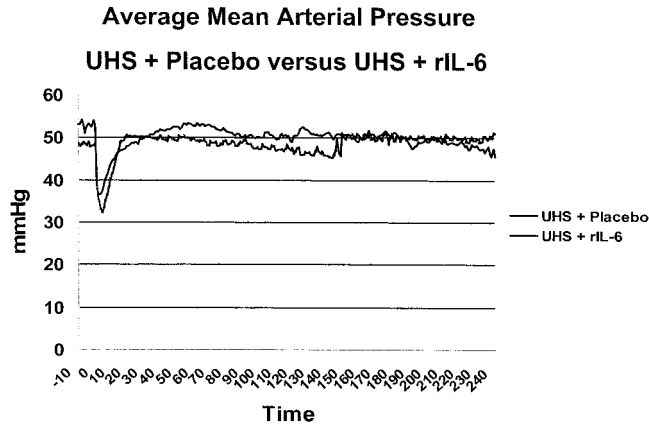


Fig. 1. There were no statistically significant differences in the overall mean arterial pressure between the uncontrolled hemorrhagic shock (UHS) plus recombinant interleukin-6 (rIL-6) group and the UHS plus placebo group.

increase, the UHS plus rIL-6 group a 21-fold increase, and the UHS plus placebo group an 83-fold increase (Fig. 2).

TNF α mRNA Levels

As compared with the sham condition, lung TNF α mRNA production in the UHS plus rIL-6 infusion group increased 6.25-fold, whereas UHS plus placebo TNF α mRNA levels increased 9-fold. As compared with the control group, the sham group showed a 4-fold increase, the UHS plus rIL-6 group a 25-fold increase, and the UHS plus placebo a 36-fold increase. Both the UHS plus placebo and the UHS plus rIL-6 TNF α mRNA levels were statistically increased, as compared the with sham levels ($p < 0.03$ and $p < 0.02$, respectively). There was no statistical difference in TNF α mRNA levels between the UHS plus placebo and the UHS plus rIL-6 groups (Fig. 3).

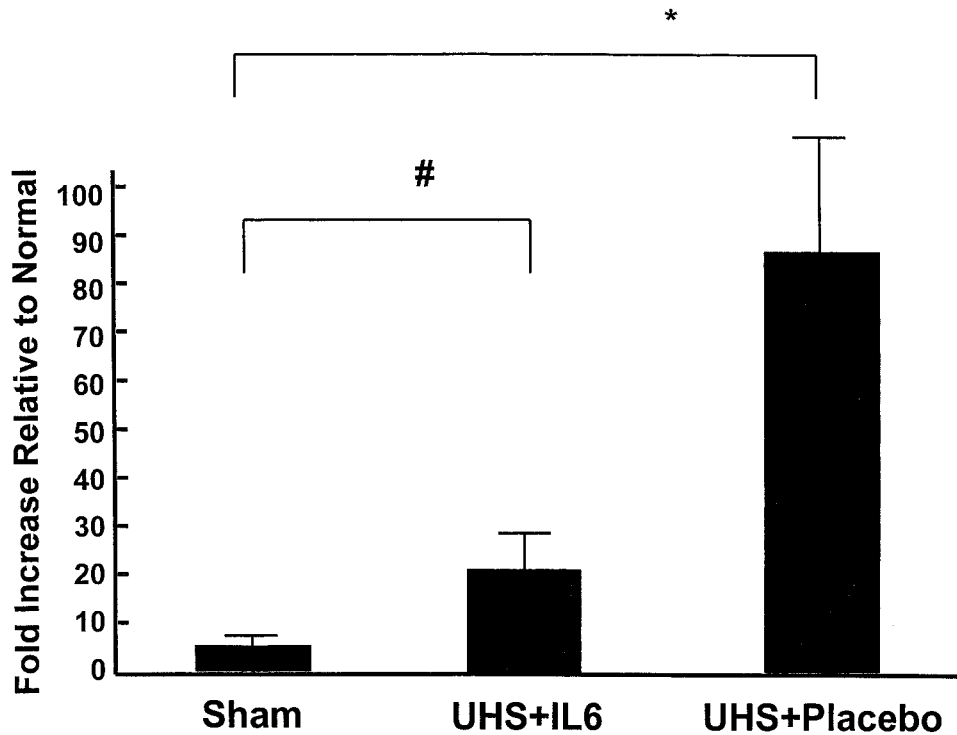


Fig. 2. Granulocyte colony-stimulating factor (G-CSF) messenger RNA (mRNA) levels in the lungs of sham, uncontrolled hemorrhagic shock (UHS) plus recombinant interleukin-6 (rIL-6), and UHS plus placebo swine. The G-CSF mRNA levels were quantified in swine lung by quantitative reverse transcription-polymerase chain reaction, as described in the Materials and Methods section, and standardized to levels detected in the normal control lung ($n = 8$). Data are presented as the mean \pm standard error of the mean of each group. Bars marked by one asterisk (*) differ significantly, $p < 0.05$. Bars marked by a number sign (#) are not statistically significant at a p value of 0.1.

