Immunotolerance during bacterial pneumonia and sepsis

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Activation of coagulation and inhibition of fibrinolysis in the human lung upon bronchial instillation of lipoteichoic acid and lipopolysaccharide

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Abstract

Pneumonia is characterized by an acute inflammatory response in the lung, which is frequently associated with changes in coagulation and fibrinolysis in the bronchoalveolar space. Here, we compared the effects of lipoteichoic acid (LTA), a major cell wall component of gram-positive bacteria, and lipopolysaccharide (LPS), in the human bronchoalveolar space. Therefore sterile saline was instilled into a lung subsegment followed by bronchoscopic instillation of either LTA (S. aureus, at a dose of either 4, 20 or 100 ng/kg body weight) or LPS (E. coli, 4 ng/kg body weight) into the contralateral lung of 23 healthy non-smoking male volunteers. Bronchoalveolar lavage fluid (BALF) was obtained six hours thereafter. Bronchial instillation of LTA or LPS activated bronchoalveolar coagulation, as reflected by increases in the levels of thrombin-antithrombin complexes, D-dimer and soluble tissue factor. Concurrently, LTA and LPS inhibited anticoagulant mechanisms, as indicated by reductions in antithrombin, Protein C and Activated Protein C concentrations together with elevated levels of soluble thrombomodulin. Both LTA and LPS administration was associated with an inhibition of pulmonary fibrinolysis, as measured by a reduction in plasminogen activator activity and elevated levels of plasminogen activator inhibitor type I. This study is the first to describe the effects of LTA on hemostasis in humans, demonstrating that LTA induces similar changes in the human bronchoalveolar space as LPS, characterized by activation of coagulation with concurrent inhibition of anticoagulant and fibrinolytic pathways.
Introduction

Alterations in coagulation and fibrinolysis frequently accompany the acute inflammatory response in the bronchoalveolar space during pneumonia. These changes, characterized by activation of coagulation and inhibition of fibrinolysis, can be considered host-protective in containing inflammation to the site of infection. However, procoagulant activity can also be disadvantageous if there is an excess of fibrin formation (one of the pathologic hallmarks of adult respiratory distress syndrome (ARDS)) and by modulating inflammatory activity, leading to excessive activation of inflammation in the alveolar compartment during pneumonia. As such, there is an extensive cross talk between coagulation and inflammation in the lung, where immune cells are important in the initiation of procoagulant pathways, mediators involved in coagulation and fibrinolysis can impact on inflammatory processes and cytokines can modulate pulmonary hemostasis.

We recently demonstrated that lipopolysaccharide (LPS), a constituent of the gram-negative bacterial cell wall, can reproduce the hemostatic alterations of pneumonia in the lungs of healthy humans when administered via the airways (either by inhalation or bronchial instillation). In addition, LPS has been used extensively in human models of systemic inflammation, revealing that intravenous injection of LPS induces a procoagulant response in the circulation. Of note, LPS is only expressed by gram-negative pathogens and activates cells via Toll-like receptor (TLR)4. In sharp contrast, knowledge of the hemostatic balance in inflammation caused by gram-positive pathogens is limited. Such knowledge is of relevance especially in the context of pneumonia considering that gram-positive bacteria are a major cause of respiratory tract infection. It is not self-explanatory that gram-positive pathogens elicit immune and procoagulant responses in the lung via similar pathways as gram-negative bacteria or LPS, since gram-positive cell wall constituents, such as lipoteichoic acid (LTA) and peptidoglycan, activate cells primarily via TLR2. Nonetheless, our laboratory previously showed that both patients and rodents with pneumonia caused by the gram-positive pathogen Strepococcus pneumoniae display pulmonary activation of coagulation and concurrent inhibition of fibrinolysis. In addition, our group recently demonstrated that intranasal application of purified LTA reproduced these findings in the lungs of mice, implicating that this cell wall constituent likely contributes to the altered hemostatic balance in gram-positive pneumonia. At present it is unknown, however, whether LTA can influence coagulation and anticoagulation in humans in vivo. Therefore, we here investigated the hemostatic effects elicited by LTA and compared these with the hemostatic effects induced by LPS in the human lung. The results from this study were obtained during a simultaneous study investigating the inflammatory response upon bronchial instillation of LTA in the human lung, of which the results have been reported previously.
Materials and Methods

Subjects

Twenty-three non-smoking healthy males (age 22.0 ± 0.5 yrs, mean ± SE), who were not on any medication, were recruited by advertising. Screening, consisting of a questionnaire, physical examination, routine blood and urine investigation, electrocardiogram and spirometry, did not reveal any abnormality. The study was approved by the institutional ethics and research committees. Written informed consent was obtained from all subjects before enrollment in the study.

