

Bacillus anthracis Cell Wall Peptidoglycan but Not Lethal or Edema Toxins Produces Changes Consistent With Disseminated Intravascular Coagulation in a Rat Model

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Background. Disseminated intravascular coagulation (DIC) appears to be important in the pathogenesis of *Bacillus anthracis* infection, but its causes are unclear. Although lethal toxin (LT) and edema toxin (ET) could contribute, *B. anthracis* cell wall peptidoglycan (PGN), not the toxins, stimulates inflammatory responses associated with DIC.

Methods and Results. To better understand the pathogenesis of DIC during anthrax, we compared the effects of 24-hour infusions of PGN, LT, ET, or diluent (control) on coagulation measures 6, 24, or 48 hours after infusion initiation in 135 rats. No control recipient died. Lethality rates (approximately 30%) did not differ among PGN, LT, and ET recipients ($P = .78$). Thirty-three of 35 deaths (94%) occurred between 6 and 24 hours after the start of challenge. Among challenge components, PGN most consistently altered coagulation measures. Compared with control at 6 hours, PGN decreased platelet and fibrinogen levels and increased prothrombin and activated partial thromboplastin times and tissue factor, tissue factor pathway inhibitor, protein C, plasminogen activator inhibitor (PAI), and thrombin-antithrombin complex levels, whereas LT and ET only decreased the fibrinogen level or increased the PAI level ($P \leq .05$). Nearly all effects associated with PGN infusion significantly differed from changes associated with toxin infusion ($P \leq .05$ for all comparisons except for PAI level).

Conclusion. DIC during *B. anthracis* infection may be related more to components such as PGN than to LT or ET.

Keywords. anthrax infection; sepsis; coagulopathy; thrombocytopenia; *Bacillus anthracis*; disseminated intravascular coagulation; peptidoglycan; lethal toxin; edema toxin.

Two outbreaks and several isolated cases of *Bacillus anthracis* infection over the past decade in the United States and Europe, as well as this bacterium's potential for

weaponization, have heightened concern about anthrax in the developed world [1, 2]. Even with aggressive treatment and support, mortality due to the inhalational, gastrointestinal, and injected or soft-tissue forms of anthrax can be very high. In humans and animals with invasive *B. anthracis* infection, coagulopathy and hemorrhagic tissue injury have been characteristic findings [3–6]. Hemorrhagic mediastinal lymphadenitis is common in inhalational disease, and disseminated intravascular coagulation (DIC) was prominent in individuals who died from anthrax during the anthrax leak in Sverdlovsk, Soviet Union (now Yekaterinburg, Russia) [3, 4]. The recent US case of gastrointestinal anthrax was complicated by hemorrhagic lymphadenitis [7]. Reports of anthrax

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in injection drug users in the United Kingdom noted thrombocytopenia, excessive tissue bleeding, and unusually high blood product requirements [1]. Even cutaneous anthrax has been associated with coagulopathy [8].

The virulence factors underlying the pathogenesis of anthrax and those potentially producing coagulopathy are complex. *B. anthracis* produces 2 binary toxins, lethal toxin (LT) and edema toxin (ET). Each toxin includes protective antigen (PA), the component necessary for uptake by host cells of the toxic moieties. LT also includes lethal factor (LF), a metalloproteinase that inactivates mitogen-activated protein kinase kinases and influences inflammasome formation [1, 9], whereas ET also includes edema factor (EF), an adenyl-cyclase that increases intracellular cyclic adenosine monophosphate to high levels [1]. Both LT and ET have actions that could contribute to coagulopathy. However, the less studied outer cell wall of *B. anthracis* is capable of making substantial contributions to coagulopathy [10]. Excessive activation of host coagulant and inflammatory pathways is interdependent and provides a basis for pathologic coagulopathy and DIC during invasive infection [11, 12]. In contrast to LT and ET, which may suppress inflammation, growing evidence suggests that *B. anthracis* cell wall components produce a robust inflammatory response [13–17]. In *in vitro* studies, peptidoglycan (PGN) has a predominant role in the inflammatory effects of *B. anthracis* cell wall [15].

At present, there has been little *in vivo* investigation regarding the individual effects of LT, ET, or *B. anthracis* cell wall components on coagulation and DIC and no investigation comparing their effects. We hypothesized that, when *B. anthracis* cell wall PGN is infused over 24 hours to simulate its release during infection, PGN would have a greater effect than LT or ET on coagulation and inflammation.

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

In 10 weekly experiments, Sprague-Dawley rats with carotid arterial and jugular venous catheters were randomly assigned to receive challenge with either *B. anthracis* cell wall PGN, LT, ET, or diluent as a 24-hour continuous infusion (Supplementary Figure 1A). A total of 135 animals were studied. Each experiment included ≥ 1 animal assigned to each of 4 challenge groups. Animals randomly selected 6 or 24 hours after initiation of infusion and all animals remaining 48 hours after infusion initiation were anesthetized and then exsanguinated for coagulation, complete blood count, chemistry, and arterial blood gas analyses and measurement of cytokine, nitric oxide,

lactate, alanine aminotransferase, aspartate aminotransferase, blood urea nitrogen, and creatinine levels. The number of animals available in each experiment for study at the 3 time points is shown in Supplementary Figure 1B. Every 2 hours during the 24-hour challenges, animals had mean arterial blood pressure and heart rate measured.

Preparation of PGN, LT, and ET

PGN was isolated and prepared from *B. anthracis* strain Δ Sterne, as previously described [15, 16]. Briefly, bacteria grown up on tryptic soy broth plates were boiled in 8% sodium dodecyl for 30 minutes and centrifuged. The pellet was washed with endotoxin-free water, subjected to DNase I and RNase A treatment, boiled in 4% sodium dodecyl for 30 minutes, and washed again 3 times with endotoxin-free water. The pellet was treated with 2 M NaCl, washed 6 times with endotoxin-free water, dried, weighed, resuspended in endotoxin-free water, and treated with HF to remove PGN-associated polysaccharide [18]. The material was then treated with a denaturing buffer (50 mM Tris [pH 8.0], 6 M guanidine HCl, and 25 mM dithiothreitol) at 60°C for 1 hour. Iodoacetamide 75 mM was added, and the preparation was incubated for 15 minutes in the dark to alkylate Cys residues. The reaction was stopped by adding dithiothreitol 40 mM. The PGN was resuspended in buffer with 50 mM Tris (pH 7.5), 1 M guanidine HCl, and 5 mM CaCl₂ and treated with 2 μ g of proteinase K. Finally, PGN was washed 3 times with endotoxin-free water, dried, weighed, and resuspended in endotoxin-free water. It required 1.7×10^{10} colony-forming units (CFU) to produce 1 mg of purified PGN. The total dose of PGN infused over 24 hours was 40 mg/kg (ie, 40 mg/kg per 24 hours).

Toxin components (PA, LF, and EF) were recombinant proteins prepared from *Escherichia coli* as previously described [19–21]. LT and ET were composed of LF or EF with PA in ratios of 1:2 on the basis of weight. The dose of LT and ET reported for experiments reflects the dose of LF or EF used. In the first 4 experiments, LF and EF doses of 0.045 and 0.3375 mg/kg, respectively, per 24 hours were used, whereas in the last 6 experiments, doses of 0.050 and 0.3750 mg/kg, respectively, per 24 hours were used.