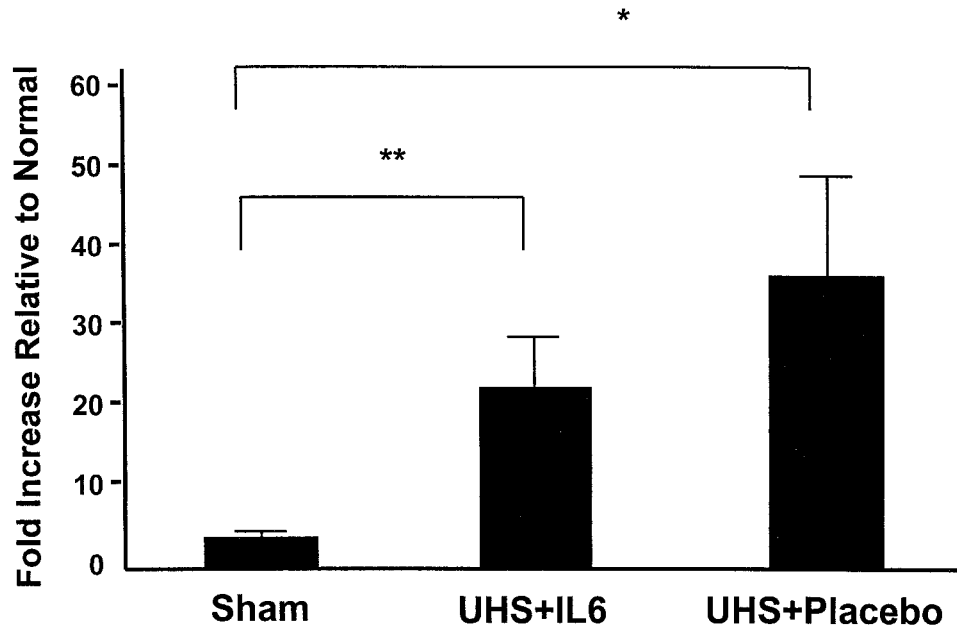


Fig. 3. Tumor necrosis factor- α (TNF α) messenger RNA (mRNA) levels in the lungs of sham, uncontrolled hemorrhagic shock (UHS) plus recombinant interleukin-6 (rIL-6), and UHS plus placebo swine. The TNF α mRNA levels were quantified in swine lung by quantitative reverse transcription-polymerase chain reaction, as described in the Material and Methods section, and standardized to levels detected in the normal control lung ($n = 8$). Data are presented as the mean \pm standard error of the mean of each group. Bars marked by one and two asterisks (* and **) differ significantly ($p < 0.03$ and $p < 0.02$, respectively).

IL-6 mRNA Levels

The endogenous production of IL-6 mRNA in the lung was evaluated. As compared with the constitutive production of IL-6 mRNA in the control group, the IL-6 mRNA level in the sham group was 0.95, the IL-6 mRNA level in the UHS plus rIL-6 group was 0.77-fold, and the IL-6 mRNA level in the UHS plus placebo group increased to 1.62-fold. The values among the groups did not reach statistical significance (Fig. 4).

ELISA Analysis for Circulating Protein Levels

Recombinant IL-6 infusion did not increase final serum levels of IL-6 nor TNF α , as compared with placebo (Table 2).

DISCUSSION

Most late deaths after trauma and hemorrhagic shock result from multiorgan dysfunction syndrome caused by dysfunctional inflammation after massive resuscitation or sepsis.¹¹ Upregulation of IL-6 correlates with multiple organ failure and mortality in numerous clinical studies.¹⁻³ As a result of the known proinflammatory IL-6 effects and the clinical correlation of high serum levels with mortality, IL-6 is widely thought to be detrimental. Remick et al.¹² recently suggested that serum IL-6 levels measured 6 hours after the induction of sepsis accurately predict mortality and suggest initiation of therapeutic interventions using high IL-6 levels to target patients with severe insults.

