Sublethal Doses of Bacillus anthracis Lethal Toxin Inhibit Inflammation with Lipopolysaccharide and Escherichia coli Challenge but Have Opposite Effects on Survival

Xizhong Cui, Yan Li, Xuemei Li, Michael Haley, Mahtab Moayeri, Yvonne Fitz, Stephen H. Leppla, and Peter Q. Eichacker

Background. On the basis of the findings of previous in vitro studies, we hypothesized that anthrax lethal toxin (LeTx) would have anti-inflammatory effects in vivo.

Methods. We investigated the effects of sublethal doses of LeTx in rats receiving intravascular challenge with lipopolysaccharide (LPS) or intratracheal challenge with Escherichia coli.

Results. In rats receiving 24-h infusions of LPS, compared with control rats, pretreatment with high or low sublethal doses of LeTx 3 h before infusion produced similar patterns of reduction in the hazards ratio (HR) of survival at 168 h (0.60 [95% confidence interval {CI}, 0.37–0.98]; P = .03, for the doses combined). LeTx increased mean arterial blood pressure throughout the period of LPS infusion (P = .01); decreased the levels of 10 of 13 cytokines assessed (i.e., interleukin [IL]–1α, IL-1β, IL-2, IL-4, IL-6, IL-10, interferon-γ, tumor necrosis factor–α, granulocyte macrophage–colony-stimulating factor, migratory inhibitory protein [MIP]–1α, MIP-2, MIP-3α, and RANTES) at 2 h; decreased all 13 cytokine levels at 8 h; decreased only 4 cytokine levels at 24 h; and decreased the plasma level of nitric oxide (NO) at 8 h and 24 h but not at 2 h (P = .02, for the effect of LeTx, across time, on both cytokine levels and the NO level). Although pretreatment with LeTx before challenge with E. coli altered mean arterial blood pressure, cytokine levels, and the NO level in a pattern similar to that noted in association with LPS infusion, it increased the HR in a pattern different from that associated with LPS (4.36 [95% CI, 0.3–63.4]; P = .04, for the effects of LeTx with LPS vs. E. coli).

Conclusion. Inhibition of inflammation with LeTx can occur in vivo, and, although beneficial with noninfectious stimuli, it may be harmful with bacteria.

Release of inflammatory cytokines by macrophages has been implicated in the pathogenesis of Bacillus anthracis lethal toxin (LeTx) [1, 2]. However, growing evidence suggests that inhibition of cellular signaling by LeTx may also have anti-inflammatory effects. Lethal factor (LF), which is the toxigenic moiety of LeTx, is a zinc protease that inactivates mitogen-activated protein kinase kinases (MAPKKs, or MEKs), therefore inhibiting downstream mitogen-activated protein kinases (MAPKs), including extracellular signal–regulated kinase (ERK), c-Jun NH2-terminal kinase/stress-activated protein kinase, and p38 [3–9]. These actions may underlie the organ dysfunction associated with LeTx [10–12]. However, they may also have anti-inflammatory effects. In macrophages, cleavage of MEK3 by LF inhibited the release of tumor necrosis factor (TNF)–α and nitric oxide (NO) by lipopolysaccharide (LPS) and interferon (IFN)–γ together and the activation of IFN regulatory factor 3 by LPS alone [13, 14]. Inhibition of MEK3 by LF also induces macrophage apoptosis [15]. Finally, in-
Figure 1. Effect of sublethal doses of anthrax lethal toxin (LeTx) on survival rates for rats challenged with LPS or Escherichia coli. Shown are the proportions of rats surviving over time after pretreatment with low or high sublethal doses of LeTx or diluent (as a control) and challenge performed 3 h later with a 24-h infusion of lethal lipopolysaccharide (LPS) [A] or intratracheal inoculation of E. coli [B].

Inhibition of ERK1/2 and p38 by LeTx impaired the release of inflammatory cytokines by dendritic cells in response to LPS in vitro and antigen-specific T and B cell immunity in vivo [16]. Thus, one role of LeTx during active B. anthracis infection may be to suppress host inflammatory responses and facilitate the infection. However, few data are available on the anti-inflammatory effects of LeTx in vivo. We previously showed that, in contrast to a 24-h infusion of LPS, administration of lethal doses of LeTx to rats did not increase the release of cytokines or NO, and TNF levels appeared to decrease [12]. In the present study, we further investigated the potential anti-inflammatory effects of LeTx in vivo. Rats pretreated with sublethal doses of LeTx were challenged with either lethal LPS 24-h infusions or intratracheal challenge with E. coli. LeTx pretreatment reduced intravascular levels of cytokines and NO and increased blood pressure in similar patterns when used with LPS and E. coli. However, in different patterns, LeTx pretreatment increased survival rates when used with LPS but decreased survival rates when used with E. coli.

