The Duration of Hypotension before the Initiation of Antibiotic Treatment Is a Critical Determinant of Survival in a Murine Model of Escherichia coli Septic Shock: Association with Serum Lactate and Inflammatory Cytokine Levels

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Background. This study was designed to examine the relationship between the timing of antibiotic treatment and both survival rates and hemodynamic/inflammatory correlates of survival in a murine model of Escherichia coli septic shock.

Methods. Surgical implantation of an E. coli (O18:K1:H7)–laced, gelatin capsule–encased fibrinogen clot was used to generate a bacteremic model of murine septic shock. Survival duration, hemodynamic responses, and circulating serum tumor necrosis factor (TNF)–α, interleukin (IL)–6, and lactate levels were assessed in relation to increasing delays in or absence of antibiotic treatment.

Results. A critical inflection point with respect to survival occurred between 12 and 15 h after implantation. When initiated at or before 12 h, antibiotic treatment resulted in ≤20% mortality, but, when initiated at or after 15 h, it resulted in >85% mortality. Physiologically relevant hypotension developed in untreated septic mice by 12 h after implantation. Values for heart rate differed between untreated septic mice and sham-infected control mice by 6 h after implantation, whereas values for cardiac output and stroke volume did not differ until at least 18–24 h after implantation. Antibiotic treatment initiated ≥12 h after implantation was associated with persistence of increased circulating serum lactate, TNF-α, and IL-6 levels.

Conclusions. The timing of antibiotic treatment relative to hypotension is closely associated with survival in this murine model of septic shock. Delay in antibiotic treatment results in the persistence of inflammatory/stress markers even after antibiotic treatment is initiated.

Although substantial effort has been expended in the development of broad-spectrum and powerful antibiotics, many basic questions regarding antibiotic treatment in septic shock have not been effectively addressed. Key among these is the potential importance of the rapidity of effective antimicrobial administration relative to the deterioration of key hemodynamic variables during the development of septic shock. Although it is known that appropriate antibiosis is a cornerstone of effective therapy for life-threatening bacterial infections [1, 2], questions about how rapidly such antibiotics must be administered and what hemodynamic responses to infection signal critical inflection points in mortality have not been addressed, to our knowledge, in animal models or in human disease.

To address this issue, a murine model of septic shock induced by the implantation of an Escherichia coli–impregnated fibrinogen clot was developed to mimic human disease. This model was used to examine the effect...
that the timing of the initiation of antibiotic treatment has relative to hemodynamic/cardiovascular perturbations, as assessed by tail plethysmography for blood pressure and by Doppler echocardiography for heart rate, stroke volume, and cardiac output. In addition, the effect that antibiotic treatment initiated at varying time points has on serum levels of lactate and the proinflammatory cytokines tumor necrosis factor (TNF)-α and interleukin (IL)-6 was assessed. The specific hypothesis was that the duration of hypotension before the initiation of antibiotic treatment would be the physiologic variable most closely correlated with mortality. Specifically, we anticipated that there would be a rapid upward inflection in the mortality risk coincident with the onset of hypotension consistent with early septic shock.

**MATERIALS AND METHODS**

**Murine model.** This study was approved by the Animal Use Committee of Rush University and conformed to the National Institutes of Health guidelines for animal experimentation. Male 129SV mice (16–18 weeks old and ~25 g in weight; Jackson Laboratories) were anesthetized intramuscularly with 100 mg/kg ketamine and 5 mg/kg acepromazine, and a gelatin capsule-encased 4% bovine fibrinogen clot containing ~1000 cfu of *E. coli* strain O18K:1:H7 [3] was surgically implanted into each abdominal cavity. Identical sterile capsules were implanted in control mice. Immediately after implantation, mice were resuscitated with 1 mL of prewarmed saline injected subcutaneously, and then they were injected with 0.5 mL every 6 h thereafter. In the absence of antibiotic treatment, this model yields 100% mortality in 24–60 h.

