

# *Bacillus anthracis* Edema and Lethal Toxin Have Different Hemodynamic Effects but Function Together to Worsen Shock and Outcome in a Rat Model

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(See the editorial commentary by Artenstein, on pages 471–3.)

**Introduction.** To better define the contribution of edema toxins (ETx) and lethal toxins (LeTx) to shock with *Bacillus anthracis*, recombinant preparations of each were investigated alone or together in rats.

**Methods and results.** Lethal dose ranges (0%–100% lethality) of ETx (200–800  $\mu\text{g}/\text{kg}$  as a 24-h infusion) were higher than those of LeTx (12.5–200  $\mu\text{g}/\text{kg}$ ) ( $P < .0001$ ). However, compared with LeTx, similarly lethal ETx doses produced earlier and greater reductions in mean blood pressure (MBP) and increased, rather than decreased, heart rate (HR) ( $P < .05$  for all). Combining either similar weight or lethal doses of ETx and LeTx increased the hazard ratio for death ( $\log \pm$  standard error) similar to the sum calculated with the toxin's effects alone ( $2.6 \pm 1.1$  observed vs.  $2.9 \pm 1.0$  calculated for similar weight and  $3.1 \pm 1.0$  vs.  $3.9 \pm 1.5$  for similar lethal doses;  $P = .5$  for both). Early ( $\leq 10$  h) and late during infusion, ETx and LeTx together also altered MBP and HR in patterns consistent with the sum of their individual effects.

**Conclusions.** ETx was  $\sim 10$  times less lethal than LeTx but produced greater hypotension and added to the latter's harmful effects. These findings suggest that it may be appropriate for antitoxin therapies for *B. anthracis* to target both ETx and LeTx.

*Bacillus anthracis* produces 2 exotoxins: lethal toxin (LeTx), which is composed of lethal factor (LF) and protective antigen (PA), and edema toxin (ETx), which is composed of edema factor (EF) and PA [1]. PA mediates the cellular uptake of the toxic moieties LF and EF. In this process, 7 PA molecules form a heptamer on the host cell surface to which 3 molecules of LF or EF bind [2]. The affinity of LF and EF for PA appears

to be similar [3]. LF is an endopeptidase that cleaves mitogen-activated protein kinases and disrupts intracellular signaling [4, 5]. EF is an adenylyl cyclase with activity 1000 times greater than that of eukaryotic forms of the enzyme [6]. Understanding the relative importance of these 2 toxins in the pathogenesis of *B. anthracis* infection is important for the treatment of this lethal infection and bioterrorism threat [7–10].

Several lines of evidence have suggested that, although these toxins might be synergistic, LeTx and not ETx was primarily responsible for the shock and death occurring with *B. anthracis*. In the only prior direct comparison of the toxins, which took place  $>40$  years ago, LeTx was several times more lethal than ETx [11]. In later studies, mutant *B. anthracis* strains unable to produce LeTx were 2 log units less virulent than those not producing ETx [12, 13]. Subsequent research has emphasized the study of LeTx rather than ETx.

Despite the likely importance of LeTx during infection, however, administration of it alone in animal

Received 22 June 2006; accepted 22 August 2006; electronically published 3 January 2007.

Potential conflicts of interest: M.S. owns stock options and is an employee of Human Genome Sciences. M.W.L. owns stock in and is a former employee of Human Genome Sciences. All other authors report no conflicts.

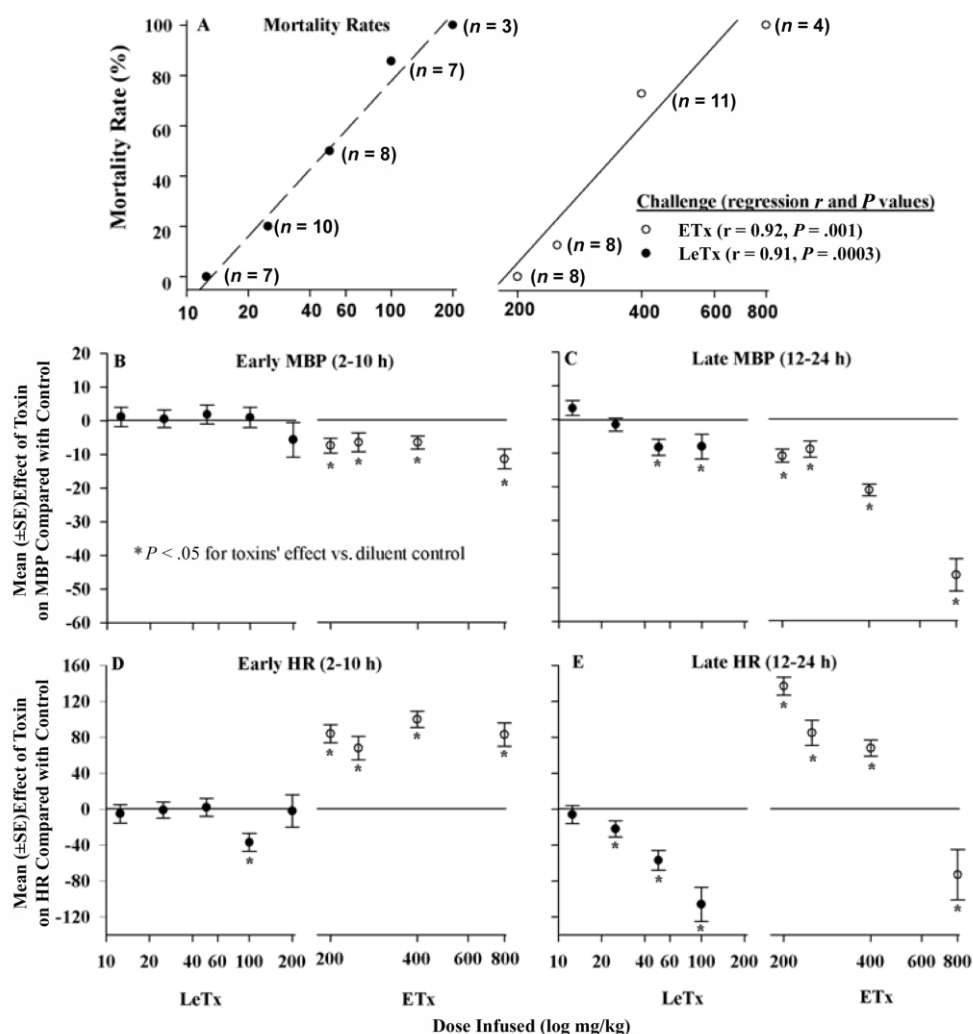
Presented in part: American Thoracic Society meeting, San Diego, 19–24 May 2006 (abstract A803, poster 107).

