THE EFFECT OF VARIOUS BIOAGENTS ON BLOOD COAGULATION

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ABSTRACT

The possibility of bioterrorism is a looming threat. Potentially deadly biological agents are being studied for their uses in today's technologically advanced warfare, and the medical community is not prepared for the results of such an attack. Many potential bioterrorist agents may have a significant impact on blood coagulation, because of their effects on immune and inflammatory pathways. Bioagents such as anthrax, plague, and glanders have pathophysiological pathways that converge to cause mortality through sepsis and coagulopathy. Therefore, we hypothesized that these agents would cause significant alterations in the coagulation profile of a patient's blood. We designed a rapid, cost-effective, clinically relevant bioassay to serve as a biomarker for pathogen-induced coagulopathy. Our studies indicated that E. coli-derived lipopolysaccharide, anthrax, glanders, and plague-generated procoagulant materials that induced a hypercoagulable state in whole blood. Furthermore, our studies point to tissue-factor activation as the pathway for LPS, anthrax, and plague; and factor VII activation for glanders. Studies in plasma indicated a difference in clotting profiles as compared to whole blood, when comparing the effects of LPS stimulation. The simple tests outlined in this study provide a thorough depiction of the clotting profile of a patient's blood sample. Therefore, these tests would be of great utility as a screening assay for blood coagulopathy. In addition, the tests also may serve effectively to detect the presence of a pathogen, and as a biomarker for bacterial action.

INTRODUCTION

Recent world concern about the threat of bioterrorist attacks has drawn attention to prevention, detection, and treatment methods for highly virulent diseases such as anthrax, plague, and glanders [1]. Plague, once thought to be a disease of the Middle Ages, is now recognized by bioterrorism experts as a potentially disastrous agent in biological warfare. The bacillus, *Yersinia pestis*, invades the lymphatic and vascular systems, causing a severe bacterimia that progresses into sepsis, disseminated intravascular coagulopathy (DIC), and eventually death [2, 3]. This final pathway of sepsis leading to shock and death is shared by *Bacillus anthracis*, the pathogen that causes anthrax, another bioterrorist weapon whose devastating potential was amply demonstrated in recent mail attacks in the U. S. [4]. *Burkholderia mallei*, the agent that produces the disease glanders, also has been extensively studied in the United States and Russia as a possible weapon of biological warfare. During World War II, glanders was utilized as a bioweapon against horses, civilians, and prisoners of war. Like plague and anthrax, glanders causes overwhelming septicemia often leading to death once the bacteria is disseminated in the vasculature [5].

Rapid detection of biological agents is the critical first step in bioterrorism prevention and response. Many common and emerging detection methods are dependent upon antibody-antigen or gene chip assays that operate via specific biochemical interactions. Genetic modification of recognition sequences or surface protein expression may therefore subvert these screening methods. Additionally, these types of tests are costly and complex, requiring highly trained technicians and expensive equipment.

The goal of this study was to create a simplified, rapid, cost-effective diagnostic test that monitors an individual's risk potential for coagulopathy to determine the presence of a pathogen. The coagulation-inflammation cascades are common pathophysiological mechanisms affected by bioterrorism agents that cause anthrax, plague, and glanders, and eventually converge to cause sepsis [2 - 5]. Thus, the vascular system may be affected by these agents in significant ways that could compromise the clotting cascade.

We further sought to elucidate the effect of these agents of bioterrorism on blood coagulation times, thereby developing a useful, clinically relevant bioassay to evaluate patient samples. To this

end, we utilized *E. coli*-derived lipopolysaccharide (LPS or endotoxin), which has potent immuno-stimulating effects comparable to those of the living bacterial agents, as a standard from which to examine their consequences on blood clotting *in vitro*.

In vitro studies were performed to determine whether a small volume of isolated, sterilized blood supernatants that had been exposed to virulent *B. anthracis*, *Y. pestis*, or *B. mallei* would elicit coagulation changes in uninfected blood. The impact on coagulation caused by these isolates from deadly agents was compared to clotting changes caused by blood exposed to endotoxin, thereby potentially creating a clinically relevant bioassay.

