The American Journal of Pathology, Vol. 169, No. 2, August 2006

DOI: 10.2353/ajpath.2006.051330

Immunopathology and Infectious Diseases

Sepsis and Pathophysiology of Anthrax in a Nonhuman Primate Model

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Studies that define natural responses to bacterial sepsis assumed new relevance after the lethal bioterrorist attacks with *Bacillus anthracis* (anthrax), a spore-forming, toxigenic gram-positive bacillus. Considerable effort has focused on identifying adjunctive therapeutics and vaccines to prevent future deaths, but translation of promising compounds into the clinical setting necessitates an animal model that recapitulates responses observed in humans. Here we describe a nonhuman primate (*Papio c. cynocephalus*) model of *B. anthracis* infection using infusion of toxigenic *B. anthracis* Sterne 34F2 bacteria (5×10^5 to 6.5×10^9 CFU/kg). Similar to that seen in human patients, we observed changes in vascular permeability, disseminated intravascular coagulation, and systemic inflammation. The lung was a primary target organ with serosanguinous pleural effusions, intra-alveolar edema, and hemorrhagic lesions. This animal model reveals that a fatal outcome is dominated by the host septic response, thereby providing important insights into approaches for treatment and prevention of anthrax in humans. ***(Am J Pathol 2006, 169:433–444; DOI: 10.2353/ajpath.2006.051330)***

Bacillus anthracis, a zoonotic toxigenic gram-positive, spore-forming rod, is the cause of clinical anthrax disease. There has been a significant resurgence in biomedical anthrax-related research because of the bioterrorism attacks in the United States.[1–6] As a result, the genomic sequence of *B. anthracis* has been completed,[7] exotoxin crystal structures solved,[8,9] and cellular toxin receptors identified.[10,11] The virulence of *B. anthracis* bacilli is primarily governed by products of two large plasmids that code for secreted exotoxins and an exterior capsule.[12,13] The capsule, composed of poly-γ-D-glutamic acid, has anti-phagocytic properties and contributes to bacterial dissemination.[14] Under the control of the *atxA* gene product,[15] the bacteria produce exotoxin components: protective antigen (PA) serves as a conduit for translocation of lethal factor (metalloprotease) and edema factor (adenylate cyclase) into the cell for toxicity and injury.[16]

However, the pathophysiology of anthrax as a septic disease is less well defined. Sepsis is defined as a host systemic inflammatory response to infection and is complicated in severe sepsis with organ dysfunction, hypoperfusion, and coagulation abnormalities.[17] Clinical and pathology data from the victims of anthrax bioterrorism,[1,18] as well as a 1979 inadvertent release of military-grade anthrax spores in Russia,[19,20] show evidence of concomitant pulmonary edema, inflammation, and disseminated intravascular coagulation (DIC). To mimic anthrax, considerable work in animal models, including rhesus monkeys and chimpanzees, has been done using administration of spores by various routes, including aerosol.[21–24] These studies investigated important spore dose-response relationships and subsequent pathology observations were consistent with a general consensus that *B. anthracis* introduced by the respiratory route results in a fulminating septicemia rather than a primary pulmonary infection.[22] However, a consistent picture of pathophysiology progression is difficult to ascertain from these inhalational models. There is significant individual variation in gross and microscopic pathology of rhesus monkeys after challenge,[24] probably attributable to dose-response issues because it is difficult to know how many of the inhaled spores actually result in infection. Although organ hemorrhage, edema, and inflammatory infiltrates were noted in some animals, a systematic analysis of

Supported by the National Institutes of Health (grants 1R01 AI058107 and 1U19 AI062629 to S.K., and RR020143 to D.J.S.-K.).

Accepted for publication May 4, 2006.

The work was performed at: Oklahoma Medical Research Foundation and the University of Oklahoma Health Sciences Center.

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inflammatory or coagulation biomarkers was not available. These observations are further compounded by the current paradigm, based on toxic murine models, which describes anthrax pathogenesis as being governed by exotoxin bioactivities and host inflammatory or coagulopathic responses as playing little role.[25–27] These disparities gain importance when extrapolating experimental data to patients because vaccine development and clinical management decisions are based on an understanding of disease pathogenesis.

The current study addresses whether the pathogenesis of the bacteremic phase of anthrax is governed by predominately noninflammatory pathways as suggested by toxic murine models or is represented by uncompensated inflammation and coagulation responses to the infection. We have adapted our nonhuman primate model of *E. coli* sepsis that has been extensively characterized[28,29] and has served as the basis[30] for clinical studies that culminated in Food and Drug Administration approval of an adjunct therapy for patients with severe sepsis.[31] We chose infection by infusion of bacteria for reproducible dosing, because with a high *B. anthracis* spore infection dose, the onset of bacteremia is rapid, with dissemination within 24 ~ 48 hours,[14,32] and overwhelming.[23] This approach mimics the bacteremia stage during which patients become sick and seek medical attention. Unencapsulated *B. anthracis* 34F2 Sterne strain was used because this strain produces toxin in quantities similar to the natural fully virulent strains.[33] The results illustrate the physiological, hemostatic, cellular, and inflammatory responses to anthrax, as well as distinctive lung pathology that may be a unique feature of anthrax.

Materials and Methods

Animals

Infusion methods were essentially identical to those used for *E. coli*[34] and Shiga toxin 1.[35] *Papio c. cynocephalus* or *Papio c. anubis* baboons were purchased from the breeding colony maintained at the University of Oklahoma Health Sciences Center (Dr. Gary White, Director). Baboons were free of tuberculosis, weighed 6 to 8 kg, had leukocyte concentrations of 5000/mm^3 to 14,000/mm^3, and hematocrits exceeding 36%. T0-hour blood samples were drawn from the cephalic vein catheter followed by bacteria infusion for 2 hours. Levofloxacin infusion (7 mg/kg) was initiated at T4 hours and repeated daily. Infusion studies were performed at the University of Oklahoma Health Sciences Center. All experiments were approved by the Institutional Animal Care and Use Committee and the Institutional Biosafety Committee of the Oklahoma Medical Research Foundation and the University of Oklahoma Health Sciences Center.

Bacteria

Vegetative bacteria germinated from *Bacillus anthracis* 34F2 Sterne strain spores (Colorado Serum Co., Boulder, CO) were washed and resuspended in sterile saline for infusion. Live bacteria were quantitated using the BacTiter-Glo microbial cell viability assay (Promega, Madison, WI). In preliminary studies, a standard curve of viable bacteria (BacTiter-Glo) versus viable bacteria obtained by traditional plating methods (CFU/ml) was established. This relationship was very reproducible (r = 0.99; n = 3), permitting use of the luminescence assay for determining viable bacteria counts, rather than counting colonies on plates, which can be difficult with *B. anthracis* because of chaining. CFU/kg dosage was calculated by reference to this standard curve.