Financial support: Trans-National Institutes of Health/Food and Drug Administration (biodefense program grant).

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The Journal of Infectious Diseases 2007;195:572–80

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**Figure 1.** A, Effect of increasing doses of either edema toxin (ETx; white circles) or lethal toxin (LeTx; black circles) alone as 24-h infusions on mortality. B–D, Effects of these infusions on mean blood pressure (MBP) and heart rate (HR), compared with those of diluent controls (data not shown), averaged over the early time points before initial lethality (B and D, 2–10 h) or later time points (C and E, 12–24 h). Toxin doses are shown over a log scale.

models does not produce the spectrum of injury found clinically with *B. anthracis* infection [14–17]. This has prompted further consideration of the contribution of ETx [18–20]. In the first study testing the in vivo effects of ETx since its original description, purified preparations appeared to be more lethal in mice than was found in preceding investigations with LeTx [18]. As noted, however, the rapid intravenous administration of ETx did not simulate toxin release during live bacterial infection [18]. Furthermore, ETx caused bradycardia rather than the tachycardia predicted on the basis of its potent adenyl cyclase activity [21, 22]. Finally, ETx and LeTx were not compared directly or in combination.

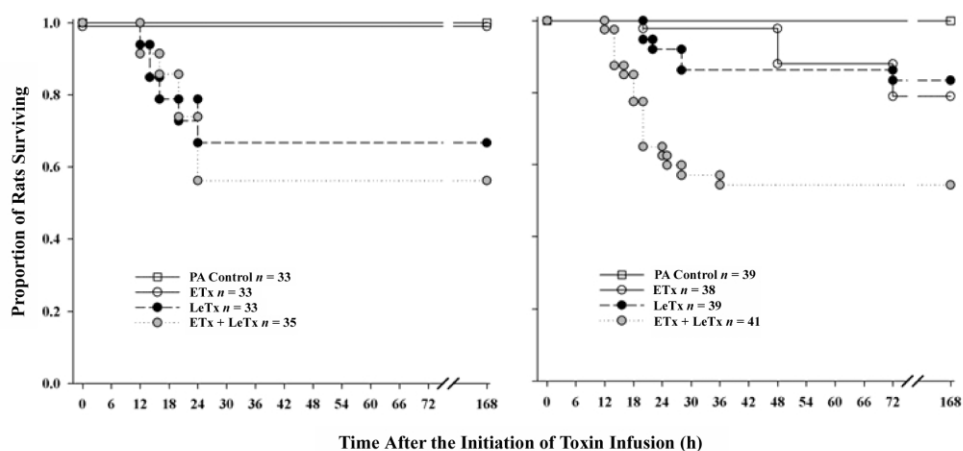
We previously used 24-h infusions of LeTx in rats to better define this toxin's pathogenic effects [14]. The present investigation used similar infusions to directly compare the effects

of recombinant preparations of ETx and LeTx. First, the dose-response effects of the 2 toxins alone were investigated (study 1). Then the effects of the toxins alone or together were compared (study 2). Two different types of doses were investigated (i.e., either similar weight or similar lethal doses), because it is unclear in what proportions the 2 toxins are produced during actual infection.