METHODS

Control and Endotoxin Supernatants

This study used 24-hour-old citrated whole blood (CWB) obtained from the clinical laboratory of the University Hospital. Samples of whole blood (n = 3) were created from the combination of three individual vials. Each of these three samples was separated into two aliquots. One of the two aliquots of each set was then spiked with a final concentration of 10 µl/mL physiologic saline (control), and the other aliquot with 10 µl/mL containing 10 µg (the same final concentration) of *E. coli*-derived endotoxin. After two hours of incubation at 37° C, the cultures were subjected to three freeze-thaw cycles to break open blood cells and isolate any tissue factor that had been generated during the incubation with the agents. The blood was spun at 1200 rpm for 10 minutes and the top layer (supernatant) was separated to remove any remaining blood cells, to produce three pairs of unstimulated (control) and stimulated (LPS-infected) blood supernantants.

Bioagent Supernatants

Bacillus anthracis Vollum 1B, *Burkholderia mallei* 23344, and *Yersinia pestis* Co92 were obtained from the U. S. Department of Defense. Whole blood obtained from healthy donors was infected with each of the strains at an MOI of 1:1 (1 bacteria per 1 monocyte). The cultures were incubated at 37° C for 3 hours and 8 hours (1 hour and 3 hours for plague). At each time point, aliquots of the cultures were subjected to three freeze-thaw cycles. The blood was spun at 1200 rpm for 10 minutes and the top layer was removed and filtered (0.22 µm) to remove any remaining bacterial cells. Control aliquots of donor bloods were also prepared and subjected to the same freeze-thaw and filtering cycles. Samples of the filtered supernatant were plated and incubated for a week to ensure sterility.

Whole Blood Clotting Studies:

Human citrated whole blood (CWB) samples were obtained from the University Hospital's clinical lab with prior IRB approval. Samples were spiked with uninfected (control) and endotoxin (infected) supernatants (n = 10) at a final concentration of 2%. The aliquots were inverted to ensure homogeneity, incubated at 37° C for 10 minutes, and mixed with calcium to initiate clotting at a concentration of 32.5 µL of 0.1M $CaCl_2$ in 300 µL blood. Clotting times were recorded using an Amelung KC4A Micro and a Sonoclot Coagulation Analyzer. The procedure was repeated with uninfected and infected *B. anthracis* (n = 7) and *B. mallei* (n = 15) supernatants collected at the 3-hour time point to obtain final concentrations of 0-3% supernatant. Additionally, clinical assays to detect levels of essential clotting factor VII were conducted in bloods treated with *B. mallei* supernatants.

The procedure was altered for *Y. pestis* to include studies with mercuric ion, as the addition of the ion would activate latent membrane-bound tissue factor by the monocyte and is thus a marker for monocyte activity and DIC. Studying the effect of *Y. pestis* on tissue factor action would help elaborate the pathophysiological damage to the blood coagulation system. CWB samples (n = 10) were spiked with uninfected (control) and *Y. pestis* (infected) supernatants at a final concentration of 2%, with and without mercuric ion (final concentration 0.005% Hg^{2+}).

Platelet-Poor Plasma Clotting Studies

Day-old citrated whole blood (CWB) obtained from the clinical laboratory of the University Hospitals was centrifuged at 3300 rpm (1600 g) for 10 minutes to obtain platelet-poor plasma (PPP). Samples of PPP (n = 10) were created from the combination of three individual vials, and were spiked with control (uninfected) and endotoxin (infected) supernatants. Clotting times (in seconds) were determined in the same manner as above.