As determined by the chromogenic limulus amoebocyte lysate assay (Clonogen, Germantown, MD), the lipopolysaccharide content of the PGN preparation was 0.127 ng/mg, while the contents of the PA, LF, and EF preparations were 0.001, 0.002, and 0.006 ng/ μ g, respectively. On the basis of the average size of the animals used for study, the amount of lipopolysaccharide administered during 24-hour infusion of PGN, LT, and ET would have been 5.07, 0.24, and 3.62 ng/kg, respectively. In a past investigation, compared with diluent, 24-hour lipopolysaccharide infusions in doses up to 8 ng/kg were completely nonlethal and did not produce significant changes in hemodynamic parameters, arterial blood gas findings, complete blood counts, cytokine levels, or nitric oxide level [17].

Chemical Characterization of PGN

The phosphorus level was estimated by the method of Chen et al [22], the protein level was estimated using the method of Lowry et al [23], and neutral sugar level was measured by the anthrone assay [24]. Amino acid composition was determined by gas chromatography–mass spectrometry after hydrolysis with 6 N HCl at 150°C for 1 hour and derivatization to volatile N-heptafluorobutyl isobutyl esters of amino acids [25], using a Hewlett-Packard apparatus (model HP 6890) with a type HP-5 glass capillary column (0.32 mm by 30 m) and temperature programming at 8°C/minute, from 125°C to 250°C in the electron ionization (106 eV) mode. Standards of amino acids were hydrolyzed, derivatized, and analyzed in the same way. Capillary electrophoresis–mass spectrometry analysis was performed using a 4000 Q-Trap mass spectrometer (Applied Biosystems/Sciex, Concord, Canada) via a capillary electrophoresis–mass spectrometry interface with a 90-cm length of bare fused-silica capillary, using 15 mM ammonium acetate in deionized water (pH 7.0). A sheath solution (isopropanol-methanol, 2:1) was delivered at a flow rate of 1.5 μ L/minute. The orifice voltage was set at –110 V. Nuclear magnetic resonance experiments were performed on a Varian Inova 500 MHz (1 H) spectrometer with a 3-mm gradient probe at 25°C, using an acetone internal reference (2.225 parts per million for 1 H and 31.45 parts per million for 13 C).

Laboratory Measurements

Hemodynamic, complete blood count, arterial blood gas, cytokine, and nitric oxide measurements were performed as previously described [26, 27]. Arterial blood samples were prepared, and activated partial thromboplastin time, prothrombin time, and fibrinogen levels were measured with a biphasic transmittance method as previously described [28]. An enzyme-linked immunosorbent assay was used to measure tissue factor, tissue factor pathway inhibitor, protein C (NovaTeinBio, Cambridge, MA), anti-thrombin III (Genway Biotech, San Diego, CA), plasminogen activator inhibitor (America Diagnostic, Stamford, CT), and thrombin antithrombin complexes (Siemens Healthcare, Newark, DE). Chemistry analysis was conducted with the Drew Trilogy Analyzer (Diamond Diagnostics, Holliston, MA).

Statistics

Survival time distributions were compared between treatment groups, using a stratified Cox proportional hazard model to account for cycle effect. Mortality rates in the PGN, LT, and ET groups were compared using a χ^2 test. For other variables, linear mixed models were used to compare treatment effects. Random effects were used to account for cycle effects and repeated measurements of each animal (if applicable). Interactions between treatments were tested first. If nonsignificant, the interaction term was dropped from the model, and main effects

for treatments were estimated. For clarity in figures, serial effects of PGN, LT, or ET (ie, challenge minus control) are shown. Standard residual diagnostic analyses were used to check model assumptions. Logarithm transformation (Log) was used when necessary. Two-sided *P* values of <.05 were considered statistically significant. SAS, version 9.3 (Cary, NC), was used for all analyses unless otherwise noted.

RESULTS

Effect of Challenges on Survival and Hemodynamic Measures

Compared with a control challenge (phosphate-buffered saline infused over 24 hours), PGN, LT, and ET challenge infusions each decreased the proportion of animals surviving over the 48-hour study period and increased the hazard ratio (HR) of death in patterns that reached or approached significance (PGN: HR, 11.9 [95% confidence interval {CI}, 1.54–92.0; *P* = .02]; LT: HR, 7.36 [95% CI, 0.94–57.9; *P* = .06]; and ET: HR, 6.86 [95% CI, 0.88–53.5; *P* = .07]; Figure 1A and 1B). Effects on the HR of death did not differ significantly among the 3 challenge infusions (*P* = .87).

During challenges, ET produced progressive reductions in mean arterial blood pressure and early increases in heart rate, compared with control (*P* < .0001 for the interaction with time for both; Figure 1C and 1D). PGN and LT produced a small increase and decrease in heart rate, respectively, but these differences did not reach significance (*P* = .13 for both; Figure 1C and 1D).

Effect of Challenges on Measures of Coagulation and Fibrinolysis

Because laboratory measures other than hemodynamic data required the euthanization of animals for blood sampling, comparisons of survivors and nonsurvivors were not possible. However, with all 3 challenge infusions, lethality among most nonsurvivors occurred between 6 and 24 hours after the start of infusion (14 of 14 nonsurvivors in the PGN group, 10 of 10 in the LT group, and 9 of 11 in the ET group; Figure 1A). Therefore, while the effects of each challenge on coagulation and other measures described below were analyzed over all 3 observation points (ie, 6, 24, and 48 hours), comparisons were only made among challenge infusions for the 6-hour time point, when changes may have best reflected mechanisms associated with the onset of lethality.

Of the 3 challenge infusions, PGN most consistently altered coagulation measures (Figure 2A–J and Supplementary Table 1). Across the 3 observation points, compared with control, PGN decreased platelet counts and increased tissue factor, tissue factor pathway inhibitor, protein C, and plasminogen activator inhibitor levels in patterns that did not differ over time and were significant overall (*P* \leq .01 averaged over time; Figure 2). In addition, PGN increased prothrombin and activated partial thromboplastin

