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Bacillus anthracis cell wall produces injurious inflammation but paradoxically decreases the lethality of anthrax lethal toxin in a rat model

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Abstract

Objectives—The in vivo inflammatory effects of the *Bacillus anthracis* cell wall are unknown. We therefore investigated these effects in rats and, for comparison, those of known inflammatory stimulants, *Staphylococcus aureus* cell wall or lipopolysaccharide (LPS).

Method and Results—Sprague–Dawley rats ($n = 103$) were challenged with increasing *B. anthracis* cell wall doses (10, 20, 40, 80, or 160 mg/kg) or diluent (control) as a bolus or 24-h infusion. The three highest bolus doses were lethal (20–64% lethality rates) as were the two highest infused doses (13% with each). Comparisons among lethal or nonlethal doses on other measured parameters were not significantly different, and these were combined for analysis. Over the 24 h after challenge initiation with lethal bolus or infusion, compared to controls, ten inflammatory cytokines and NO levels were increased and circulating neutrophils and platelets decreased ($P \leq 0.05$). Changes with lethal doses were greater than changes with nonlethal doses ($P \leq 0.01$). Lethal bolus or infusion doses produced hypotension or hypoxemia, respectively ($P \leq 0.05$). The effects with *B. anthracis* cell wall were similar to those of *S. aureus* cell wall or LPS. However, paradoxically administration of *B. anthracis* cell wall or LPS decreased the lethality of concurrently administered *B. anthracis* lethal toxin ($P < 0.0001$ and 0.04, respectively).

Conclusion—*B. anthracis* cell wall has the potential to produce inflammatory injury during anthrax infection clinically. However, understanding why cell wall or LPS paradoxically reduced lethality with lethal toxin may help understand this toxin's pathogenic effects.

Keywords

Bacillus anthracis; Cell wall; Cardiopulmonary dysfunction; Cytokine release; Inflammation

Introduction

The influence of *Bacillus anthracis* cell wall on inflammation and tissue injury has not been tested in vivo. The structure and inflammatory effects of cell wall components differ among gram-positive bacteria. While *Staphylococcus aureus* cell wall components have consistently been shown to stimulate an inflammatory response, those of other gram-positives have been more variable [1–3]. In this regard, *B. anthracis* cell wall has been shown to stimulate production of tumor necrosis factor (TNFα), interleukin 1 (IL-1β) and IL-6 in peripheral blood mononuclear cells [4]. In cultured epithelial cells *B. anthracis* cell wall activates NF-kB via TLR2 and TLR6 [5]. Finally, infection with *B. anthracis* in vitro results in NOD2-associated IL-1β production [6].

Based on such in vitro results, we hypothesized that *B. anthracis* cell wall in vivo has the potential to produce systemic inflammation and organ dysfunction. To test this, increasing doses of *B. anthracis* cell wall administered either as an intravenous bolus or, to simulate its gradual release during infection, as a 24-h infusion were investigated in a rat model [7, 8]. For comparison, infusions of similar weight doses of cell wall from a *S. aureus* strain pathogenic for humans and of similarly lethal doses of lipopolysaccharide (LPS) were also studied. Finally, to investigate the effects of cell wall on the lethal effects of anthrax lethal toxin (LeTx), animals were challenged with these components either alone or in combination, and survival was assessed. We hypothesized that the potentially injurious effects of the cell wall would likely add to the lethal effect of LeTx.

Materials and methods

Animal care

The protocol used in this study was approved by the Animal Care and Use Committee of the Clinical Center of the National Institutes of Health.

Study design

Sprague–Dawley rats (n = 103) with carotid arterial and jugular venous catheters were randomized to receive *B. anthracis* cell wall in a total dose of 10, 20, 40, 80, or 160 mg/kg or diluent (control), as either a bolus injection or 24-h continuous infusion [7, 8]. All animals received equivalent volumes of cell wall or diluent. Immediately before challenge and at 15-min intervals in the first hour, 2-h intervals from 2 to 8 h and 4-h intervals from 12 to 24 h after the initiation of cell wall challenge, mean arterial blood pressures (MBP) and heart rates (HR) were measured. At 4, 8 and 24 h, arterial blood was collected in all animals for blood gas (ABG), complete blood count (CBC), plasma cytokine and nitrite/nitrate (NO) measures. Animals had similar volumes (0.5 ml) of blood drawn and normal saline replaced at each time point, and were observed for 168 h. For comparison to *B. anthracis* cell wall, additional animals (n = 84) were challenged with 24-h infusions of either similar doses of *S. aureus* cell wall, doses of LPS (0.25, 0.5 and 1 mg/kg per 24 h) producing a similar range of lethality rates [7, 8] or diluent (control), all in equivalent volumes.