**Mortality studies.** Mice were administered 30 mg/kg ampicillin subcutaneously at 6-h intervals starting 0, 6, 12, 15, 18, or 24 h (n = 10–15 mice/group) after implantation. One group of mice (n = 15) did not receive antibiotic treatment. Mice were followed for 96 h to determine survival rates.

**Hemodynamic studies.** Mean blood pressures and heart rate were noninvasively assessed before mice underwent anesthesia, after the administration of anesthesia, immediately after implantation, then every 3 h for 24 h, and then every 6 h until death or the end of the study (96 h after implantation). In addition, mice underwent echocardiographic assessment to determine stroke volume and cardiac output after the administration of anesthesia; at 6, 9, and 12 h after implantation; and then every 6 h until death or the end of the study. Mice were maintained under constant light ketamine/acepromazine anesthesia.

Systolic, diastolic, and mean arterial pressures, as well as heart rate, were measured noninvasively using volume pressure recording, a tail cuff method that measures tail blood volume (XPB1000 device and DasyLab for Windows; version 8; Kent Scientific) [4]. Echocardiographic assessment was performed using a Series 5500 machine (Hewlett-Packard) [5, 6].

**Blood bacterial, lipopolysaccharide (LPS), cytokine, and lactate studies.** Quantitative blood cultures were assessed using a standard serial dilution technique. Serum lactate levels were assessed using a spectrophotometric assay (DU-64; Beckman), in accordance with the instructions of the assay manufacturer (Sigma Biodiagnostics), to detect the conversion to pyruvate and H₂O₂ by lactate oxidase (absorbance was measured at 540 nm). Serum LPS levels were assayed using an automated quantitative limulus amebocyte lysate assay (Associates of Cape Cod) [7]. Serum TNF-α and IL-6 levels were assessed using a commercially available EIA in accordance with the instructions of the manufacturer (R&D Systems).

**Statistical analysis.** Survival rates were examined by log-rank analysis, except where otherwise specified. Sequential hemodynamic variables, blood bacterial counts, and levels of serum LPS, lactate, and cytokines were assessed using repeated-measures analysis of variance, with comparison of individual points by the least-squares method. All statistical tests were performed as 2-tailed assessments.

**RESULTS**

**Mortality studies.** The survival rate in this murine model of *E. coli* septic shock was clearly dependent on the rapidity with which antibiotic treatment was initiated, with stepwise increased mortality observed with each delay in the time line (P < .0001, log-rank analysis). The critical inflection point for increased mortality was between 12 and 15 h after implantation (figure 1). In mice receiving antibiotic treatment 12 h after implantation and those receiving treatment 15 h after implantation, the 96-h survival rate was 80% and 13.3%, respectively. In mice receiving antibiotic treatment 0, 6, or 12 h after implantation, the 96-h and cumulative survival rates did not differ significantly from one another (P > .3 for all), and all were significantly higher than those in mice receiving antibiotic treatment 15 h after implantation (P < .0005 for all). The survival rate in mice receiving antibiotic treatment 18 h after implantation was significantly lower than that in mice receiving antibiotic treatment earlier, including those receiving it 15 h after implantation (P = .0012). Although 96-h mortality was 100% in both groups, the cumulative survival duration in mice receiving antibiotic treatment 18 h after implantation was marginally longer than that in untreated septic mice (P = .0456). In addition to the differences it caused in total survival and cumulative survival duration, earlier antibiotic treatment also delayed the onset of mortality in each group in a stepwise manner (P < .0001, Wilcoxon analysis) (figure 1).

**Hemodynamic studies.** Heart rates were initially low, compared with published normal values, for mice in all groups, likely because of the anesthesia that was required for implantation (figure 2A). During the course of the study, heart rates in untreated septic mice were marginally increased, compared with...
groups included untreated septic mice ( ) and mice implanted with an identical sterile capsule (sham infected; ).