Interleukin-6 is a 21-kD cytokine produced by numerous cells. Endothelial cells, hepatocytes, fibroblasts, macrophages, neutrophils, and T and B lymphocytes all are capable of producing IL-6. Interleukin-6 affects a variety of biologic functions including immunoglobulin production and the

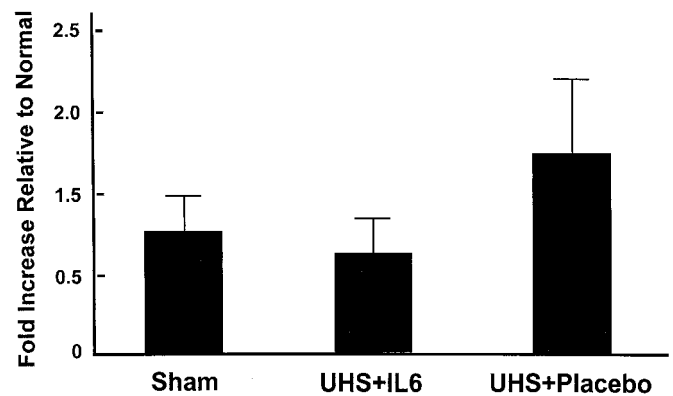


Fig. 4. Endogenous interleukin-6 (IL-6) messenger RNA (mRNA) levels in the lungs of sham, uncontrolled hemorrhagic shock (UHS) plus recombinant IL-6 (rIL-6), and UHS plus placebo swine. The IL-6 mRNA levels were quantified in the swine lung by quantitative reverse transcription-polymerase chain reaction, as described in the Material and Methods section, and standardized to levels detected in the normal control lung ($n = 8$). Data are presented as the mean \pm standard error of the mean of each group. There are no statistically significant differences between the experimental groups.

Table 2 ELISA Data: IL-6 and TNF-alpha Final Serum Levels in UHS + rIL-6 vs. UHS + Placebo

	IL-6 (pg/mL)	TNF-alpha (pg/mL)
UHS + rIL-6	1.4 (0.64)	1.3 (0.2)
UHS + Placebo	1.6 (0.92)	0.91 (0.36)
<i>p</i> -value	0.86	0.3

Mean (+/-SEM).

acute-phase response. Specifically, IL-6 stimulates PMN production. It is essential for the inflammatory response.¹³⁻¹⁶ Circulating IL-6 stimulates neutrophil production under physiologic stress. Interleukin-6 also is required for local production of CXC chemokines and neutrophil infiltration.¹⁷ These properties of IL-6 result in both pro- and antiinflammatory effects. The balance of the pro- and antiinflammatory effects of IL-6 is controversial. Many of the proinflammatory IL-6 effects are secondary to activation of B-cells and induction of chemokines and adhesion molecule expression in endothelial cells.¹⁷⁻¹⁹ Conversely, stimulation with rIL-6 causes decreased expression of the primary proinflammatory cytokines IL-1B, TNF α , and IL-12 from macrophages, astrocytes, and fibroblasts. The data showing downregulation of major proinflammatory mediators suggest that the paracrine effects from local endogenous production of IL-6 primarily serve an antiinflammatory role.

Consequently, numerous reports demonstrate that exogenously administered rIL-6 has significant beneficial antiinflammatory effects. Intraperitoneal injection of rIL-6 has been shown to inhibit TNF α production and to protect against LPS lethality.²⁰ Recombinant IL-6 also is protective against mortality in a model of staphylococcal enterotoxin-induced toxic shock.²¹ Administration of rIL-6 for a rat liver ischemia-reperfusion injury decreased hepatocellular damage and abrogated TNF α production.²² In a rat model of controlled hemorrhagic shock, intravenous administration of rIL-6 during resuscitation markedly decreased liver and lung injury and reduced PMN infiltration.⁴ Instillation of rIL-6 into an endotoxin-induced tracheal injury significantly decreased PMN infiltration and reduced concomitant markers of inflammation.²³ Interleukin-6 knockout mice have increased reperfusion injury after hepatic ischemia, which is reversed by pretreatment with rIL-6.¹⁴ Interestingly, IL-6 knockout mice treated with LPS produce three times more TNF α than wild-type animals, suggesting that IL-6 may be an important negative regulator of TNF α production.¹⁴ Additionally, rIL-6 has been shown to prevent production of TNF α and to induce the release of soluble TNF α receptor p55, which is responsible for binding and neutralizing circulating TNF α .²⁴

Granulocyte colony-stimulating factor is a 20- to 25-kD glycoprotein produced by a variety of stimulated cells, including macrophages,^{25,26} fibroblasts, and endothelial and astroglial cells.²⁷⁻³⁰ The primary cell target of G-CSF is the neutrophil, with G-CSF increasing the number and function

of circulating neutrophils. Findings show that G-CSF binds to a high-affinity receptor of 130- to 150-kD on neutrophils and has a wide range of effects including enhancement of endothelial adhesion,^{31,32} chemotaxis,³³ chemokinesis,³⁴ degranulation, priming for reactive oxygen intermediate (ROI) production,³⁵⁻³⁷ and survival.³⁸ In addition, G-CSF stimulates a variety of neutrophil effector functions.^{27,28}