MATERIALS AND METHODS

Animal care. The protocol used in the present study was approved by the Animal Care and Use Committee of the Clinical Center of the National Institutes of Health. During the study, every effort was made to minimize animal suffering.

Study design. In one set of experiments, 115 Sprague-Dawley rats (weight, 180-250 g) with carotid arterial and jugular venous
catheters that had been previously placed (i.e., 72 h before the study) [12] were transiently anesthetized with isoflurane. The rats were then randomized to receive either an intravenous injection of LeTx with low or high sublethal doses (see below) of LF given in combination with protective antigen (PA) dissolved in 0.5 mL of PBS with rat albumin (25 mg/mL) or diluent only (as a control). Venous catheters were attached to syringe pumps for later infusion of LPS, and arterial catheters were connected to transducers. Three hours after treatment with LeTx or diluent was administered, a 24-h infusion of *E. coli* 0111:B4 LPS (10 mg/kg body weight in PBS with 25 mg/mL rat albumin run at a rate of 0.5 mL/h) was initiated. Before and during LPS infusion, the rats had mean arterial blood pressure (MBP) and heart rate measured every 2 h. At 2, 8, and 24 h after initiation of LPS infusion, rats from each group were randomly selected to have measurements of either arterial blood gases and complete blood counts or plasma levels of cytokines and NO performed. All rats had similar quantities of blood (0.5 mL) drawn at each time point. The rats were observed for a total of 168 h.

In a second set of experiments, 118 rats were randomly assigned to receive an intravenous injection of LeTx (PA + LF dose, 6.25 + 3.13 μg/kg) or diluent (as a control). Three hours later (at "0 h"), the rats were anesthetized; received intratracheal challenge with *E. coli* (60 × 10⁴ cfu/kg), as described elsewhere [17]; and then underwent the same procedures described above. For 48 of the 118 rats, laboratory measurements were performed as described above, but measurement began 2 h after inoculation with *E. coli*, because earlier monitoring was prevented by inoculation procedures. The remaining rats were randomly selected, anesthetized so quantitative blood cultures could be obtained, and then killed so lung lavage could be performed, with cell, protein, and quantitative bacterial measurements at 8 or 24 h after inoculation. To simulate clinical practice, beginning 6 h after *E. coli* inoculation, the rats were treated with ceftriaxone...
Table 1. Plasma levels of cytokines and nitric oxide (NO) in rats at 2, 8, and 24 h during a 24-h infusion of lipopolysaccharide (LPS) started 3 h after treatment with a low or high sublethal dose of lethal toxoid (LeTx) or a diluent (control).

<table>
<thead>
<tr>
<th>Cytokine level</th>
<th>2 h</th>
<th>8 h</th>
<th>24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low</td>
<td>High</td>
<td>Low</td>
</tr>
<tr>
<td>IL-1α</td>
<td>5.46 ± 0.12</td>
<td>5.43 ± 0.21</td>
<td>5.57 ± 0.16</td>
</tr>
<tr>
<td>IL-1β</td>
<td>6.55 ± 0.16</td>
<td>6.26 ± 0.19</td>
<td>6.13 ± 0.48</td>
</tr>
<tr>
<td>IL-2</td>
<td>6.77 ± 0.15</td>
<td>6.75 ± 0.20</td>
<td>6.81 ± 0.18</td>
</tr>
<tr>
<td>IL-4</td>
<td>2.06 ± 0.72</td>
<td>1.18 ± 0.62</td>
<td>0.99 ± 0.53</td>
</tr>
<tr>
<td>IL-6</td>
<td>8.73 ± 0.09</td>
<td>8.69 ± 0.15</td>
<td>7.87 ± 0.61</td>
</tr>
<tr>
<td>IL-10</td>
<td>6.77 ± 0.16</td>
<td>6.41 ± 0.14</td>
<td>6.41 ± 0.24</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>7.28 ± 0.19</td>
<td>7.15 ± 0.17</td>
<td>6.97 ± 0.11</td>
</tr>
<tr>
<td>TNF-α</td>
<td>7.28 ± 0.17</td>
<td>7.11 ± 0.16</td>
<td>6.60 ± 0.52</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>5.92 ± 0.63</td>
<td>6.52 ± 0.25</td>
<td>4.87 ± 0.72</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>7.04 ± 0.13</td>
<td>7.60 ± 0.23</td>
<td>6.77 ± 0.33</td>
</tr>
<tr>
<td>MIP-3α</td>
<td>6.56 ± 0.08</td>
<td>6.43 ± 0.11</td>
<td>6.08 ± 0.47</td>
</tr>
<tr>
<td>MIP-2</td>
<td>9.03 ± 0.23</td>
<td>8.84 ± 0.18</td>
<td>8.30 ± 0.52</td>
</tr>
<tr>
<td>RANTES</td>
<td>7.01 ± 0.06</td>
<td>6.86 ± 0.15</td>
<td>6.92 ± 0.16</td>
</tr>
<tr>
<td>NO level</td>
<td>3.72 ± 0.11</td>
<td>3.73 ± 0.12</td>
<td>3.99 ± 0.12</td>
</tr>
</tbody>
</table>