Control was stable throughout the study period (figure 2). The mean arterial pressure in septic mice did not decrease to a level consistent with shock by 9 h after implantation. However, the mean arterial pressure in sham-infected control mice (and from baseline values) continued to diverge until all septic mice died, by 48–54 h after implantation.

Similarly, cardiac output in sham-infected control mice were not significantly lower than baseline values (measured before implantation) until 24 h after implantation (P < .005). Stroke volumes in sham-infected control mice increased transiently for 18–48 h after implantation. During the same period, there was a marked decrease in stroke volumes in septic mice. Similarly, cardiac output in sham-infected control mice remained relatively stable throughout the study (figure 2C). Septic mice exhibited a decrease in cardiac output, despite fluid resuscitation. The first significant difference occurred 24 h after implantation, and cardiac output in the 2 groups continued to diverge until all septic mice died, by 48–54 h after implantation.

The mean arterial pressure in sham-infected control mice was stable throughout the study period (figure 2D). The mean arterial pressure in septic mice began to statistically differ from that in sham-infected control mice (and from baseline values) by 9 h after implantation. However, the mean arterial pressure in septic mice did not decrease to a level consistent with shock (<65 mm Hg) until 12 h after implantation. The mean arterial pressure in septic mice continued to decrease between ~24 and 52 h after implantation, until all mice died.

**Serum lactate levels.** In all mice, serum lactate levels were found to be within the normal range (<2 mmol/L) immediately and 6 h after implantation. Lactate levels were also normal in sham-infected control mice at all other time points. A trend toward increased lactate levels (4/6 samples with lactate levels >2.0 mmol/L), coincident with the onset of hypotension and consistent with early septic shock, was noted in septic mice at 12 h after implantation (mean ± SE, 2.18 ± 0.17 vs. 1.68 ± 0.09 mmol/L for sham-infected control mice at the same 12-h time point [P = .074] and 1.63 ± 0.15 mmol/L for septic mice immediately after implantation [P = .078]) (figure 3A). Between 12 and 24 h after implantation, significant and sequentially increasing lactate levels were noted in septic mice. In the separate set of experiments depicted in figure 4A, pretreatment lactate levels at 12 h after implantation were significantly increased, compared with baseline levels (1.45 ± 0.22 vs. 2.55 ± 0.21 mmol/L; P = .0028; baseline data not shown). Pretreatment lactate levels at 18 h after implantation were similarly increased.

In mice receiving antibiotic treatment 6 h after implantation, lactate levels were normal at baseline and did not increase significantly after treatment (figure 4A). The slightly increased lactate levels observed in mice receiving antibiotic treatment 12 h after implantation decreased to normal levels by 6 h after the treatment. However, in mice receiving antibiotic treatment 18 h after implantation, the markedly increased lactate levels continued to increase during the next 6 h after treatment (figure 4A).

**Blood bacterial counts and serum LPS levels.** To examine other factors potentially associated with a sharp inflection in mortality, bacterial blood counts and serum LPS levels were assessed. A stepwise increase in blood bacterial counts was noted (table 1). In septic mice, there was a 20-fold increase in bacterial counts between 6 and 12 h and an additional 14-fold increase between 12 and 18 h after implantation (n = 8 mice/group). Blood cultures performed 6 h after the initiation of antibiotic treatment consistently demonstrated ~2–3 log-fold decreases in bacterial counts. Assessment of blood cultures performed immediately after mice died consistently failed to detect E. coli if the survival duration was at least 30 h.