## 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.

**Study design.** In all studies, ETx and LeTx were formulated by combining doses of either EF or LF with PA in ratios of



**Figure 2.** Effect of 24-h infusions of similar weight (A) or similar lethal (B) doses of edema toxin (ETx) and lethal toxin (LeTx) alone or in combination or of protective antigen (PA) control on the proportion of rats surviving over time

1:2 on the basis of weight. Because the molecular weights of PA, EF, and LF are similar [1, 11, 22], all toxin doses are reported as the weight of either EF or LF used (weight doses). When PA alone was used as a control, its dose is reported. In initial experiments (study 1), Sprague-Dawley rats ( $n = 97$ ) weighing 180–250 g with previously placed carotid arterial and jugular venous catheters were randomized to receive as 24-h infusions (0.5 mL/h) either ETx in a dose of 25, 100, 200, 250, 400, 800, or 1600  $\mu\text{g}/\text{kg}$ ; LeTx in a dose of 12.5, 25, 50, 100, 200, or 400  $\mu\text{g}/\text{kg}$ ; or diluent (control). Immediately before and at 2-h intervals during ETx or LeTx infusion, mean arterial blood pressure (MBP) and heart rate (HR) were measured. Rats were observed for 168 h.

A second study (study 2) then compared the effects of ETx and LeTx infused alone or together. In one set of experiments comparing equivalent-weight doses of toxin, rats ( $n = 134$ ) were randomized to receive ETx (50  $\mu\text{g}/\text{kg}$ ) or LeTx (50  $\mu\text{g}/\text{kg}$ ) alone or together or to receive only PA (200  $\mu\text{g}/\text{kg}$ ) as a control. In another set of experiments that compared toxin doses producing similar 20% lethality, rats ( $n = 157$ ) were randomized to receive ETx (300  $\mu\text{g}/\text{kg}$ ) or LeTx (30  $\mu\text{g}/\text{kg}$ ) alone or together or to receive only PA (660  $\mu\text{g}/\text{kg}$ ) as a control. All rats received equivalent volumes of PA or toxin. Measures were performed as in study 1. Also, at 8 and 24 h after the initiation of either toxin or control infusions, rats were randomly selected to have either arterial blood gas (ABG) and complete blood count (CBC) or plasma cytokine and nitric oxide (NO) measured. All rats had similar volumes (0.5 mL) of blood drawn and normal saline replaced at each time point.

**Toxin preparations.** Toxin components (PA, LF, and EF) were recombinant proteins prepared and purified from *Escherichia coli* by standard chromatographic techniques described elsewhere [22–24]. Endotoxin levels were  $<0.1$  EU/mg protein for each component as based on testing with a limulus ame-

bocyte lysate assay. Rat albumin (25  $\mu\text{g}/\text{mL}$ ) was used to maintain the stability of PA, LF, and EF in PBS.

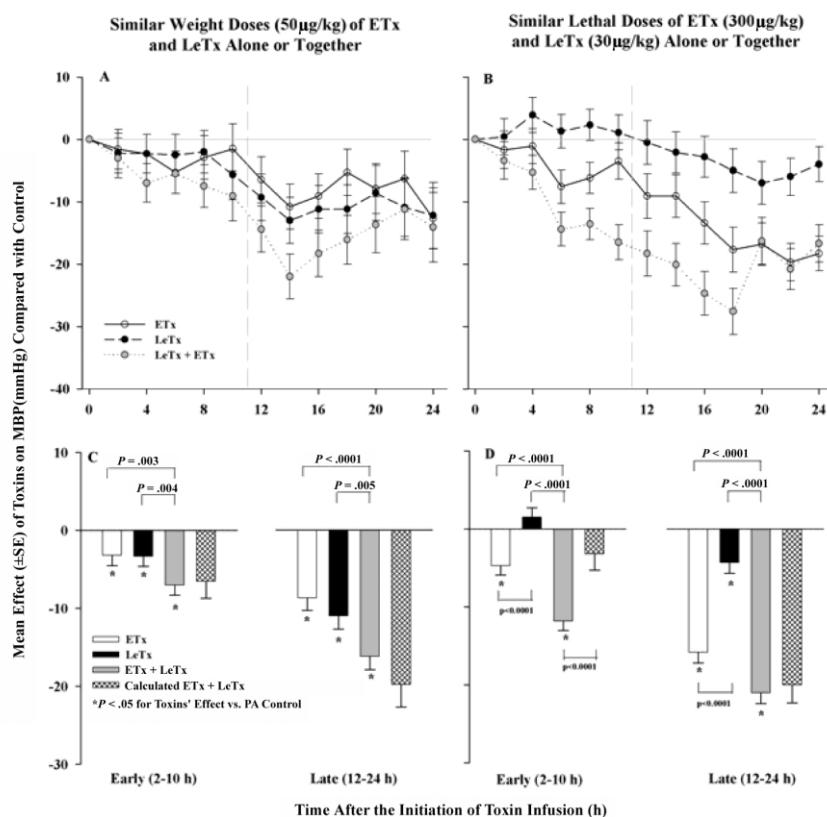
**Hemodynamic and blood measures.** As described elsewhere [14], catheters were attached to arterial and central venous access ports on each rat and were then connected to syringe pumps or pressure transducers. MBP and HR data and serial CBCs and levels of ABGs, cytokines (tumor necrosis factor- $\alpha$ , interleukin [IL]-1 $\alpha$  and -1 $\beta$ , IL-2, IL-6, IL-10, interferon [IFN]- $\gamma$ , 2 migratory inhibitory proteins [MIP-1 $\alpha$  and MIP-3 $\alpha$ ], and RANTES), and NO were measured.