Study design

Ten milliliters of sterile saline was instilled into a lung subsegment (either the right middle lobe or lingula) followed by instillation of either LTA (from Staphylococcus aureus (DSM 20233) or LPS (from Escherichia coli; United States Pharmacopeial Convention, Lot G, Bureau of Biologics, United States Food and Drug Administration, Rockville, MD) into the contralateral lung, using a flexible video bronchoscope. The subjects were randomized to left or right lungs for saline or LTA / LPS instillation. The LTA preparation was >99% pure; no contamination with lipopeptides was detected using photometric measurements (UV absorption) and NMR examination. LTA contained <50 pg endotoxin/mg LTA as determined by the Limulus amoebocyte lysate assay. Since LTA had never been administered to humans before, we performed a dose escalating study with LTA: four subjects received LTA 4 ng/kg, followed by four subjects receiving 20 ng/kg. After this initial study and based on the degree of inflammatory responses, seven subjects received 100 ng/kg bodyweight LTA. Moreover, eight subjects received LPS at a dose of 4 ng/kg body weight, which was identical to that used in previous pulmonary endotoxin instillation studies. Vital variables were measured on an hourly basis; the volunteers did not have any complaints and were completely ambulant; they walked around in the vicinity of the study unit during the investigation.

Bronchoalveolar lavage

A bilateral bronchoalveolar lavage (BAL) was performed 6 hours post-challenge in a standardized fashion according to the guidelines of the American Thoracic Society, using a flexible video bronchoscope. Seven successive 20 ml aliquots of pre-warmed 0.9% saline were instilled in the saline-challenged subsegment of the lung and each aspirated immediately with low suction. This procedure was repeated in the LPS- or LTA-challenged subsegment of the contralateral lung. The recovery of BAL fluid was comparable in each group (all groups had a mean recovery of 75-85 ml); therefore, similar to our previous investigations, no corrections for dilution of BAL fluid were made.
Analysis of tissue factor mRNA

Alveolar macrophages were purified by autoMACS using CD71 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). Differential cell counts revealed a purity of isolated macrophages of >95% in all groups. After isolation alveolar macrophages were dissolved in RNAeasy lysis Buffer (buffer RLT, QIAGEN, Hilden, Germany) and stored at -80°C until used for RNA isolation. Tissue factor (TF) mRNA was determined using multiplex ligation dependent probe amplification as described before.11

Flow cytometry

Expression of CD142 (TF) on alveolar macrophages in BALF was determined by flow cytometric analysis using fluorochrome-conjugated mouse anti-human CD14, CD15, HLA-DR and CD142 antibody (BD Pharmingen, San Diego, CA) in combination with isotype controls. Alveolar macrophages were selected using side scatter (SSC), HLA-DR and CD14 (HLA-DR+/CD14+ cells were defined as macrophages). Using this gate definition macrophages can be discriminated from neutrophils (HLA-DR-/CD15+) and lymphocytes (SSC low/HLA-DR+).

Assays

Thrombin-antithrombin complexes (TATc), soluble TF, tissue-type plasminogen activator (tPA), soluble thrombomodulin (TM), urokinase type plasminogen activator (uPA), intact tissue factor pathway inhibitor (TFPI) and D-dimer concentrations were measured using specific commercially available ELISAs (TATc: Behringwerke AG, Marburg, Germany; uPA, soluble TF and TFPI: American Diagnostics, Greenwich, CT; tPA and soluble TM: Diagnostica Stago, Asnières-sur-Seine, France; D-dimer: Diagnostica Stago, Roche, Almere, the Netherlands). Antithrombin (AT), plasminogen activator (PA) activity, and PAI-1 activity were measured by automated amidolytic techniques.28-30 Activated protein C (APC) was measured with an enzyme capture assay using monoclonal antibody HAPC 1555 and chromogenic substrate Spectrozyme PCa (American Diagnostica, Greenwich, CT). Protein C (PC) activity was determined with an amidolytic assay using chromogenic substrate S2366 (Chromogenix, Milan, Italy).