LPS levels were at the lower limit of detection in all mice before implantation (figure 5). Sham-infected control mice also had minimal LPS levels at baseline and at 6-h intervals until 24 h after implantation (data not shown). LPS levels in septic mice receiving antibiotics 0 h after implantation also remained at the lower limit of detection to at least 6 h after implantation (figure 5). In all other septic mice (antibiotic treatment initiated 6, 12, or 18 h after implantation), LPS levels obtained immediately before the initiation of antibiotic treatment were ele-
Measurements were taken of heart rate (HR; A), stroke volume (SV; B), cardiac output (CO; C), and mean arterial pressure (MAP; D). Group comparisons were made using repeated-measures analysis of variance; point comparisons were made using the least-squares method. * , vs. sham-infected control mice at the equivalent time point; † , vs. sham-infected control mice at the equivalent time point. Error bars indicate SEs.

In mice receiving antibiotic treatment 6, 12, or 18 h after implantation, TNF-α levels were increased before the initiation of antibiotic treatment, compared with baseline levels (P < .001) (figure 4B). There were significant intergroup differences in TNF-α levels (P < .0001 overall): mice receiving antibiotic treatment 18 h after implantation had significantly higher TNF-α levels than did mice receiving it 6 h after implantation (P < .01). Different responses were noted in cytokine levels after antibiotic treatment, and these differences were dependent on when the treatment was initiated. At 3 h after the initiation of antibiotic treatment, there were modest increases (P < .05) in TNF-α levels in mice receiving treatment 6 or 12 h after implantation but no change in the levels in mice receiving treatment 18 h after implantation. At 6 h after the initiation of antibiotic treatment, TNF-α levels were significantly decreased in mice receiving treatment 6 or 12 h after implantation, but levels in mice receiving treatment 18 h after implantation were substantially unchanged. At 6 h after the initiation of antibiotic treatment, TNF-α levels were substantially higher in mice receiving antibiotic treatment 18 h after implantation than in mice receiving it earlier (P < .01).

IL-6 levels were substantially (but similarly) increased immediately before the initiation of antibiotic treatment in mice receiving treatment 6 h or later after implantation, compared with the levels found in each group before implantation (P < .001) (figure 4C). At 3 h after the initiation of antibiotic treatment, IL-6 levels in mice receiving treatment 6 or 12 h after implantation had decreased significantly, whereas the levels in mice receiving treatment 18 h after implantation were substantially unchanged. This pattern persisted at 6 h after the initiation of antibiotic treatment, with IL-6 levels close to normal values in mice receiving treatment 6 h after implantation. IL-6 levels were significantly increased both 3 and 6 h after the initiation of antibiotic treatment in mice receiving treatment 18 h after implantation, compared with those in mice receiving treatment 6 or 12 h after implantation (minimum P < .01) (figure 4C). Similarly, IL-6 levels at the same 3- and 6-h time points after the initiation of antibiotic treatment were higher in mice receiving treatment 12 h after implantation than in mice receiving treatment 6 h after implantation (minimum
Duration of Shock before Antibiotics

**Figure 3.** Mean serum lactate (A), tumor necrosis factor (TNF)-α (B), and interleukin (IL)-6 (C) levels in untreated septic mice (white inverted triangles) and sham-infected control mice (black circles; n = 8 mice/group). Group comparisons were made using repeated-measures analysis of variance; point comparisons were made using the least-squares method. Septic mice vs. sham-infected control mice, in all cases. †, P < .0001 vs. sham-infected control mice at the equivalent time point. Error bars indicate SEs.

**DISCUSSION**

In this study, we made several novel observations regarding mortality in a murine model of *E. coli* septic shock that may be relevant to spontaneous human disease. First, although death occurred between ~24 and 48 h after the septic insult (implantation of an *E. coli*–laced, gelatin capsule–encased fibrinogen clot) in this model, the critical events and interventions that determine mortality occurred much earlier. Second, there was a critical upward inflection point in mortality >12 h af-

**Figure 4.** Mean serum lactate (A), tumor necrosis factor (TNF)-α (B), and interleukin (IL)-6 (C) levels in mice given antibiotic treatment 6 (black bars), 12 (light gray bars), or 18 (dark gray bars) h after implantation (n = 6 mice/group). Highly significant differences in responses between groups existed (P < .0001). Group comparisons were made using repeated-measures analysis of variance; point comparisons were made using the least-squares method. *P < .05, vs. septic mice before antibiotic treatment at the same treatment initiation time point; †P < .0001, vs. septic mice before antibiotic treatment at the same treatment initiation time point. Error bars indicate SEs.