**Statistical analysis.** Linear regression tested the relationship between toxin doses and mortality rate, and a rank test compared the dose ranges of ETx and LeTx producing 0%–100% mortality. The lethal effects of the toxins alone or together were analyzed using a Cox proportional-hazards model and are presented as the log of the hazard ratio for death  $\pm$  SE. All other laboratory measures were analyzed using analysis of variance. Data were averaged over variables when justified on the basis of statistical analysis to increase power and reduce animal use. Effects were calculated by subtracting the mean of the control group from the mean of the challenge group. Data were log transformed where appropriate. For all laboratory measures, the observed effects of ETx and LeTx together were compared with the sum of the effects of the toxins alone. All results are expressed as means  $\pm$  SEs, and  $P \leq .05$  was considered to indicate significance.

## RESULTS

### Increasing Doses of ETx or LeTx Alone (Study 1)

**Survival.** Increasing doses of either ETx or LeTx increased mortality rates [ $y = -4.1 + 1.8 \log(x)$  for ETx and  $y = -1.04 + 0.9 \log(x)$  for LeTx;  $r = 0.9$  and  $P \leq .001$  for each] (figure 1A). However, the range of doses over which lethality was



**Figure 3.** Serial effects on mean blood pressure (MBP) of similar weight (A) or similar lethal (B) doses of edema toxin (ETx) and lethal toxin (LeTx) alone or together, compared with those of protective antigen (PA) control (data not shown). These effects were compared at early (2–10 h) or late (12–24 h) time points before or after mortality was first evident in experiments (C, similar weight doses of toxin; D, similar lethal doses of toxin). The calculated effects of ETx and LeTx together were determined by adding the effects of each toxin alone. Significant differences are denoted by the brackets and *P* values.

first evident and then increased was higher and broader for ETx (200–800 µg/kg) than for LeTx (12.5–200 µg/kg) ( $P < .0001$  for the 2 ranges). Time to death in nonsurvivors did not differ significantly between toxins ( $31 \pm 6$  vs.  $20 \pm 6$  h for ETx and LeTx, respectively;  $P =$  not significant [NS]).

**Hemodynamics.** Measurement time influenced the effects of toxin over the course of the 24-h period of infusion on both MBP and HR ( $P \leq .05$ ). Therefore, changes in MBP and HR with increasingly lethal doses of ETx versus LeTx were compared during early (2–10 h) or late (12–24 h) time periods (i.e., before and after mortality was first evident in experiments, respectively). Time did not alter the effects of toxin doses during each of these periods ( $P =$  NS), so data were averaged (figure 1).

Early, compared with controls (data not shown), all doses of ETx, but not LeTx, decreased MBP significantly ( $P \leq .05$  for each ETx dose), and the effects of ETx were different from those of LeTx ( $P < .0001$  comparing the changes in each toxin alone averaged over the number of doses analyzed) (figure 1B). Late, both toxins decreased MBP, but these decreases were

greater for more lethal doses of each ( $P \leq .0004$  for the influence of dose with each toxin), and for ETx compared with LeTx ( $P < .0001$  averaged over doses) (figure 1C).

Early, compared with controls, 1 higher LeTx dose decreased HR significantly ( $P < .0001$ ), whereas all ETx doses increased it ( $P < .0001$  for each); overall, the effects of LeTx and ETx were very different ( $P < .0001$  averaged over doses) (figure 1D). Late, 3 of 4 LeTx doses decreased HR significantly, and these reductions increased with more lethal doses ( $P < .05$  for each effect) (figure 1E). By contrast, the 3 lower doses of ETx increased HR ( $P < .05$  for each), but these increases were reduced with more lethal doses, and the most lethal ETx dose reduced HR ( $P < .0001$  for the decreasing effect of more lethal ETx doses) (figure 1E).