Statistical analysis

Values are expressed as means ± SE. Data were checked for normal distribution and equal variances of the residuals. If Shapiro-Wilk was >0.90 data were considered normally distributed. Statistical comparisons of differences between saline and LTA (4 ng/kg and 20 ng/kg) were made by Wilcoxon sign rank test, since the sample size of these groups (N=4) was too small for normal distribution. Statistical comparisons between saline and LTA (100 ng/kg) and saline and LPS groups were made by paired
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T-test, since data were - with the exception of D-dimer, TF mRNA and uPA - normally distributed. The latter three parameters were statically compared by Wilcoxon sign rank test, since data were not normally distributed. These analyses were performed using SPSS (version 12.0.1; SPSS Inc., Chicago, IL). A P-value of less than 0.05 was considered to represent a statistically significant difference.

Results

Activation of coagulation upon bronchial LTA instillation

Instillation of LTA resulted in a marked dose-dependent activation of bronchoalveolar coagulation, as reflected by increased BALF levels of the thrombin generation marker TATc (P<0.001 versus saline instilled side; Figure 3.1A). The enhanced procoagulant activity in the pulmonary segment upon LTA instillation was accompanied by increased levels of the fibrin degradation product D-dimer (P<0.001 versus saline-instilled; Figure 3.1B). These findings paralleled the coagulation activation after LPS instillation, which also was associated with increases in TATc and D-dimer concentrations in BALF (both P<0.001 versus saline; Figure 3.1A and 3.1B). Of note, a LTA dose of 100 ng/kg was required to induce similar changes in pulmonary coagulation as LPS at a dose of 4 ng/kg.

Effect of LTA on the TF pathway

Since TF is considered pivotal for the initiation of coagulation, we measured the effect of LTA and LPS on TF protein, i.e. soluble TF, TF surface expression on alveolar macrophages and TF mRNA, as well as on the levels of TFPI (Figure 3.2). Both LTA and LPS elicited release of soluble TF in BALF (both P<0.001 versus saline; Figure...
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3.2A). Remarkably, LTA (P<0.05) but not LPS induced enhanced expression of TF mRNA in alveolar macrophages (Figure 3.2B), whereas neither challenge altered TF expression at the surface of these cells (Figure 3.2C). Moreover, neither LTA nor LPS altered TFPI levels in BALF (Figure 3.2D).

Figure 3.2 Effect of LTA and LPS on the tissue factor pathway.
Concentrations of soluble tissue factor (A) and tissue factor pathway inhibitor (D) were measured in BALF 6 hours post challenge. mRNA encoding for tissue factor (B) was analyzed by multiplex ligation-dependent probe amplification (MLPA) after isolation from alveolar macrophages purified from bronchoalveolar lavage fluid 6 hours post challenge. Expression of tissue factor on the surface of macrophages was measured by flow cytometry (C). Open bars indicate measurements after saline instillation, dark grey bars indicate LTA instillation (4, 20 or 100 ng/kg bodyweight, N=4 for 4 and 20 ng/kg, N=7 for 100 ng/kg) and light grey bars indicate LPS instillation (4 ng/kg bodyweight, N=8). Data are means ± SE. *P<0.05, **P<0.01, ***P<0.001 vs. saline.

LTA inhibits anticoagulant mechanisms
Activation of pulmonary coagulation was accompanied by a concurrent inhibition of anticoagulant mechanisms in the lung after instillation of both LTA and LPS. Indeed, LTA induced a dose-dependent reduction in BALF AT levels, which became significant at a LTA dose of 20 ng/kg (P<0.05 versus saline), decreasing further at a LTA dose of 100 ng/kg (P<0.001 versus saline; Figure 3.3). Similarly, LPS administration resulted in lower BALF AT concentrations (P<0.001 versus saline). In addition, both LTA and LPS inhibited the function of the PC system, as reflected by decreased levels of PC and APC.
and accompanied by increased levels of soluble TM (all P<0.001 versus saline; Figure 3.4A-C). Notably, the effect of LTA on APC and soluble TM levels was already apparent at a dose of 20 ng/kg (P<0.05 versus saline).

