Activation of plasminogen activator inhibitor implicates protease InhA in the acute-phase response to *Bacillus anthracis* infection

Myung-Chul Chung, Shelley C. Jorgensen,† Taissia G. Popova, Jessica H. Tonry, Charles L. Bailey and Serguei G. Popov

National Center for Biodefense and Infectious Diseases, George Mason University, 10900 University Blvd, Manassas, VA 20110, USA

Anthrax is a zoonotic disease caused by *Bacillus anthracis*. The infection is associated with inflammation and sepsis, but little is known about the acute-phase response during disease and the nature of the bacterial factors causing it. In this study, we examined the levels of the acute-phase proteins (APPs) in comparative experiments using mice challenged with spores and a purified *B. anthracis* protease InhA as a possible factor mediating the response. A strong increase in the plasma levels of APPs such as haptoglobin and serum amyloid A was observed during infection. Protein and mRNA levels of plasminogen activator inhibitor (PAI)-1 in the liver were also increased concurrently with bacterial dissemination at 72 h post-infection. Similar effects were observed at 6 h post injection with InhA. Induction of hepatic transforming growth factor-β1, a PAI-1 inducer, was also found in the liver of InhA-injected mice. PAI-1 elevation by InhA resulted in an increased level of urokinase-type plasminogen activator complex with PAI-1 and a decreased level of D-dimers indicating inhibition of blood fibrinolysis. These results reveal an acute liver response to anthrax infection and provide a plausible pathophysiological link between the host inflammatory response and the pro-thrombotic haemostatic imbalance in the course of disease through PAI-1 induction in the liver.

**INTRODUCTION**

Anthrax is a highly dangerous zoonotic disease caused by *Bacillus anthracis*. It is characterized by septicaemia and toxemia caused by disseminated, proliferating bacteria and their secreted factors including lethal and oedema toxins. Death of infected people and animals follows the onset of anthrax septic shock, which has recently become the subject of intense studies aimed at developing effective therapeutic modalities against anthrax. One of the key questions relevant to anthrax septic shock is the role of the host response in this terminal condition (Sherer *et al.* 2007).

Bacterial infections typically induce an inflammatory response manifested in the release of pro-inflammatory cytokines as a result of innate recognition of bacterial antigens at the initial stage of infection, as well as tissue injury during the disease progression (Lin & Karin, 2007; Sutterwala *et al.*, 2007). Pro-inflammatory cytokines are known to trigger changes in the plasma levels of acute-phase proteins (APPs) produced by the liver (Tilg *et al.*, 1997). Our previous data revealed increased expression of several cytokines, chemokines and apoptotic genes in the liver of Sterne spore-challenged mice (Popov *et al.*, 2004).

We hypothesize that cytokine induction in the liver coincides with acute inflammation; however, the acute-phase response to anthrax infection remains uncharacterized. In this study, we examined the levels of APPs in comparative experiments in mice challenged with the toxigenic, lethal Sterne strain versus the non-toxigenic, non-lethal delta-Sterne strain. The circulating concentrations of APPs are related to the severity of the underlying condition and therefore provide a means of evaluating the presence and extent of disease processes (Grüys *et al.*, 1993, 1994; Kushner & Mackiewicz, 1987; Thompson *et al.*, 1992).

Many APPs are now known to play beneficial roles in mediating complex inflammatory responses and restoration of homeostasis (Rocha *et al.*, 1998). Levels of several APPs, including haemopexin (HPX), haptoglobin (HPG), serum amyloid A (SAA) and C-reactive protein (CRP), are

**Abbreviations:** APP, acute-phase protein; CRP, C-reactive protein; HPG, haptoglobin; HPX, haemopexin; InhA, neutral metalloprotease immune inhibitor A; PAI-1, plasminogen activator inhibitor-1; SAA, serum amyloid A; TGF, transforming growth factor; uPA, urokinase-type plasminogen activator.

Supplementary tables are available with the online version of this paper.
typically elevated in both serum and plasma as a result of infection-mediated inflammation (Duan, 2005; Wait, 2005). Plasminogen activator inhibitor-1 (PAI-1), another APP, is an essential regulator in physiological thrombotic and fibrinolytic processes in the blood vessels. PAI-1 expression is regulated by many intrinsic factors such as cytokines, growth factors, hormones and lipids, and by extrinsic factors such as physical injury and DNA-damaging agents (Biemond et al., 1995; Lee & Huang, 2005). During severe anthrax infection or sepsis, a rise in PAI-1 levels is strongly associated with the systemic inflammatory response (Liu, 2008). Hence increased PAI-1 levels are highly suggestive as a marker for pathogenesis in numerous pathogenic infections and during sepsis (Kruithof et al., 1988).