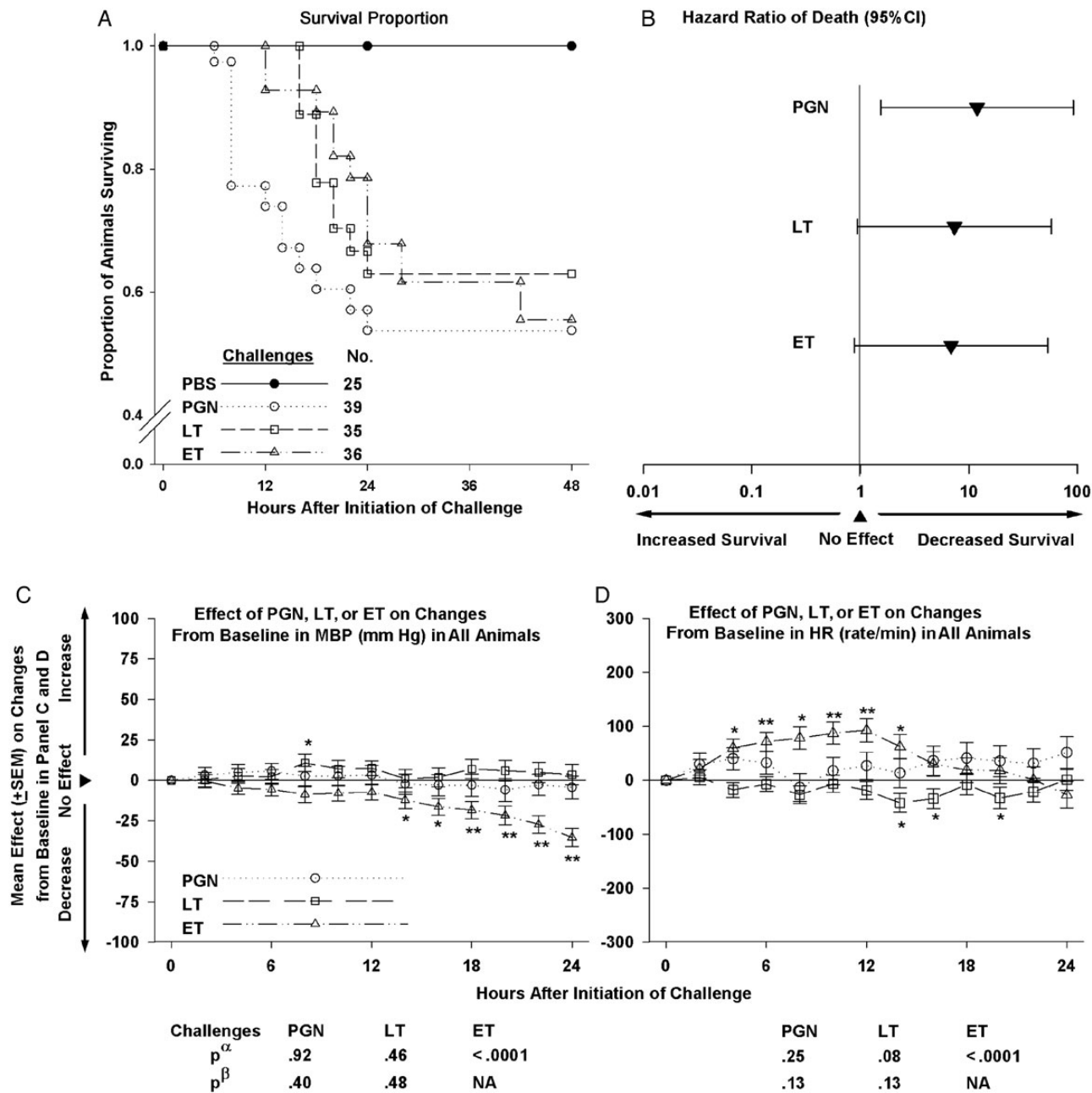


Figure 1. A, Survival over time among animals that received phosphate-buffered saline (PBS; control), peptidoglycan (PGN), lethal toxin (LT), or edema toxin (ET). B, Hazard ratio of death associated with each challenge component, compared with control. C and D, Mean difference (\pm standard error of the mean [SEM]) in mean arterial blood pressure (MBP) and heart rate (HR) between rats in each challenge group and control rats after initiation of infusion. P^{α} is the level of significance for the interaction between time and the effect of each challenge on changes in either MBP and HR, whereas P^{β} is the level of significance for the overall effect of each challenge across time. If there was a significant time interaction for the effect of a challenge (ie, $P^{\alpha} \leq .05$), then the significance of the overall effect of a challenge was designated as not applicable (NA). The individual effects of the challenges that were significant are designated by asterisks (* $P \leq .05$; ** $P \leq .0001$). Abbreviation: CI, confidence interval

times and the thrombin antithrombin complex level and decreased the fibrinogen level early ($P \leq .02$ for the time interaction). LT decreased the platelet count and fibrinogen level overall ($P = .01$ averaged over time) and increased the plasminogen activator inhibitor level later ($P \leq .05$ for the time interaction). ET

decreased the fibrinogen level early and decreased the platelet count and increased the fibrinogen and thrombin antithrombin complex levels later ($P \leq .02$ for the time interaction).

When challenges were directly compared 6 hours after the start of challenge infusions and before the onset of lethality in

