Bactericidal antibiotics temporarily increase inflammation and worsen acute kidney injury in experimental sepsis

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Objective: **To explore the relationships among bactericidal antimicrobial treatment of sepsis, inflammatory response, severity of acute kidney injury, and outcomes.**

Design: **Controlled laboratory experiment.**

Setting: **University laboratory.**

Interventions: **Sepsis was induced by cecal ligation and puncture in 52 rats and was treated with either bactericidal antibiotics (ampicillin/sulbactam) or placebo (saline). Serial blood specimens were obtained after cecal ligation and puncture for serum creatinine, interleukin-6, and neutrophil gelatinase-associated lipocalin concentrations. RIFLE (Risk, Injury, Failure, Loss, End-stage kidney disease) criteria were used to assess severity of acute kidney injury. All animals were observed for survival up to 1 wk. In a separate experiment, six healthy animals were given antibiotics and renal function was assessed. Another 12 animals were euthanized 2 days after laparotomy for kidney histology.**

Measurements and Main Results: **Survival in the placebo group was 50% compared with 81.8% in the antibiotic group ($p < .05$). Most animals (93%) without antibiotics developed acute kidney injury, of which 39% exhibited greater than a threefold rise in serum creatinine (RIFLE-F). Furthermore, survival decreased as acute kidney injury severity increased. Surprisingly, all antibiotic-treated animals developed acute kidney injury, of which 68.6% reached RIFLE-F. However, renal dysfunction was less persistent in these animals. Patterns of plasma interleukin-6 were similar to creatinine with higher concentrations seen earlier in antibiotic-treated animals but with faster resolution. Interleukin-6 concentration at 24 hrs was independently associated with the development of RIFLE-F. Histologic findings were consistent with functional parameters showing that antibiotics worsened acute kidney injury.**

Conclusion: **In polymicrobial sepsis, bactericidal antibiotics resulted in more inflammation and more severe acute kidney injury. However, resolution of inflammation and acute kidney injury was faster with antibiotics and correlated best with survival. These results suggest that transient worsening of renal function may be an expected consequence of sepsis therapy. These findings also question the value of peak severity of acute kidney injury as a primary end point and suggest that resolution of acute kidney injury may be more appropriate. (Crit Care Med 2012; 40:000–000)**

Key Words: acute kidney injury; acute renal failure; sepsis

Acute kidney injury (AKI) often complicates sepsis in critically ill patients (1, 2) and is also an important predictor for outcome even in noncritically ill patients (3). In general, sepsis and likelihood of death appear to correlate with severity of AKI (4, 5). However, observations in humans reflect both the degree of functional impairment as measured by serum creatinine as well as the severity of the underlying insult causing the impairment. It is assumed that measures that improve kidney function will reduce kidney injury or, conversely, therapies that worsen kidney function acutely must also increase kidney injury. These assumptions were analogous to the way heart failure was approached 20 yrs ago, because interventions for inhibition of cardiac contractility were contraindicated in congestive heart failure. The introduction of β blockers as a treatment for heart failure shattered the existing paradigm that only measures to increase cardiac function would be beneficial (6). Our current AKI paradigm is similarly constrained and we are evaluating treatments solely on the basis of the degree to which maximum functional impairment is affected. Under this paradigm, even therapies that shorten AKI duration but transiently result in worse function would be rejected.

Furthermore, the mechanisms of sepsis-induced AKI remain controversial (7–10). We have shown, in humans with community-acquired pneumonia, that AKI severity is associated with the degree of inflammatory response as measured by pro- and anti-inflammatory cytokine activation (11). If, in sepsis, AKI occurs as a result of the cytotoxic effects of cytokines and other inflammatory mediators, then experimental models of sepsis showing

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greater cytokine activation should result in worse AKI (12).

Bactericidal antibiotics are known to increase inflammation acutely by release of bacterial toxins (13, 14). However, timing and appropriateness of antibiotics have also been shown to influence outcome in humans with sepsis (15) and are recommended as critical components of sepsis care bundles (15, 16). We hypothesized that bactericidal antibiotics would result in increased inflammation and worse kidney function while still leading to improved survival and ultimately better organ function. This question is important because it asks whether AKI therapies can be solely evaluated on the basis of maximum functional impairment.