Infusion Procedures

Briefly, the baboons were fasted for 24 hours before the study, with free access to water. They were immobilized the morning of the experiment with ketamine (14 mg/kg, i.m.) and sodium pentobarbital administered through a percutaneous catheter in the cephalic vein of the forearm to maintain a light level of surgical anesthesia (2 mg/kg, approximately every 20 to 40 minutes). This catheter was also used to infuse the *B. anthracis* bacteria and sterile saline to replace insensible loss. An additional percutaneous catheter was inserted into the saphenous vein in one hind limb and the catheter advanced to the inferior vena cava; this catheter was used for sampling blood. Baboons were orally intubated and positioned on their left side on a heat pad. Our typical infusion protocol involved blood draw at T0, followed immediately by bacteria infusion at the appropriate concentration for 2 hours, typically at 0.2 ml/minute.

Monitoring and Sampling Procedures

Blood samples were taken at various time points for assay purposes and to confirm bacteremia. Except for samples taken for colony counts, blood samples were collected into 1/100 vol of 5000 U/ml penicillin and 500 μg/ml streptomycin to kill circulating vegetative bacteria. Bacteremia was confirmed by traditional plating methods using blood obtained at T+2 hours just after finishing the infusion and T+4 hours before antibiotics. Colony counts varied according to the loading dose. For a 10^8 CFU/kg challenge, colony counts were near 10^4 CFU/ml at T+2 hours and 200 CFU/ml at T+4 hours. Colony counts on blood sampled between days 2 to 7 were consistently negative. Blood pressure and rectal temperature were measured with a Critikon monitor (Critikon, Inc., Tampa, FL) and a YSI thermometer (Yellow Springs Instrument Co., Yellow Springs, OH), respectively.

Metabolic and Cytokine Assays

Complete blood counts and hematocrits were determined and blood smears were done for differential counts. Routine blood chemistries, fibrinogen, fibrin degradation products (FDP), and activated partial thromboplastin times (APTT) were determined.[34,36,37] Fibrin degradation products and APTT assays were run on-line during the experiments. Plasma interleukin (IL)-1β levels

were determined by enzyme-linked immunosorbent assay (ELISA) using the hIL-1Beta/IL-1F2 DuoSet kit (R&D Systems, Minneapolis, MN). Plasma IL-6 levels were determined by ELISA.[36] D-dimer levels were determined by ELISA (Diagnostica Stago, Asnières, France). Other cytokines were quantitated by flow cytometry-based Multiplex assay (Dr. J. Connolly, Ph.D., Baylor Institute for Immunology Research, Dallas, TX).

Tumor Necrosis Factor (TNF)-α ELISA

Microtiter plates were coated with 50 μl of 1 μg/ml of goat anti-human TNF-α (anti-hTNF-α/TNFSF1A, R&D Systems), washed, and blocked. Plasma samples were diluted at least 1:50 and incubated in the wells for 2 hours at room temperature. Wells were washed, and bound antigen was detected with 0.2 μg/ml of biotinylated goat anti-human TNF-α (R&D Systems) followed by streptavidin-horseradish peroxidase and TMB substrate (1 Step Ultra TMB; Pierce, Rockford, IL). The reaction was stopped with 2 mol/L H_2SO_4 and OD_{450nm} was determined. Linear standard curves were prepared using recombinant human TNF-α (R&D Systems); the assay was sensitive to 15 pg/ml TNF-α.

Protein C ELISA

Wells of 96-well microtiter plates were coated with 50 μl of 10 μg/ml goat anti-human protein C polyclonal antibody as a capture antibody, and an anti-human protein C HPC4 murine monoclonal antibody conjugated with biotin (EX-Link Sulfo-NHS-LC-biotin, final 4 μg/ml; Pierce) was the detection antibody. Antibodies were obtained from Dr. Charles Esmon (Cardiovascular Biology Research, Oklahoma Medical Research Foundation). Wells were coated overnight, washed, and blocked, and samples (50 μl of 1:2000) were incubated at 37°C for 1 hour. Wells were washed, incubated with detection antibody (4 μg/ml, 1.5 hours, room temperature) followed by streptavidin-horseradish peroxidase (1:8000, 1 hour, AMDEX streptavidin-horseradish peroxidase; Amersham Pharmacia Biotech, Arlington Heights, IL). Color was developed with TMB substrate. The reaction was stopped with 2 mol/L H_2SO_4 and the OD_{450nm} was determined. Standard curves were made from dilution of normal human plasma or baboon pooled plasma and results expressed as percentage of normal for that species. The human and baboon standard curves were parallel and linear (data not shown). Baboons have slightly lower protein C levels compared to humans; the protein C antigen in a normal baboon plasma pool (from five animals) was 66.5 ± 1.2% of the human protein C level.

PA ELISA

Anthrax PA was quantitated by standard ELISA methods using goat anti-PA as coating antibody (1 μg/ml), biotinylated-goat anti-PA as detection antibody (1 μg/ml), and purified recombinant PA as standards (0 to 25 ng/ml; List Biologicals, Campbell, CA). Bound antigen from plasma (1:50) was detected with streptavidin-horseradish peroxidase and TMB substrate (450 nm). The assay was sensitive to 3 ng/ml PA antigen.

Terminal dUTP Nick-End Labeling (TUNEL) Assay

Apoptotic cells were visualized using an *in situ* fluorescence TUNEL assay (Roche, Indianapolis, IN), according to the manufacturer's instructions.

Histopathology

At necropsy, the gross appearance of the major organs was examined, and specimens were collected within 1 hour of death. Tissues were fixed in 10% neutral buffered formalin for at least 24 hours, processed by standard methods, and embedded in paraffin. Sections were stained with hematoxylin and eosin (H&E) or phosphotungstic acid (PTAH) for routine histopathology. Congestion, white cell influx, hemorrhage, thrombosis, and necrosis on blinded samples were quantified by Dr. Stanley Kosanke (Department of Pathology, School of Medicine, University of Oklahoma Health Sciences Center) as described.[38] Tissues were rated according to the severity of the histopathological lesions. The scale ranged from 0 to + 4, with 4 being the most severe.

Immunohistochemistry

Tissues were processed as described.[39] Tissues from saline-treated control animals[37] were treated identically to those obtained in the current anthrax study. Tissues were fixed (4% paraformaldehyde), cryoprotected (5% sucrose, mounted in Tissue-Tek OCT compound), and snap-frozen in liquid nitrogen-cooled isopentane. Tissue cryosections were treated with 0.1 mol/L glycine in phosphate-buffered saline (PBS) for 15 minutes to block free aldehyde groups and with 3% bovine serum albumin and 5% normal goat serum in PBS plus 0.1% saponin, for 30 minutes at room temperature to block nonspecific binding sites. For double-immunofluorescence labeling, specimens were incubated with mixtures of monoclonal (mAb; 10 μg/ml) and polyclonal antibodies (20 μg/ml) for 1 hour at 20°C or overnight at 4°C. The following antibodies were used: anti-tissue factor mAb, (clone TF9-10H10; gift from Dr. James H. Morrissey, Department of Biochemistry, University of Illinois at Urbana-Champaign, Urbana, IL), anti-nitrotyrosine mAb (LabVision Corp., Fremont, CA), anti-CD68 mAb (DAKO, Carpinteria, CA), rabbit polyclonal IgGs against human tissue factor pathway inhibitor (TFPI), and human inducible nitric oxide synthetase (iNOS; NeoMarkers Inc., Fremont, CA). The sections were washed 3 × 10 minutes in PBS/saponin and incubated for 1 hour at 20°C with combinations of appropriate detection antibodies conjugated with fluorescein isothiocyanate or Cy3 (Jackson ImmunoResearch Laboratories, West Grove, PA) diluted 1:100 in 1% bovine serum albumin in the same buffer. After washing as

above, segments were mounted in Vectashield (Vector Laboratories, Burlingame, CA) containing TO-PRO-3 iodine (Molecular Probes, Eugene, OR) as a nuclear counterstain.