**NOTE.** Data are mean log (µg/mL) ± SEM (for cytokine levels) or mean log (µmol/L) ± SEM (for the NO level). GM-CSF, granulocyte macrophage-colony-stimulating factor; IFN, interferon; IL, interleukin; MIP, migratory inhibitory protein; ND, not detectable; TNF, tumor necrosis factor.

*a The n. of samples tested is the same at each time point and in each group for each cytokine, but not for NO. The number of samples assessed to determine NO levels were 14 (control group), 12 (low-dose sublethal LeTx), and 16 (high-dose sublethal LeTx) at 2 h; 13 (control group), 7 (low-dose sublethal LeTx), and 12 (high-dose sublethal LeTx) at 8 h; and 3 (control group), 6 (low-dose sublethal LeTx), and 11 (high-dose sublethal LeTx) at 24 h.

*b The low and high sublethal doses of LeTx were 3.13 ± 1.56 µg/kg and 6.25 ± 3.13 µg/kg (protective antigen + lethal factor dose), respectively.

(100 mg/kg administered intramuscularly every day for 4 days; Roche Laboratories).

**LeTx pretreatment.** LPS-free recombinant PA and LF were prepared for LeTx as described elsewhere [12, 18, 19]. Previously, an intravenously administered bolus of LeTx caused dose-dependent increases in the mortality rate. The lowest lethal dose of LeTx that was studied included PA and LF concentrations of 25 and 12.5 µg/kg, respectively. A lower dose of LeTx, which included PA and LF concentrations of 12.5 and 6.25 µg/kg, respectively, did not cause death [12]. In the present study, we tested this lower dose of LeTx and 3 successive dilutions of the dose, designating 2 of them as high sublethal doses (PA + LF dose, 12.5 + 6.25 µg/kg and 6.25 + 3.13 µg/kg) and the remaining 2 as low sublethal doses (PA + LF dose, 3.13 + 1.56 µg/kg and 1.56 + 0.78 µg/kg).

**LPS infusion and intratracheal E. coli challenges.** LPS (Sigma) diluted in PBS was administered at 10 mg/kg over 24 h in a volume of 12 mL at a rate of 0.5 mL/h [12]. Intratracheal challenge with E. coli 0111:B4 was administered as described elsewhere [17].

**Laboratory measurements.** Hemodynamic measurements and blood sample collection were performed as described elsewhere [12]. Arterial blood samples were collected for arterial blood gas measurement, complete cell counts, or quantitative cultures. Lung lavage with cell count and bacterial culture was performed as described elsewhere [17].

**Measurement of cytokine and nitrite/nitrate levels.** The cytokines TNF-α, IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, IFN-γ, GM-CSF, 3 migratory inhibitory proteins (MIP-1α, MIP-2, and MIP-3α), and RANTES were measured using a multiplexed sandwich ELISA (SearchLight Rat Cytokine Array; Pierce). For measurement of nitrate/nitrite levels, plasma was precipitated, the supernatant was heated, and the resulting NO from nitrate, nitrite, and S-nitrosyl components was measured as described elsewhere [12].