Well studied as recombinant human G-CSF (rhG-CSF), G-CSF is in wide clinical use secondary to Food and Drug Administration (FDA) approval for the treatment of neutropenia caused by acute myelogenous leukemia and chemotherapy. Numerous studies evaluating treatment with rhG-CSF in rodent models of experimental sepsis show a decreased mortality rate. Several investigators have examined the therapeutic use of exogenous rhG-CSF in large animal models of septic shock and two hit models of hemorrhage followed by sepsis.^{39,40} Patton et al.³⁹ and Davis et al.⁴⁰ showed an increased production of circulating PMNs, containment of intraabdominal abscesses, and no evidence of increased PMN activity causing exacerbation of tissue injury. The laboratory of the current authors has previously reported contradictory data in a rodent model of hemorrhagic shock.⁴ Intravenous infusion of rhG-CSF during resuscitation significantly increased PMN infiltration into the lungs 2.4-fold, as compared with placebo, and was associated with a significant increase in interstitial edema and pneumocyte swelling reflected by a 42% increase in alveolar cross-sectional area ($p < 0.01$). In the same animals, a decrease in liver injury was noted secondary to rhG-CSF infusion. The role of endogenous G-CSF in healthy non-neutropenic tissues is unclear.⁴¹ Endogenous G-CSF production in the lung of the current uncontrolled hemorrhage model suggests that subsequent PMN recruitment likely contributes to lung injury and subsequent acute respiratory distress syndrome.

The current data adds to the literature suggesting that IL-6 plays a significant role as a local antiinflammatory mediator. In this study, rIL-6 infusion significantly blunted G-CSF mRNA expression in the lung from hemorrhaged animals. Decreased G-CSF mRNA levels likely abrogate neutrophil recruitment to the lung, consequently diminishing neutrophil-mediated injury. Tumor necrosis factor- α mRNA levels also were comparatively decreased, although this decrease did not reach statistical significance because of the large standard errors and the small sample size in this heterogeneous population of large animals.

The possible mechanisms by which exogenous rIL-6 decreases PMN infiltration and subsequent tissue damage involve upregulated suppressor of cytokine signaling 3 production and protein inhibitor of activated signal transducer and activator of transcription production, ultimately resulting in downregulation of proinflammatory cytokine expression. Further investigations of these potential mechanisms are necessary.

Study Advantages and Limitations

The swine model described in this study fulfills a need for a model of hemorrhagic shock that is uncontrolled and occurs in an animal near in size to humans. The need for such a model arises from the desire to validate, if possible, findings obtained in rodent HS models, and to establish a model for preclinical testing of interventions designed to reduce dysfunctional inflammation and multiple organ dysfunction syndrome. Although large animal models are clinically relevant, a wide variability in each individual animal's response to hemorrhage and resuscitation is observed. Herein lies the significance and crux of this clinical model. Secondary to the large variation in reaction to the UHS model, large SEMs along with relatively small sample sizes may result in type 2 statistical error.

Large animal studies are more costly than studies using rodent models. Consequently, the current sample size and data time points were limited by economics. A single point in time 4 hours after injury was studied. No data on inflammation from later time points are given, and survival information is not addressed. The small sample size emphasizes the possibility of a type 2 error. The goal of this study was to recapitulate a complex clinical scenario with multiple variables, uncontrolled hemorrhage, hypovolemia, tissue destruction (liver laceration), coagulopathy, and hypothermia. Although the authors succeeded in creating a clinically relevant large animal model of UHS, their study suffers from the inherent inter-animal variability of a large animal, as compared with rodent models, and the prohibitive costs of large animal investigations, which severely limit the sample size. The finding that TNF α is blunted in the rIL-6 plus UHS animals suffers from lack of power combined with large SEMs.

This study was limited by the decision to focus on only three cytokines and to examine primarily mRNA expression. The investigations were focused on the cytokines IL-6 and G-CSF because published reports¹⁶ support an essential role for each in a murine model of controlled hemorrhagic shock. Tumor necrosis factor- α was included because of its central proinflammatory role. Other well-known proinflammatory cytokines, such as IL-1 beta, were not examined in this study. The entire swine genome has yet to be sequenced. This currently limits ability to examine the mRNA expression of some potential crucial mediators such as SOCS3, which along with other relevant regulators of negative feedback in the proinflammatory cascade, has not yet been sequenced from the *sus scrofa* genome. Another disadvantage of using the swine model is that there is a relative paucity of immunologic reagents. As a result, confirmatory studies at the protein level are limited.

The current study design also was limited by a lack of confirmatory studies regarding neutrophil infiltration into the lungs. Histologic analysis of formalin-fixed lung tissue for MPO staining was not performed because there were no formalin-fixed tissues. Attempted MPO enzymatic assays on the frozen lung tissue did not yield any contributory information.