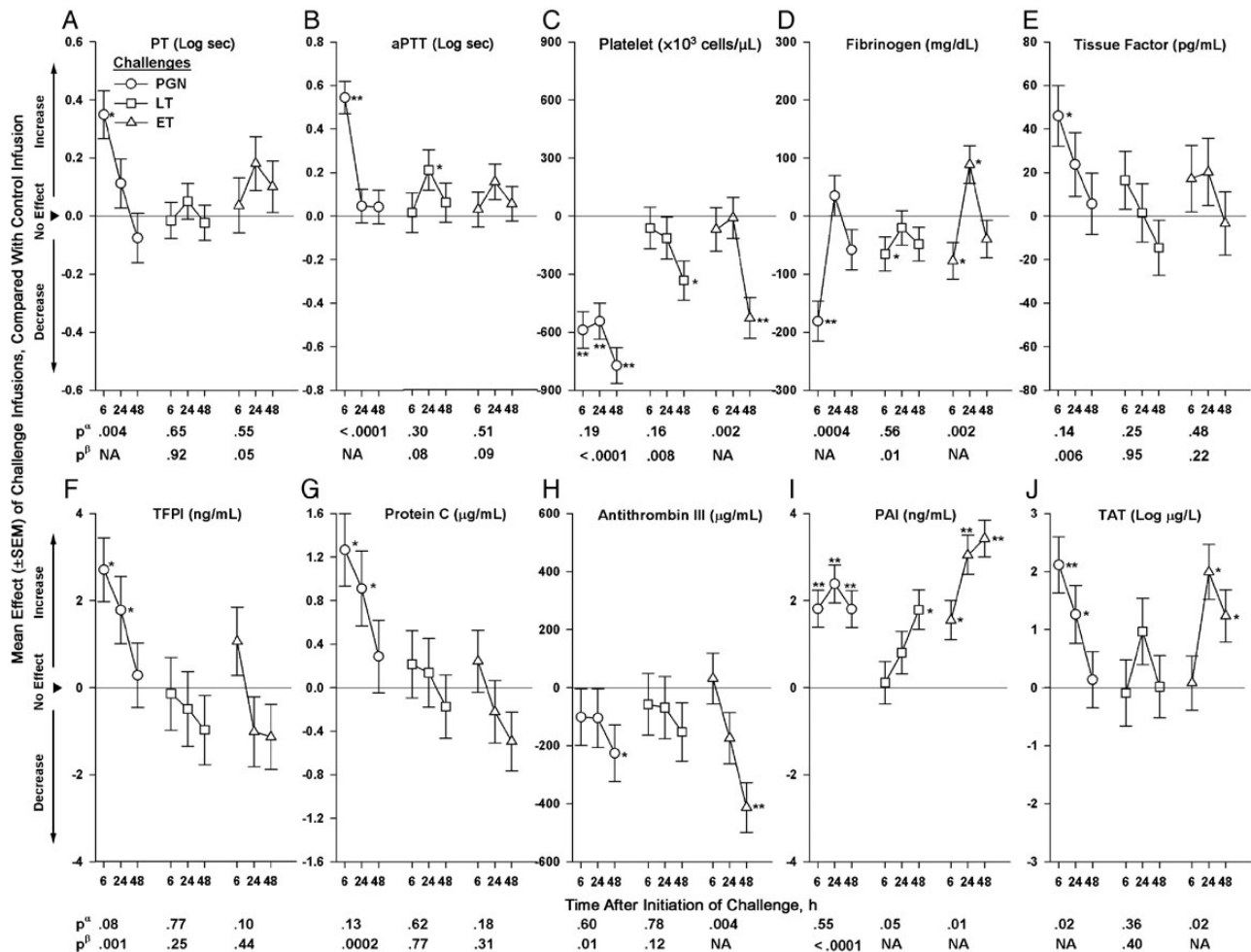


Figure 2. Mean difference (\pm standard error of the mean [SEM]) between peptidoglycan (PGN), lethal toxin (LT), and edema toxin (ET) challenge infusions and phosphate-buffered saline control infusion in log prothrombin time (PT), log activated partial thromboplastin time (aPTT), and platelet, fibrinogen, tissue factor, tissue factor pathway inhibitor (TFPI), protein C, antithrombin III, plasminogen activator inhibitor (PAI), and log thrombin antithrombin (TAT) levels 6, 24, and 48 hours after the initiation of challenge infusions. P^α is the level of significance for the interaction between time and the effect of the challenges on each measure, whereas P^β is the level of significance for the overall effect of each challenge across time. If there was a significant time interaction (ie, $P^\alpha \leq .05$), then the significance of the overall effect of a challenge was designated as not applicable (NA). The individual effects of the challenges that were significant are designated by asterisks (* $P \leq .05$; ** $P \leq .0001$).

most animals, PGN produced greater changes in all parameters, compared with either LT or ET ($P \leq .03$ for all comparisons), except the antithrombin III level, when comparing PGN to LT or ET, and the plasminogen activator inhibitor level, when comparing PGN to ET (Figure 3A–J). LT and ET had similar effects on all parameters except for the plasminogen activator inhibitor level, which was greater with ET ($P = .0005$).

Effect of Challenges on Cytokine and Nitric Oxide Levels

Of the 3 challenge infusions, PGN also altered cytokine and nitric oxide levels most consistently (Figure 4A–J and Supplementary Table 1). Across all observation points, PGN increased interleukin-6 (IL-6), interferon gamma (IFN γ), and regulated

on activation, normal T cell expressed and secreted (RANTES) levels significantly, compared with control ($P \leq .02$ averaged over time). In addition, PGN increased interleukin-1 beta (IL-1 β), interleukin-10 (IL-10), tumor necrosis factor alpha (TNF α), macrophage inflammatory proteins-1 alpha (MIP-1 α), MIP-2, monocyte chemoattractant protein-1 (MCP-1), and nitric oxide levels early ($P \leq .04$ for the time interaction). LT increased the RANTES level overall, the nitric oxide level early, and the IL-10 level later ($P \leq .05$ averaged over time or for the time interaction). ET increased the IL-10, MCP-1, RANTES, and nitric oxide levels overall ($P \leq .05$ averaged over time) and increased the IFN- γ level later in a pattern approaching significance ($P = .06$ for the time interaction).

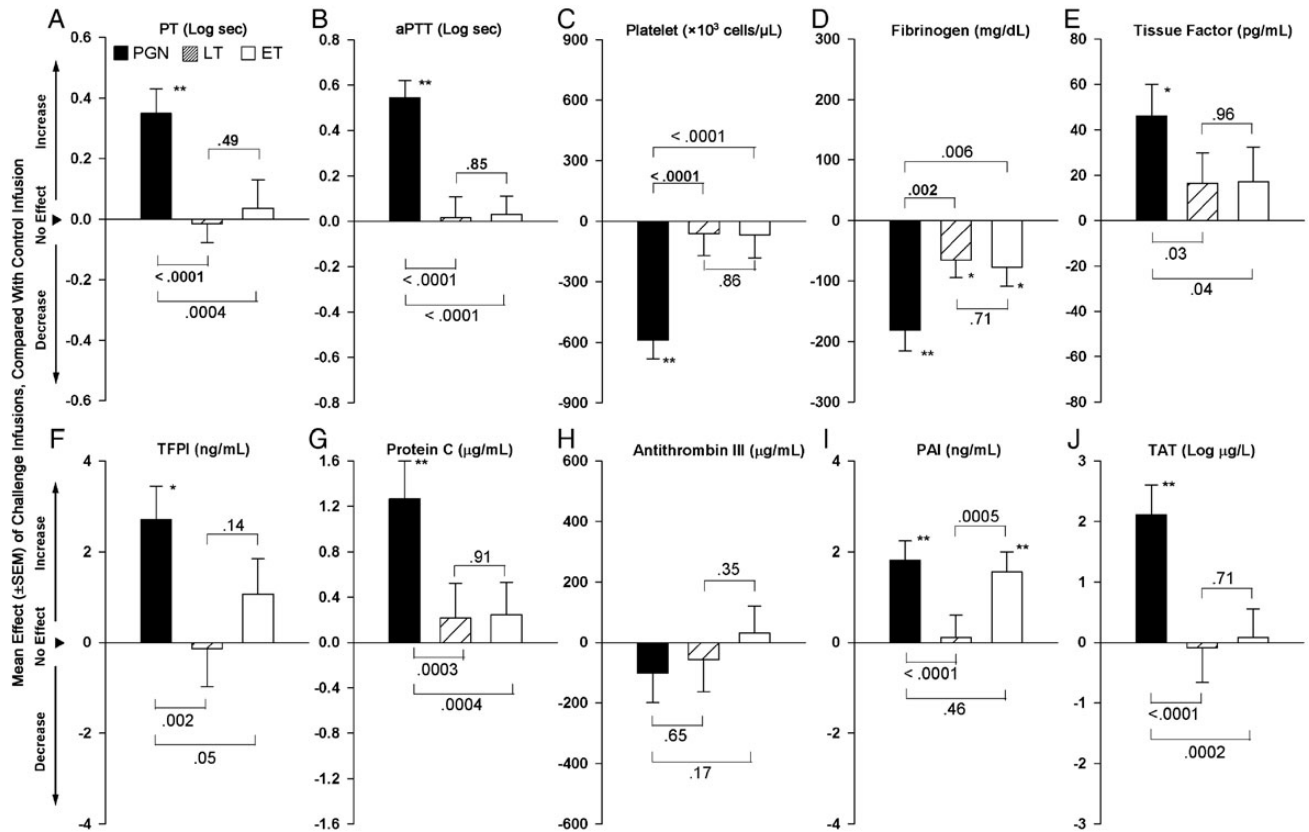


Figure 3. Mean difference (\pm standard error of the mean [SEM]) between peptidoglycan (PGN), lethal toxin (LT), and edema toxin (ET) challenge infusions and phosphate-buffered saline control infusion in log prothrombin times (PT), log activated partial thromboplastin times (aPTT), and platelet, fibrinogen, tissue factor, tissue factor pathway inhibitor (TFPI), protein C, antithrombin III, plasminogen activator inhibitor (PAI), and log thrombin antithrombin (TAT) levels 6 hours after the initiation of challenge infusions. Levels of significance comparing challenges are shown by the brackets, whereas individual effects of the challenges that were significant are designated by asterisks (* $P \leq .05$; ** $P \leq .0001$).