Figure 3.3 Instillation of LTA or LPS reduces antithrombin levels. Concentrations of antithrombin were determined in BALF 6 hours after instillation of saline (open bars) in a lung segment and either LTA (4, 20 or 100 ng/kg bodyweight, N=4 for 4 and 20 ng/kg, N=7 for 100 ng/kg; dark grey bars) or LPS (4 ng/kg bodyweight, N=8; light grey bars) in the contralateral lung. Data are means ± SE. *P<0.05, *** P<0.001 vs. saline.

Figure 3.4 Instillation of LTA or LPS inhibits the Protein C pathway. Concentrations of soluble thrombomodulin (A), protein C (B) and activated protein C (C) were determined in BALF 6 hours after instillation of saline (open bars) in a lung segment and either LTA (4, 20 or 100 ng/kg bodyweight, N=4 for 4 and 20 ng/kg, N=7 for 100 ng/kg; dark grey bars) or LPS (4 ng/kg bodyweight, N=8; light grey bars) in the contralateral lung. Data are means ± SE. *P<0.05, ***P<0.001 vs. saline.
Decreased fibrinolysis upon LTA instillation

Instillation of LTA resulted in an inhibition of fibrinolysis in the lung, as quantified by a reduction in PA activity in BALF (P<0.01 versus saline; Figure 3.5A) together with an increase in PAI-1 activity (P<0.001 versus saline; Figure 3.5D). The concentrations of tPA and uPA antigen increased upon LTA instillation (both P<0.001 versus saline; Figure 3.5B and 3.5C). LTA only significantly influenced these fibrinolytic mediators at the highest dose given. Administration of LPS induced changes that resembled those elicited by LTA.

Figure 3.5 Instillation of LTA or LPS results in inhibition of fibrinolysis.
Plasminogen activator activity (A) and concentrations of tissue-type plasminogen activator (B), urokinase-type plasminogen activator (C) and plasminogen activator inhibitor 1 (D) were determined in BALF 6 hours after instillation of saline (open bars) in a lung segment and either LTA (4, 20 or 100 ng/kg bodyweight, N=4 for 4 and 20 ng/kg, N=7 for 100 ng/kg; dark grey bars) or LPS (4 ng/kg bodyweight, N=8; light grey bars) in the contralateral lung. Data are means ± SE. *P<0.05, **P<0.01, ***P<0.001 vs. saline.

Discussion

Bacterial pneumonia is one of the most common infectious diseases and the leading cause of sepsis. In light of the intimate cross-talk between coagulation and inflammation and the prominent causative role of gram-positive pathogens in the pathogenesis of pneumonia, we were interested to investigate the hemostatic effects
of LTA, an important proinflammatory component of gram-positive bacteria, in the human lung. Following our previous study on the LTA induced pulmonary inflammatory response in the same subjects\textsuperscript{23}, we here report a detailed analysis of the \textit{in vivo} effects of LTA on coagulation in humans. Our results demonstrate that LTA instillation into a pulmonary segment results in a procoagulant state, manifested by activation of coagulation, a depressed anti-coagulant system and inhibition of fibrinolysis.