The historical problem with anticytokine therapy has been its applicability to humans. The current data shows that this large animal model recapitulates the documented beneficial response to exogenously administered rIL-6 discovered in rodent models. Although rodent models have provided new and additional insight into dysfunctional inflammation, determining that the molecular events described in rodents also occur in larger animals that can be sampled and evaluated more extensively and systematically than patients is an important step in understanding what occurs in hemorrhagic shock–trauma patients. In summary, the fundamental role of IL-6 remains controversial despite its discovery more than a decade ago. Is IL-6 our friend or foe? Are systemic IL-6 levels in critically ill patients a mediator or a marker of injury?⁴²

CONCLUSION

Exogenous rIL-6 blunts lung mRNA levels of the proinflammatory cytokine G-CSF. The administration of rIL-6 does not increase the local expression of IL-6 or TNF α mRNA in the lung. In fact, rIL-6 administration also appears to decrease TNF α mRNA production, although this finding does not achieve significance. Additionally, rIL-6 infusion does not appear to cause systemic toxicity. Elucidation of the specific mechanisms through which IL-6 exerts its antiinflammatory influence could ultimately establish specific therapies to abrogate the initiation of the systemic inflammatory response syndrome and inhibit ensuing multiple organ failure.

REFERENCES

1. Casey LC, Balk RA, Bone RC. Plasma cytokine and endotoxin levels correlate with survival in patients with the sepsis syndrome. *Ann Intern Med.* 1993;119:771–778.
2. Meduri GU, Headley S, Kohler G, et al. Persistent elevation of inflammatory cytokines predicts a poor outcome in ARDS. Plasma IL-1 beta and IL-6 levels are consistent and efficient predictors of outcome over time. *Chest.* 1995;107:1062–1073.
3. Arnalich F, Garcia-Palomero E, Lopez J, et al. Predictive value of nuclear factor kappaB activity and plasma cytokine levels in patients with sepsis. *Infect Immun.* 2000;68:1942–1945.
4. Meng ZH, Dyer K, Billiar TR, Tweardy DJ. Distinct effects of systemic infusion of G-CSF vs. IL-6 on lung and liver inflammation and injury in hemorrhagic shock. *Shock.* 2000;14:41–48.
5. Moore EE, Cogbill TH, Jurkovich GJ, Shackford SR, Malangoni MA, Champion HR. Organ injury scaling: spleen and liver (1994 revision). *J Trauma.* 1995;38:323–324.
6. Holcomb JB, Pusateri AE, Harris RA, et al. Dry fibrin sealant dressings reduce blood loss, resuscitation volume, and improve survival in hypothermic coagulopathic swine with grade V liver injuries. *J Trauma.* 1999;47:233–240, discussion 240–232.
7. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem.* 1987;162:156–159.
8. Gloster SE, Strom AD. *Sus scrofa* granulocyte-colony stimulating factor mRNA, complete cds. *Direct Submission, NCBI, NIH.* 1996.
9. Choi CS, Lin GF, Molitor TW, Murtaugh MP. Complete nucleotide sequence of a cDNA encoding porcine tumor necrosis factor-alpha. *Anim Biotechnol.* 1991;2:97–105.