When the challenge infusions were directly compared at 6 hours, PGN produced significantly greater changes in all parameters, compared with either LT or ET ($P \leq .01$ for all; Figure 5A–J). LT and ET had similar effects on all parameters except for the IL-10 level, which was greater with ET ($P = .002$).

Effect of Challenges on Other Measures

Across all observation points, PGN increased the alanine aminotransferase level and decreased the bicarbonate (HCO_3^-) level overall, compared with control ($P \leq .002$ averaged over time; Figure 6A–H and Supplementary Table 1). In addition, PGN decreased the white blood cell, neutrophil, and lymphocyte counts and increased the hemoglobin and lactate levels early ($P \leq .002$ for the time interaction). LT increased the white blood cell count and hemoglobin level overall ($P \leq .03$ averaged over time) and increased the aspartate aminotransferase level later ($P = .02$ for the time interaction). ET decreased the lymphocyte and HCO_3^- levels and increased the lactate and aspartate aminotransferase levels overall ($P \leq .05$ averaged over

time). ET also decreased the hemoglobin level and increased the neutrophil count, alanine aminotransferase level, and blood urea nitrogen level later (Supplementary Table 1; $P \leq .04$ for the time interaction).

At 6 hours, PGN produced greater changes in the white blood cell count, neutrophil count, alanine aminotransferase level, and lactate level, compared with either LT or ET ($P \leq .01$ for all comparisons); greater changes in the lymphocyte count, compared with LT ($P < .0001$); and greater changes in the hemoglobin level, compared with ET ($P = .02$) (Figure 7A–H). LT and ET had similar effects on all parameters except for white blood cell and lymphocyte counts, which ET decreased more ($P \leq .01$).

PGN Composition

The *B. anthracis* PGN preparation was negative for proteins, neutral sugars, and phosphorus. Amino acid gas chromatography–mass spectrometry analyses detected alanine, glutamic acid, and diaminopimelic acid in the molar ratio of 1.6:1.0:1.0.

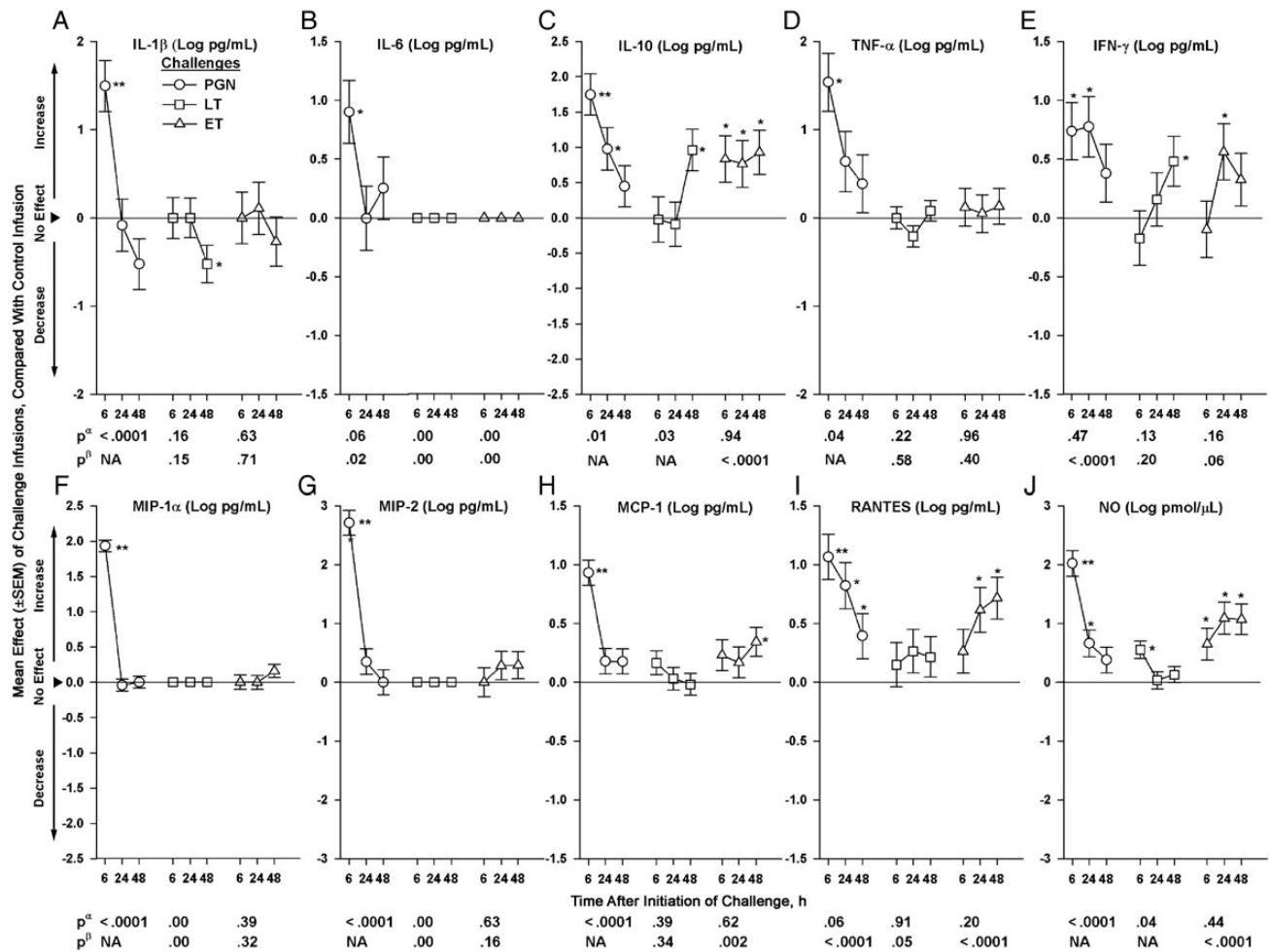


Figure 4. Mean difference (\pm standard error of the mean [SEM]) between peptidoglycan (PGN), lethal toxin (LT), and edema toxin (ET) challenge infusions and phosphate-buffered saline control infusion in log interleukin 1 β (IL-1 β), log interleukin 6 (IL-6), log interleukin 10 (IL-10), log tumor necrosis factor α (TNF- α), log interferon γ (IFN- γ), log macrophage inflammatory proteins-1 α (MIP-1 α), log macrophage inflammatory proteins-2 (MIP-2), log monocyte chemoattractant protein-1 (MCP-1), log regulated on activation, normal T cell expressed and secreted (RANTES), and log nitric oxide (NO) levels 6, 24, and 48 hours after the initiation of challenge infusions. P^α is the level of significance for the interaction between time and the effect of the challenges on each measure, whereas P^β is the level of significance for the overall effect of each challenge on these measures across time. If there was a significant time interaction (ie, $P^\alpha \leq .05$), then the significance of the overall effect of a challenge was designated as not applicable (NA). The individual effects of the challenges that were significant are designated by the asterisks (* $P \leq .05$; ** $P \leq .0001$).