RESULTS

Both the Amelung KC4A Micro and the Sonoclot Coagulation Analyzer report clotting times by a mechanism that allows them to monitor increasing blood viscosity as fibrin, the end product of the coagulation cascade, is produced. The Sonoclot is a miniviscometer that not only provides the clotting time, but also plots a graph describing clot viscosity as a function of time. It thus provides a means by which the entire coagulation profile of a blood sample can be evaluated, including pathways of thrombosis, platelet function, and fibrinolysis.
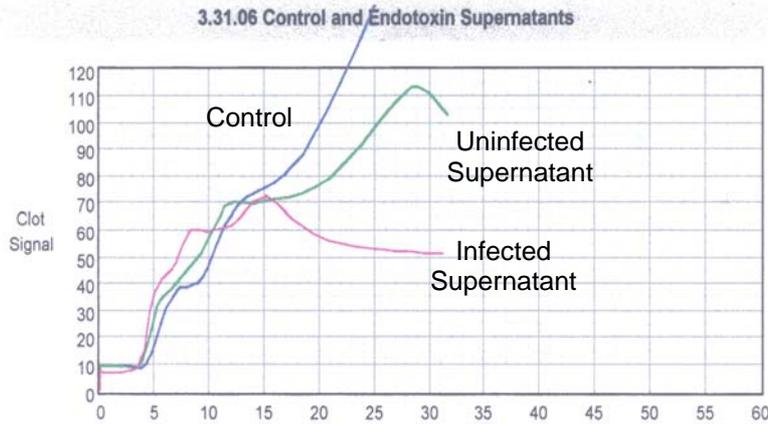

Figure 1. Sonoclot tracings of the effect of endotoxin. Measurements of clot viscosity as a function of time, when comparing CWB spiked with uninfected supernatant to CWB spiked with endotoxin-infected supernatant. The clotting action of unadulterated CWB is marked as 'Control.'

CWB behaved differently under varying conditions, as described by Figure 1. When unadulterated, CWB has a very typical linearly increasing clotting profile. When spiked with unstimulated supernatant, the clotting profile of CWB is similar to the control, but generates a clot with decreased viscosity. CWB spiked with LPS-stimulated supernatant induces a more rapid clot formation, but the clot thus generated is of a decreased overall strength as compared to the other two samples.
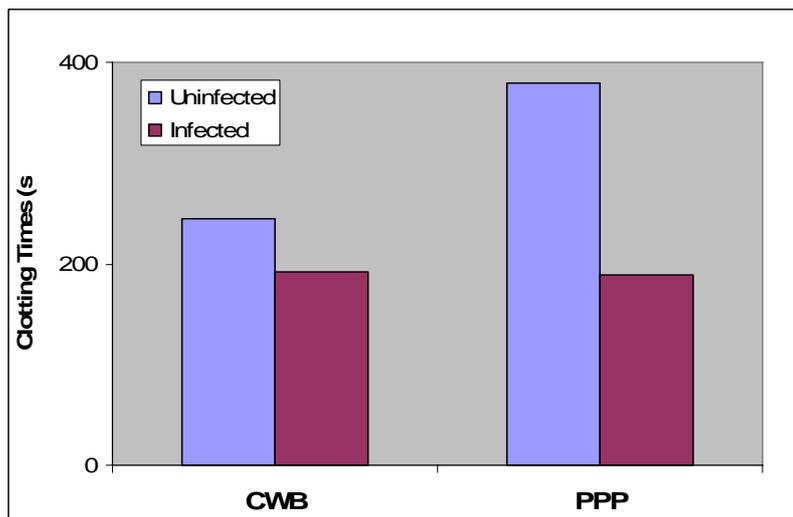

Figure 2. Comparison of the Effect of LPS on CWB and PPP. Mean clotting times generated by the addition of supernatant samples both infected and uninfected by *E. coli*-derived LPS. Clotting times were obtained in both CWB and PPP.

CWB spiked with LPS-infected and uninfected supernatant demonstrates different magnitudes of change than does PPP under identical stimuli, as shown in Figure 2. The shortening of the clotting time in CWB averaged 52 seconds, while in PPP the shortening averaged 191 seconds.

These differences were found to be very significantly different in magnitude, with a two-tailed p value of 0.0008.