10. Mathialagan N, Bixby JA, Roberts RM. Expression of interleukin-6 in porcine, ovine, and bovine preimplantation conceptuses. *Mol Reprod Dev.* 1992;32:324–330.
11. Moore FA, Sauaia A, Moore EE, Haenel JB, Burch JM, Lezotte DC. Postinjury multiple organ failure: a bimodal phenomenon. *J Trauma.* 1996;40:501–510, discussion 510–502.
12. Remick DG, Bolgos GR, Siddiqui J, Shin J, Nemzek JA. Six at six: interleukin-6 measured 6 hours after the initiation of sepsis predicts mortality over 3 days. *Shock.* 2002;17:463–467.
13. Kopf M, Baumann H, Freer G, et al. Impaired immune and acute-phase responses in interleukin-6-deficient mice. *Nature.* 1994; 368:339–342.
14. Fattori E, Cappelletti M, Costa P, et al. Defective inflammatory response in interleukin-6-deficient mice. *J Exp Med.* 1994;180:1243–1250.
15. Dalrymple SA, Slattery R, Aud DM, Krishna M, Lucian LA, Murray R. Interleukin-6 is required for a protective immune response to systemic *Escherichia coli* infection. *Infect Immun.* 1996;64:3231–3235.
16. Meng ZH, Dyer K, Billiar TR, Tweardy DJ. Essential role for IL-6 in postresuscitation inflammation in hemorrhagic shock. *Am J Physiol Cell Physiol.* 2001;280:C343–C351.
17. Romano M, Sironi M, Toniatti C, et al. Role of IL-6 and its soluble receptor in induction of chemokines and leukocyte recruitment. *Immunity.* 1997;6:315–325.
18. Heinrich PC, Behrmann I, Muller-Newen G, Schaper F, Graeve L. Interleukin-6-type cytokine signalling through the gp130/Jak/STAT pathway. *Biochem J.* 1998;334(Pt 2):297–314.
19. Barton BE. IL-6: insights into novel biological activities. *Clin Immunol Immunopathol.* 1997;85:16–20.
20. Barton BE, Jackson JV. Protective role of interleukin-6 in the lipopolysaccharide–galactosamine septic shock model. *Infect Immun.* 1993;61:1496–1499.
21. Barton BE, Shortall J, Jackson JV. Interleukins 6 and 11 protect mice from mortality in a staphylococcal enterotoxin-induced toxic shock model. *Infect Immun.* 1996;64(3):714–718.
22. Camargo CA, Jr., Madden JF, Gao W, Selvan RS, Clavien PA. Interleukin-6 protects liver against warm ischemia/reperfusion injury and promotes hepatocyte proliferation in the rodent. *Hepatology.* 1997;26(6):1513–1520.
23. Ulich TR, Yin S, Guo K, Yi ES, Remick D, del Castillo J. Intratracheal injection of endotoxin and cytokines. II. Interleukin-6 and transforming growth factor beta inhibit acute inflammation. *Am J Pathol.* May 1991;138(5):1097–1101.
24. Tilg H, Trehu E, Atkins MB, Dinarello CA, Mier JW. Interleukin-6 (IL-6) as an anti-inflammatory cytokine: induction of circulating IL-1 receptor antagonist and soluble tumor necrosis factor receptor p55. *Blood.* Jan 1 1994;83(1):113–118.
25. Ernst TJ, Ritchie AR, Demetri GD, Griffin JD. Regulation of granulocyte- and monocyte-colony stimulating factor mRNA levels in human blood monocytes is mediated primarily at a post-transcriptional level. *Journal of Biological Chemistry.* 1989; 264:5700–5710.
26. Oster W, Lindemann A, Mertelsmann R, Hermann F. Granulocyte-macrophage colony-stimulating factor (CSF) and multilineage CSF recruit human monocytes to express granulocyte CSF. *Blood.* 1989; 73:64–70.
27. Demetri GD, Griffin JD. Granulocyte colony-stimulating factor and its receptor. *Blood.* 1991;78:2791–2808.
28. Tkatch LS, Tweardy DJ. Human granulocyte colony-stimulating factor (G-CSF), the premier granulopoietin: biology, clinical utility, and receptor structure and function. *Lymphokine & Cytokine Research.* 1993;12(6):477–488.
29. Tweardy DJ, Mott PL, Glazer EW. Monokine modulation of human astroglial cell production of granulocyte colony-stimulating factor and granulocyte-macrophage colony-stimulating factor. I. Effects of IL-1 alpha and IL-beta. *Journal of Immunology.* 1990;144(6):2233–2241.
30. Tweardy DJ, Glazer EW, Mott PL, Anderson K. Modulation by tumor necrosis factor-alpha of human astroglial cell production of granulocyte-macrophage colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor (G-CSF). *Journal of Neuroimmunology.* 1991;32(3):269–278.
31. Kerst JM, de HM, van dSCE, et al. Recombinant granulocyte colony-stimulating factor administration to healthy volunteers: induction of immunophenotypically and functionally altered neutrophils via an effect on myeloid progenitor cells. *Blood.* 1993; 82(11):3265–3272.
32. Yuo A, Kitagawa S, Ohsaka A, et al. Recombinant human granulocyte colony-stimulating factor as an activator of human granulocytes: potentiation of responses triggered by receptor-mediated agonists and stimulation of C3bi receptor expression and adherence. *Blood.* 1989;74(6):2144–2149.
33. Wang JM, Chen ZG, Colella S, et al. Chemotactic activity of recombinant human granulocyte colony-stimulating factor. *Blood.* 1988;72(5):1456–1460.
34. Smith WB, Gamble JR, Vadas MA. The role of granulocyte-macrophage and granulocyte colony-stimulating factors in neutrophil transendothelial migration: comparison with interleukin-8. *Experimental Hematology.* 1994;22(3):329–334.
35. Kitagawa S, Yuo A, Souza LM, Saito M, Miura Y, Takaku F. Recombinant human granulocyte colony-stimulating factor enhances superoxide release in human granulocytes stimulated by the chemotactic peptide. *Biochemical & Biophysical Research Communications.* 1987;144(3):1143–1146.
36. Yuo A, Kitagawa S, Ohsaka A, Saito M, Takaku F. Stimulation and priming of human neutrophils by granulocyte colony-stimulating factor and granulocyte-macrophage colony-stimulating factor: qualitative and quantitative differences. *Biochemical & Biophysical Research Communications.* 1990;171(1):491–497.
37. Nathan CF. Respiratory burst in adherent human neutrophils: triggering by colony-stimulating factors CSF-GM and CSF-G. *Blood.* 1989;73(1):301–306.
38. Adachi S, Kubota M, Wakazono Y, et al. Mechanism of enhancement of neutrophil survival by granulocyte colony-stimulating factor and adenine. *Experimental Hematology.* 1993; 21(9):1213–1218.
39. Patton JH, Jr., Lyden SP, Ragsdale DN, Croce MA, Fabian TC, Proctor KG. Granulocyte colony-stimulating factor improves host defense to resuscitated shock and polymicrobial sepsis without provoking generalized neutrophil-mediated damage. *J Trauma.* May 1998;44(5):750–758; discussion 758–759.
40. Davis KA, Fabian TC, Ragsdale DN, Trentham LL, Croce MA, Proctor KG. Granulocyte colony-stimulating factor and neutrophil-related changes in local host defense during recovery from shock and intra-abdominal sepsis. *Surgery.* Aug. 1999;126(2):305–313.
41. Quezado ZM, Eichacker P. Q. Prophylactic granulocyte colony-stimulating factor in the critically ill: carefully balancing the benefits and risks. *Crit Care Med.* Sep. 2002;30(9):2162–2164.
42. Biffi WL, Moore EE, Moore FA, Peterson VM. Interleukin-6 in the injured patient. Marker of injury or mediator of inflammation? *Ann Surg.* Nov. 1996;224(5):647–664.