MATERIALS AND METHODS

Experimental Protocol. After approval by the Animal Care and Use Committee of the University of Pittsburgh, we anesthetized 52 adult (24–28 wks old, weight 400–600 g), healthy, male, Sprague-Dawley rats with intraperitoneal injection of pentobarbital sodium (40 mg/kg). Cecal ligation and puncture (CLP) was performed with a predetermined 25% ligated length of cecum and 20-gauge needle: two punctures inferior to the ileocecal valve. This protocol is associated with a mortality of approximately 50% to 60% at day 7 (17). The abdomen was closed and 20 mL/kg lactated Ringer's solution was given subcutaneously for resuscitation. Topical anesthetic was applied to the surgical wound and rats were returned back to their cages and allowed food and water *ad libitum*.

Eighteen hrs after CLP, animals were returned to the laboratory and assigned to either: group 1 (n = 22) given ampicillin/sulbactam (125 mg/kg every 12 hrs) starting 18 hrs after CLP and continued for 3 days; or group 2 (n = 30), which were given saline injections as a placebo. A jugular vein catheter was also placed to draw blood and survival time was assessed up to 7 days. To exclude any possible effects of antibiotics on measures of renal function, we gave the same dose and courses of therapy to another six healthy (laparotomy but no CLP) animals as a control and obtained the same measurements. Another 12 animals (four CLP, four CLP + antibiotics, and four healthy + antibiotics) were euthanized 2 days after laparotomy for kidney histology.

Measurements and Calculations. Blood (0.8 mL) was drawn from a central venous catheter at 18 hrs, 24 hrs, 48 hrs, 72 hrs, 5 days, and 7 days after CLP. Similar time points were obtained for healthy control animals. To ensure that the maximum blood loss for each animal was controlled below the 20% total blood volume, we staggered sample collections so that only four time points were obtained in each animal. Therefore, data that were not collected for serum creatinine (Cr) were imputed by averaging the values before and after the missing value. The isolated plasma was kept at −80°C for subsequent interleukin (IL)-6, neutrophil gelatinase-associated lipocalin (NGAL), and Cr measurements. Survival time was recorded in days starting from CLP.

Plasma IL-6 was measured with an enzyme-linked immunosorbent assay (R & D Systems, Minneapolis, MN). Plasma NGAL was determined using an enzyme-linked immunosorbent assay (BioPorto Diagnostics, Gentofte, Denmark). Plasma Cr was detected with a Cr enzymatic assay kit (BioVision Technologies, Mountain View, CA). The severity of AKI was assessed using the serum creatinine portion of the RIFLE (Risk, Injury, Failure, Loss, End-stage kidney disease) criteria (18), which classified risk, injury, and failure on the basis of maximum Cr increase of 150%, 200%, and 300%, respectively, over the 7 days after CLP.

Evaluation of Kidney Histology. Rat kidneys were fixed in 10% neutral-buffered formalin, dehydrated in graded anhydrous absolute ethanol, and embedded in paraffin. Histologic sections (5 μm) of kidney were stained with hematoxylin–eosin and periodic acid Schiff. We considered the morphologic changes indicating acute tubular necrosis as the loss of brush border, the vacuolization of tubular epithelial cells, and the presence of intratubular debris.

To explore if the inflammatory response produces organ-specific effects, we also evaluated liver injury with alanine aminotransferase. Alanine aminotransferase was determined using a lactic dehydrogenase–nicotinamide adenine dinucleoacid Hydrogen coupled assay (Pointe Scientific Inc., Canton, MI) from 20 animals (13 from the antibiotics-treated group and seven from the placebo group).

Statistical Analysis. Descriptive data were expressed as means ± SE. The analysis of variance and unpaired Student's *t* test were applied to compare the normally distributed variables within and between groups. Mann-Whitney *U* test was used to compare the non-normally distributed data. Categorical variables were expressed as proportions and compared using the chi-square test. The association between plasma IL-6 and severity of AKI was examined with logistic regression analysis. The survival analysis was assessed by Kaplan-Meier statistics and compared using log rank test. A two-sided $p < .05$ was considered statistically significant.