	CWB				PPP			
Supernatant	1	2	3	Mean ± SD	1	2	3	Mean ± SD
Uninfected	256 ± 95	247 ± 67	231 ± 58	**244 ± 13**	382 ± 59	373 ± 58	385 ± 48	**380 ± 6**
Infected	205 ± 57	206 ± 67	166 ± 42	**192 ± 23**	192 ± 48	204 ± 74	169 ± 25	**189 ± 18**

Table 1. Effect of Endotoxin on CWB and PPP clotting times. Mean clotting times generated by three supernatant samples, either infected or uninfected by bacterial endotoxin. Clotting times were obtained in both citrated whole blood and platelet-poor plasma.

As shown in Table 1, whole blood and plasma differ in terms of clotting behavior. Supernatant infected with LPS in both CWB and PPP caused a significant decrease in the clotting time, as compared to the effect of unstimulated supernatant (two-tailed paired t-tests; $p < 0.01$). When compared to each other, however, CWB and PPP acted differently: the clotting time of whole blood spiked with uninfected supernatant was significantly decreased as compared to platelet-poor plasma (two tailed paired t-test; $p < 0.001$). However, two-tailed paired t-tests indicate that the clotting time of CWB spiked with endotoxin-infected supernatant was not significantly different from that of PPP under identical stimulus.

Final Concentration of Supernatant	1%	2%	3%
Uninfected	274 ± 46	397 ± 194	456 ± 155
Infected	222 ± 42	235 ± 95	342 ± 87

Table 2. Effect of Anthrax on CWB clotting times. Mean clotting times generated by varying concentrations of *B. anthracis*. All clotting times were obtained by addition of infected and uninfected supernatant to citrated whole blood.

Based on Table 2, a direct relationship was observed between the increasing concentration of uninfected supernatant in blood and the increasing clotting time. Additionally, supernatant infected with *B. anthracis* demonstrated a similar relationship to the mean clotting time. Two tailed paired t-tests indicate a significant decrease in clotting time between 1% supernatant uninfected and infected groups ($p = 0.016$) and 2% supernatant uninfected and infected groups ($p = 0.049$). Paired t-tests between 3% supernatant uninfected and infected groups approaches significance at $p = 0.058$. Furthermore, samples taken from 8 hours of infection were found to be statistically significant versus relevant controls ($p < 0.05$).

Final Concentration of Supernatant	1%	2%	3%
Uninfected	351 ± 143	475 ± 212	510 ± 175
Infected	290 ± 133	312 ± 77	366 ± 185

Table 3. Effect of Glanders on CWB clotting times. Mean clotting times generated by varying concentrations of *B. mallei*. All clotting times were obtained by addition of infected and uninfected supernatant to citrated whole blood.

As depicted in Table 3, *B. mallei* demonstrated a direct relationship between the concentration of both infected and uninfected supernatants and the mean clotting times. Two tailed paired t-tests indicate a significant decrease in clotting time between 1% supernatant uninfected and infected groups ($p = 0.0051$); 2% supernatant uninfected and infected groups ($p = 0.0209$); and 3% supernatant uninfected and infected groups ($p = 0.0314$). Furthermore, samples taken from 8 hours of infection were found to be statistically significant versus relevant controls at 1-3% concentrations ($p < 0.05$). Additionally, when the *B. mallei*-infected supernatant was added to clinical test plasma, a decreased level of Factor VII was found, as compared to uninfected supernatant.

Character of Supernatants	2% Supernatant	Mercuric Ion & 2% Supernatant
Uninfected	406 ± 80	302 ± 69
Infected	332 ± 59	245 ± 21

Table 4. Effect of Plague on CWB clotting times. Mean clotting times generated by *Y. pestis* under varying conditions in citrated whole blood. Infected and uninfected supernatants were used at a 2% concentration, with and without mercuric ion.