#### Similar Weight or Lethal Doses of ETx and LeTx Alone or Together (Study 2)

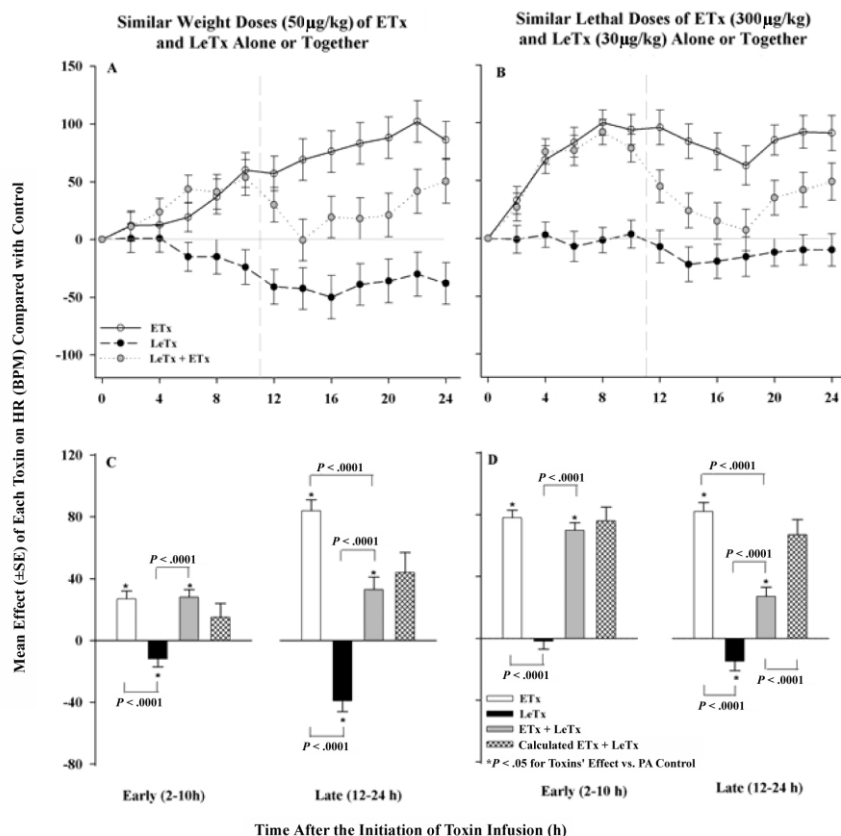
**Survival.** The observed increases in mortality rate (log normal of the hazard ratio of death  $\pm$  SE) with ETx and LeTx

together were not significantly different from those calculated with their effects alone for either similar weight doses ( $2.6 \pm 1.1$  vs.  $2.9 \pm 1.0$ ;  $P = .53$  for observed and calculated, respectively) or similar lethal doses ( $3.1 \pm 1.0$  vs.  $3.9 \pm 1.5$ ;  $P = .55$ , respectively) (figure 2A and 2B, respectively). Under the assumption of similar results with further experiments testing similar weight or lethal toxin doses, an additional 648 or 1244 rats would have been required, respectively, to demonstrate that differences between the observed and calculated effects of the toxins together were different at the  $P = .05$  level (Fisher's exact test).

**Hemodynamics.** Time did not significantly alter ( $P = NS$ ) the effects of toxin alone or together on MBP and HR during either early (2–10 h) or late (12–24 h) time periods during infusion, and these were averaged as described in study 1 (figures 3 and 4). Compared with controls (data not shown), similar weight doses of ETx and LeTx alone both decreased MBP early and late ( $P < .05$  for all) in similar patterns (figures 3A and 3C). Together, the 2 toxins decreased MBP more than either alone over both periods ( $P \leq .05$  for all) but never significantly

differently ( $P = NS$ ), compared with the sum of their effects alone. Similar lethal doses of ETx and LeTx alone decreased MBP early and late ( $P \leq .05$  for all), except for early LeTx (figure 3B and 3D). Reductions in MBP were greater with ETx, compared with LeTx, for each time period ( $P < .0001$  for both early and late). Together, these doses of the 2 toxins decreased MBP more than either toxin alone early and late ( $P \leq .05$  for all). Although these decreases with the toxins together were greater than the sum of their individual effects early ( $P < .0001$ ), they were not different late ( $P = NS$ ).

Compared with controls, both early and late similar weight and lethal doses of ETx alone increased HR, whereas LeTx decreased it ( $P \leq .05$  for all), except for the early lethal LeTx dose (figure 4A–4D). Throughout, increases in HR when ETx was used were greater and different than those when LeTx was used ( $P < .0001$  for all times). Together, both similar weight and lethal doses of toxin always produced effects on HR that were either intermediate between the increases with ETx alone and decreases with LeTx alone or not significantly different ( $P = NS$ ) from the sum of the effects of each alone.



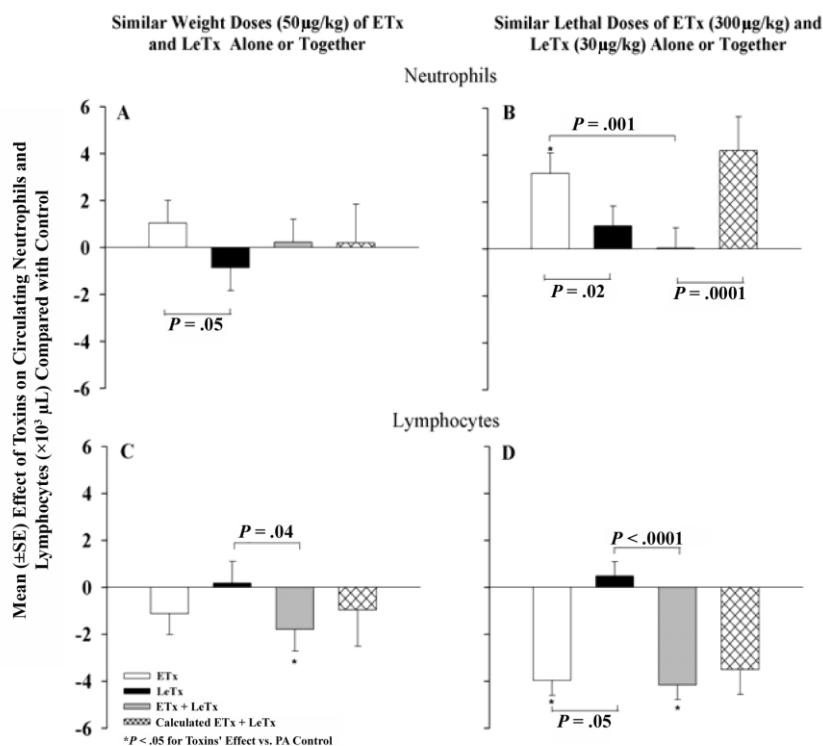
**Figure 4.** Serial effects on heart rate (HR) of similar weight (A) or similar lethal (B) doses of edema toxin (ETx) and lethal toxin (LeTx) alone or together, compared with those of protective antigen (PA) control (data not shown). The analysis and presentation of these data are similar to those in figure 3. BPM, beats per minute.