RESULTS

Antibiotics and AKI. Although antibiotics significantly improved 7-day survival compared with placebo (81.8% vs. 50% $p = .01$; Fig. 1), antibiotics did not improve kidney function acutely. Most animals (93%) not receiving antibiotics and all animals treated with antibiotics developed AKI. However, maximum functional impairment was greater in animals receiving antibiotics. Of animals developing AKI, 68.6% of antibiotic-treated animals and 39% of placebo-treated animals reached RIFLE-F (Fig. 2) ($p < .05$). None of the healthy animals that received antibiotics developed AKI. Figure 3 shows the kidney histology using hematoxylin–eosin and periodic acid Schiff stains under light microscopy with original magnification of ×400. Loss of brush border was evident as was mild dilation of the tubular lumen 48 hrs after CLP. Vacuolization was seen after CLP in almost all tubule cells (Fig. 3*A*). These pathologic changes were even worse at 48 hrs in the

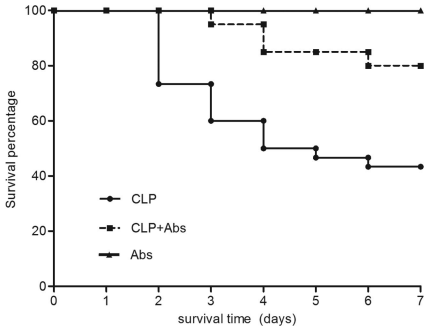

Figure 1. Kaplan-Meier survival plots for all animals. Cecal ligation and puncture (*CLP*) (n = 30), animals with cecal ligation and puncture treated with saline; *CLP + Abs* (n = 22), animals with cecal ligation and puncture treated with antibiotics; antibiotics (*Abs*) (n = 6): animals without cecal ligation and puncture treated with antibiotics; $p < .05$, CLP vs. CLP + Abs.

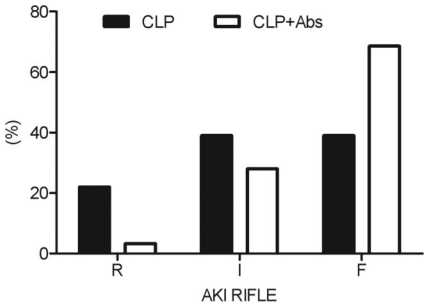

Figure 2. Distribution of severity of acute kidney injury. RIFLE categories, *R, I, F*, acute kidney injury RIFLE classes Risk, Injury, and Failure. Cecal ligation and puncture (*CLP*) (n = 30): animals with cecal ligation and puncture treated with saline; *CLP + Abs* (n = 22): animals with cecal ligation and puncture treated with antibiotics (*Abs*); $p < .05$, CLP vs. CLP + Abs regarding RIFLE-F. *AKI*, Acute kidney injury; *RIFLE*, Risk, Injury, Failure, Loss, End-stage kidney disease.