Another important aspect of anthrax septic shock is the nature of B. anthracis pathogenic factors involved in the modulation of the inflammatory response. Available evidence indicates that this process is complex and likely represents a concerted action of toxins (Chung et al., 2008a; Dong et al., 2003; Kruithof et al., 1988; Tilg et al., 1997) and other pathogenic factors such as bacterial wall components, phospholipases and the pore-forming hae-molysin (Heffernan et al., 2007; Lee & Huang, 2005; Park et al., 2004; Wait, 2005). Our recent data suggest that B. anthracis broad-spectrum metalloprotease InhA might play an inflammatory role based on its proteolytic activity toward plasma and the extracellular matrix (Chung et al., 2006, 2008b), similar to proteases in other pathogenic infections (Gutierrez et al., 2008; Komori et al., 2001). Here we present evidence in favour of InhA serving as a pathogenic factor contributing to anthrax-mediated inflammation and thrombosis through PAI-1 induction in the liver and consequent impairment of fibrinolysis.

METHODS

Animals. Female 9-week-old DBA/2 mice were purchased from Jackson Laboratory. Mice were maintained at Biocon, The George Mason University Institutional Animal Care and Use Committee and the Biocon Animal Care and Use Committee/Institutional Review Board approved all protocols prior to animal experiments.

Preparation of spore-challenged mouse plasma. Mice (n=5–10 per group) were challenged intraperitoneally with 5 × 10^6 c.f.u. spores of B. anthracis non-encapsulated Sterne strain 34F2 (pXO1+, pXO2−) or the non-toxigenic delta-Sterne strain (pXO1+, pXO2−). Approximately 200–500 µl blood was drawn via the retro-orbital sinus into 20 µl 0.5 M EDTA and centrifuged at 900 g for 10 min to obtain platelet-poor plasma. Mice were euthanized by cervical dislocation immediately following the blood draw and the livers were collected to measure APPs. Fifty per cent mortality took place at 72 h post Sterne challenge. At this time point, all surviving animals were euthanized. There was a 100 % survival rate in delta-Sterne spore-challenged mice.

Protease injection and preparation of mouse plasma. InhA was prepared as described previously (Chung et al., 2006). Mice were challenged intravenously with a small dose of purified InhA (1 unit per mouse). One unit is defined as the amount of enzyme required to liberate 1 µmol t-leucine equivalents from collagen in 5 h at 37 °C, pH 7.5. The injected dose is expected to result in a similar collagenolytic activity in blood to that observed in bacterial culture supernatants (0.8–2 units ml⁻¹). Enzyme activity was determined by a standard curve using a collagenase from Clostridium histolyticum from the EnzChek Gelatinase/Collagenase Assay kit (Invitrogen) and its purity was estimated to be ~90 % by a Coomassie blue stained gel. Platelet-poor plasma was prepared as described above.

ELISAs. Organ homogenates were prepared by extraction with Tris-buffered saline containing 1 % Triton X-100 at 4 °C overnight. For the quantitative determination of proteins, the following ELISAs were used according to manufacturer’s instructions: mouse ELISA kits (Life Diagnostics) for HPX, HPG, SAA and CRP; a mouse PAI-1 total antigen kit (Innovative Research) for PAI-1; OpTEA cytokine kits (BD Biosciences) for IL-1β, IL-6 and TNF-α; and a Quantikine immunos assay kit (R&D Systems) for TGF-β1. For D-dimer ELISA, D-dimer antibodies DD1 and DD4 (Abcam) were used as a capture and a detection antibody, respectively, under the same conditions as for the commercial kit (BD Biosciences). For determination of the urokinase-type plasminogen activator (uPA)–PAI-1 complex, wells of a 96-well microtitre plate (Nunc MaxiSorp) were coated with anti-uPA antibody (Abcam) in 50 mM carbonate buffer (pH 9.0) and incubated overnight at 4 °C. The uPA–PAI-1 complex was detected with anti-PAI-1 antibody and the corresponding secondary antibody under the same conditions as for the commercial kit (BD Biosciences).