Mass spectrometry confirmed the presence of PGN components (Supplementary Figure 2): a mass to charge ratio (m/z) of 455.1 atomic mass units corresponded to an N-acetylated disaccharide, glucosamine-muramic acid, which is a carbohydrate subunit of the PGN chain; a m/z of 826.8 atomic mass units represented the disaccharide substituted with a peptide chain containing alanine, diaminopimelic acid, and glutamic acid; and a m/z of 897.7 atomic mass units contained additional alanine. Proton nuclear magnetic resonance spectrum analysis did not detect any additional compounds, confirming that the preparation contained pure PGN of a published structure [29, 30].

DISCUSSION

In this rat model, the investigated doses of *B. anthracis* cell wall PGN, LT, and ET produced similar levels of lethality. Each challenge also produced evidence of hemodynamic or organ dysfunction or tissue hypoperfusion. Of the 3 challenge infusions, however, PGN produced changes in laboratory measures most consistent with the coagulopathy associated with invasive anthrax infection.

By 6 hours after initiation of PGN infusion, PGN had produced significant increases in the prothrombin and activated

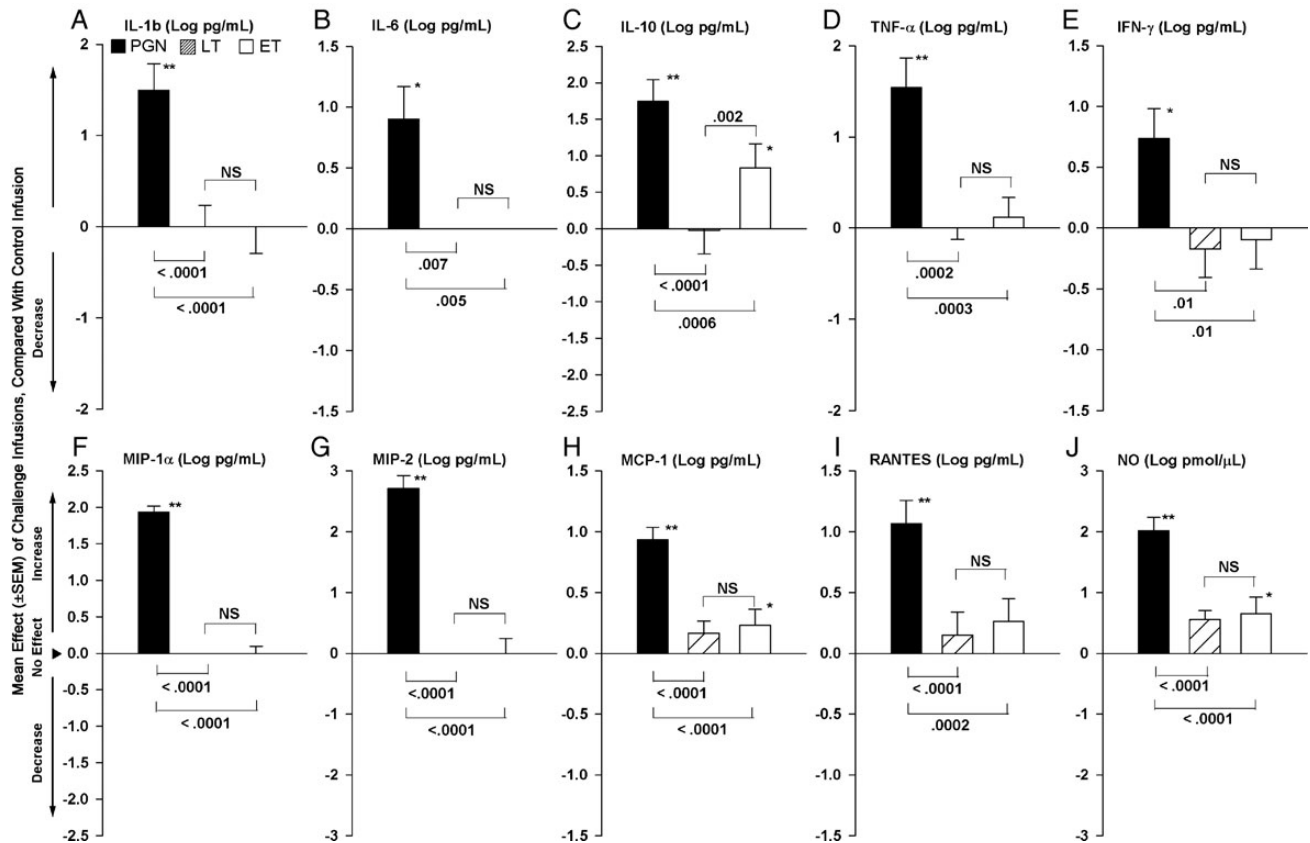


Figure 5. Mean difference (\pm standard error of the mean [SEM]) between peptidoglycan (PGN), lethal toxin (LT), and edema toxin (ET) challenge infusions and phosphate-buffered saline control infusion in log interleukin 1 β (IL-1 β), log interleukin 6 (IL-6), log interleukin 10 (IL-10), log tumor necrosis factor α (TNF- α), log interferon γ (IFN- γ), log macrophage inflammatory proteins-1 α (MIP-1 α), log macrophage inflammatory proteins-2 (MIP-2), log monocyte chemoattractant protein-1 (MCP-1), log regulated on activation, normal T cell expressed and secreted (RANTES), and log nitric oxide (NO) levels 6 hours after the initiation of challenge infusions. Levels of significance comparing challenges are shown by the brackets, whereas individual effects of the challenges that were significant are designated by asterisks (* $P \leq .05$; ** $P \leq .0001$). Abbreviation: NS, nonsignificant.

partial thromboplastin times and significant decreases in the circulating platelet count and the fibrinogen level, changes that, together, characterize DIC [31]. At this same time, the level of tissue factor, the primary stimulus for dysregulated fibrin production during sepsis-associated DIC, was increased with PGN, as were levels of tissue factor pathway inhibitor and protein C, mediators released to counter the effects of excessive tissue factor. Although the antithrombin III level was not elevated, the level of thrombin antithrombin complexes was increased. Finally, the level of plasminogen activator inhibitor, which opposes tissue factor-induced fibrin deposition, was increased with PGN. Increases in tissue factor, thrombin antithrombin complex, and plasminogen activator inhibitor levels all occurred with DIC in patients with sepsis or other conditions [12].

In addition, PGN produced significant increases in levels of inflammatory cytokines, including TNF- α , IL-1 β , and IL-6. Such cytokine release may stimulate coagulation during infection, and sepsis and could have contributed to the DIC-like

picture PGN produced [11, 12]. However, factors released during the activation of coagulation, like tissue factor and thrombin, can also stimulate inflammatory cytokines [11, 12]. The extent to which increases in inflammatory cytokine levels may have added to or resulted from coagulopathy with PGN is unclear.