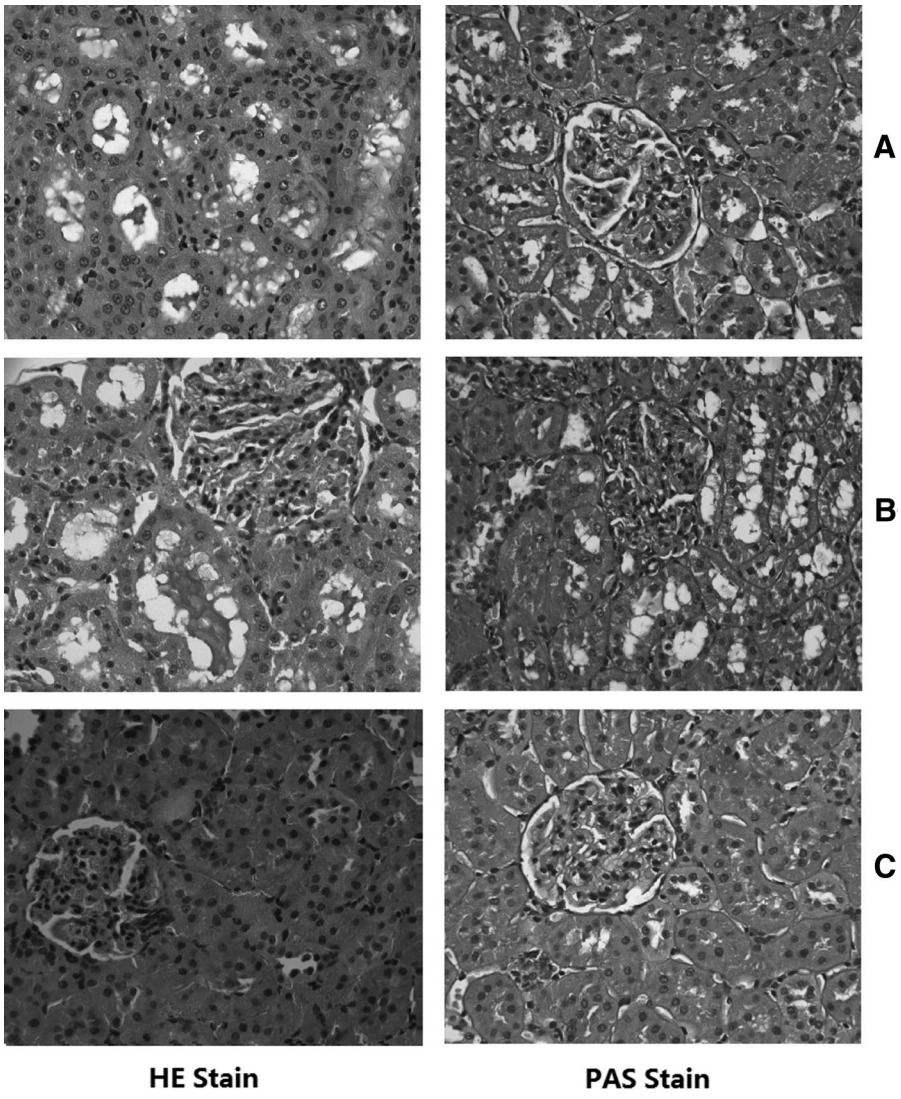

Figure 3. Kidney histology after 48 hrs of laparotomy. Histologic sections (5 μm) of kidney were stained with hematoxylin–eosin (*HE*) and periodic acid Schiff (*PAS*). *A*, *CLP*: animals with cecal ligation and puncture treated with saline; (*B*) *CLP + Abs*: animals with cecal ligation and puncture treated with antibiotics; (*C*) *Abs*: animals without cecal ligation and puncture treated with antibiotics.

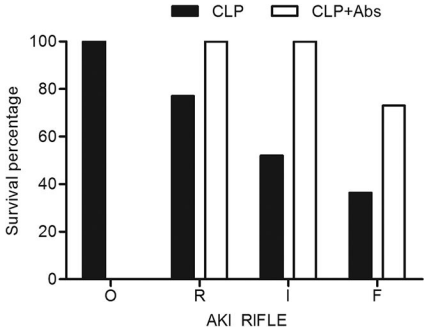

Figure 4. Survival of animals by severity of acute kidney injury (RIFLE class). No acute kidney injury, *O*. Acute kidney injury RIFLE classes R, I, and F. *CLP*, Cecal ligation and puncture; *Abs*, antibiotics; *AKI*, acute kidney injury; *RIFLE*, Risk, Injury, Failure, Loss, End-stage kidney disease.

antibiotic-treated CLP animals (Fig. 3*B*). There was no obvious kidney injury in healthy animals treated with antibiotics (Fig. 3*C*).

Outcomes by Severity of AKI. When we compared the survival rates and severity of AKI, we found that mortality increased with increasing severity of AKI (Fig. 4). However, 73% of animals with RIFLE-F died in the placebo group compared with only 36.4% in the antibiotic-treated group. When we compared the Cr concentration over time between the survivors and nonsurvivors, we found that the Cr concentration increased with time in the nonsurvivors of both groups, whereas in all the survivors, the Cr concentration increased temporarily and then gradually recovered (Fig. 5).

Biomarker Patterns With Time. Figure 6*A* shows the relationship between inflammatory response as measured by IL-6 over time and by group. IL-6 levels were not different before antibiotics or placebo in septic animals, but in both groups, levels were significantly increased compared to healthy control animals at 18 hrs. At 24 hrs (6 hrs after antibiotics were given), IL-6 levels peaked in the antibiotic-treated group and were significantly greater than placebo-treated animals (370.80 vs. 561.46 pg/mL, $p < .05$). Although inflammation resolved quickly in antibiotic-treated animals, it persisted in placebo-treated animals such that the IL-6 levels were much greater in this group starting at 72 hrs (336.79 vs. 133.76 pg/mL, $p < .05$) persisting throughout the 7 days.