**Statistical analysis.** An SAS software program (version 8.0; SAS) was used for analysis. To minimize the use of rats, when statistical analysis justified the averaging of data on the basis of the LeTx dose or the time of measurement, data were pooled to increase the sensitivity of analyses. A Cox proportional hazards model was used for all survival analysis of LPS infusion and E. coli challenge individually. With LPS, no differences were noted when the effects of low and high doses of LeTx were compared with the effects of the diluent control, and data were
Figure 3. Effect of sublethal doses of anthrax lethal toxin (LeTx) on plasma levels of cytokines and nitric oxide (NO) noted in association with lipopolysaccharide (LPS) challenge. Effects of pretreatment with low and high sublethal doses of LeTx, as demonstrated by serial measurements (mean ± SEM) of the plasma levels of 13 different cytokines (A) and NO (B) performed at 2, 8, and 24 h after the initiation of a 24-h infusion of lethal LPS. The effects of low and high doses of sublethal LeTx pretreatment on these parameters were similar, and data on these effects are combined for presentation in this figure. Each arrow denotes the direction and amount of change noted, compared with changes noted for control rats (see Materials and Methods). *Statistically significant (P < .05) for the effect of sublethal LeTx on an individual parameter at a particular time point. **P values for effects associated with sublethal LeTx that changed significantly over the 24-h observation period. The P value in panel A denotes the differing effect of LeTx on the proportion of cytokine levels that were decreased, comparing measurements obtained at each of the 3 time points. GM-CSF, granulocyte macrophage–colony-stimulating factor; IFN, interferon; IL, interleukin, MIP, migratory inhibitory protein; TNF, tumor necrosis factor.

combined for survival analysis. Differences in the effect on survival of LeTx pretreatment with LPS versus E. coli were tested using the Mantel-Haenszel $\chi^2$ test. Survival effects are presented as the HR of survival (95% confidence interval [CI]). For all other laboratory parameters with LPS challenge, a 3-way analysis of variance (ANOVA) accounting for type (toxin vs. diluent) and dose of pretreatment and time of measurement was used. As with survival, the effects of low and high doses of LeTx were similar for all parameters, and data on these effects were combined for analysis. With E. coli challenge, 2-way ANOVA that accounted for treatment type and time of measurement was used for analysis of laboratory parameters. With both LPS infusion and E. coli challenge, a Tukey test was also used to compare the effects of treatment on laboratory parameters at individual time points. Treatment effects were calculated by subtracting the mean laboratory value for the control group from the mean laboratory value for the treatment group. With both LPS infusion and E. coli challenge, the proportion of cytokine levels that decreased in association with LeTx pretreatment was compared across the 3 time points and between the 2 challenges by use of a $\chi^2$ test. Data were log-transformed, where appropriate, for analysis. All results were expressed as
mean values ± SEMs, and $P \leq .05$ was considered to denote statistical significance.

**RESULTS**

**Effects of Pretreatment with Sublethal LeTx before LPS Challenge**

**Survival rates.** In rats treated with diluent only (control rats), LPS challenge resulted in lethality beginning at 4 h after initiation of LPS infusion (figure 1A). Each of the 2 low and 2 high doses of LeTx pretreatment produced effects on the survival rate that did not differ significantly ($P$ value was not significant [NS]), and data on these effects were combined at each dose level for presentation. Similar reductions in mortality rates were noted among rats treated with high (HR of survival, 0.60 [95% CI, 0.37–0.98]; $P = .04$) and low (HR of survival, 0.71 [95% CI, 0.36–1.42]; $P = .33$) doses of LeTx, compared with control rats, and, overall, this reduction was statistically significant (HR of survival, 0.60 [95% CI, 0.37–0.98]; $P = .03$, for the doses combined) (figure 1A).

**Hemodynamic measurements.** In control rats, hemodynamic measurements performed after LPS challenge revealed a decrease in the MBP and an increase in the heart rate over the 24 h of infusion, compared with hemodynamic measurements performed at baseline (figure 2). Low and high doses of LeTx had similar ($P = \text{NS}$; data not shown) effects on hemodynamic measurements, and data demonstrating these effects were combined for presentation. The effects of LeTx on MBP and heart rate did not differ over time compared with the diluent control.
(P = NS, for the effect of time). At 2 h, before death occurred among rats in any of the study groups, LeTx-treated rats had a significantly increased MBP, compared with control rats (mean ± SEM for the 2 doses combined, 6.1 ± 2.8 mm Hg; P = .03). Over the 24 h of LPS infusion, the MBP and the heart rate were both significantly increased by LeTx pretreatment (mean ± SEM, 9.6 ± 1.3 mm Hg and 23.1 ± 5.8 beats per minute [bpm]; P < .001, for both parameters averaged across the 24 h) (figure 2).