In order to investigate the effects of *B. anthracis* cell wall on the lethal effects of LeTx, animals (n = 55) were challenged with 24-h infusions of either cell wall (40 mg/kg per 24 h) or LeTx (100 μg/kg protective antigen (PA) + 50 μg/kg lethal factor (LF) over 24 h) alone or together or diluent. For comparison, additional animals (n = 82) were challenged with LPS (0.1 mg/kg per 24 h) or the same dose of LeTx alone or together. Survival was assessed at 168 h.

Cell wall, LeTx and LPS preparations

B. anthracis cell wall (Sterne strain) was prepared based on previously reported methods and obtained from List Biological Laboratories (Campbell, CA) [4, 9]. *S. aureus* cell wall was prepared in the laboratory of one of the authors (JS) using similar methods. For both *B. anthracis* and *S. aureus* cell wall, agarose gel electrophoresis with ethidium eromide staining and SDS-PAGE with comassie blue staining did not show any detectable DNA/RNA or protein contamination, respectively.

Based on kinetic chromogenic limulus amoebocyte lysate assays performed [Lonza (Basel, Switzerland) or Clogen Laboratories (Germantown, MD)], endotoxin was undetectable in either *B. anthracis* or *S. aureus* cell wall preparations. The lowest level of endotoxin detection by these assays is <0.1 EU/mg cell wall (or 0.01 ng/mg cell wall). Challenge (24 h infusion) in rats (n = 6) with a total dose of LPS comparable to what would be received if the highest cell wall dose tested (160 mg/kg) was contaminated with LPS at a level of 0.01 ng/mg cell wall did not alter any parameter measured in this study compared to diluent control (n = 6).

Cell wall preparations were diluted with 1 × phosphate buffered saline (PBS) to deliver doses of 10, 20, 40, 80 and 160 mg/kg bw when administered either as an infusion over 24 h in 12 ml at a rate of 0.5 ml/h or as a bolus over 2 min in a volume of 0.7 ml. LPS from *Escherichia coli* 0111:B4 (Sigma–Aldrich, St. Louis, MO) and lethal toxin were prepared and administered as previously described [10].

Laboratory measures

Arterial blood pressure, heart rate, ABG, lactate, and CBC measures and samples for cytokine and NO levels were obtained as previously described [7]. Cytokines [IL-1β, IL-6, IL-10 TNFα, migratory inhibitory protein 1α (MIP-1α), regulated on activation, normal T cell expressed and secreted (RANTES), granulocyte macrophage-colony stimulating factor (GM-CSF) and interferon γ (INFγ)] were measured using standard kits [Searchlight® Proteome Array Multiplex system (Pierce Biotechnology, Rockford, IL, or Cytokine Multiplex Immunoasssy Kit, Millipore, Danvers, MA)]. Plasma nitrite/nitrate (NO) was measured with a fluorometric assay kit (Cayman Chemical, Ann Arbor, MI).

Statistics

Kaplan–Meier survival curves were used to show the survival effect of individual doses of cell wall or LPS. All other parameters were analyzed with repeated measures ANOVA using PROC MIXED in Statistical Analysis System Version 9.1 software (SAS Institute, Inc., Cary, NC), and least squares means and associated standard errors were reported. For each of the three challenges (*B. anthracis* or *S. aureus* cell wall or LPS), comparison among nonlethal doses or among lethal doses on measured parameters did not show significant differences throughout. Therefore, to increase the power to detect the effects of cell wall or LPS, nonlethal doses were combined into one group and lethal doses into another for comparison to respective control (diluent) groups. All results are expressed as least square means ± SEM, and two-sided P values ≤ 0.05 were considered significant. For clarity of presentation in some figures in which both the challenge effect and challenge effect versus time interaction were significant, a single P value is shown and corresponds to the less significant comparison. However, in these cases it is also denoted that each effect reached significance.

Results

B. anthracis cell wall bolus

While the two lower *B. anthracis* bolus cell wall doses (10 and 20 mg/kg) did not worsen survival, the three higher ones (40, 80 and 160 mg/kg) did (Fig. 1a) ($P = 0.003$ comparing lethal doses to control, Wilcoxon test). Compared to controls, over the 24 h following challenge lethal cell wall doses decreased MBP and increased lactate ($P < 0.0001$ and $P = 0.02$, respectively), although the effects on MBP varied with time ($P < 0.0001$ for the interaction between cell wall effect and time) (Fig. 1). While both nonlethal and lethal doses increased heart rate ($P \leq 0.01$ for each compared to controls), they had variable effects on A-aO_2 ($P \leq 0.02$ for the interaction between cell wall effect and time for each) (Fig. 1). Compared to controls, both nonlethal and lethal cell wall doses first decreased (4 and 8 h) and then increased (24 h) circulating neutrophils and lymphocytes ($P \leq 0.03$ for the interaction between cell wall effect and time for all) but decreased platelets throughout ($P = 0.001$ and $P < 0.0001$ compared to controls, respectively) (Fig. 1). Changes in neutrophils (decreases and then increases) and platelets were greater with lethal compared to nonlethal cell wall doses ($P \leq 0.001$ for each).