Changes in plasma Cr (Fig. 6*B*) over time were similar to those of IL-6 but increased more slowly. Serum Cr peaked at approximately 48 hrs in the antibiotic-treated CLP animals and then recovered gradually; however, it increased more slowly and remained elevated longer in placebo-treated animals. The peak serum creatinine concentration was observed 48–72 hrs after peak IL-6 concentrations, suggesting a temporal relationship between inflammation and onset of AKI. At 7 days, the Cr in the antibiotic-treated group was significantly lower than that of the placebo group (0.38 vs. 0.62 mg/dL, $p < .05$). There was no significant change in Cr with time in healthy animals treated with antibiotics.

Plasma NGAL increased with time in all groups as shown in Figure 6*C*. Eighteen hrs after CLP, plasma NGAL had increased nearly tenfold compared with healthy animals, reaching a peak (approximately 15-fold) at 24 hrs, and remained significantly elevated for the entire week. Although NGAL from antibiotic-treated animals gradually decreased after 48 hrs, pNGAL concentrations remained significantly elevated in placebo-treated animals beginning at 96 hrs and up to 7 days (Fig. 6*C*, $p < .05$).

Association Among IL-6, AKI Severity, and Outcome. In logistic regression analysis, the plasma IL-6 concentration at 24 hrs of CLP was independently associated with AKI severity (RIFLE-F vs. others). The odds ratio was 1.28 (95% confidential interval, 1.09–1.89, $p < .05$). To determine the contributions of IL-6 to the predictive model for AKI severity, the areas under the receiver operating characteristic curve were measured for the lo-

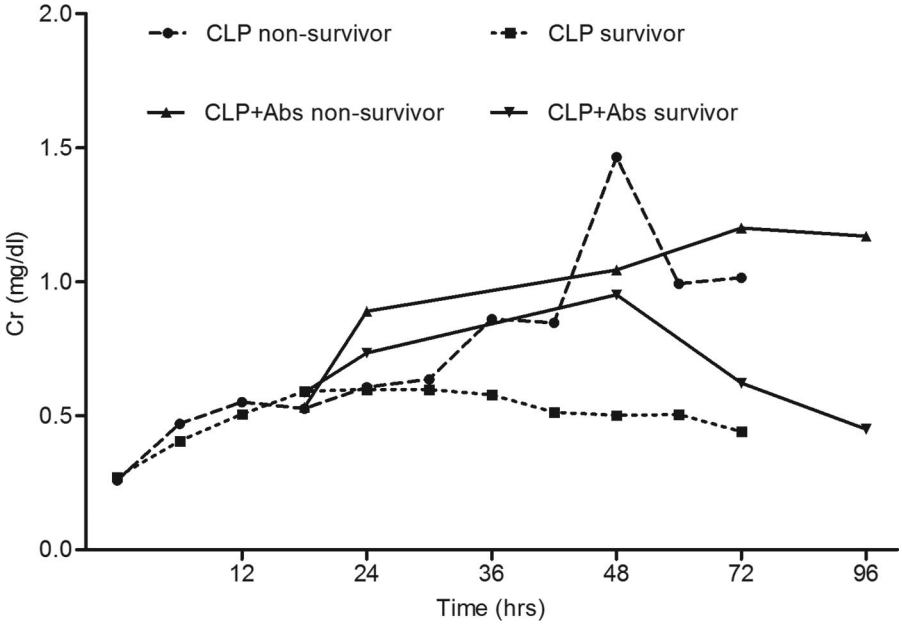

Figure 5. Serum creatinine patterns over time for survivors and nonsurvivors in two groups (mean, mg/dL). *CLP*, Cecal ligation and puncture; *Abs*, antibiotics; *Cr*, creatinine.