**Measurement of plasma levels of cytokines and NO.** In control rats, except for IL-2, all cytokine levels were higher at 2 and 8 h, compared with levels noted at 24 h, whereas NO levels were greater at 8 and 24 h than at 2 h (table 1). This effect of time was significant for TNF-α, IL-1α, IL-1β, IL-4, IL-6, IFN-γ, GM-CSF, MIP-1α, MIP-2, MIP-3α, and RANTES (P ≤ .05, for the change in each parameter across time) and for the NO level (P < .0001). Low and high doses of LeTx had similar (P = NS) effects on levels of individual cytokines and on the NO level, and data on these effects were combined for presentation (figure 3) (table 1). Compared with control rats, rats receiving LeTx pretreatment had decreased levels of 10 of 13 cytokines at 2 h (none of the decreases were significant) and decreased levels of all 13 cytokines at 8 h (4 cytokine levels [IL-6, IL-10, IFN-γ, and MIP-2] demonstrated decreases that were statistically significant or that approached statistical significance; P ≤ .08) (figure 3). At 24 h, however, 9 cytokine levels were increased with LeTx, and 4 cytokine levels were decreased (only the GM-CSF level had decreased more than was noted at earlier time points). Overall, the decreases in most cytokine levels noted at 2 and 8 h with LeTx, followed by later increases noted at 24 h, occurred in a pattern that was highly significant (P = .0005, for the change in the effect of LeTx pretreatment on the proportion of decreased cytokine levels when 3 time points after LPS challenge were compared). After 2 h, LeTx pretreatment was associated with increasing reductions in NO levels (P = .02, for the change in the effect of LeTx on NO levels over time) (figure 3).

**Other laboratory measurements.** In control rats, LPS challenge increased the total numbers of white blood cells (WBCs), neutrophils, and lymphocytes and decreased the total number of platelets in the period from 2 to 24 h after challenge (P ≤ .05, for the change in each parameter across time; data not shown). Low and high doses of LeTx pretreatment had similar (P = NS) effects on complete blood count and arterial blood gas data, and these data were combined for analysis. Compared with the diluent control, LeTx caused increases (mean ± SEM) in total WBC counts at 8 h (1.49 ± 0.70 × 10^9 cells/mL) that were different from decreases noted at 2 h (−0.63 ± 0.70 ×

### Table 2. Plasma levels of cytokines and nitric oxide (NO) in rats at 2, 8, and 24 h after intratracheal challenge with *Escherichia coli* (60 × 10^9 cfu/kg) administered 3 h after a high sublethal dose of lethal toxin (LeTx) or diluent (control).

<table>
<thead>
<tr>
<th>Measurement</th>
<th>2 h</th>
<th>8 h</th>
<th>24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n = 24)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sublethal dose of LeTx</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>(n = 24)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean plasma level (± SEM), by time after initiation of <em>E. coli</em> challenge in each study groupa</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytokine level</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-1α</td>
<td>2.58 ± 0.41</td>
<td>2.81 ± 0.48</td>
<td>3.07 ± 0.41</td>
</tr>
<tr>
<td>IL-1β</td>
<td>0.87 ± 0.59</td>
<td>1.48 ± 0.78</td>
<td>2.28 ± 0.85</td>
</tr>
<tr>
<td>IL-2</td>
<td>0.72 ± 0.49</td>
<td>1.23 ± 0.53</td>
<td>0.91 ± 0.48</td>
</tr>
<tr>
<td>IL-4</td>
<td>ND</td>
<td>ND</td>
<td>0.25 ± 0.25</td>
</tr>
<tr>
<td>IL-6</td>
<td>5.92 ± 1.12</td>
<td>5.53 ± 1.10</td>
<td>8.46 ± 0.53</td>
</tr>
<tr>
<td>IL-10</td>
<td>5.94 ± 0.19</td>
<td>5.95 ± 0.36</td>
<td>5.40 ± 0.29</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>3.69 ± 0.53</td>
<td>3.13 ± 0.68</td>
<td>4.99 ± 0.67</td>
</tr>
<tr>
<td>TNF-α</td>
<td>4.56 ± 0.82</td>
<td>4.52 ± 0.97</td>
<td>3.46 ± 0.52</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>ND</td>
<td>ND</td>
<td>0.30 ± 0.30</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>6.61 ± 0.40</td>
<td>6.57 ± 0.42</td>
<td>6.41 ± 0.39</td>
</tr>
<tr>
<td>MIP-3α</td>
<td>2.63 ± 0.71</td>
<td>2.63 ± 0.71</td>
<td>5.47 ± 0.32</td>
</tr>
<tr>
<td>MIP-2</td>
<td>4.95 ± 0.71</td>
<td>4.95 ± 0.69</td>
<td>5.37 ± 0.59</td>
</tr>
<tr>
<td>RANTES</td>
<td>5.28 ± 0.22</td>
<td>5.12 ± 0.19</td>
<td>5.79 ± 0.35</td>
</tr>
<tr>
<td>NO level</td>
<td>3.94 ± 0.09</td>
<td>4.02 ± 0.11</td>
<td>4.97 ± 0.21</td>
</tr>
<tr>
<td>NOTE. Data are mean log (pg/mL) ± SEM for the cytokine levels or mean log (μmol/L) ± SEM for the NO level. GM-CSF: granulocyte macrophage-colony-stimulating factor; IFN: interferon; IL: interleukin; MIP: migratory inhibitory protein; ND: not detectable; TNF: tumor necrosis factor.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a The no. of samples tested is the same at each time point and in each group, for assessment of both cytokine levels and the NO level.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>b The sublethal dose of LeTx was 6.25 ± 3.13 μg/kg (protective antigen + lethal factor dose).</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 5. Effect of sublethal doses of anthrax lethal toxin (LeTx) on plasma levels of cytokines and nitric oxide (NO) with *Escherichia coli* challenge. Effects of pretreatment with a high sublethal dose of LeTx on 13 different plasma levels of cytokines (A) and NO (B), as demonstrated by serial measurements performed at 2, 8, and 24 h after intratracheal *E. coli* challenge. Each arrow denotes the direction and amount of change, compared with that noted with the control (see Materials and Methods). ■ absence of detectable change with LeTx treatment. *Statistically significant (P < .05) for the effect of sublethal LeTx on an individual parameter at a particular time point. **Differing effect of LeTx on the proportion of cytokine levels that were decreased, comparing measurements performed at each of the 3 time points. GM-CSF, granulocyte macrophage–colony-stimulating factor; IFN, interferon; IL, interleukin, MIP, migratory inhibitory protein; TNF, tumor necrosis factor.