At 6 hours and immediately prior to the onset of lethality with the challenge infusions, neither LT nor ET had produced significant changes in any measures of coagulation, with the exception of the fibrinogen level (which decreased with both toxins) and the plasminogen activator inhibitor level (which increased with ET). Although LT and ET were associated with later changes in some coagulation measures, these changes were not in a pattern consistent with DIC. Also, these changes occurred later and were unlikely to have contributed to lethality. Finally, neither LT nor ET stimulated the type of inflammatory cytokine response potentially contributing to or resulting from coagulopathy. Consistent with the present findings, neither toxin stimulated tissue factor production in human vascular

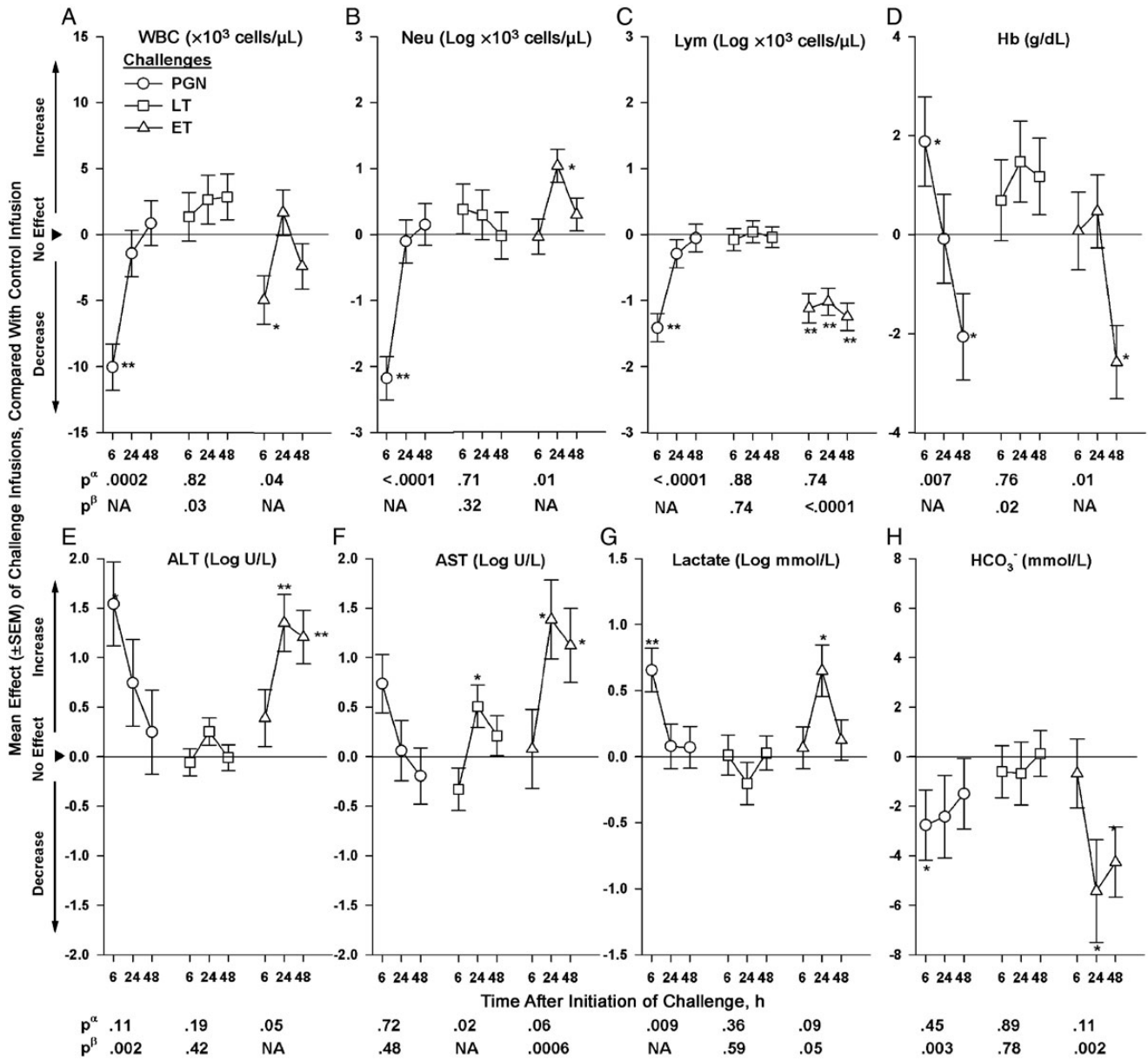


Figure 6. Mean difference (\pm standard error of the mean [SEM]) between peptidoglycan (PGN), lethal toxin (LT), and edema toxin (ET) challenge infusions and phosphate-buffered saline control infusion in white blood cell (WBC), log neutrophil (Neu), and log lymphocyte (Lym) counts and hemoglobin (Hb), log alanine amino transferase (ALT), log aspartate amino transferase (AST), and log lactate and bicarbonate (HCO_3^-) levels 6, 24, and 48 hours after the initiation of challenge infusions. P^α is the level of significance for the interaction between time and the effect of the challenges on each measure, whereas P^β is the level of significance for the overall effect of each challenge on these measures across time. If there was a significant time interaction (ie, $P^\alpha \leq .05$), then the significance of the overall effect of a challenge was designated as not applicable (NA). The individual effects of the challenges that were significant are designated by the asterisks (* $P \leq .05$; ** $P \leq .0001$).

endothelial cells [32]. Also, in mice, while LT challenge produced later reductions in the platelet count, histologic analysis of multiple organs did not suggest DIC [33].

Although LT and ET did not consistently alter coagulation in the present study, each may still contribute to bleeding during infection. Both toxins have been shown to increase endothelial permeability sufficiently to allow extravasation of

blood cells [34]. Each has also been shown in vitro to impair platelet aggregation or binding in mouse or rabbit cells [35, 36]. However, human platelet counts were not altered by LT or ET and displayed limited expression of the tumor endothelial marker 8 and capillary morphogenesis gene 2 receptors necessary for PA binding [37]. However, LT challenge in mice in a study different from the one noted above did produce changes in the