Compared to controls, while both nonlethal and lethal cell wall bolus doses significantly increased IL-1β, IL-6, MIP-1α, and GM-CSF levels ($P \leq 0.04$ for all) over the 24 h after initiation of challenge, these changes except for GM-CSF were significantly greater with lethal compared to nonlethal doses ($P \leq 0.01$ for all) (Fig. 2). Furthermore, only lethal cell wall significantly increased IL-10, IFNγ and RANTES across all time points ($P \leq 0.02$ for all compared to controls) or TNFα at early time points ($P = 0.03$ for the interaction between cell wall effect and time) (Fig. 2). Finally, both nonlethal and lethal cell wall doses increased NO levels significantly ($P < 0.0001$ compared to controls for all) (Fig. 2). However, these

increases were greatest at 8 h ($P < 0.0001$ for the interaction between the effects of both nonlethal and lethal cell wall doses and time), and they were always greater with lethal cell wall doses ($P = 0.001$).

B. anthracis cell wall infusion

When administered as 24-h infusions, none of the three lower *B. anthracis* cell wall doses (10, 20 and 40 mg/kg) worsened survival, but the two higher doses did (Fig. 3), although these changes were not statistically significant (P = ns comparing lethal cell wall doses to control). Compared to controls, while neither nonlethal or lethal cell wall decreased MBP consistently during the 24-h challenge infusion, lethal dose increased heart rate and lactate levels ($P \leq 0.003$ for each) (Fig. 3). However, both nonlethal and lethal cell wall doses worsened gas exchange and increased the A-aO_2 ($P \leq 0.0003$ for each compared to controls) (Fig. 3). Furthermore, both nonlethal and lethal doses decreased circulating neutrophil, lymphocyte and platelet counts ($P \leq 0.01$ for all compared to controls) during the 24-h infusion (Fig. 3). These changes in A-aO_2 and circulating cells were significantly greater with lethal compared to nonlethal cell wall doses ($P \leq 0.01$).

Compared to controls, although nonlethal cell wall consistently increased all cytokine levels throughout the 24-h infusion period, these changes did not reach significance. In contrast lethal cell wall significantly increased all cytokines measured at almost all time points ($P \leq 0.03$ compared to controls) (Fig. 2). These changes were all significantly greater with lethal compared to nonlethal doses ($P \leq 0.01$) except for IFNγ. Finally, although not as great as with cell wall administered as a bolus, both nonlethal and lethal infused cell wall doses increased NO levels, but these were only significant with the latter ($P = 0.0002$) (Fig. 2).

S. aureus cell wall and LPS infusions

While the two lower *S. aureus* doses (10 and 20 mg/kg) did not worsen survival, the three higher doses (40, 80 and 160 mg/kg) did (Figure E1), although these changes were not statistically significant (P = ns comparing lethal cell wall doses to control). Compared to controls, neither nonlethal nor lethal *S. aureus* cell wall doses had consistent effects on MBP or HR (Figure E1). However, compared controls, both nonlethal and lethal doses increased lactate levels ($P < 0.0001$ for each) and lethal dose increased A-aO_2 ($P = 0.04$), although all these changes were greater at earlier time points ($P \leq 0.04$ for the interaction between cell wall effect and time) (Figure E1). Both nonlethal and lethal *S. aureus* doses decreased neutrophils ($P \leq 0.005$ and 0.002 compared to controls for each, respectively) and platelets and lethal dose decreased lymphocytes ($P = 0.0006$ compared to controls) (Figure E1). Changes were greater with neutrophils earlier and with platelets later ($P \leq 0.002$ for the interaction between cell wall effect and time), and changes in all three cell types were significantly greater with lethal cell wall doses ($P \leq 0.001$ for all). Both nonlethal and lethal *S. aureus* cell wall doses significantly increased IL-1β, IL-10, TNFα, MIP-1α and NO levels ($P \leq 0.01$ compared to controls for all) (Figure E2), although these changes were not consistently greater with the lethal doses.

The lowest dose of infused LPS (0.25 mg/kg) did not alter survival, but the two higher ones (0.5 and 1.0 mg/kg) did (Figure E1), although these changes were not statistically significant (P = ns comparing lethal cell wall doses to control). While only the nonlethal LPS dose reduced MBP at later time points ($P = 0.0003$ for the interaction between the effect of LPS and time) and neither dose had consistent effects on HR, both doses increased lactate levels ($P \leq 0.004$ for each compared to controls) (Figure E1). LPS did not increase A-aO_2 (Figure E1). Comparing controls, both doses decreased neutrophils early and increased them late ($P < 0.0001$ for the interaction between cell wall effect and time) and decreased lymphocytes ($P \leq 0.03$ for each) and platelets ($P \leq 0.001$ for each) at all time points (Figure E1). Both LPS

doses significantly increased IL-1β, IL-6, IL-10, TNFα and MIP-1α levels (all $P \leq 0.05$), and lethal doses increased IFNγ and RANTES ($P \leq 0.002$), and decreased GM-CSF levels ($P = 0.001$) (Figure E2). Both LPS doses also increased NO levels ($P \leq 0.003$), and these increases were significantly ($P = 0.001$) greater with lethal doses (Figure E2).