**CBC, ABG, cytokines, and NO measures.** Measurement time (8 vs. 24 h) did not alter the effects ( $P = \text{NS}$ ) of the toxins alone or together on any blood measures, with the exception of hemoglobin and NO levels. Therefore, these times were averaged over for all measures except these 2. Compared with controls (data not shown), similar weight and lethal doses of ETx alone increased the numbers of circulating neutrophils ( $P < .05$  for similar lethal dose) in patterns different from those of LeTx alone ( $P \leq .05$  for all) (figure 5A and 5B). Similar lethal doses of the toxins together had effects that were less than those calculated from the toxins alone ( $P = .001$ ). Both doses of ETx alone decreased the numbers of circulating lymphocytes ( $P < .05$  for similar lethal dose), and, with similar lethal doses, these decreases were different from the changes when only LeTx was used ( $P < .0001$ ) (figure 5C and 5D). Both doses of the 2 toxins together had effects on lymphocytes that were not significantly different from the sum of their effects alone ( $P = \text{NS}$ ).

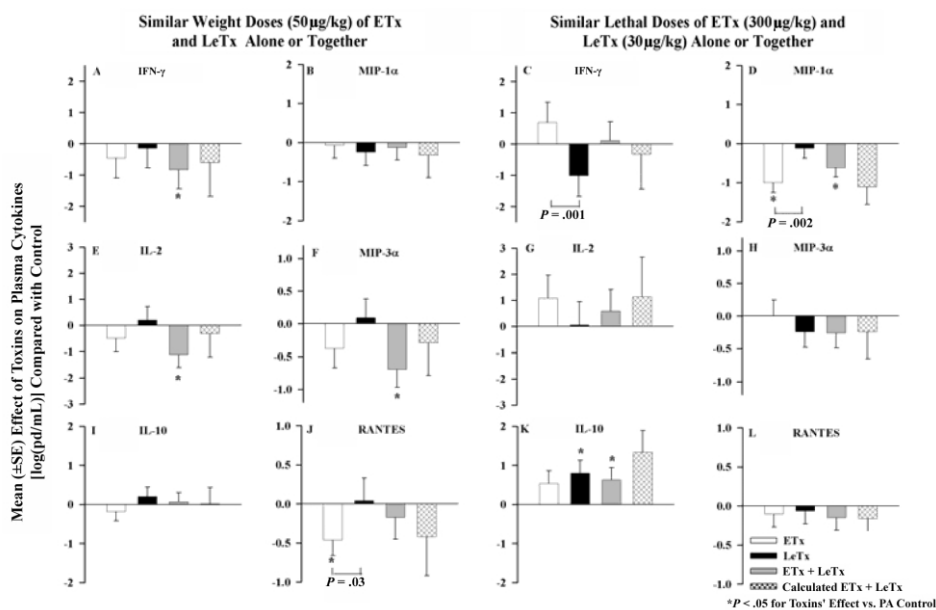
Compared with controls, similar weight and lethal doses of ETx alone reduced levels of RANTES and MIP-1 $\alpha$ , respectively ( $P \leq .05$  for both) and in patterns different from changes when LeTx alone was used ( $P \leq .03$  for each) (figure 6D and 6J).

Similar lethal doses of ETx increased and LeTx decreased levels of IFN- $\gamma$  in patterns that were different ( $P = .001$ ) (figure 6C). Similar weight doses of ETx and LeTx together decreased levels of IFN- $\gamma$ , MIP-3 $\alpha$ , and IL-2 (figure 6A, 6E, and 6F), and similar lethal doses of the 2 toxins together decreased MIP-1 $\alpha$  and increased IL-10 levels significantly (all  $P \leq .05$ ) but not differently from the sum of the effects of the toxins alone (figure 6D and 6K).