DISCUSSION

Dr. Joe H. Patton Jr. (Detroit, MI): I commend the authors on a nice study and an excellent presentation. Dr. Brundage and colleagues have presented us with a well-designed clinically relevant large animal study that mimics their previous work in rodents, and one that addresses an

interesting paradox: Is IL-6 a friend or foe in the inflammatory response to hemorrhagic shock and resuscitation?

Interleukin-6 is generally perceived to act as a proinflammatory mediator in response to shock and sepsis, as evidenced by a strong positive correlation between adverse clinical outcomes and increased levels of the cytokine. In fact, recent work from the authors' group using the same model has shown an increased production of endogenous IL-6 in the liver that is proportionate to the level and severity of shock. They concluded from this recent study that the increase in proinflammatory IL-6 contributed to dysfunctional inflammation and end organ damage. The current study, on the other hand, suggests a possible antiinflammatory role for exogenously administered IL-6 via a decrease in the endogenous production of the proinflammatory mediator G-CSF.

Unfortunately, through the data presented today, I am not convinced that the functional abrogation of the inflammatory response has occurred. As we showed in Dr. Fabian's laboratory, that exogenous G-CSF does not necessarily promote neutrophil-mediated damage in animals subjected to a two-hit hemorrhage-sepsis insult. Therefore, I believe some functional outcome data are required. Certainly, the conclusions of this paper would be strengthened with data regarding lung injury or the lack thereof. Do you have any BAL myeloperoxidase or histologic data to confirm your assumptions? I also am surprised not to see an increase in endogenous IL-6 production in your placebo group, as compared with the sham animals, because this did in fact occur in the liver, as demonstrated in your previous work.

Can you explain the discrepancy between the organ systems? Does it relate to the site of injury, perhaps exemplifying the distinction between autocrine and paracrine effects of the cytokine? Did you look at NF kappa B or stat 3 activities in the lung?

I appreciate the variability inherent in large animal models. This double-edged sword often confounds the data, but perhaps amplifies the clinical relevance of any positive conclusions. Regarding the model, did the fluid requirements of the various groups differ? If so, would the amount or type of resuscitation used affect the results?

You controlled for mean arterial pressure as an end point for your resuscitation. Therefore, it is not a surprise that this variable was not different among study groups. But did you look for any more subtle variables of systemic physiologic change between the study groups?

Also, regarding your model, a group in Sweden published data in 2002 showing that hypothermia itself may adversely affect the production of endogenous IL-6 in a pig model of hemorrhagic shock. Why did you include this variable in the development of the model?

I enjoyed the paper very much, and I look forward to further work from your group. I thank the Program Committee for the invitation to discuss this work and the Association for the privilege of the floor.

Dr. James Tyburski (Detroit, Michigan): We looked at IL-6 levels in patients from their pulmonary artery IL-6 levels

and arterial ones and found a gradient. That gradient was quite large when the patients' lungs were injured (i.e., the lungs themselves became the producer of IL-6 in these injured patients). I wonder if you could comment about the possibility of the lungs as a source in light of your findings when you gave the exogenous IL-6.

Dr. Michael A. West (Chicago, Illinois): This was a very nice presentation and an interesting model. I just have one very simple question. I am confused as to why, when you infused IL-6, you did not see a significantly higher level of IL-6 statistically in the serum if the IL-6 being infused was available to do something. I would have expected that you may have seen a measurable increase in IL-6 levels as a positive control.

Dr. Susan I. Brundage (Stanford, California, closing): Thanks, Dr. Patton, for your very thorough and thoughtful review. As discussed, this data represents only the cytokine expression.