Reverse-transcriptase (RT)-PCR. Total RNAs were prepared from frozen liver sections using an RNeasy mini kit (Qiagen). Random-primed cDNA was prepared from 5 µg total RNA using Platinum PCR Supermix (Invitrogen). Specific primers were: 5′-GGG GTA TGT GGG TGA CGA GG (sense) and 5′-GGG AGA GCA GTA GCA AT A GCC CTC GTA AGA T (antisense) for β-actin; 5′-GGT GCC TTC TCT CCC TAT G (sense) and 5′-CTC TGA GAA GTC CAC CTG T (antisense) for PAI-1; and 5′-TG CCC CAC ACC ATT CCA GGG (sense) and 5′-GCC AAT CTG CAC ATA GCA CC (antisense) for uPA. After amplification, PCR products were separated on a 1.7 % agarose gel and stained with ethidium bromide. Photographs were scanned and analysed by densitometry.

Immunofluorescence staining. Livers of infected mice were harvested at 24, 48 and 72 h post-infection and frozen in liquid nitrogen. Frozen livers were embedded into Tissue-Tek OCT compound (Sakura Finetek) and stored at −80 °C. Liver cryosections (8 µm) were fixed in acetone/methanol (1 : 1) for 5 min, air-dried and blocked for 30 min in a 10 % PBS solution of non-immune goat serum matching the goat origin of the secondary antibody. Blocked slides were probed for 1 h with 100-fold PBST (PBS containing 0.05 % Tween 20)-diluted serum from rabbits immunized by a subcutaneous challenge with B. anthracis Sterne spores. After washing three times with PBST, the slides were stained with a goat secondary anti-rabbit antibody fluorescently labelled with Alexa-546 (Invitrogen). The bacteria were visualized under a Nikon Eclipse 90i fluorescence microscope.

Statistical analysis. All data were expressed as means ± standard deviation. Comparisons between groups were carried out using Student’s t-test. Statistical significance was determined by one-way analysis of variance (ANOVA) prior to Student’s t-test. Significance was set at P-values less than 0.05.

RESULTS

Markers of inflammation/acute-phase responses in the plasma of spore-challenged mice

To determine whether B. anthracis infection induces the changes in the blood levels of APPs, mice (five per group)
were inoculated intraperitoneally with a dose (5 × 10^6 c.f.u. per mouse) of the toxigenic *B. anthracis* Sterne strain spores or the attenuated delta-Sterne strain. This route of inoculation results in a systemic disease characterized by a haematogenic spread of bacteria to the liver and other organs (Popov et al., 2004). We expected that for a particular APP sensitive to the anthrax disease processes, the initial responses to bacterial challenge might be similar between both *B. anthracis* strains due to their close genetic similarity; however, the follow-up responses would reveal differences in the progression of infection. The non-lethal challenge would result in the elimination of the pathogen and convalescence, in contrast to the progression of disease and the onset of moribund condition in the case of lethal challenge. The results presented in Fig. 1 are consistent with this suggestion. Among four tested APPs, HPG and SAA were the most sensitive indicators of bacterial presence as early as 24 h post-challenge, while a change in the levels of CRP was detectable only at the peak of mortality corresponding to 72 h post-challenge (Fig. 1 and supplementary Table S1 in JMM Online). SAA ELISA of Sterne-challenged plasma showed the most notable mean 60-fold and 125-fold increases at 48 h and 72 h post-challenge, respectively. We previously reported that PAI-1 levels were increased 2- to 3-fold at 24–72 h post-challenge for both strains (Chung et al., 2008a). These results demonstrate an early acute-phase response indicative of inflammation and tissue injury during the course of *B. anthracis* infection.

**Acute-phase reactants in the InhA-injected mice**

Next, we investigated which pathogenic factor may be involved in the acute-phase response. As mentioned above, the tissue-damaging and haemorrhagic activities of *B. anthracis* metalloproteases suggested them as potential candidates. This hypothesis was tested using purified InhA as one of the most abundant metalloproteases secreted by *B. anthracis* into circulation during infection (Chitlaru et al., 2007). ELISA analysis of plasma from InhA-injected mice revealed increased levels of all tested APPs as early as 6 h post InhA injection (for SAA and CRP) (Fig. 2 and Supplementary Table S2). In agreement with the results in spore-challenged mice, SAA showed the highest mean 120-fold increase at 24 h post-injection (Fig. 2c). As expected, the bolus InhA injections caused earlier and more transient responses compared to the gradual accumulation of the enzyme after spore challenge. These results confirmed that anthrax metalloprotease InhA may be considered a host response-modulating factor in the murine anthrax model. Interestingly, PAI-1 levels significantly increased at 6 h post InhA injection (Fig. 2e). This led us to further investigate a role of InhA in induction of PAI-1 and the consequences.