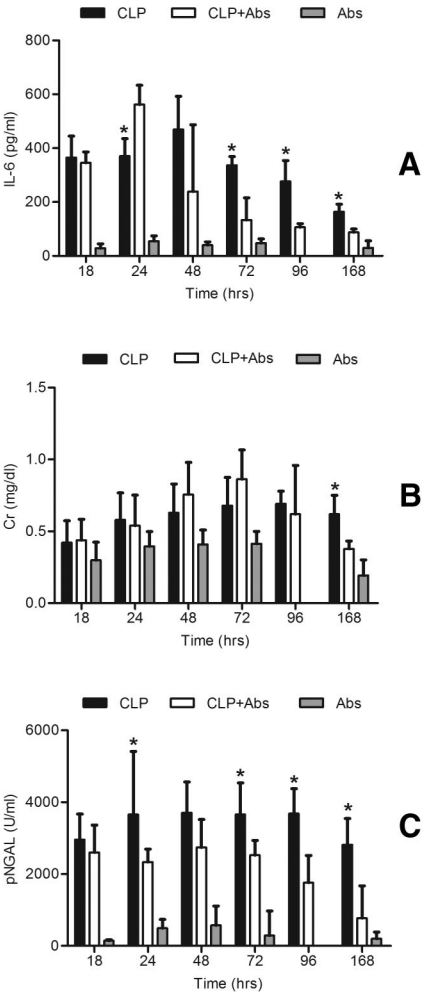

Figure 6. Interleukin-6, plasma creatinine, and neutrophil gelatinase-associated lipocalin changes with time (mean ± SE). *CLP* (n = 30): animals with cecal ligation and puncture treated with saline; *CLP + Abs* (n = 22): animals with cecal ligation and puncture treated with antibiotics; *Abs* (n = 6): animals without cecal ligation and puncture treated with antibiotics; *$p < .05$, CLP vs. CLP + Abs. *A,* Interleukin-6 (pg/mL); (*B*) creatinine (mg/dL); (*C*) neutrophil gelatinase-associated lipocalin (IU/mL). *CLP*, Cecal ligation and puncture; *Abs*, antibiotics; *IL-6*, interleukin-6; *Cr*, creatinine; *pNGAL*, neutrophil gelatinase-associated lipocalin.

gistic regression model based on the IL-6 concentration at 24 hrs after CLP. The area under the receiver operating characteristic curve was 0.79 (95% confidential interval, 0.61–0.91, $p < .05$). The median IL-6 concentration at 24 hrs after CLP in RIFLE-R, RIFLE-I, and RIFLE-F were 270.75, 414.24, and 563.62 pg/mL, respectively ($p < .05$, RIFLE-F vs. RIFLE-I; RIFLE-F vs. RIFLE-R; Fig. 7*A*). In all survivors, the median IL-6 levels decreased rapidly with time, whereas the levels were still high with time in all nonsurvivors (Fig. 7*B*).

Association Between IL-6 and Liver Injury. To explore if the inflammatory response produces organ-specific effects, we analyzed the relationship between median IL-6 levels and median alanine aminotransferase levels. Figure 8 demonstrated that changes of alanine aminotransferase (Fig. 8*A*) and IL-6 (Fig. 8*B*) with time were consistent.

DISCUSSION

The main finding of this study was that although treatment with bactericidal antibiotics improved survival in CLP-induced sepsis, it neither prevented AKI nor limited its severity as measured by maximum RIFLE stage using serum creatinine. Indeed, AKI severity was actually greater in antibiotic-treated animals, although the duration was attenuated. Although this finding seems paradoxic, it is consistent with observations in humans. First, it has been known for many years that bactericidal antibiotics can release toxins as they kill bacteria causing inflammation and clinical symptoms (e.g., fever, chills). The classic Jarisch-Herxheimer reaction has been described with spirochetes, but similar reactions have been described with multiple other infections (13) and are probably quite common in sepsis (14). The downstream mechanism appears to be the activation of inflammatory cytokines, especially IL-6, IL-8, and tumor necrosis factor (19). In our study, we chose to measure only one cytokine to minimize blood loss and chose IL-6 as the representative cytokine (20, 21); and we found that IL-6 increased early after antibiotics. We chose ampicillin/sulbactam in our study because it is active against a wide range of bacterial groups and used in various infections, including intra-abdominal, skin, lower respiratory tract, and gynecologic infections. It is also a commonly used bactericidal antibiotic. The "spike" in IL-6 induced by antimicrobials (ampicillin/sulbactam) was most likely related to rapid bacterial cell death resulting in endotoxin release (22, 23), although it is also known that antimicrobials have immunomodulating properties (24) and macrophage activation with IL-6 release may also be a "side effect" of antimicrobials (25, 26).