At 24 h, similar weight doses of LeTx increased and similar lethal doses of ETx decreased levels of hemoglobin ( $P < .05$  for each) (figure 7A and 7B). Both doses of the 2 toxins together increased levels of hemoglobin significantly at 8 and 24 h ( $P \leq .05$ ), except for similar lethal doses at 8 h, which decreased levels ( $P < .05$ ). These combined effects were not different from the sum of their effects alone for similar weight doses but were for similar lethal doses ( $P \leq .04$  early and late). The similar lethal dose of ETx decreased levels of NO at 8 h ( $P \leq .05$ ) (figure 7C and 7D). Together, similar weight doses of the toxins increased levels of NO at 8 h and decreased them at 24 h, and similar lethal doses decreased levels at 8 h ( $P \leq .05$  for all). None of these effects were different from the sum of the effects of the toxins alone ( $P = \text{NS}$ ). There were no significant changes



**Figure 5.** A and C, Effects of similar weight doses of edema toxin (ETx) and lethal toxin (LeTx) alone or together on circulating neutrophils and lymphocytes averaged over the course of the 8- and 24-h measurement times. B and D, Effects of similar lethal doses of ETx and LeTx. The calculated effects of ETx and LeTx together were determined by adding the effects of each toxin alone. Significant differences are denoted by the brackets and  $P$  values. PA, protective antigen.



**Figure 6.** A, B, E, F, I, and J, Effects of similar weight doses of edema toxin (ETx) and lethal toxin (LeTx) alone or together on interferon (IFN)- $\gamma$ , macrophage inflammatory protein (MIP)-1 $\alpha$ , interleukin (IL)-2, MIP-3 $\alpha$ , IL-10, and RANTES, respectively, averaged over 8- and 24-h measurement times. C, D, G, H, K, and L, Effects of similar lethal doses of ETx and LeTx. The calculated effects of ETx and LeTx together were determined by adding the effects of each toxin alone. Significant differences are denoted by the brackets and *P* values. PA, protective antigen.

in any of the other blood measures, including arterial oxygenation, throughout the study (*P* = NS for all comparisons).

## DISCUSSION

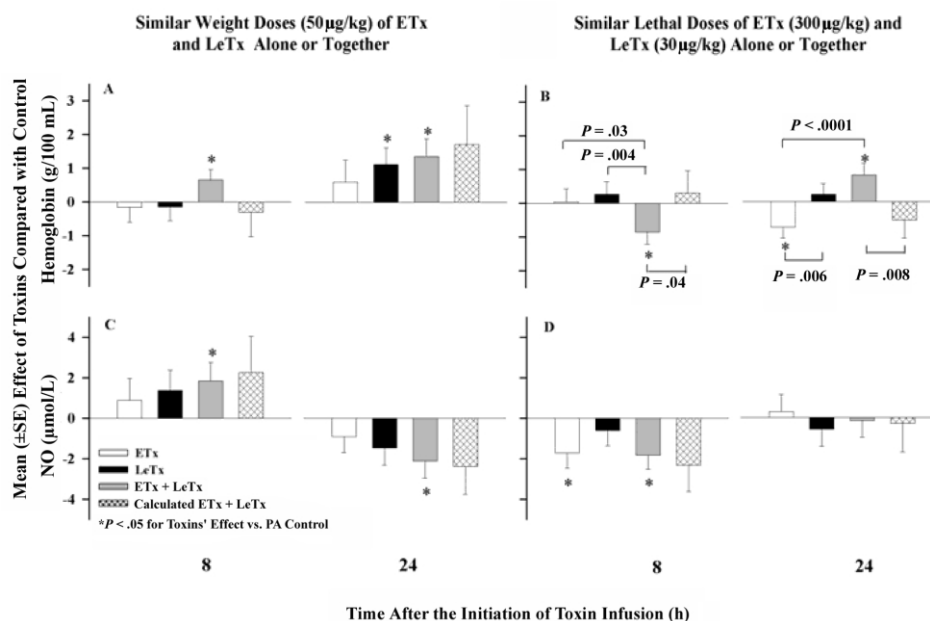
To our knowledge, the present study is the first to have directly compared the effects of recombinant preparations of *B. anthracis* ETx and LeTx alone or together on survival and hemodynamic function. Early studies suggested that LeTx was primarily responsible for the lethal effects of *B. anthracis* and that the 2 toxins worked synergistically [11–13]. In the present study, however, the lethal dose range of ETx was only 10 times greater than that of LeTx. In addition, ETx produced greater and earlier reductions in blood pressure than LeTx, and the 2 toxins together had effects that were additive.

Although both LeTx and ETx produced hypotension, its onset and the changes in HR associated with it differed between the 2 toxins. The mechanisms underlying the hypotensive effects of LeTx are unclear. Recent studies and the present study have suggested that it is not due to excessive inflammatory cytokine or NO release [14, 17]. Instead, LeTx may alter endothelial cell function and vascular permeability via noninflammatory mechanisms [25–27]. Consistent with this and similar to the results of previous studies, LeTx increased levels of hemoglobin at later time points [14]. These likely reflect hemoconcentration related to the extravasation of fluid and may be a basis for similar changes observed in patients with *B. anthracis*

infection [9, 16]. Decreases in HR seen when LeTx was used could also contribute to shock.