As negative controls for polyclonal antibody staining, primary antibodies were replaced with equivalent amounts of rabbit nonimmune serum. The anti-nitrotyrosine antibody specificity was confirmed by control experiments showing loss of antibody recognition after competition with excess (10 mmol/L) 3-nitrotyrosine (not shown). Anti-digoxigenin mAb, a hapten antigen that occurs only in plants, was used as negative control for mAb staining.

Specimens were examined using a Nikon C1 confocal laser-scanning unit equipped with a three-laser launcher (488, 543, and 633 nm emission lines) installed on an Eclipse TE200-U inverted microscope (Nikon, Melville, NY). Images were taken with either a ×20 plan achromat objective (NA 0.46) or a ×60 apochromat oil immersion objective (NA 1.4).

Statistical Analyses

Data were analyzed for differences between dosage groups using the Student's *t*-test, assuming equal variance. APTT, D-dimer, elastase, and cyto/chemokine data were transformed to natural log before analysis to account for unequal variances. A P value <0.05 was considered to be significantly different.

Results

In all animals, except those that received the lowest doses, we observed coagulopathy, increased vascular permeability, and inflammation, in which the severity of the response was commensurate with the extent of the bacterial challenge. Eleven animals were studied; data are grouped according to the $\log_{10}$ bacterial load.

Mortality

Survival times after challenge with *B. anthracis* Sterne strain were dose-dependent (Figure 1). A 7-day survivor was considered to be a permanent survivor. Infusion of 5×10^5 and 5×10^7 CFU/kg was sublethal, and the upper limit of a sublethal dose was near 6×10^7 CFU/kg. Bacterial exotoxin production was confirmed by increases in PA (Figure 2), which is required for cellular intoxication.[40] PA antigen decreased to baseline after antibiotic treatment began.

Lung Pathology

Pathological changes in the lungs after $\geq 10^7$ CFU/kg were consistent with acute lung injury. Macroscopic findings (Figure 3, A and B) included widespread hemorrhagic lesions and frothy edematous fluid from the trachea. Large volumes of serosanguinous pleural fluid (40 ~ 60 ml) were found at necropsy in all animals that received a lethal challenge. Microscopic findings included congestion, hemorrhage, intra-alveolar edema, fibrin, neutrophilic influx, and hyaline membrane formation (Figure 3, C–F), with the severity being proportionate to the challenge dose (Figure 3G).

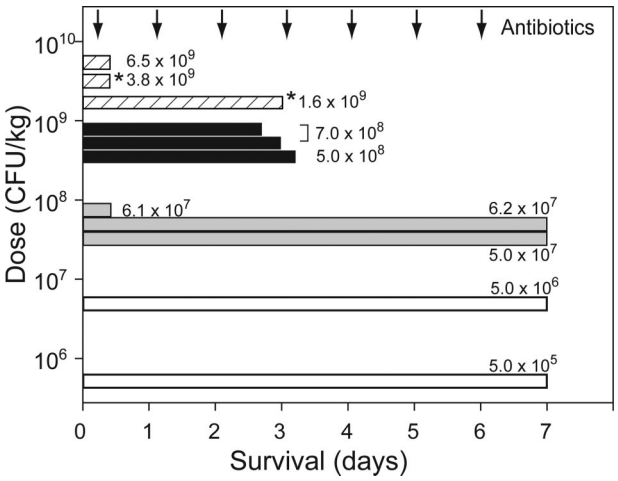

Figure 1. Mortality. Animals were infused with *B. anthracis* Sterne bacteria in doses ranging from 5×10^5 CFU/kg to 6.5×10^9 CFU/kg. All animals except two (*1.6×10^9 and *3.8×10^9 CFU/kg) received antibiotic treatment to more closely mimic a clinical setting.

Coagulopathy

Early aggressive hemostatic changes were apparent by loss of fibrinogen, prolongation of APTT clotting times, elevated D-dimer, and decreased platelets (Figure 4, A–D). D-dimer changes indicated fibrin formation and fibrinolysis, and the sensitivity of this marker was indicated by continuing elevated levels after 10^5 to 10^6 CFU/kg when the animals appeared to be otherwise normal. Fibrinogen increases by 24 hours reflected the acute phase response. A consumptive coagulopathy typical of overt DIC[41] was evident at 12 to 24 hours ($\geq 10^7$ CFU/kg)

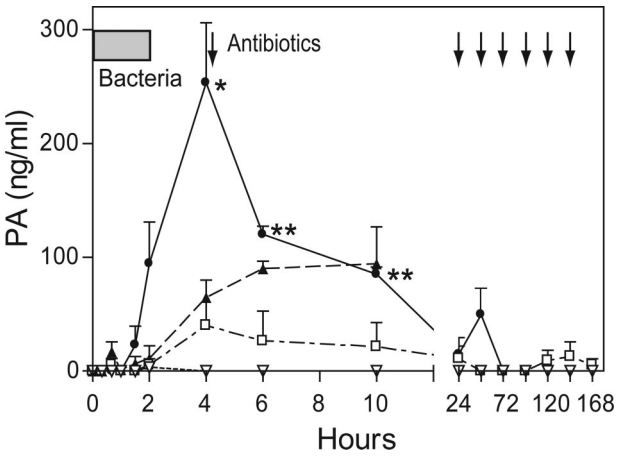

Figure 2. Toxemia. Plasma levels of anthrax toxin PA were determined by ELISA after infusion of bacteria. Challenge dose in CFU/kg: 10^5 to 10^6 (▽, $n = 2$); 10^7 (□, $n = 3$); 10^8 (●, $n = 3$); and 10^9 (▲, $n = 3$). *$P < 0.05$; **$P < 0.01$.