**B. anthracis** infection and InhA upregulate PAI-1 in the liver

Although plasma PAI-1 levels were strongly induced by the intravenous InhA injection (Fig. 2e), there was a slight increase of PAI-1 in plasma after spore challenge (Chung et al., 2008a). We therefore hypothesized that during infection PAI-1 may be produced and differentially accumulate locally in one or several target organs. PAI-1 levels in spore-challenged mice were significantly increased in the kidney (5-fold), spleen (2.4-fold) and the liver (15-fold), but no increase was determined in the heart or lung (Fig. 3a and Supplementary Table S3). The PAI-1 level in

---

**Fig. 1.** APP levels are increased in plasma of *B. anthracis* spore-challenged mice. Mice were intraperitoneally challenged with 5×10^6 c.f.u. per mouse of Sterne (■) and delta-Sterne (□) spores. The proteins in plasma obtained from infected mice (n=5) at 24, 48 and 72 h post-challenge were analysed by a sandwich ELISA. Two separate control groups (n=5) were treated with equal volumes of PBS. (a) HPX; (b) HPG; (c) SAA; and (d) CRP. Error bars represent SEM. *P <0.05 and **P <0.01, compared to control treatments.
the liver of the InhA-injected mice was also significantly increased (35-fold) at 6 h post-injection (Fig. 3b).

To investigate whether PAI-1 upregulation was dependent on the transcription of the gene, semiquantitative RT-PCR was employed using total RNA extracted from the liver of spore-challenged or InhA-injected mice. A 30-fold increase in PAI-1 gene transcription was revealed in the liver of Sterne spore-challenged mice at 72 h (Fig. 4a). A similar increase was found in the liver of InhA-injected mice (Fig. 4b). The pathological changes at the level of PAI-1 gene transcription and protein expression coincide with the terminal condition of challenged animals and correlate with the progressive bacterial accumulation in the liver at 72 h post Sterne challenge (Fig. 4c). In agreement with the pro-thrombotic nature of the PAI-1 activity, the livers of moribund mice demonstrated aggregates of bacilli completely blocking the lumen of capillaries (Fig. 4c, panel iv).

**InhA induces cytokine expression in the liver**

Cytokines, including IL-1β, IL-6 and TNF-α, are the primary mediators of the APP response. Their release by a variety of inflammatory cells (Baumann & Gauldie, 1994; Steel & Whitehead, 1994) is known to modulate PAI-1 expression and secretion (Birgel et al., 2000). Therefore, we determined whether the induction of these cytokines by
InhA takes place in the liver. Indeed, ELISA of cytokines showed a statistically reliable but moderate increase in IL-1β (2.7-fold), IL-6 (2-fold), and TNF-α (2-fold) along with a more significant increase in TGF-β1 (6-fold) at 6 h post-injection (Fig. 5 and Supplementary Table S4). Since TGF-β1 is an effective inducer of PAI-1 expression (Stearns-Kurosawa et al., 2006), we suggest that TGF-β1 induction by InhA serves as an upstream signalling mechanism for the induction of PAI-1.

**Fibrinolysis impairment is mediated by InhA**

PAI-1 is a major physiological inhibitor of both urokinase-type and tissue-type plasminogen activators (uPA and tPA, respectively). It plays an important role in determining the net fibrinolytic activity in vivo (Kruithof, 1988; Vassalli et al., 1991). To examine whether elevated PAI-1 levels modulate uPA activity in circulation, we measured the level of the uPA–PAI-1 complex in the plasma of InhA-injected mice and found that it was increased at 6 h post-injection.
(Fig. 6a). At this time point, gene expression of uPA in the liver determined by semiquantitative RT-PCR was not increased (Fig. 6b). Together, these data indicate that activity of circulating uPA was inhibited by PAI-1 at the post-transcriptional level. Finally, we wanted to confirm that the increased level of PAI-1 was accompanied by decreased fibrinolysis. For this purpose, we measured the amount of circulating D-dimers representing the breakdown products of the fibrin mesh and found that the decrease in plasma coincided with the formation of uPA–PAI-1 complex at 6 h post InhA injection (Fig. 6c). This indicates that InhA may play a role in modulation of fibrin degradation during bacterial infection, probably through regulation of PAI-1 levels.