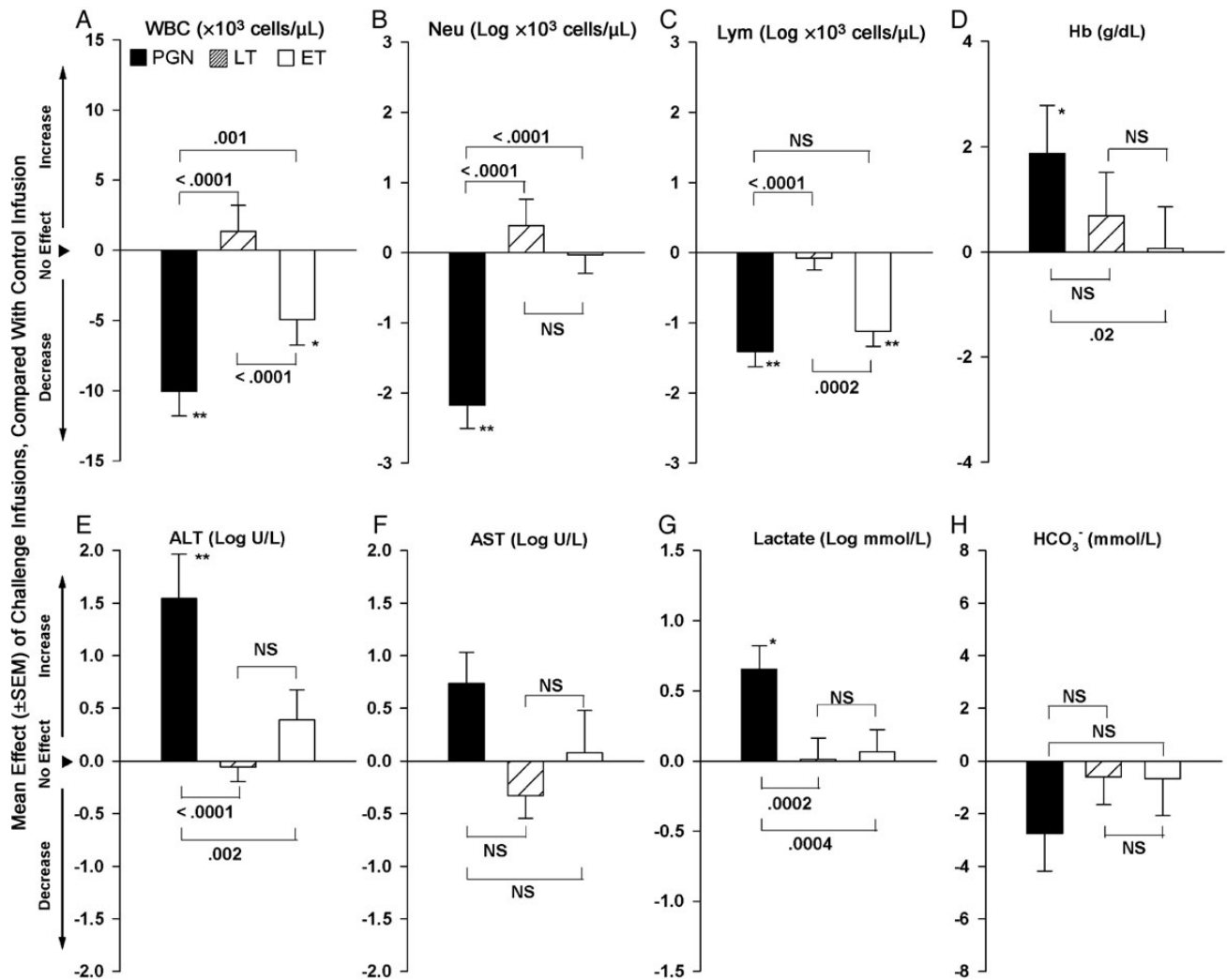


Figure 7. Mean difference (\pm standard error of the mean [SEM]) between peptidoglycan (PGN), lethal toxin (LT), and edema toxin (ET) challenge infusions and phosphate-buffered saline control infusion in white blood cell (WBC), log neutrophil (Neu), and log lymphocyte (Lym) counts and hemoglobin (Hb), log alanine amino transferase (ALT), log aspartate amino transferase (AST), and log lactate and bicarbonate levels 6 hours after the initiation of challenge infusions. Levels of significance comparing challenges are shown by the brackets, whereas individual effects of the challenges that were significant are designated by asterisks (* $P \leq .05$; ** $P \leq .0001$). Abbreviation: NS, nonsignificant.

fibrinogen level, activated partial thromboplastin time, and prothrombin time consistent with DIC, but the changes were not significant [38].

Mechanisms underlying the coagulopathic effects of *B. anthracis* PGN in the present study are unclear. However, these effects are consistent with those noted with PGN isolated from other bacteria types. Peptidoglycan from *Staphylococcus aureus* increases tissue factor expression in human monocytes and umbilical vein endothelial cells [39, 40]. Also, after the breakdown *Mycobacterium tuberculosis* cell wall, the PGN component elicited the strongest tissue factor expression in human monocyte-derived macrophages as compared to other components (eg, arabinogalactan, lipomannan, mycolic acid methyl esters, phosphatidylinositol mannoside, and lipoarabinomannan) [41].

Components besides PGN may also contribute to platelet dysfunction and coagulopathy during *B. anthracis* infection. The metalloproteases InhA and Npr599 degrade von Willebrand factor and its regulator ADAMTS13 (the 13th member of ADIntegrin-like And Metalloprotease with ThromboSpondin type 13 motifs), stimulate plasminogen activator inhibitor-1 production, deplete antithrombin via shedding of neutrophil elastase and syndecan (a heparin-like molecule), and break down thrombin-activatable fibrinolysis inhibitor [42–45]. Determining which components play the biggest role during DIC with anthrax, how they work together, or whether there are other components contributing to DIC requires further study.

In the present study, it required 1.7×10^{10} CFU *B. anthracis* to produce 1 mg of purified PGN. Based on the dose of PGN

administered to each animal (40 mg/kg administered over 24 hours), this would have represented a total dose of 68×10^{10} CFU per kg of body weight or 8.9×10^9 CFU per mL of blood in a 250-g animal with a typical blood volume of 17 mL. While these *B. anthracis* concentrations are similar to those lethal in guinea pigs challenged with subcutaneous bacteria (5 to 15×10^9 CFU per mL of blood [46]), they are higher than those measured after a lethal bacterial infusion in baboons (1×10^4 CFU per mL of blood) [6].

It is unlikely that microangiopathy related to administration of a large particle number contributed to the effects of PGN. Only 6 hours after initiation of PGN infusion (when 25%, or 10 mg/kg, of the total dose had been administered), changes in coagulation and inflammatory mediator release were well established and highly significant. These changes had certainly started earlier, after an even smaller concentration of PGN. Also, in other studies, administration of PGN from nonpathogenic *Bacillus subtilis* in a dose of 10 mg/kg did not significantly alter inflammatory mediator release in rats [47]. Finally, the effects that *B. anthracis* PGN had on inflammatory cytokine production in the present study are consistent with the effects it had in vitro [15].

Our findings provide one basis for the coagulopathy noted in patients with severe anthrax infection. Also, in light of the high bacterial loads and potential for large reservoirs of cell wall products during anthrax, these findings suggest that therapies modulating the coagulopathic and inflammatory effects of PGN might be considered [46, 48–50].

This study has limitations. First, the need to exsanguinate animals for measurements prevented analysis of a correlation between levels of coagulopathy and lethality. Ideally, the predictive value of samples obtained at 6 hours might have been analyzed. Second, examination of interactions between PGN, LT, and ET would be of interest. However, such study would require examination of the challenges paired and then combined together, and the necessary resources would be prohibitive. Importantly, while PGN decreased the platelet count and increased inflammatory cytokine levels in this study, in prior studies involving rats, LT and ET together did not. Third, additional measures of organ function, such as myocardial, urinary, or adrenal measures, might have been informative but weren't possible in small animals. However, results of liver function, blood urea nitrogen, and creatinine tests are provided. Finally, although mass spectrometry showed that the PGN preparation had a structure consistent with published data, the possibility that its biological activity was diminished during purification cannot be excluded.

In conclusion, the present findings support the possibility that *B. anthracis* cell wall PGN makes an important contribution to the coagulopathy, DIC, and lethality occurring during severe anthrax in patients. While LT and ET may be important therapeutic targets for anthrax, identifying agents to counteract

the pathogenic effects of components like PGN may be relevant, as well.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (<http://jid.oxfordjournals.org/>). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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