Effects of Sublethal LeTx before *E. coli* Challenge

Survival rates. Intratracheal challenge with *E. coli* in rats receiving diluent pretreatment resulted in a mortality rate similar to that associated with LPS challenge (figure 1A and 1B). Compared with control rats, rats pretreated with a high sublethal dose of LeTx (6.25 and 3.13 μg/kg of PA and LF, respectively) before intratracheal challenge with *E. coli* increased the HR of survival (4.36 [95% CI, 0.3–63.4]), although not significantly (P = .28) (figure 1B). However, compared with the same dose of LeTx pretreatment that increased the survival rate associated with LPS challenge, pretreatment with *E. coli* reduced the survival rate in a significantly different pattern (P = .04, for the survival rates for control rats and rats receiving LeTx with LPS [29% and 43%, respectively] vs. the survival rates for control rats and rats receiving LeTx with *E. coli* [33% and 21%, respectively]).

Hemodynamic measurements. In control rats, *E. coli* challenge first increased and then decreased the MBP and the heart
Figure 6. Effect of sublethal doses of anthrax lethal toxin (LeTx) on oxygenation and the leukocyte count in lung lavage fluid with Escherichia coli challenge. Serial measurements (mean ± SEM) of the partial arterial pressure of oxygen (\(P_{A_O_2}\)) (A) performed at 2, 8, and 24 h (\(n = 24\) and 24 at 2 h, 24 and 22 at 8 h, and 16 and 16 at 24 h for control and LeTx-treated groups, respectively) and of the leukocyte count in lung lavage fluid (B) at 8 and 24 h (\(n = 12\) and 11 at 8 h and 14 and 11 at 24 h for control and LeTx-treated groups, respectively) after intratracheal challenge with \(E.\ coli\) in rats receiving pretreatment with a high sublethal dose of LeTx or diluent (as a control). On the basis of data averaged over 2 time points, pretreatment with LeTx resulted in significant increases in the \(P_{A_O_2}\) (\(P < .006\)) and nonsignificant decreases in the lung leukocyte count (\(P = .20\)). *Statistically significant (\(P < .05\)) for the effect of sublethal LeTx on the \(P_{A_O_2}\) at 8 h. NA, not available.

rate over 24 h (figure 4). Over this period, compared with control rats, rats receiving LeTx pretreatment had a significantly increased MBP (mean ± SEM, 4 ± 0.5 mm Hg; \(P < .0001\), averaged across 24 h), but they did not have a significantly increased heart rate (mean ± SEM, 4 ± 1 bpm; \(P = .26\)) (figure 4). Increases in MBP and heart rate occurring with LeTx pretreatment with \(E.\ coli\) challenge were less than the increases occurring in association with LeTx pretreatment with LPS challenge (\(P = .0002\) and \(P = .04\), for the differing effect of similar doses of LeTx pretreatment with LPS vs. \(E.\ coli\) challenge on the 2 parameters, respectively) (figures 2 and 4).