DISCUSSION

The inflammatory response is an unspecific reaction in the animal or human body to trauma, injury or infection. The liver is a major site for synthesis of inflammatory and procoagulant proteins by hepatocytes. Histopathological evidence indicates that the liver is one of the critical target organs of *B. anthracis* infection and anthrax lethal toxin (Popov et al., 2004; Moayeri et al., 2003). In this report, we determined the amounts of the inflammatory and acute-phase markers expected to be triggered by the proinflammatory cytokines in the liver parenchyma and in the blood circulation. We found that HPG and SAA are sensitive blood markers of anthrax infection as early as 24 h post-challenge. However, an increased level of CRP, a classic marker of the acute-phase response, was observed only at the late stage of infection. SAA is an important biomarker for both inflammation and disease, and its level in blood has been shown to increase multi-fold during bacterial lipopolysaccharide exposure (Hoffman & Benditt, 1982). There was a steady increase in the SAA level after the virulent Sterne challenge, indicating that the observed changes are due to the pathogenic process during the course of disease rather than a mere response to bacterial presence, as might be expected in the case of the non-virulent delta-Sterne strain. HPG exerts a broad range of anti-inflammatory activities and acts indirectly as a bacteriostatic agent to facilitate immediate haemoglobin clearance by macrophages (Ascenzi & Fasano, 2007; Tolosano et al., 2002). HPG can also be produced by neutrophils upon activation (Theilgaard-Mønch et al., 2006). Detoxification after liver injury is the most important physiological role of HPX. Its transient release into circulation peaking at 48 h post Sterne-challenge may reflect a functional collapse of the liver before death (Moayeri et al., 2003). Overall, the APP responses observed in our experiments are consistent with the haemolytic, haemorrhaging and tissue-damaging activities of anthrax virulence factors such as phospholipases, pore-forming toxin and metalloproteases, including lethal toxin (Heffernan et al., 2007; Lee & Huang, 2005; Park et al., 2004; Steel & Whitehead, 1994; Wait, 2005).

Recently, Kastrup et al. (2008) reported that clustered *B. anthracis* cells initiated blood coagulation and InhA deletion mutants prolonged the clotting time. This suggests that InhA expressed in clustered bacteria may induce blood coagulation through direct activation of coagulation factors such as prothrombin and factor X. Here we show that anthrax metalloprotease InhA is a potential pathogenic factor capable of inducing the APPs, as could be expected from its similarity to metalloproteases produced by other micro-organisms (Beaufort et al., 2008; Komori et al., 2001; Narasaki et al., 2005). InhA is also a strong liver-specific inducer of PAI-1, likely through the activation of TGF-β expression (Birgel et al., 2000). Thus InhA may play a role in the cascade of pro-thrombotic events including inhibition of uPA and consequent decrease of the fibrinolysis parameters are lowered in InhA-injected mice. (a) uPA–PAI-1 complex formation measured by an ELISA as described in Methods. (b) Hepatic uPA expression determined by RT-PCR as described for Fig. 4 using uPA-specific primers. (c) D-dimer levels in plasma of InhA-injected mice measured by a sandwich ELISA. *P < 0.05 and **P < 0.001, compared to control treatment.
thrombolytic activity of plasmin. Considerable similarities in the APP responses of mice to the Sterne spore versus InhA challenge led us to surmise that InhA may play a significant role in inflammation and impairment of PAI-1-mediated fibrinolysis during anthrax infection.

This report describes the acute liver response to anthrax infection and provides a plausible pathophysiological link between the host inflammatory response and the prothrombotic haemostatic imbalance in the course of disease. In future studies with InhA mutant proteins and bacteria, we plan to address the mechanism of APP induction by InhA. This analysis may also help identify potential pharmacological measures to improve the patient’s outcome in B. anthracis-induced sepsis.

ACKNOWLEDGEMENTS

We thank Bryan Millis (George Mason University) for technical assistance. This work was supported by the US Department of Defense grant DAMD 17-03-C-01220 and the US Department of Energy grant DE-FC52-FO4NA25455.

REFERENCES