Second, we have recently shown a strong correlation between IL-6 expression and AKI severity in sepsis-induced AKI in humans (11). It is therefore not surprising that an increased acute inflammatory response should result in worse renal function. Although the mechanistic link between cytokine activation and AKI is not well understood, emerging evidence suggests that the inflammatory milieu may lead to renal cell dysfunction in a variety of ways (27). However, this inflammatory response is not organ-specific, because we also showed the consistent liver injury occurred with the increased IL-6. Finally,

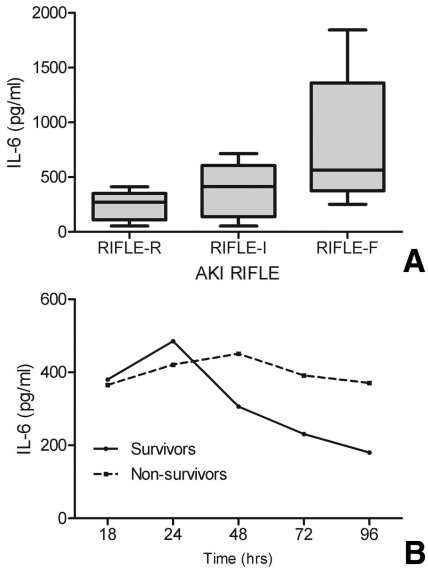

Figure 7. Relationship among interleukin-6 levels, acute kidney injury severity, and outcome. *A*, Box plot summaries of interleukin-6 level at 24 hrs of cecal ligation and puncture in those with acute kidney injury RIFLE-R, RIFLE-I, and RIFLE-F (pg/mL). $p < .05$, RIFLE-F vs. RIFLE-I, RIFLE-F vs. RIFLE-R. *B*, Median interleukin-6 (pg/mL) changes with time in all survivors and nonsurvivors. *IL-6*, Interleukin-6; *RIFLE*, Risk, Injury, Failure, Loss, End-stage kidney disease; *AKI*, acute kidney injury.

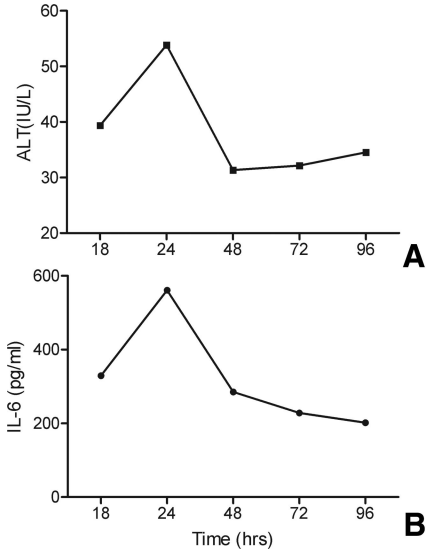

Figure 8. Relationship between interleukin-6 and liver injury. Median interleukin-6 (*IL-6*; pg/mL) changes with time in 20 rats (13 from the antibiotics-treated group and seven from the placebo group). *A*, Median alanine aminotransferase (*ALT*; IU/L) changes with time in 20 rats (13 from the antibiotics-treated group and seven from the placebo group).

the duration of AKI appears to be a predictor of long-term outcome in patients with AKI. A study by Coca and colleagues (28) reported that in diabetics with postoperative AKI the duration of renal impairment was independently associated with decreased survival and recovery. Our results are similar in many ways. As shown in Figure 6, animals treated with antibiotics had a shorter duration of AKI by both RIFLE (serum Cr) and plasma NGAL. Indeed, animals not receiving antibiotics had still not completely recovered by day 7. As shown in Figure 5, surviving animals exhibited evidence of renal recovery relatively early (by 36–48 hrs). Animals that did not resolve AKI early did not survive. Of course, unlike the clinical situation, our study design would have accentuated this effect because we did not provide renal replacement therapy. Nevertheless, our results show clear evidence that a shorter duration of AKI is associated with survival as was seen in the study by Coca et al (28).

Our results are also consistent with the vast majority of epidemiologic studies that found a relationship between severity of AKI and survival. In our study, like in clinical studies (1, 2), survival decreased as severity of AKI increased. However, our study was also able to extend these prior results because we could specifically control for resolution of infection. Our antibiotic-treated animals represent early and effective control of infection, whereas nontreated animals represent the extreme case of inadequate control. In this way, our results demonstrate the importance of adequate treatment of infection (appropriate antibiotics and source control) for the successful resolution (although not prevention) of AKI. These findings have potentially important implications for future studies of therapies for AKI, especially regarding end points. Like antibiotics, a drug might be effective in limiting the course of AKI and hence improving outcome while simultaneously worsening renal function temporarily.