Measurements of plasma levels of cytokines and NO. In control rats challenged with \(E.\ coli\), all cytokine levels were greatest at 8 h, compared with levels noted at 2 and 24 h, with the exception of the TNF-\(\alpha\) and IL-10 levels, which were greatest at 2 h, compared with levels noted at other time points (\(P = .05\), for the effect of time on IL-6, IL-10, TNF-\(\alpha\), MIP-1\(\alpha\), MIP-2, MIP-3\(\alpha\), and RANTES), whereas NO levels increased during the period from 2 to 24 h (\(P < .0001\), for the change over time) (table 2). Pretreatment with LeTx had effects on cytokine levels that differed significantly when levels measured at 2, 8, and 24 h after challenge were compared (\(P = .02\), for the differences in the effect of LeTx pretreatment on the proportion of cytokines for which decreases were noted when plasma measurements obtained at the 3 time points after \(E.\ coli\) challenge were compared). At 2 and 24 h, these effects were variable (i.e., at 2 h, LeTx was associated with increases in 5 cytokine levels, decreases in 6 cytokine levels, and no
changes in 2 cytokine levels, whereas, at 24 h, there were increases in 6 cytokine levels, decreases in 5, and no changes in 2 cytokine levels) (figure 5) (table 2). However, at 8 h after E. coli challenge, LeTx pretreatment was associated with decreases in all 13 cytokine levels, just as had been noted with LPS challenge. From 2 to 24 h after challenge, these changes in cytokine levels noted in association with LeTx pretreatment before E. coli challenge were similar to those noted in association with LPS challenge (P = NS). From 2 to 24 h after E. coli challenge, LeTx pretreatment caused increasing reductions in NO levels, in a pattern similar to the one noted in association with LPS challenge; however, overall, this effect was not significant (P = .46, for the change in the effect of LeTx on NO levels measured at 2, 8, and 24 h after challenge) (figure 5) (table 2).

Other laboratory measurements. In control rats, E. coli decreased the mean arterial pressure of oxygen (Pao2), decreased the WBC, neutrophil, and lymphocyte counts, and increased lactate levels at 2, 8, and 24 h after intratracheal challenge (P < .0001, for each parameter averaged over these time points) (data for Pao2, are shown in figure 6). At 2, 8, and 24 h after challenge, compared with control rats, rats receiving LeTx pretreatment had greater decreases in platelet counts (mean ± SEM, 760 ± 29 vs. 628 ± 45 × 10^9 cells/mL [control vs. LeTx pretreatment], for these time points combined) and greater increases in Pao2 (mean ± SEM, 77 ± 4 vs. 85 ± 3 mm Hg) (P = .006, for each parameter averaged over these time points) (figure 6). Compared with control rats, rats receiving LeTx pretreatment had greater decreases in total leukocyte concentrations in lung lavage fluid at 8 and 24 h (i.e., the 2 time points when these measurements were performed), although this difference was not statistically significant (log [cells × 10^9/mL] ± SEM, 1.68 ± 0.11 vs. 1.47 ± 0.14, averaged over the 2 time points; P = .20) (figure 6). Other laboratory parameters, including bacteria counts in the blood and lungs, were not altered significantly (P = NS, for all) with LeTx treatment.

DISCUSSION

We previously showed that, in rats, LeTx challenge produced levels of shock and lethality similar to those associated with lethal LPS, but it did not increase levels of inflammatory cytokines or NO [12]. The present study demonstrates that not only does LeTx not elicit the pattern of inflammatory mediator release associated with other types of bacterial shock, it can also suppress this response. In the present study, reductions in LPS- and E. coli–stimulated release of cytokines and NO occurring in association with administration of sublethal doses of LeTx are consistent with findings of in vitro studies that have shown that LF inactivates MAPKs and inhibits activation of downstream MAPKs. This inactivation inhibited LPS-stimulated release of cytokines and NO in vitro [13, 14]. Because excessive production of inflammatory cytokines and NO is implicated in the pathogenesis of shock due to LPS challenge, their inhibition with sublethal doses of LeTx provides a basis for the improved hemodynamic function and survival that we noted in animals challenged with LPS [12, 20–26]. These findings are also consistent with the findings of other in vivo studies in which synthetic inhibitors of the same MAPKs targeted by LF reduced the release of cytokines by LPS [27].