We will be looking at MPO, histology, and protein expression, so that data will be forthcoming. In addition, the data on NF kappa B and stat 3 will be forthcoming.

Regarding the question about endogenous production of IL-6 in the liver versus the lung, the model published in the *Journal of Surgical Research* specifically looked at the liver. In this case, we have chosen to look at the lung because our interest was acute respiratory distress syndrome. We will be looking at the liver and the terminal ileum in the same animal model, so we will have that data.

About the resuscitation of the animals, we did resuscitate them with lactated Ringer's solution. There was no difference in the administration of fluid volumes.

Moreover, we did choose the animal model of hypothermia and coagulopathy. In our prior experiments, when we did not have that variable, the blood loss was approximately half. We wanted to have a more severe animal model of shock. That is why it is important to note that the sham group was an appropriate sham group because they did undergo the hypothermic and dilutional coagulopathy, yet they had nowhere near the proinflammatory cytokine expression observed in the uncontrolled hemorrhagic shock groups.

Dr. Tyburski, I think your question echoed by Dr. Patton is really the crux of our research. What is the role of IL-6? Perhaps at a paracrine level, IL-6 is functioning in an antiinflammatory manner. What happens is that our immune system gets overwhelmed and tries to overcompensate by producing IL-6. This is done systemically, and the systemic production of IL-6 really is a marker showing that IL-6 is not able to do its paracrine job initially.

Finally, Dr. West, that was a question of ours too. Why are serum IL-6 levels not different? I think probably what is occurring here is that the serum is measured 4 hours after injury, and at that point, the infusion has been diluted out by the amount of fluid the animals have received. They all have bled approximately 1 L, which is one third of their blood volume, and these differences at the end experiment probably reflect the short half-life of IL-6 in the serum. Thanks for your questions and your attention.

Trauma, 5th Edition, Edited by Ernest E. Moore, MD, David Feliciano, MD, and Kenneth L. Mattox, MD, McGraw-Hill, New York, 2004, 1469 pp, ISBN: 0-07-137069-2

In this, the fifth edition of *Trauma*, Moore, Feliciano, and Mattox have provided us with a comprehensive and updated text on the care and management of trauma victims. As has been the case for nearly 20 years of continuous publication, this is one text that should be on every trauma surgeon's bookshelf. As noted by Dr. Ben Eisenman in his forward, this book continues to be written by the Who's Who in trauma and provides a comprehensive reference source not only for the trauma practitioner, but also for all in surgery who may be called upon to treat trauma victims in the realities of the 21st century. The strengths of this edition are, as in previous editions, the depth and breadth of topics and information. As has become the trademark for this book, it is a comprehensive while user friendly and highly practical reference. The text remains divided into six primary areas, dealing with an overview of trauma, generalized approaches to the trauma patient, management of specific injuries, special problems, management of complications after trauma, and closes with a section on medical, legal, and legislative issues in trauma. A hallmark of *Trauma* is its attention to important details, and in this fifth edition the editors have streamlined their work with a reduction in the number of pages and a limiting of the chapter references. A feature that has been expanded in the current edition is the commentary on each of the chapters. In general these are well done and insightful and in many cases provide complimentary information or clarifying thoughts.

Important additions to this fifth edition are the combining of trauma system triage and transport into a single chapter, which logically links each of these areas where previously they were separated. The inclusion of a chapter on trauma outcomes is appropriate and necessary information for all who manage trauma care. The inclusion of a chapter on rural trauma is long overdue as a component of any standard text in trauma, and this one is particularly well done. The chapter on surgeon-performed ultrasound in trauma and in the surgical critical care unit is an excellent addition and well done by one of the leaders in this area. Combining the upper extremity and hand chapters into a single chapter is clearly done and provides thorough coverage of this area. A new chapter, titled *Surgical Care of Victims of Conflict*, is a timely addition to the text and provides a ready reference for those who might be called upon in a crisis to deal with such patients. The chapter *Genetic and Genomic Aspects of the Immunoinflammatory Response* is an excellent primer and nicely introduces the clinician to basic principles involved and analytical techniques used in the field of molecular biology. It provides information that the clinician needs to become familiar with as this area moves from the basic science laboratory to the patient's bedside. The chapter *The Convergence of Trauma, Medicine and the Law* is one that all need to read to understand the contexts in which our patients live and we work. This is further complimented by the editorial comment of the senior editor. Many chapters have new authors and substantially updated information. The chapter on *Kinematics of Trauma* has been completely redone and treats this area in a clear and concise manner. This is also the case for the chapter on indications for laparotomy.

The fifth edition of *Trauma* is certainly a worthwhile addition to anyone's library. It remains a comprehensive text that is clearly written, well illustrated, and includes significant new and important information. The thoughtful use of chapter editorial commentaries provides a balanced view of each of the topic areas and adds further value to this publication.

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