P < .01) (figure 4C). Figure 6 shows a summary of the time sequence of major hemodynamic and serum cytokine/lactate responses in untreated septic mice.
ter implantation. At or before the 12-h point, intervention with effective antibiotic treatment resulted in minimal mortality (≤20%). When antibiotic treatment was initiated at or after the 15-h point, mortality was substantial (86.7%–100%) (figure 1). Finally, this upward inflection in mortality was most closely associated with the transition to an unrecoverable state was a novel observation in the context of septic shock. That this difference in response was closely linked to the duration of hypotension in experimental septic shock was novel. That this difference in response to infection in low-mortality animal models of infection. However, the observation in the present study that this decrease in mortality was not. Indeed, lactate levels in mice receiving antibiotic treatment 18 h after implantation continued to increase. The survival study demonstrated that, despite continued antibiotic treatment, mortality in mice receiving antibiotic treatment 18 h after implantation was 100% (figure 1).

These findings suggest that, between 12 and 15–18 h after implantation, the development of shock—defined as hypotension associated with increasing lactic acidosis—drives a rapid increase in mortality in this model. This narrow temporal window associated with the transition to an unrecoverable state was a novel observation in the context of septic shock.

Another novel observation was that inflammatory cytokine responses differed depending on the time that antibiotic treatment was initiated. For example, antibiotic treatment initiated either 6 or 12 h (but not 18 h) after implantation resulted in an increase in TNF-α levels 3 h after treatment (figure 4B). This increase may have occurred as a consequence of antibiotic-mediated LPS release from killed bacteria in mice receiving treatment 6 or 12 h after implantation. At 18 h after implantation, however, pretreatment circulating LPS levels were 50–100 times higher than in mice receiving treatment 6 or 12 h after implantation (figure 5), and additional TNF-α release might have been inhibited by feedback mechanisms.

Furthermore, although TNF-α and IL-6 levels at 6, 12, and 18 h after implantation were all increased, the initiation of antibiotic treatment 6 or 12 h after implantation was associated with a decrease in circulating levels of both cytokines at 3 and/or 6 h after treatment, compared with those measured immediately before the initiation of treatment (figure 4B and 4C). In contrast, levels of these cytokines were largely unchanged after antibiotic treatment if it was initiated 18 h after implantation.

Other researchers, including Coopersmith et al. [8], have shown that increased serum TNF-α and IL-6 levels at 6, 12, and 18 h after implantation were all increased, the initiation of antibiotic treatment 6 or 12 h after implantation was associated with a decrease in circulating levels of both cytokines at 3 and/or 6 h after treatment, compared with those measured immediately before the initiation of treatment (figure 4B and 4C). In contrast, levels of these cytokines were largely unchanged after antibiotic treatment if it was initiated 18 h after implantation. This difference in the inflammatory cytokine response occurred despite uniform increases in circulating LPS levels at the same time points after the initiation of antibiotic treatment (figure 5) and uniform decreases in blood bacterial counts (table 1).

Table 1. Blood bacterial counts before and after antibiotic treatment.

<table>
<thead>
<tr>
<th>Time after implantation</th>
<th>Before antibiotic treatment</th>
<th>6 h after antibiotic treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 h</td>
<td>$7.75 \times 10^7 \pm 1.25 \times 10^7$</td>
<td>$3.76 \times 10^7 \pm 0.91 \times 10^7$</td>
</tr>
<tr>
<td>12 h</td>
<td>$1.55 \times 10^6 \pm 0.15 \times 10^7$</td>
<td>$4.53 \times 10^7 \pm 0.98 \times 10^7$</td>
</tr>
<tr>
<td>18 h</td>
<td>$2.21 \times 10^6 \pm 0.25 \times 10^7$</td>
<td>$6.83 \times 10^7 \pm 1.25 \times 10^7$</td>
</tr>
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NOTE. Data are means ± SD.