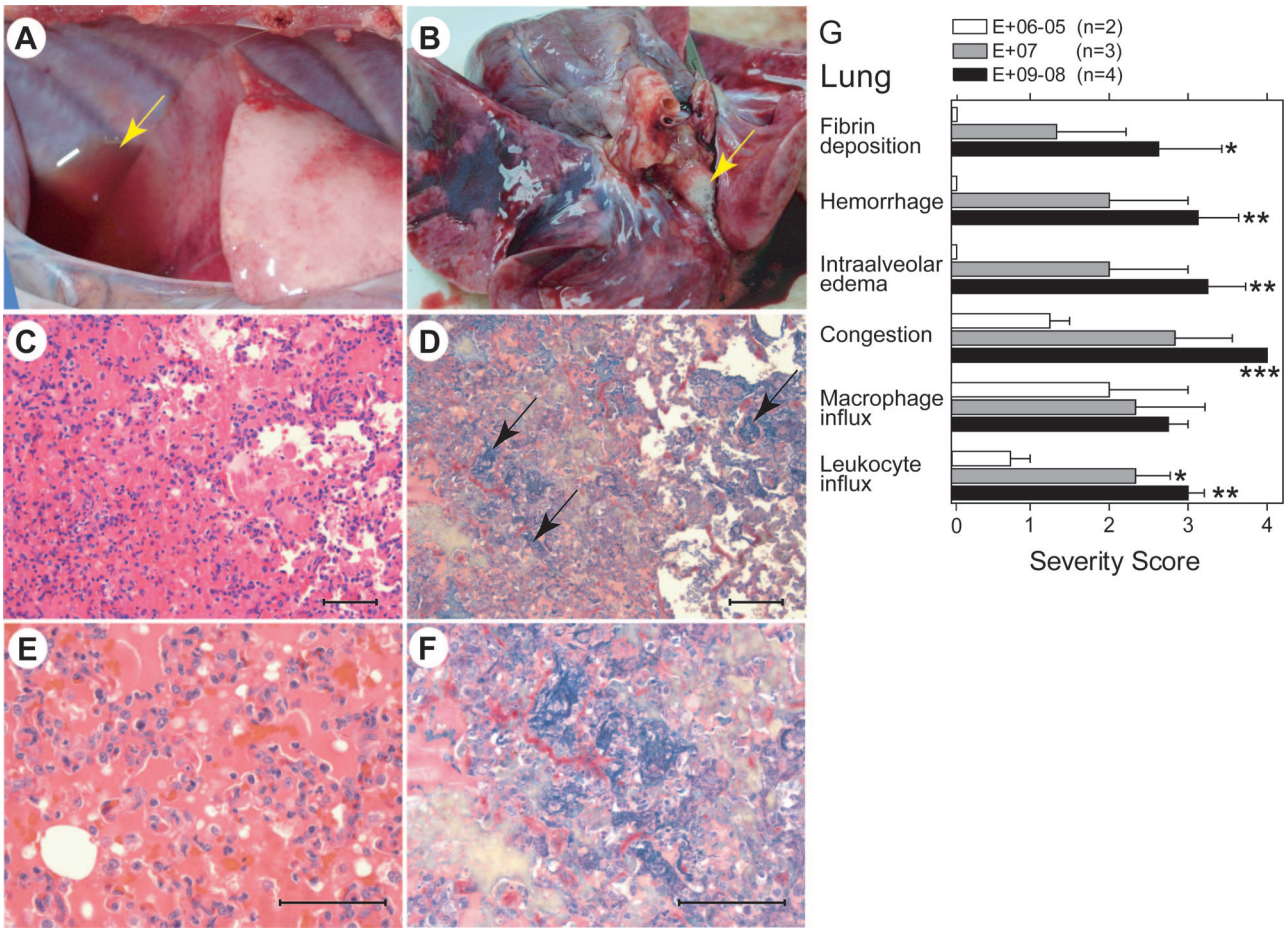

Figure 3. Lung pathology. **A:** Pleural cavities contained prominent serosanguinous pleural effusions (**arrow**). **B:** Hemorrhagic lesions and frothy edematous fluid from the trachea (**arrow**) is consistent with permeability edema. **C:** Widespread capillary leakage in lung and intra-alveolar edema, 72-hour survival (H&E). **D:** Fibrin aggregation (**arrow**) with generalized epithelial and endothelial damage, 72-hour survival (PTAH). **E:** Higher magnification view of lung (H&E). **F:** Higher magnification view of lung (PTAH). **G:** Severity of lung injury on blinded samples was graded by a pathologist. Tissues were rated according to the severity of the histopathological lesions. The scale ranged from 1 to +4, with 4 being the most severe. Data were grouped according to the dose range. Mean ± SEM. Significant differences from the low-dose challenge (10^5 to 10^6 CFU/kg) were determined: $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Scale bars = 100 μm. Original magnifications: × 200 (**C**, **D**); ×400 (**E**, **F**).

when fibrinogen levels recovered to ≈80%, but APTT clotting times remained prolonged (45 to 65 seconds) and platelets low.

The protein C pathway down-modulates coagulation and inflammation,[42] and reduced protein C levels are associated with a poor prognosis in patients.[31,43] In the *B. anthracis*-challenged baboons, protein C levels decreased, indicating consumption (Figure 5). The early rise in protein C antigen was likely due to hemoconcentration, not increased synthesis.

Vascular Permeability

Increased vascular permeability was reflected by changes in mean systemic arterial pressure, respiration, and hematocrit (Figure 6, A–C). Hemoconcentration attributable to fluid exiting from the vasculature to extravascular spaces was accompanied by increased respiration rates.

Cellular Responses

Cellular responses were typical of a septic challenge, were less dose-dependent, and were similar to our previous studies in the *E. coli* baboon model[29] (Figure 7). Changes in white cell populations reflected the expected margination of white cells and subsequent granulopoietic responses to the infection.

Inflammation

Similar to patients and most animal models, there was an early, transient hyperinflammatory response to *B. anthracis* with increased cytokine and neutrophil elastase levels (Figure 8). Some differences were observed from comparison of cytokine/chemokine data from *B. anthracis*-treated baboons (Figure 9) and from earlier experiments with baboons that received 10^8 CFU/kg (low sublethal), 10^9 CFU/kg (high sublethal), or 10^{10} CFU/kg (lethal) *E. coli* 086:K61H. Analyses from *E. coli*-treated baboons

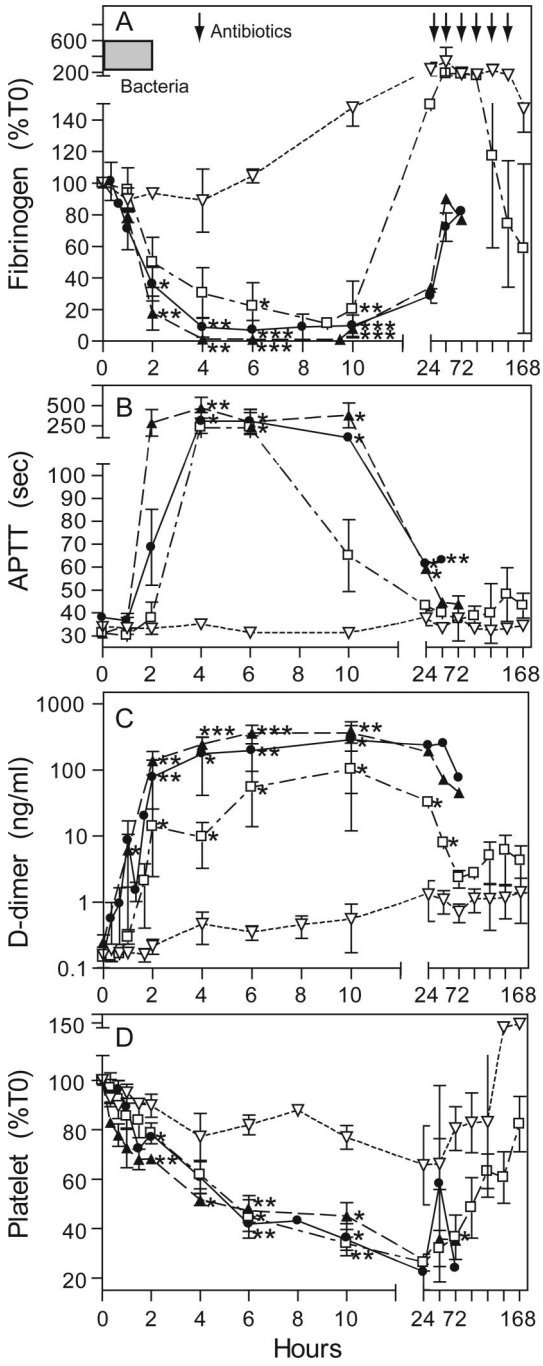

Figure 4. Coagulopathy. Hemostatic changes are apparent by loss of fibrinogen (**A**), prolongation of APTT clotting times (**B**), elevated D-dimer (**C**), and reduced platelets (**D**). Mean ± SEM. Challenge dose in CFU/kg: 10^5 to 10^6 (▽, $n = 2$); 10^7 (□, $n = 3$); 10^8 (●, $n = 3$); and 10^9 (▲, $n = 3$). Significant differences from the low-dose challenge (10^5 to 10^6 CFU/kg) were determined: $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