The time course of LPS-stimulated release of cytokines and NO with LeTx pretreatment differed, however. Although the early reductions in cytokine levels noted at 2 and 8 h were less pronounced or reversed at 24 h, decreases in NO levels were greatest at 24 h. This finding suggests that the effects of LeTx on signaling or other cellular events, although of shorter duration for the cytokines measured, persisted for NO. Consistent with this effect on cytokines, in in vitro studies performed in the laboratories of M.M. and S.H.L., LeTx treatment of macrophages resulted in MAPKK inactivation that increased from 2 to 8 h and was associated with reductions in stimulated cytokine release. By 24 h, however, resynthesis of MAPKK had occurred, and cytokine release was again intact. Presumably, in the present in vivo experiment, LeTx resulted in the inhibition of other cellular mechanisms causing the late reductions in NO levels that were noted.

Similar to the effects noted with LPS challenge, sublethal doses of LeTx pretreatment markedly decreased cytokine levels at 8 h with E. coli challenge. The pattern of reductions in NO levels noted in association with LeTx pretreatment with E. coli challenge was also similar to that noted for LeTx treatment with LPS. Despite these similarities, although LeTx pretreatment increased the survival rate with LPS infusion, it decreased the survival rate with E. coli challenge in patterns that differed significantly. Also, although LeTx pretreatment increased the MBP with E. coli challenge, these increases were not as great as the increases in MBP associated with LPS infusion, and they were not associated with any change in the heart rate. These differences in the effects of LeTx pretreatment on the survival rate and hemodynamic measurements comparing E. coli with LPS may relate to the dose of LeTx used. Alternatively, however, these findings suggest that suppression by LeTx of components in the inflammatory and innate immune response, although beneficial during a noninfectious challenge like LPS, aggravate the effects of live bacterial infection. In a previous study, although suppression of inflammation with TNF-directed antibodies was beneficial in animals challenged with LPS, it was not beneficial with bacterial infection [28]. Similar anti-TNF therapies decreased the survival rate in another model of live bacterial peritonitis, and TNF administration was beneficial [29]. Thus, although LPS is frequently used as a septic stimulus, it may not result in the full spectrum of sequelae that the host contends with during bacterial infection. Although LeTx pretreatment did not increase bacteria counts after E. coli challenge.
in the present study, the use of antibiotics or the timing of collection may have confounded microbiological analysis. However, LeTx was associated with decreases in lung leukocyte recruitment, although not significantly. This recruitment, which is mediated by many of the cytokines that had decreased levels after LeTx pretreatment, is critical for microbial clearance. In addition to being associated with decreased numbers of leukocytes in the lung, LeTx was also associated with significant increases in oxygenation, providing further evidence that pretreatment reduced lung inflammation. A previous study demonstrated that, although inhibitors of pulmonary leukocyte recruitment improved lung function in this *B. anthracis* model, the survival rate was worsened [30]. Leukocyte recruitment is likely important in host defense during *B. anthracis* infection as well.

Although sublethal LeTx pretreatment only transiently decreased cytokine levels in association with LPS and *E. coli* in the present study, during *B. anthracis* infection, LeTx production would persist and increase with a growing bacterial burden. This persistence could result in continued inhibition of an innate response and could contribute both to the establishment of early infection and to the high bacterial loads noted in association with later *B. anthracis* infection [31]. It is of interest that monoclonal antibody against LeTx improved the survival rate and reduced the bacteria counts in *B. anthracis*-challenged rabbits [32].

Two other reasons provide a basis for the differing effects of sublethal LeTx with LPS infusion versus *E. coli* challenge. First, death occurred earlier with LPS than with *E. coli*. Thus, although LPS was likely cleared after discontinuation of its infusion, bacterial replication with intratracheal *E. coli* challenge resulted in a more prolonged lethal stimulus. The duration of the beneficial effects of sublethal doses of LeTx may have been insufficient to reduce this later lethality associated with *E. coli*. Second, we previously showed that reductions in the heart rate during sepsis in rats are associated with a worsened outcome [12]. In the present study, although LeTx increased heart rates with LPS challenge, such increases did not occur with *E. coli*.

In conclusion, these findings provide in vivo evidence that LeTx may inhibit components in the early inflammatory response. Although this inhibition was beneficial with LPS, it appeared to be harmful with *E. coli*. Extrapolated clinically, these anti-inflammatory effects of LeTx could contribute to the establishment and persistence of *B. anthracis* infection. Thus, therapies that selectively inhibit LeTx might not only limit its cytotoxic effects but might also augment clearance of *B. anthracis*.

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