At a 2% concentration, supernatant infected with plague demonstrated a decrease in clotting time as compared to uninfected supernatant (Table 4). Two tailed paired t-tests indicate a significant decrease in clotting time between both infected and uninfected plague groups at 2% ($p = 0.0078$). Mercuric ion, which stimulates the tissue factor pathway of coagulation, is also shown significantly to decrease the clotting time of the infected group versus uninfected at $p = 0.023$.
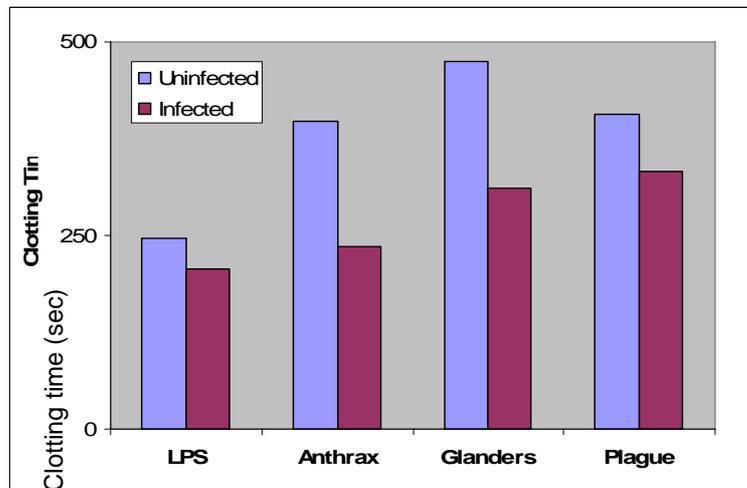

Figure 3. Comparative Effect of Bioagents on CWB Clotting Times. Mean clotting times generated by the addition of supernatant samples both infected and uninfected by various bioagents. Clotting times were obtained with a final concentration of 2% supernatant in CWB.

As clearly shown in Figure 3, a significant decrease in mean clotting time was demonstrated by all bioagent samples when infected (agent-stimulated) supernatant was added to CWB at a final concentration of 2%. Anthrax-infected supernatants did not induce a clotting time that significantly differed from LPS-infected supernatants (paired t-test). However, as compared to LPS, clotting times significantly differed in glanders-infected and plague-infected supernatants (paired t-tests, $p < 0.05$). LPS demonstrates a smaller decrease from uninfected to infected than do the other bioagents.

DISCUSSION AND CONCLUSIONS

Early detection of biological threats poses a considerable problem for public health authorities. Our studies demonstrate that the rapid, inexpensive clotting assay described may be effective as a first-line screening tool for agents of bioterrorism. For each pathogen, exposure was consistently detected, at low concentrations in blood after just 10 minutes of incubation. Detection was indicated by a significant shortening of the clotting time as compared to uninfected blood.

In citrated whole blood, addition of a supernatant laced with *E. coli*-derived LPS caused a significant decrease in the clotting time as compared to the control (Table 1). This indicates that the two hour incubation with LPS must generate a material that accelerates the coagulation process. Previous studies [6] indicate that this substance may be tissue factor that is generated by the monocyte, an immune cell. Thus, the LPS stimulation of the coagulation and inflammatory process may occur through the immune system—specifically through the monocyte. However, the decrease in clotting time caused by infected supernatant was less than in other anthrax, plague, and glanders. We believe that this may occur because the supernatant of the other agents was derived from live bacteria, which may induce a more virulent coagulopathy.

Whole blood spiked with *Bacillus anthracis* supernatants behaved in a comparative manner. As indicated by significantly shortened clotting times, anthrax-infected supernatant also induced a strongly procoagulant state compared to unstimulated supernatant, in a manner similar to that of LPS (Table 2). In fact, no significant difference was found between the clotting times generated by LPS-infected and anthrax-infected supernatants in whole blood. This may indicate a similarity in mechanism of action, and thus *B. anthracis* may also stimulate the coagulation system through the immune-inflammatory response system—perhaps through the monocyte as well.

Our results indicate that *B. mallei*, which causes glanders, may act in a somewhat different manner. While infected supernatants followed the same trend of shortening the clotting times of whole blood, the hypercoagulable state thus caused was different from that caused by LPS (Table 3). Paired t-tests demonstrate a significant difference between the mean clotting times generated by LPS-stimulated and *B. mallei*-stimulated supernatants. Our study indicates that one reason for this discrepancy may be a divergent effect on the clotting cascade. While LPS may stimulate the monocyte, we found that *B. mallei* may be related to the upregulation of clotting factor VII to factor VIIa (activated).