Influence of *B. anthracis* cell wall on the lethal effects of LeTx

Infusion with diluent or *B. anthracis* cell wall alone produced no lethality, while anthrax LeTx alone was very lethal (68% lethality rate). Unexpectedly, this same dose of LeTx in combination with cell wall produced no lethality ($P < 0.0001$ comparing LeTx alone to cell wall with LeTx on survival) (Fig. 4). To explore this interaction further, animals were challenged with LeTx alone or in combination with LPS (0.1 mg/kg). This LPS dose, as with the anthrax cell wall dose employed, was just below the dosage range associated with lethality in the model and had been shown previously to produce cytokine and NO release. Consistent with *B. anthracis* cell wall, LPS in combination with LeTx resulted in a significant reduction in lethality compared to LeTx alone ($P = 0.04$) (Fig. 4).

Discussion

These in vivo findings are the first showing that *B. anthracis* cell wall produces a systemic inflammatory response with organ dysfunction and, at higher doses, lethality. Whether administered rapidly or slowly, both lethal and nonlethal cell wall doses increased intravascular cytokines and NO levels. Lethal cell wall doses produced greater changes in these mediators than nonlethal ones. While early increases in six cytokines and chemokines later decreased following cell wall bolus, all eight increased with cell wall infusion remaining elevated throughout. Along with its effects on cytokines and chemokines, *B. anthracis* cell wall reduced circulating neutrophils, lymphocytes and platelets. This is consistent with the observation that intravascular inflammation caused by microbial components increases adhesion molecule expression with adherence of circulating leukocytes and platelets to the endothelium [11, 12]. Reductions in these cells were in almost all cases greater with lethal compared to nonlethal cell wall doses.

Although both bolus and infusion of *B. anthracis* cell wall doses activated systemic inflammation, the physiologic basis for their lethal effects appeared different. Lethal bolus doses caused persistent hypotension and increases in lactate but not hypoxemia (i.e., unchanged A-aO_2). In contrast, lethal infused cell wall doses, although increasing heart rate and lactate levels, did not reduce blood pressure, but worsened oxygenation (i.e., increased A-aO_2). One basis for these differences is that bolus *B. anthracis* cell wall caused greater increases in NO than infusion. NO is closely associated with hypotension during sepsis [13]. On the other hand, reductions in circulating leukocyte number persisted throughout cell wall infusion, whereas these reversed with a bolus. Excessive and persistent activation of circulating leukocytes is implicated in septic lung injury [14, 15]. The effects of *B. anthracis* cell wall infusion were very similar to those noted with *S. aureus* cell wall.

Although LeTx is closely associated with the pathogenesis of *B. anthracis* infection, its isolated effects occur independent of excessive inflammation [7, 10, 16–18]. Studies in baboons demonstrated however that 2-h infusions of increasing *B. anthracis* (Sterne strain) concentrations produced dose-ordered decreases in blood pressure, neutrophils and platelets and increases in respiratory rate, TNFα, IL-1β and MIP-1α [19] and lung injury. These findings are very consistent with those noted with cell wall alone in this rat model. In combination these findings suggest that patients with *B. anthracis* sepsis may benefit from adjunctive therapies neutralizing toxin as well as cellular components like cell wall.

It required 1×10^{10} CFU bacteria to produce 1 mg of cell wall for these experiments. Thus, lethal doses of *B. anthracis* cell wall (40–160 mg/kg) were equivalent to infection with 40–160×10^{10} CFU/kg bw or 6–24×10^{9} CFU/ml blood (based on an estimated rat blood volume of 17 ml per 250 g animal). While these circulating *B. anthracis* counts are equivalent to ones lethal in guinea pigs, (5–15×10^{9} CFU/ml blood [20]), they are higher than those noted in baboons [19].

Challenge with LPS produced lethality and inflammatory change at much lower doses than *B. anthracis* cell wall. Separation of LPS from other gram-negative cell wall components may have increased its stimulatory effects. Whether challenge with individual components of *B. anthracis* cell wall (i.e., peptidoglycan or lipotechoic acid) would increase their inflammatory effects requires study. Breakdown of peptidoglycan chain into its muramyl subunits by bacterial autolysins might increase the chain's stimulatory capacity during infection [21, 22]. The large bacterial burden in patients and animals with *B. anthracis* could provide a high concentration of cell wall or its components [20, 23–25].