were performed on stored samples; no new animals were challenged with *E. coli* for this study. After *B. anthracis*, IL-8 responses were less dose-dependent, MCP-1 levels were up to sevenfold higher, and MIP-1 increases were more transient.

There were interesting differences in IL-12 levels, a heterodimer of p40 and p35 that functions in innate and adaptive immunity. IL-12p70 levels were similar after *B.*

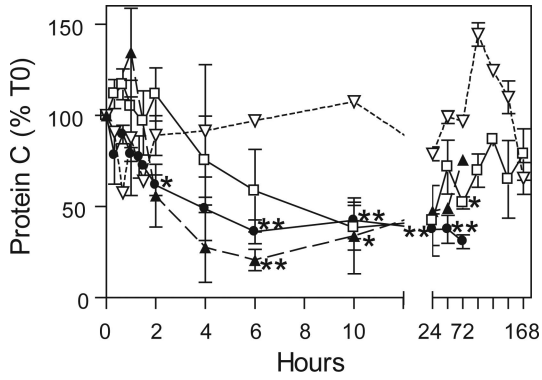

Figure 5. Protein C levels. Citrated plasma was collected at the times indicated, and protein C antigen levels were determined by ELISA as described in the Materials and Methods. Normal (100%) was defined using pooled normal baboon plasma. Challenge dose in CFU/kg: 10^5 to 10^6 (▽, $n = 2$); 10^7 (□, $n = 3$); 10^8 (●, $n = 3$); and 10^9 (▲, $n = 3$). Significant differences from the low-dose challenge (10^5 to 10^6 CFU/kg) were determined: $^*P < 0.05$, $^{**}P < 0.01$.

anthracis or *E. coli* challenge (Figure 9, G and H), indicating similar p35 production.[44] IL-12p40 is typically overexpressed after inflammatory stimuli, as was observed in the baboons after *E. coli* challenge (Figure 9J) with an inverse dose-dependent relationship, in agreement with earlier data.[45] However, little IL-12p40 was generated in *B. anthracis* animals (Figure 9I), suggesting a profound immune dysfunction at this level.

Immunohistochemistry

Molecular changes at the tissue level provide insight into pathways that respond to *B. anthracis*. Compared to a saline-treated control, expression of tissue factor (TF), the tightly regulated initiator of extrinsic coagulation,[46] was higher on lung mononuclear cells after *B. anthracis* (Figure 10, A and B; green). In contrast, expression of tissue factor pathway inhibitor (TFPI), the constitutively available inhibitor of extrinsic coagulation, was markedly lower (Figure 10, A and B; red). Both hemostatic molecules are modulated by inflammatory cytokines.[46,47] Elevated TF expression with concomitant decreases in TFPI inhibitory capacity is consistent with the DIC indicated by the cellular and physiological markers.

Pulmonary oxidative damage was indicated by marked expression of inducible nitric oxide synthetase (iNOS, red) on $CD68^+$ mononuclear infiltrates (Figure 10, C and D; green) and increased protein tyrosine nitration (Figure 10, E and F; red). Little cellular apoptosis was observed in the lung (not shown), despite infiltration of $CD68^+$ cells. Moderate apoptosis was observed in the spleen (Figure 10, G and H; green) and splenic capsule, similar to that observed previously in macaques.[48]

Discussion

The current data demonstrate that infection with toxigenic *B. anthracis* elicits many features of a septic disease; therefore, the lethal role of the host response to the septic challenge with anthrax has likely been underestimated.

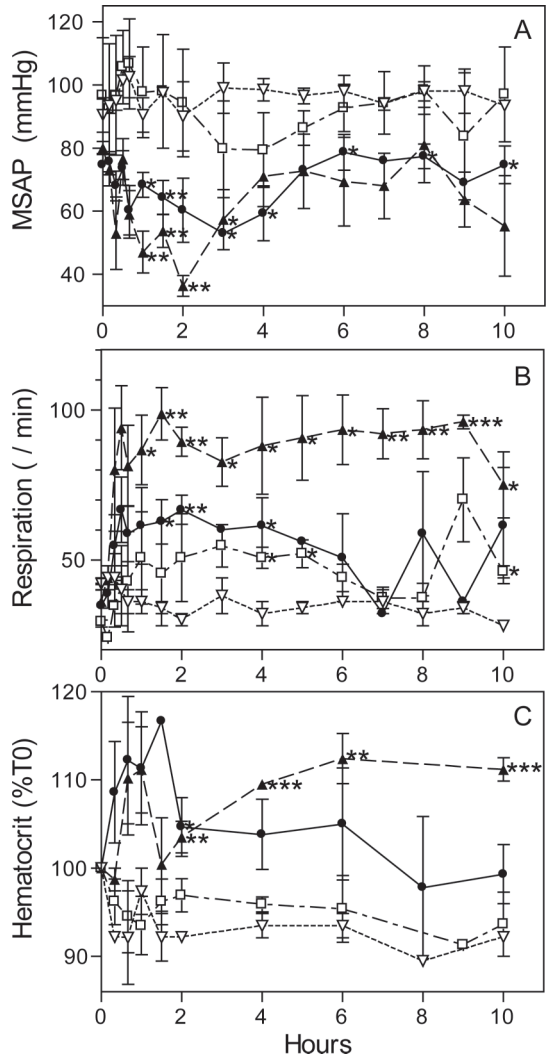

Figure 6. Vascular permeability. **A–C:** Increased vascular permeability led to reduced mean systemic arterial pressure (**A**) accompanied by corresponding increases in respiration (**B**) and hematocrit (**C**). Responses were dose-dependent. Mean ± SEM. Challenge dose in CFU/kg: 10^5 to 10^6 (▽, $n = 2$); 10^7 (□, $n = 3$); 10^8 (●, $n = 3$); and 10^9 (▲, $n = 3$). Significant differences from the low-dose challenge (10^5 to 10^6 CFU/kg) were determined: $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