Compared with LeTx, hypotension when ETx was used, in both nonlethal and lethal doses, was greater or occurred earlier. As with LeTx, these changes were not associated with excessive cytokine or NO production. In contrast to LeTx, however, levels of hemoglobin were not increased with ETx, which suggests that it did not produce substantial extravasation of fluid. It is very likely, however, that hypotension with ETx is, in part, related to the adenylyl cyclase activity of EF [6, 28]. The recombinant ETx preparation investigated stimulated adenylyl cyclase in CHO-K1 cells, just as ETx prepared from live *B. anthracis* did previously [6, 23]. In the present in vitro studies, the efficient delivery of EF to the cytosol by PA binding caused rapid increases in levels of intracellular cAMP. In vivo uptake of EF and stimulation of cAMP in vascular smooth-muscle cells that express PA receptor would cause sequestration of free cytosolic calcium by the sarcoplasmic reticulum and dephosphorylation of the myosin light chain, followed by vascular dilation [28, 29].

In support of this, in the present study, ETx was associated with pronounced increases in HR that would be expected with increased levels of cAMP in cardiac pacemaker cells [21]. This tachycardia was greater than the compensatory one associated with shock caused by lipopolysaccharide in this rat model [14]. It is noteworthy that, in a recent clinical analysis, patients with



**Figure 7.** Effect on hemoglobin and plasma nitric oxide (NO) of similar weight (*A* and *C*, respectively) or similar lethal (*B* and *D*, respectively) doses of edema toxin (ETx) and lethal toxin (LeTx) alone or together, compared with that of protective antigen (PA) control (data not shown) at 8 and 24 h. The calculated effects of ETx and LeTx together were determined by adding the effects of each toxin alone. Significant differences noted when comparing these effects are denoted by the brackets and *P* values.

inhalational anthrax had more tachycardia than patients with other types of infection [16].

Different from the results of the present study, in recent investigations in mice, rapid intravenous administration of ETx in doses producing 100% lethality decreased the HR at the time of death [18]. These ETx doses were also less than LeTx doses producing 100% lethality in the same model. Thus, the dose and method of administration, as well as the species used, may influence the effects of ETx and LeTx. In the present study, however, the infusion of ETx in <100% lethal doses and over time resulted in the tachycardic response predicted on the basis of EF's adenyl cyclase effects. Such infusions may better simulate the gradual increase in toxin that likely occurs during infection.

Like lethality, both the MBP and HR effects of ETx and LeTx were additive. These findings suggest that, to be most effective clinically, therapies to reverse the hemodynamic dysfunction associated with toxin production during *B. anthracis* infection should target both ETx and LeTx. In fact, because the molar weights of EF and LF are similar, these findings suggest that, during advanced infection, therapies inhibiting the effects of LeTx alone may fail if ETx has reached levels only 10 times greater than the lowest lethal doses of LeTx.

As in a previous study, in the present study, LeTx had little effect on circulating neutrophil and lymphocyte counts [6]. By contrast, ETx increased neutrophil counts and decreased lym-

phocyte counts. These changes may be because stimulation of intracellular cAMP by ETx has been shown in vitro to inhibit the adhesion of neutrophils [30] while stimulating apoptosis and blocking the proliferation of lymphocytes [31]. In an in vivo model, such changes would have increased neutrophil counts while decreasing lymphocyte counts. Interestingly, LeTx appeared to counteract the effect of ETx on neutrophils but not on lymphocytes. It is worthwhile noting, however, that differences in species might alter the effects of these toxins on circulating cells, just as they might on hemodynamics. In contrast to the limited effects that LeTx infusion has on circulating leukocytes in rats, LeTx reduced mononuclear cell counts and increased neutrophil counts in BALB/cJ and C57BL/6J mice [17]. These changes persisted in both strains later, except for the decreases in mononuclear cell counts in C57BL/6J mice, which became increases later. Furthermore, although ETx increased neutrophil counts in BALB/cJ mice, it did not cause the decreases in lymphocyte counts observed in rats [18].

To test the effects of ETx and LeTx in combination, 2 different types of doses (i.e., same weight and same lethality) were used in the present study. Together, these doses had effects that were additive. At present, however, it is unknown how the rates of production and release of PA, EF, and LF compare during live bacterial infection. Because the 2 factors react competitively and similarly with PA, differences in their production rates could result in effects that favored one over the other. For



example, even small increases in the rate of production of LF, compared with that of EF, could cause greater effects of the former over the latter factor. To add to the complexity of this picture, it has now been shown that ETx can itself induce anthrax toxin–receptor expression and that this effect varies among cell types from different species [32]. Such an effect when present could accelerate the physiological effects of ETx and might provide a basis for the more-rapid changes in MBP and HR noted with the toxin in the present study.

In conclusion, the present findings suggest that ETx could play a substantial role in the pathogenesis of the shock and lethality occurring during *B. anthracis* infection. However, to what extent this rat model, which incorporated continuous infusions of ETx and LeTx, reproduces the deranged physiological changes that occur during anthrax itself is debatable. In fact, other components of this microbe, such as its cell wall, may contribute to the changes observed during actual infection. The present findings do raise the possibility that therapies designed to neutralize the toxigenic component of *B. anthracis* should probably target both LeTx and ETx to be most beneficial.

## Acknowledgments

We thank Jing Feng and Yu Cao for technical support and Jennifer Candotti for manuscript preparation.

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