Different from our hypothesis, the lethal effects of LeTx were reduced rather than increased when administered with anthrax cell wall. This decrease did not appear to result from nonspecific binding of toxin by cell wall, since administration of LPS in a 400-fold lower dose than cell wall (0.1 vs. 40 mg/kg) also significantly reduced the lethal effect of LeTx. Of note, intraperitoneal lipotechoic acid administration protected mice from intravenous LeTx challenge [26]. Lethal factor, the toxigenic moiety of LeTx, is a protease that inhibits mitogen-activated protein kinase pathways important in adaptive host stress responses [8, 20]. This inhibition may contribute to the pathogenic effect of LeTx. Therefore, it is possible that stimulation of these pathways by cell wall or LPS prior to inhibition by LeTx may have contributed to protective host responses.

The present findings suggest that excessive stimulation of host inflammation by cell wall may contribute to the pathogenesis of *B. anthracis*. If effective methods to modulate host inflammation are developed for sepsis, they may be applicable for *B. anthracis*. Importantly, despite unexpected beneficial effects when cell wall was combined with LeTx, understanding this interaction may provide insights both into the pathogenic effects of LeTx as well as the treatment of anthrax sepsis itself.

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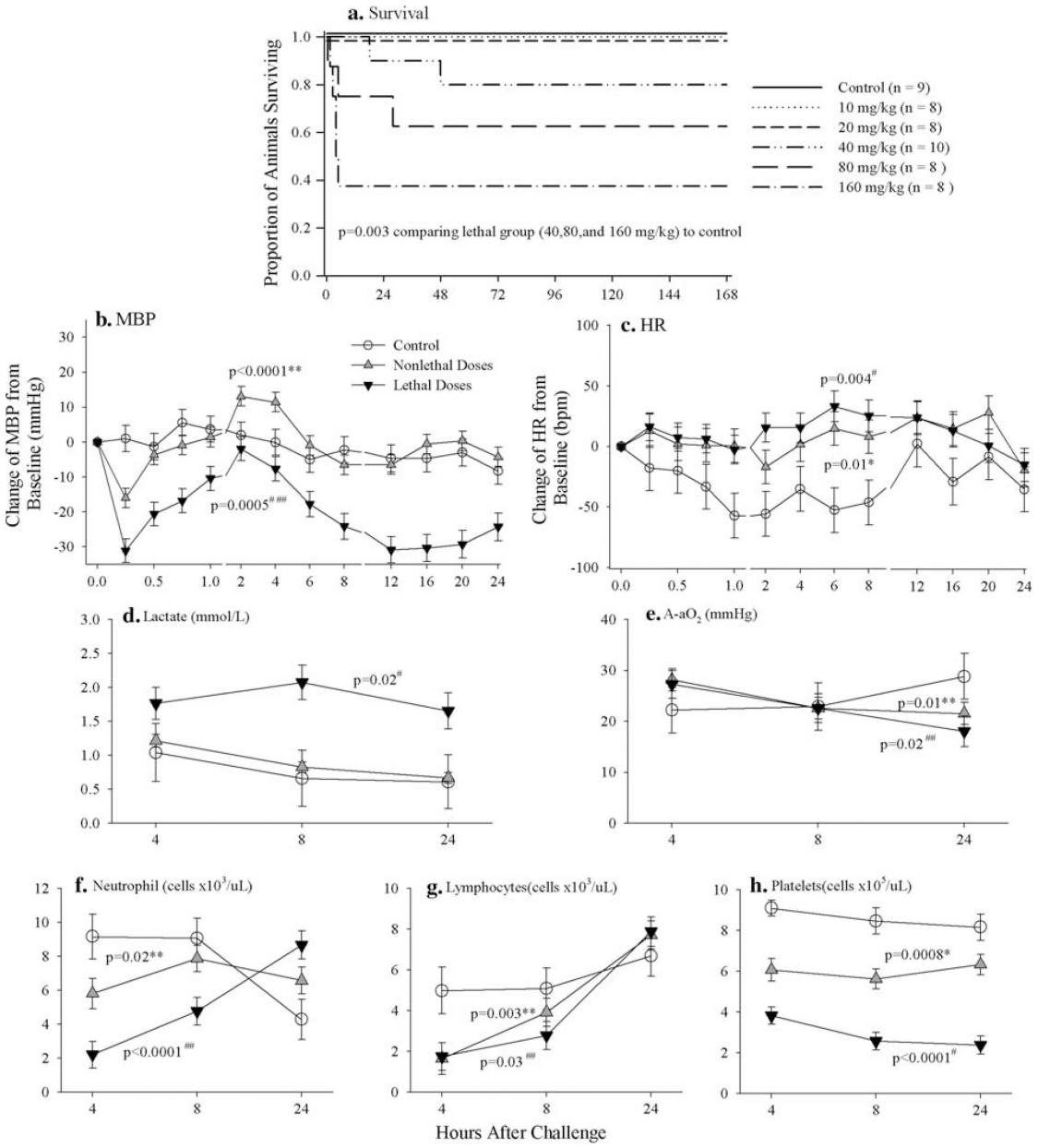
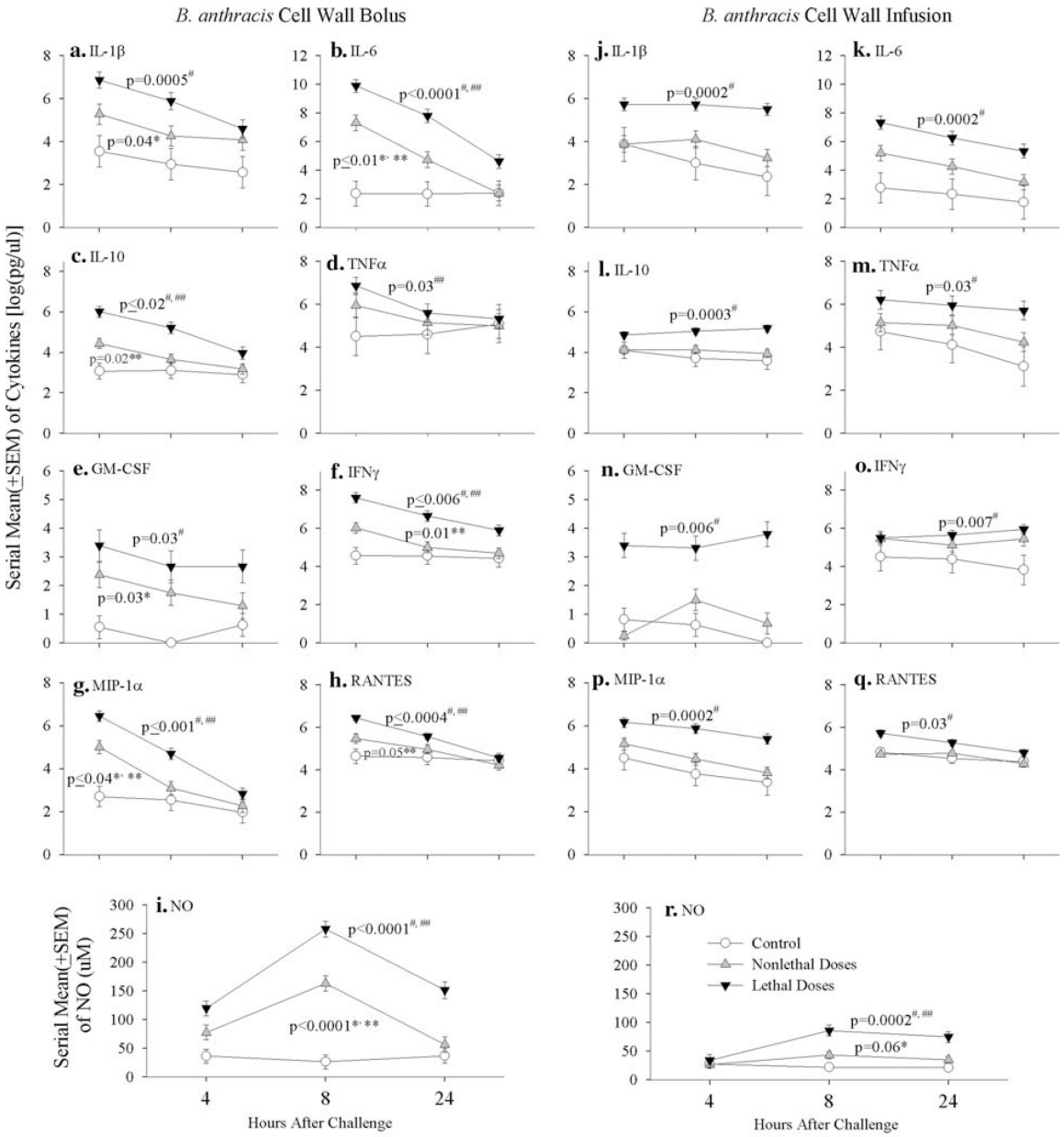


* p values for the effect of the nonlethal cell wall doses vs control
** p values for the interaction between the effect of the nonlethal cell wall doses and time
\# p values for the effect of the lethal cell wall doses vs control
\#\#p values for the interaction between the effect of the lethal cell wall doses and time

Fig. 1.