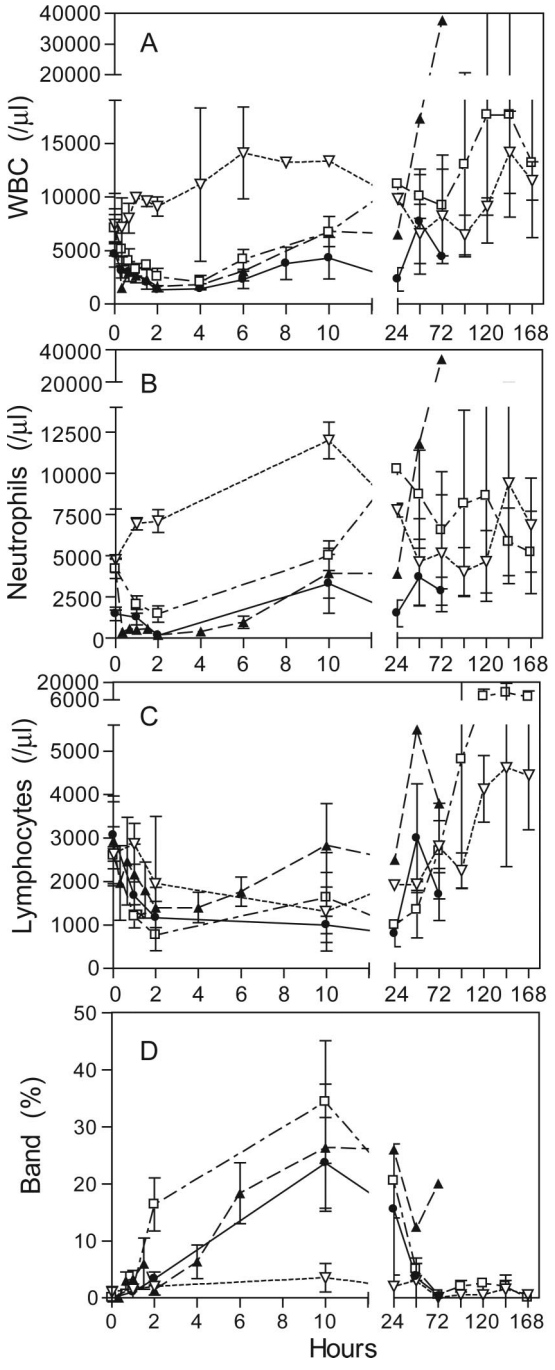

Figure 7. Cellular responses. **A–C:** The changes in WBC counts (**A**) reflect ongoing neutropenia (**B**) and lymphocytopenia (**C**). **D:** The increase in bands is a typical granulopoietic response to septic challenge. There was no clear dose dependence, but the magnitude of the changes reflected the severity of the challenge. Mean ± SEM. Challenge dose in CFU/kg: 10^5 to 10^6 (▽, $n = 2$); 10^7 (□, $n = 3$); 10^8 (●, $n = 3$); and 10^9 (▲, $n = 3$).

When infected, people do not die from spores or toxin alone. They die from complications elicited by the vegetative bacteria; this includes exotoxin activities as well as their own uncompensated inflammatory and coagulopathic responses to the septic challenge.

Massive volumes of serosanguinous pleural effusions were observed in all fatal cases in the 2001 attacks[1,18] and in our baboon model, the lung was also severely affected. The hemorrhagic lesions, intra-alveolar edema, and hyaline membrane formation were consistent with acute lung injury, and large volumes of serosanguinous pleural fluid were found at necropsy in all animals after a lethal challenge. In three communities of wild chimpanzees in the Tai National Park, Ivory Coast, eight of which died of anthrax,[49] histopathology also revealed lung edema and significant hemorrhages presenting as ecchymoses in multiple organs, including the lungs. In macaques exposed to aerosolized spores of virulent *B. anthracis* Ames strain, hemorrhagic lesions in the lung were prominent, although it was not clear whether lung injury was of airway origin or from the bloodstream.[48] The current data demonstrate that the acute lung injury and pulmonary effusions can be directly attributed to bloodborne *B. anthracis* bacteria.

Prominent pleural effusions observed both in the baboon model and in bioterrorism-associated cases are not

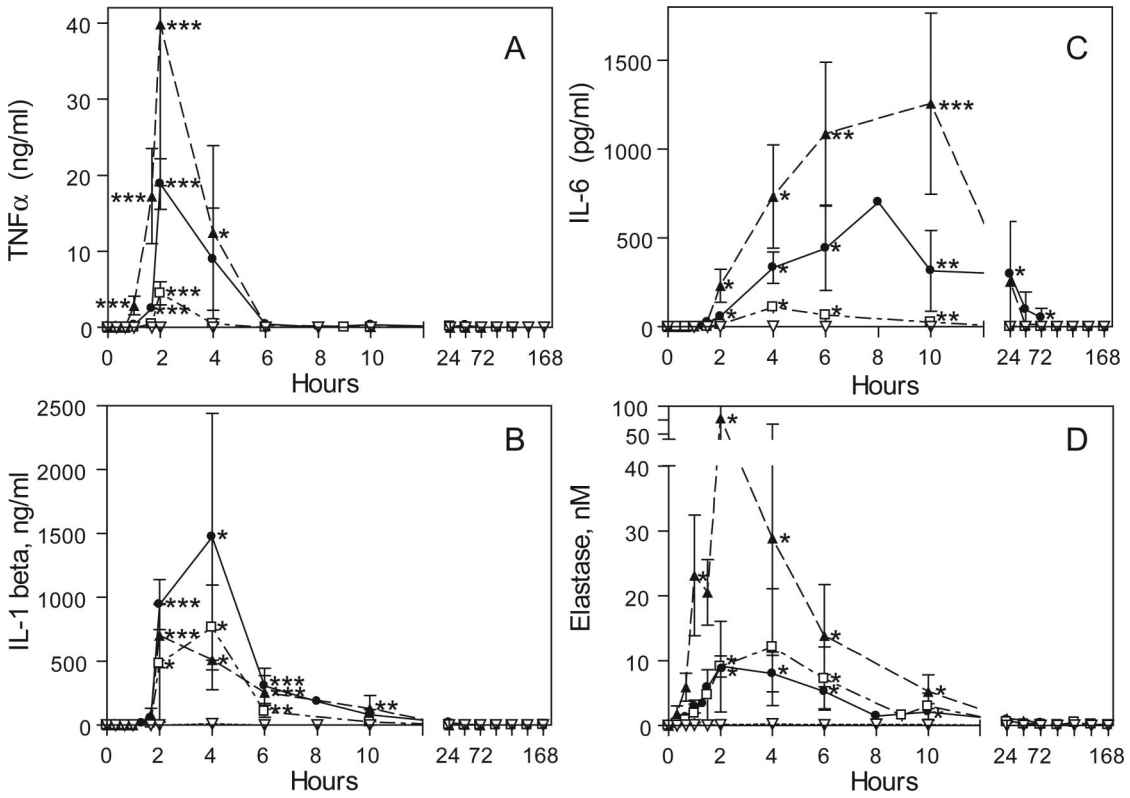

Figure 8. Systemic inflammatory responses. **A–D:** Increases in TNF-α (**A**), IL-1β (**B**), IL-6 (**C**), and neutrophil elastase (**D**) occurred after bacterial challenge. Changes were relatively early, transient, and similar to responses observed in other animal models of sepsis. Mean ± SEM. Challenge dose in CFU/kg: 10^5 to 10^6 (∇, $n = 2$); 10^7 ($\square$, $n = 3$); 10^8 ($\bullet$, $n = 3$); and 10^9 ($\blacktriangle$, $n = 3$). Significant differences from the low-dose challenge (10^5 to 10^6 CFU/kg) were determined: $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