When comparing control and *B. mallei*-stimulated supernatants in clinical test plasmas, it was found that the concentrations of the latter decreased the levels of factor VII present, a necessary clotting factor. Factor VII was detected at lower amounts because it had been converted to factor VIIa (activated). Factor VIIa, when complexed with tissue factor, has increased procogulant activity in blood. Thus, based on our preliminary laboratory data, we hypothesize that *B. mallei* induces coagulopathy by potentiating Factor VII activity.

Yersinia pestis was seen to be the most devastating of the studied agents on blood clotting, and caused a severe and rapid coagulopathy even *in vitro*. Like the other agents studied, supernatants that had been incubated with plague bacteria contained one or more substances that induce a very strongly procoagulant state in whole blood, as indicated by the shortened clotting times when compared to unstimulated supernatants (Table 4).

Furthermore, addition of mercuric ion potentiated the effect of *Y. pestis*-stimulated supernatants on whole blood. Bloods spiked with mercuric ion and plague-infected supernatants significantly decreased the clotting times, indicating that the ion played an important role in exacerbating the effect of the bacteria alone. As mercuric ion activates latent tissue factor, this suggests tissue factor activation as part of the pathogenesis of plague. Our findings may thus indicate one of the reasons plague is capable of causing mortality within a short time: the bacteria may strongly activate the coagulation pathway through tissue factor, which would result in widespread sepsis and disseminated intravascular coagulopathy. Further studies are required to investigate the effect of *Y. pestis* on tissue factor stimulation and blood coagulopathy.

In addition, our studies demonstrated a difference in the coagulation profiles of whole blood and plasma. While LPS-stimulated supernatants caused a shortening in both CWB and PPP clotting times as compared to unstimulated supernatants, the magnitude of the changes differed. In whole blood, stimulated supernatant caused a shortening of about 50 seconds, while in plasma the shortening approached 200 seconds (Table 1). This discrepancy was found to be extremely significant (p = 0.0008), which indicates both a difference in the reaction to LPS stimulation, and in clotting

ability between the two substances. PPP was thus seen to have a more sensitive reaction to LPS stimulation, possibly because plasma lacks the cellular constituents capable of absorbing or mitigating the deleterious substances generated by LPS in the supernatant samples. Thus, the presence of cells in whole blood may have 'blunted' the coagulopathic effects of the LPS-stimulated supernatant. This hypothesis, as well, requires further study to examine the individual substances produced and their effects.

These findings on the very significant differences between clotting action in whole blood and plasma are of special note, since all lab tests performed in hospitals are done on plasma exclusively. As evidenced by this study, plasma does not behave in a manner similar to whole blood—and therefore any tests performed on it do not give a correlated picture of the patient's blood coagulopathy.

The simple tests performed in this study give a much more elucidated portrait of the clotting profile. In comparison to other detection techniques, our test required no purification of sample to be analyzed; filtration procedures were carried out to remove risk of infection to the technician. Therefore, these tests would be of enormous utility as a screening assay for blood coagulopathy and the presence of a pathogen. Clotting assays like ours are clinically relevant because patients infected with these bioagents often die of blood coagulopathies, such as sepsis or disseminated intravascular coagulopathy (DIC).

Future studies will focus on further determining the unique pathways of coagulation activation for each pathogen to increase the specificity of our assay. Furthermore, correlation of our studies to clinical case studies or animal studies may help assess the potential utility of coagulation monitoring in the treatment of these diseases. Additionally, we have not excluded the possibility that viral agents exposed to human blood in a similar manner may produce comparable results, and would investigate a potential similarity in mechanism of coagulopathy.

While these are all *in vitro* studies, the effects seen may be more dramatic *in vivo*, or complexed with other physiological conditions. However, the utility of this *in vitro* study may be important, as it may serve as a biomarker (diagnostic test) for bacterial action on blood coagulation.

ACKNOWLEDGEMENTS

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