a Compares the effects of *B. anthracis* cell wall administered as a bolus in doses of 10, 20, 40, 80 or 160 mg/kg to a diluent (control) on the proportion of animals surviving. Based on the results shown in **a**, **b–h** show the effects of either nonlethal doses (10 and 20 mg/kg) or lethal doses (40, 80 and 160 mg/kg) of *B. anthracis* cell wall bolus combined compared to control on: the serial mean (±SEM) changes from baseline in mean arterial blood pressure (MBP, mmHg, **b**) and heart rate (HR, BPM, **c**) measured q15 min from 0 to 1 h, q2 h from 2 to 8 h and q4 h from 12 to 24 h and serial mean (±SEM) arterial lactates (**d**), arterial–

alveolar oxygen gradients ($A\text{-}aO_2$, **e**) and circulating neutrophils (**f**), lymphocytes (**g**) and platelets (**h**) measured at 4, 8 and 24 h

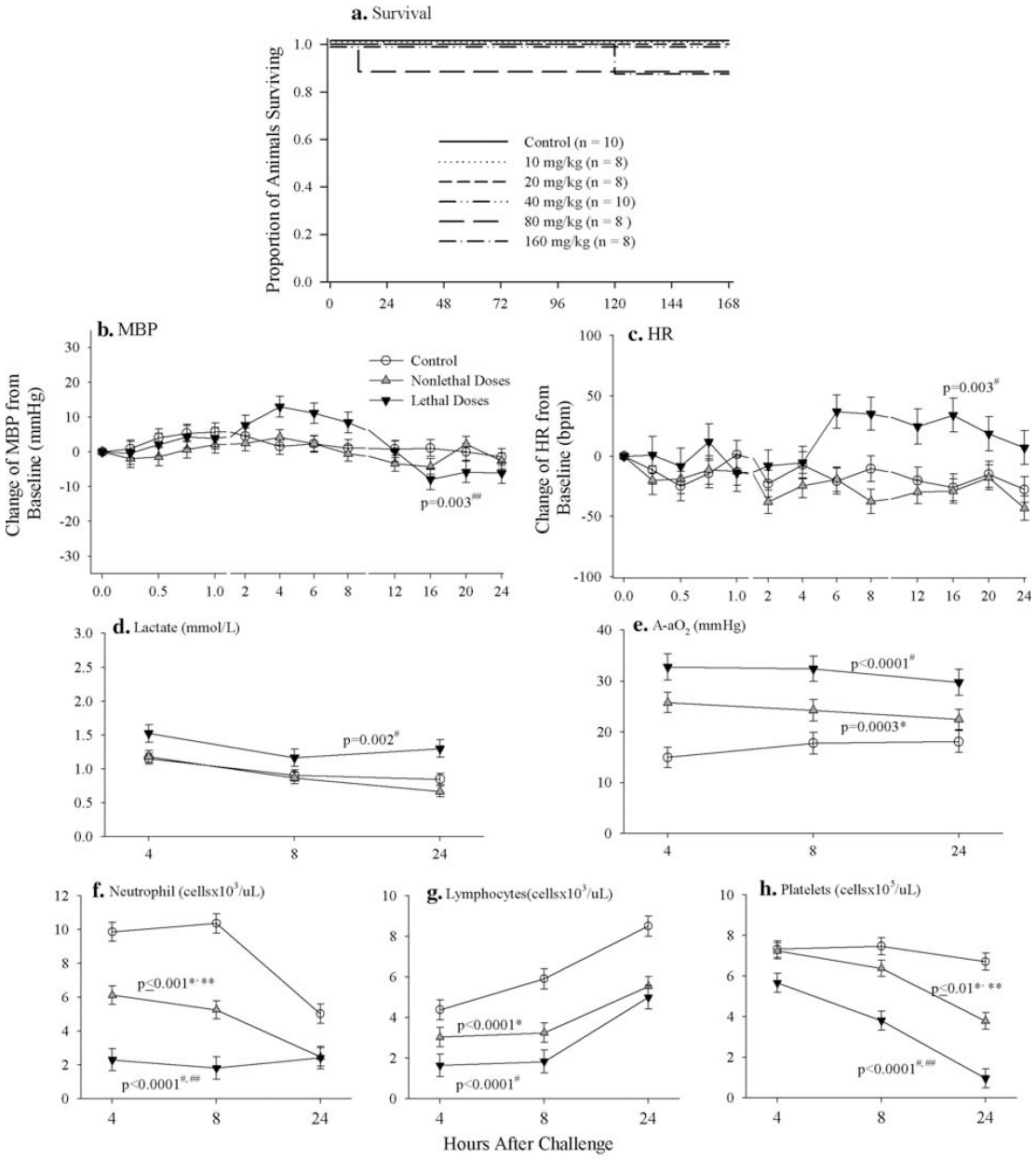


* p values for the effect nonlethal cell wall doses vs control
** p values for the interaction between the effect of nonlethal cell wall doses and time
# p values for the effect of lethal cell wall doses vs control
p values for the interaction between the effect of lethal cell wall doses and time

Fig. 2.