The results of our study also lend support to the concept that plasma NGAL is an early predictor for AKI (29). We found that NGAL increased before Cr in animals developing AKI. However, given that most of our animals developed AKI, it is not possible to adequately test the discrimination of this biomarker. Importantly, NGAL levels began to decline after 48 hrs in the antimicrobial-treated animals, whereas they remained elevated in the placebo group. Even at day 7, there was still significantly increased plasma NGAL in placebo-treated animals, whereas this biomarker had nearly normalized in the antimicrobial-treated group. To the extent that NGAL represents kidney damage, placebo-treated animals continued to manifest damage 1 wk after CLP. Finally, although the onset of NGAL activation in the plasma appeared similar to that of IL-6, NGAL remained elevated in placebo-treated animals, as did Cr, even after IL-6 began to normalize. This suggests that NGAL may be a useful marker for resolution of AKI because we have recently shown in critically ill patients with sepsis-induced AKI (30).

Another interesting finding is that there was a small increase of NGAL without concomitant increase of IL-6 or Cr after antibiotic injection in the healthy animals. To better understand the mechanisms of this effect, we injected saline into four healthy animals and obtained similar results (data not shown). Thus, it appears that this very small increase in plasma NGAL was simply the result of the overall stress reaction. NGAL is also synthesized in peritoneal mesothelial cells and is induced by the peritoneal and gut damage (31). Released by neutrophils on activation, NGAL is also a marker of bacterial infection and systemic inflammation (32, 33).

There are important limitations to this study. First, this study was conducted in CLP-induced septic rats. These animals only received antimicrobials and limited supportive care (fluid resuscitation). Surgical source control of infection, inotropes/vasopressors, mechanical ventilation, and renal replacement therapies were not applied to these animals. Thus, the course of sepsis and AKI may not be synonymous with the clinical scenario in humans. Second, we did not collect urine; therefore, we lack urine output data to integrate into the RIFLE categories or to examine urine biomarkers. These factors may have contributed to a misclassification of some cases of AKI and may have influenced risk estimates of AKI. Urine NGAL may be a more specific marker of AKI compared with plasma NGAL (34). We studied only one time point (18 hrs after CLP) for initiation of antibiotic therapy because this time point has been shown to correspond to the time when symptoms are fully manifest and therefore likely (35) simulates when a patient would seek medical attention. Furthermore, the concentrations of most mediators reach peek levels between 18 and 24 hrs (17, 36). We did not measure plasma endotoxin levels and furthermore, we tested only one of many possible an-

timicrobial treatments, and the antibiotic-induced endotoxin release may be dependent on the class of antibiotics used. For instance, a study by Vianna et al (37) demonstrated in a similar model of sepsis that at 6 hrs after CLP imipenem-treated animals showed endotoxin plasma concentrations higher than those observed in animals treated with ciprofloxacin plus clindamycin or the animals that received no antibiotic treatment.

In summary, this study demonstrates that bactericidal antibiotics transiently increase inflammation and do not prevent AKI, although they significantly improved outcome. Resolution of inflammation and AKI, however, were more common and faster in animals receiving antibiotics. The induction of AKI during sepsis is strongly correlated and temporally related to activation of inflammatory mediators. Mortality was associated with failure to recover from, rather than development of, AKI in this sepsis model. Thus, these results question the value of peak severity of AKI as a primary end point and suggest that resolution of AKI may be more appropriate.

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Since this article does not have an abstract, we have provided the first 150 words of the full text.

KEYWORDS: aminoglycosides, endotoxins, gentamicins.

Since the start of 1998, FDA and CDC have received approximately 130 reports of endotoxin-like reactions—characterized by fever, rigors, and/or hypotension—following intravenous administration of two brands of gentamicin sulfate. About 70 of the reported cases were temporally associated with once-daily dosage of one product that was voluntarily withdrawn from the market in December. The other product, also voluntarily withdrawn, was associated with more than 60 endotoxin-like reactions reported this year. FDA is investigating these cases.

A number of injectable and parenteral drug products, including gentamicin and other aminoglycosides, contain endotoxin at levels that do not cause symptoms at the approved dosage. For gentamicin, this level is 1.7 endotoxin units per milligram. However, aminoglycosides often are administered in one 24-hour dose to minimize renal toxicity and simplify dosing instead of in the three doses described in the labeling. A published survey in 1995 indicated that once-daily was the preferred regimen for …

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