normally predominant features of sepsis and are likely a unique result of *B. anthracis*. The lung pathology also differs considerably from our experience with the intravenous lethal *E. coli* model, in which acute lung injury is an inconsistent finding and pleural effusions are rarely observed (<5 ml, if at all), even at high bacteria doses.[38] The mechanism for this difference is not apparent with the current experimental approach. Dissemination would be expected to be similar between the intravenous *E. coli* and *B. anthracis* models because bacteria are infused similarly via the cephalic vein and the lung is the first capillary bed encountered; the lung is also an early target of bacteria germinated from inhaled *B. anthracis* spores.[50] There are also considerable differences in the nature of the bacteria, and gram-negative *E. coli* would be expected to propagate inflammation through Toll-like receptors with a specificity different from that recognized by toxigenic gram-positive *B. anthracis*.[51] However, there is overlap because heat-killed *B. anthracis*[52] and anthrolysin-O from *B. anthracis* Sterne strain[53] can activate Toll-like receptor 4, which is ordinarily associated with activation by lipopolysaccharide from gram-negative bacteria. Whether these differences contribute to the distinct lung pathology and cytokine profiles after *B. anthracis* challenge is not yet known.

Data from case reports of the 2001 bioterrorism victims were consistent with either overt[2,5] or probable DIC.[3] These clinical observations strongly indicate that procoagulant and inflammatory responses coincide with bacteremia and toxemia. In the baboons, we observed a significant increase in vascular permeability coincident with hemostatic imbalances manifested by thrombocytopenia, transient leucopenia, and an aggressive DIC. Histopathology confirmed the coagulopathy and fibrin deposition in the lungs. Loss of circulating protein C and cell-associated TFPI, coupled with increased tissue factor expression presents a potential molecular basis for the severe hemostatic dysfunction in the baboons and suggests that anti-coagulant adjunctive therapies may influence mortality or morbidity due to *B. anthracis* infection.

A systemic inflammatory response ensued with early transient increases in proinflammatory cytokines/chemokines. Although this hyperinflammatory response is typical after most infectious challenges, changes in IL-12p40 were notably different between *E. coli*- and *B. anthracis*-treated animals. IL-12 is a proinflammatory cytokine that bridges innate and adaptive immune responses and skews T-cell reactivity toward a Th1 response.[54] Antigen-presenting cells, such as dendritic cells and macrophages, are the primary producers of IL-12, a heterodimeric cytokine consisting of p40 and p35 subunits that arise as two different gene products.[55] p35 is constitutively transcribed, is regulated posttranslationally,[56] and is not secreted independently. In contrast, p40 production is regulated by inflammatory effectors, often to high levels, including during infection with *Neisseria meningitides*[57] or in autoimmune disease.[58] The lack of p40 in

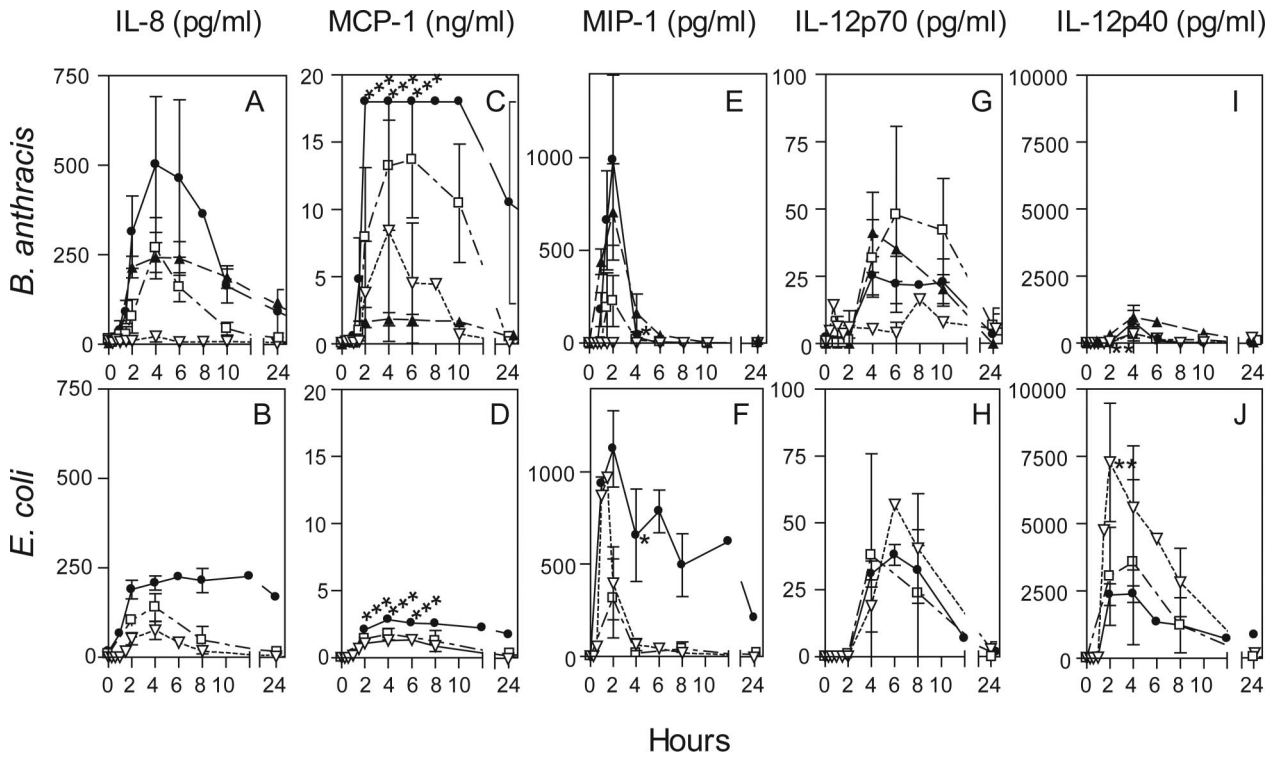

Figure 9. *B. anthracis* versus *E. coli* challenge in the baboon. Plasma levels of IL-8 (**A**, **B**), MCP-1 (**C**, **D**), MIP-1 (**E**, **F**), IL-12p70 (**G**, **H**), and IL-12p40 (**I**, **J**) were determined by assays from baboons challenged with *B. anthracis* (**top**) or *E. coli* (**bottom**). Challenge *B. anthracis* dose in CFU/kg: 10^5 to 10^6 (▽, $n = 2$); 10^7 (□, $n = 3$); 10^8 (●, $n = 3$); and 10^9 (▲, $n = 3$). Challenge *E. coli* dose in CFU/kg: 10^8 (▽, $n = 3$); 10^9 (□, $n = 2$); 10^{10} (●, $n = 3$). Mean ± SEM. **A–H:** There were significant differences between lethal challenges with 10^8 CFU/kg *B. anthracis* and 10^{10} CFU/kg *E. coli*; $^*P < 0.05$, $^{***}P < 0.001$. **I** and **J:** There were significant differences between sublethal challenge with 10^5 to 10^6 CFU/kg *B. anthracis* and 10^8 CFU/kg *E. coli*; $^{**}P < 0.01$.

the anthrax animals may have multiple consequences because p40 homodimers antagonize IL-12[59] and p40/p19 heterodimers (IL-23) have overlapping, yet distinct, functions to those of IL-12.[60] The mechanism for the paucity of p40 in the anthrax animals is not known. It may be related to whether the bacteria is gram-negative or -positive.[61] Alternatively, anthrax lethal toxin (lethal factor + PA) inhibits dendritic cell maturation[62] and kills macrophages,[63] which would selectively compromise immune responsiveness and favor bacterial survival.