This figure compares the effects of either nonlethal or lethal doses combined of *B. anthracis* cell wall administered as a bolus (**a**–**i**) or as a 24-h infusion (**j**–**r**) to their respective diluent controls on serial mean (±SEM) interleukin-1β (IL-1β, **a**, **j**), IL-6 (**b**, **k**), IL-10 (**c**, **l**), tumor necrosis factor α (TNFα, **d, m**), granulocyte macrophage-colony stimulating factor (GM-CSF, **e, n**), interferon γ (INFγ, **f, o**), migratory inhibitor protein-1α (MIP-1α, **g, p**) and RANTES (**h**, **q**) [all log (pg/ml)] and nitrite/nitrate (NO, μM) (**i, r**) at 4, 8 and 24 h. As shown in Figs. 1 and 3, nonlethal and lethal bolus doses were 10 and 20 and 40, 80 and 160

mg/kg, respectively, while nonlethal and lethal infused cell wall doses were 10, 20 and 40 and 80 and 160 mg/kg, respectively



* p values for the effect of the nonlethal cell wall doses vs control
** values for the interaction between the effect of nonlethal cell wall doses and time
# p values for the effect of lethal cell wall doses vs control;
p values for the interaction between the effect of lethal cell wall doses and time

Fig. 3.

a Compares the effects of *B. anthracis* cell wall administered as a 24-h infusion in total doses of 10, 20, 40, 80 or 160 mg/kg to a diluent (control) on the proportion of animals surviving. Based on the results shown in **a**, **b**–**h** show the effects of either nonlethal doses (10 and 20 mg/kg) or lethal doses (40, 80 and 160 mg/kg) of *B. anthracis* cell wall infusion combined compared to control on: the serial mean (±SEM) changes from baseline in mean arterial blood pressure (MBP, mmHg, **b**) and heart rate (HR, BPM, **c**) measured q15 min from 0 to 1 h, q2 h from 2 to 8 h and q4 h from 12 to 24 h and serial mean (±SEM) arterial

lactates (**d**), arterial–alveolar oxygen gradients ($A\text{-}aO_2$, **e**) and circulating neutrophils (**f**), lymphocytes (**g**) and platelets (**b**) measured at 4, 8 and 24 h

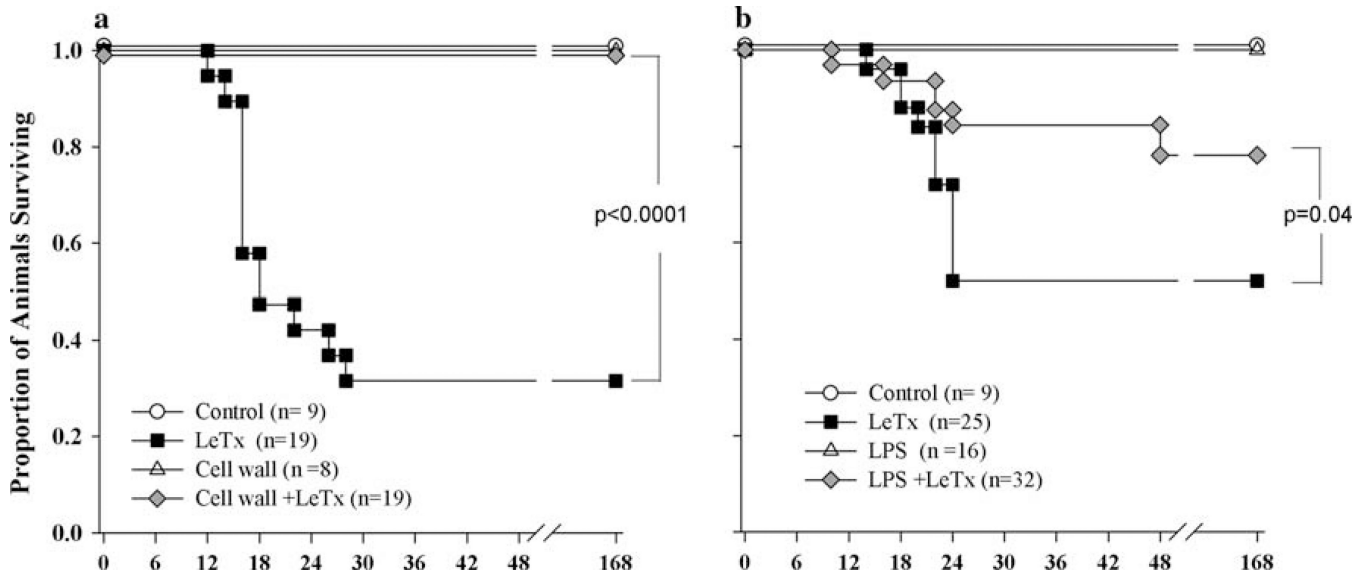


Fig. 4.

a Shows the proportion of animals surviving over time with cell wall and LeTx challenge alone or together or with diluent only (control). **b** Shows the proportion of animals surviving over time after challenge with LPS and LeTx alone or together or diluent only (control)