* P < .0001, vs. septic mice before antibiotic treatment at the same treatment initiation time point.
was related to the longer time before antibiotic treatment was initiated was consistent with the hypothesis that septic inflammatory injury, if sufficiently sustained, can propagate and generate organ failure and death despite apparent eventual eradication of the inciting organism [9]. These data also support the specific hypothesis of the present study that key mechanistic elements that drive toward death may be irrevocably initiated between 12 and 18 h after implantation, which was after the onset of physiologically significant hypotension in this model. This possibility is supported by additional observations (data not shown) suggesting that, although mortality remains universal, the onset of hemodynamic compromise is delayed and the potential window of effective therapy can be extended when the initial bacterial load is reduced.

These observations are important for 2 major reasons. First, these data help define the pathophysiologic process that drives mortality in overwhelming infection and septic shock. Our data suggest that sustained hypotension in septic shock is associated with irreversible injury leading to inevitable deterioration and death many hours after the initial injury. Several animal and human studies have suggested that prolonged hypotension associated with hemorrhagic shock results in an irreversible state of injury (Wigger’s shock model [10]) in which the reversal of hypovolemia cannot prevent further deterioration and death. This phenomenon has been linked to vascular generation of an inducible nitric oxide synthetase, a process similar to that described in septic shock [11, 12, 13]. Although shock-related irreversible injury is well accepted in the context of hemorrhage, its existence in relation to overwhelming infection is not well known.

The other important contribution of this study is the observation that the timing of effective antibiotic treatment may have potential importance for the risk of death once hypotension is present. Our data suggest that, in healthy mice, there is only a modest increase in mortality when antibiotic treatment is delayed but the hypotension of early septic shock is not yet present. However, once such hypotension is present, mortality increases rapidly. Remarkably few studies have examined the effect that delays in antibiotic treatment have on outcome in septic shock; none, to our knowledge, have examined outcome in relation to either hemodynamic or cytokine responses. For example, Fridmodt-Moller and Thomsen [14] and Knudsen et al. [15] have demonstrated that the timing of antimicrobial treatment has a critical effect on outcome in intraperitoneal inoculation of *Streptococcus pneumoniae* into mice. The degree of bacterial propagation and the survival rate were shown to be critically dependent on the timing of this treatment, with uniform mortality if treatment with penicillin was initiated 24 h after inoculation. Similarly, Greisman et al. [16] have shown that sequential delays in aminoglycoside therapy after intraperitoneal or intravenous inoculation of enteric organisms (*E. coli*, *Proteus mirabilis*, and *Klebsiella pneumoniae*) results in progressive increases in mortality from 0% to 90%–100%. Most relevant with respect to a rapid inflection in mortality, Jamieson et al. [17] have demonstrated an increase from 10% to 90% in the risk of death between 5 and 7 h after clamping of the superior mesenteric artery in a canine model of intestinal ischemia.

Our data suggest that the onset of septic shock, as indicated by hypotension and accumulation of serum lactate, is a critical inflection point for mortality. Once this point is reached, hypotension progresses, serum lactate levels increase, and increased serum levels of TNF-α and IL-6 persist despite the provision of appropriate antibiotic treatment leading to microbial eradication. These results suggest that a fundamental change in the nature of the biologic response to systemic inflammatory injury may occur between 12–18 h after the injury and 3–6 h after the onset of hypotension consistent with early septic shock that leads inevitably to death in this model. On a clinical level, our data suggest that the rapid initiation of antibiotic treatment after the onset of hypotension is a critical determinant of outcome in septic shock. Analysis of human data will be required to extend these observations to the clinical realm.

**References**