Tissue inflammation was demonstrated by pulmonary $CD68^+$ mononuclear infiltrates and iNOS expression by $CD68^+$ cells and other cell types (presumably endothelial and/or pulmonary epithelial cells). Production of nitric oxide by iNOS is important for vascular tone and antibacterial defense, but overproduction is cytotoxic, so iNOS expression is tightly regulated.[64] Increased protein tyrosine nitration in the lung reflects a shift from the signal transducing physiological actions of •NO to peroxynitrate-mediated oxidative stress and injury.[65]

The relative contribution of anthrax exotoxins toward death of the baboons remains to be established in our model. MCP-1 levels were up to sevenfold higher in the anthrax baboons compared with *E. coli*-challenged animals, and MCP-1 is induced similarly from vascular cells by both gram-positive and gram-negative bacteria,[66] suggesting that this preferential increase by *B. anthracis* may be attributable to the primate vasculature reacting to exotoxin(s). With high bacterial load, the baboons died within 10 to 12 hours when PA levels are detectable. Like patients, our primates receive antibiotics, and PA antigen was low to undetectable after 24 hours, yet animals challenged with 0.5 to 1.6 × 10^9 CFU/kg still succumbed in 3 to 4 days. Edema toxin will induce necrosis in a zebra fish model,[67] and high doses of edema toxin induce multiple organ failure secondary to vascular permeability changes in mice.[68] In the baboons, necrosis was not observed in the lungs but was present in the adrenals and in kidneys secondary to microthrombosis (not shown). However, we cannot ascribe this pathology to *B. anthracis* or exotoxins because it is also observed in baboons after challenge with *E. coli*. Species differences or choice of infective agent may contribute to these differing observations. Data from our ongoing experiments with *B. anthracis* strains defective in toxin production as well as purified toxin challenges will contribute to elucidating the relative contributions of anthrax toxins toward host mortality. Because most current vaccine approaches target the PA toxin component, understanding the *in vivo* role of anthrax toxins becomes critically important.

Collectively, the current data demonstrate that host responses to anthrax infection include compromised innate immunity coupled with uncompensated inflammatory and coagulopathic responses. Our model does not discriminate between effects of anthrax exotoxins or nontoxin bacterial products. Although the *B. anthracis* Sterne strain produces exotoxin levels approximately equivalent to naturally virulent strains,[33] it does not have the external bacterial capsule that is believed to play a role in delay-

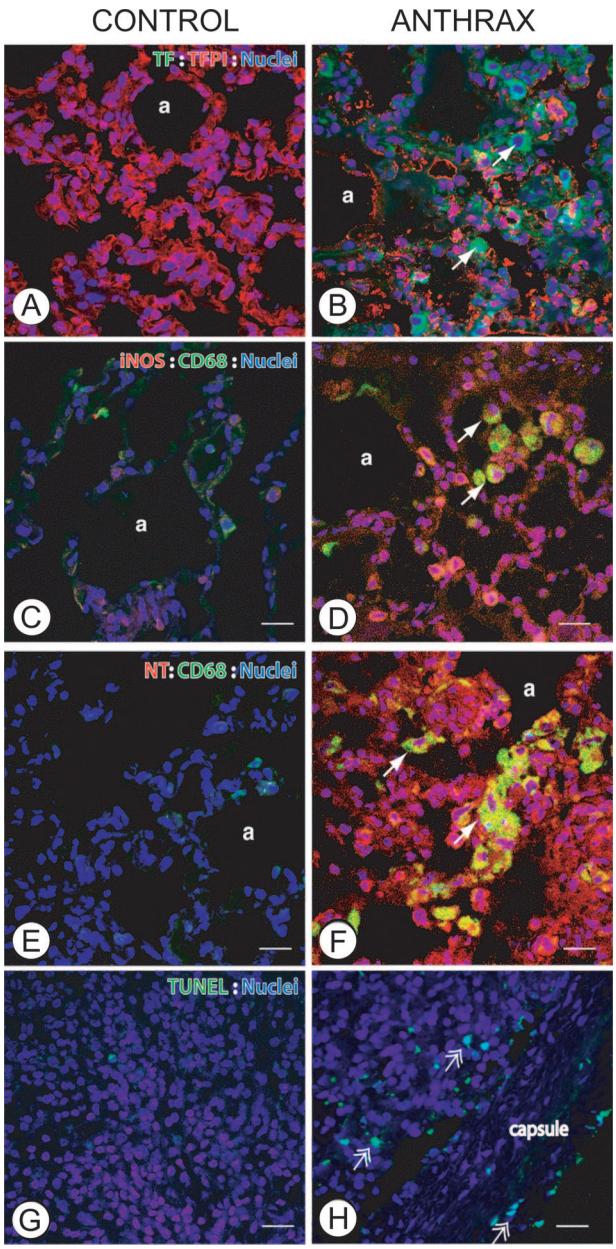

Figure 10. Immunohistochemistry. Lung tissues from a baboon that received 3.8×10^9 CFU/kg *B. anthracis* Sterne intravenously (**right**) or a saline-challenged baboon (control, **left**) were processed for immunohistochemistry as described in the Materials and Methods. Tissues were stained for TF (green) and TFPI (red) (**A**, **B**); inducible nitric oxide synthetase (iNOS, red) and CD68 (green) (**C**, **D**); protein tyrosine nitrosylation (red) and CD68 (green) (**E**, **F**). **G** and **H:** Spleen tissue was stained for apoptosis (green) by TUNEL. Nuclei, blue. a, alveolus. Scale bars = 100 μm.

ing uptake by macrophages and subsequent cytokine responses.[69] Thus, the study design may not precisely replicate the kinetics of disease progression associated with infection by spores of fully virulent *B. anthracis*. Additional baboon models are in progress to determine the effect of kinetics on disease development.

In summary, interpretation of responses to challenge with anthrax spores, toxins, or bacteria in animal models should be viewed from the perspective of human victims of anthrax in whom toxemia and bacteremia coincide. This larger perspective will accelerate development of clinically relevant animal models for rapid transition of vaccines, immunotherapeutics, and adjunct therapeutics for treatment and prevention of anthrax in humans.

Acknowledgments

We thank Glenn Peer, Brett Clough, Jamie Talbert, Kandice Swindle, Scott Freeman, and Valta Collins for their dedicated expertise; Dr. D.J. Flournoy (VA Medical Center, Oklahoma City, OK) and Dr. Gary White (Director, University of Oklahoma Health Sciences Center Baboon Research Resource, Oklahoma City, OK) for helpful discussions; and Dr. J. Donald Capra (President, Oklahoma Medical Research Foundation, Oklahoma City, OK) for his support and critical reading of the manuscript.

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