The results of the current study should be considered in the context of previously reported investigations. Patients with pneumonia or acute lung injury demonstrated increased coagulation and suppression of anticoagulant and fibrinolytic pathways in their lungs\textsuperscript{1-6,8}. In addition, similar hemostatic changes could be induced in the bronchoalveolar compartment of healthy humans by intrapulmonary delivery of LPS, either by inhalation or bronchial instillation\textsuperscript{11-13}. Considering that gram-positive bacteria are a common cause of pneumonia, we recently focused on the impact of \textit{S. pneumoniae} induced pneumonia on pulmonary coagulation, demonstrating that both patients and rodents with this infection have a net procoagulant state in their lungs\textsuperscript{3,4,20,21}. Considering that \textit{S. pneumoniae} can activate multiple TLRs, including TLR4 via its intracellular toxin pneumolysin\textsuperscript{36}, we also embarked on studies using highly purified LTA from \textit{S. pneumoniae} to examine the potency of this cell wall component to influence coagulation and fibrinolysis in the mouse lung, revealing that LTA, like LPS, concurrently activated coagulation while inhibiting fibrinolysis\textsuperscript{22}. These studies provided evidence that different stimuli (intact bacteria and purified bacterial components) produced similar alterations in local coagulation and fibrinolysis in the lungs. Considering that LPS is an established agonist of TLR4, whereas LTA specifically triggers TLR2\textsuperscript{18,19}, these experiments further suggested that stimulation of TLR4 and TLR2 affect the hemostatic mechanism in the bronchoalveolar space in a similar way. The current investigation, that directly compared the effects of LPS and LTA in the human bronchoalveolar space, firmly establishes that both bacterial constituents induce qualitatively similar hemostatic effects with the exception of TF mRNA expression in alveolar macrophages, which will be discussed later on in the Discussion. This is remarkable, since we previously reported profound differences between LTA- and LPS-induced lung inflammation in the same subjects\textsuperscript{23}. Indeed, LPS but not LTA instillation was associated with activation of neutrophils and consistent rises of the concentrations of cytokines and chemokines in BALF. Moreover, LPS but not LTA activated alveolar macrophages, as reflected by enhanced expression of ten different mRNAs encoding pro-inflammatory mediators and increased spontaneous cytokine release upon incubation ex vivo\textsuperscript{23}. Our current data suggest that such differential activation by LPS and LTA does not apply to activation of the hemostatic mechanism in the lung.
Although both LPS and LTA are approximately 1 to 2% of the dry weight of respectively *E. coli* and *S. aureus*\(^{37,38}\), much higher amounts of LTA were needed to obtain similar hemostatic results as LPS. Recently, it has been shown that the immunostimulatory potency of LTA increases dramatically when LTA was presented and coated to a surface, an effect which was not found for LPS\(^{39}\). This finding suggests that it is perhaps more interesting to focus on the mechanisms by which LTA on the surface of gram-positive bacteria is able to induce inflammation and coagulation *in vivo*, rather than to directly compare amounts of both agonists.

TF is considered a central mediator of systemic and pulmonary coagulation\(^{4,9,10,16,33}\). In the lung TF is constitutively and abundantly expressed by different cell types, including epithelial cells and macrophages\(^{40}\). Of considerable interest, LTA induced a significant rise in TF mRNA levels in alveolar macrophages. This is especially remarkable since LTA, in contrast to LPS, did not induce proinflammatory gene expression in these cells, suggesting that the induction of TF gene expression by LTA occurs via distinct mechanisms\(^{23}\). Although in our study LPS did not induce a significant rise in TF mRNA levels in alveolar macrophages, in an earlier study we did detect a rise in TF mRNA levels in alveolar macrophages upon inhalation of nebulised LPS by humans, suggesting that the dose of LPS used here was not sufficient to induce such a response\(^{11}\). In further support of the notion that LPS can induce TF mRNA in the pulmonary compartment *in vivo*, mice challenged with LPS via the airways were reported to demonstrate increased TF mRNA expression in alveolar macrophages\(^{41}\).

Neither LTA nor LPS instillation was associated with a rise in TF protein at the surface of alveolar macrophages; we previously were unable to detect such an increase upon inhalation of nebulised LPS in healthy humans\(^{11}\). In light of the established role for TF in the initiation of coagulation\(^{4,9,10,16}\), it is likely that enhanced TF protein expression did occur in LPS and LTA challenged lung segments, a presumption supported by the strong increase in soluble TF concentrations in BALF. TF could have been induced on pulmonary cell types not present in BALF (*e.g.* epithelial cells) and/or with a time course not captured by the single BALF sample obtained. In this respect it should be noted that, although a kinetic analysis over multiple time-points of especially LTA effects would have been of considerable interest and would have substantially strengthened our results, we chose not to expand the number of time points for BALF sampling (and thereby the number of human subjects) considering the invasive nature of this investigation and procedure.

The present data indicate that both LTA and LPS may further shift the alveolar hemostatic balance towards a prothrombotic state by reducing AT concentrations and by decreasing the activity of the anticoagulant PC-APC pathway. AT concentrations may have been diminished by enzymatic degradation by neutrophil degradation product elastase, impaired synthesis and increased consumption as a result of ongoing thrombin generation\(^{12}\). Suppression of PC and APC in the pulmonary compartment has previously been demonstrated in patients with pneumonia and
acute lung injury\textsuperscript{2,7,20}. The LTA- and LPS-induced decreases in the bronchoalveolar concentrations of APC and PC were accompanied by a rise in the local concentrations of soluble TM, which is in accordance with previous investigations on sterile or infectious lung injury\textsuperscript{2,13,20}. Shedding of TM from the surface of endothelial and epithelial cells is expected to reduce the availability of cell-surface TM for the activation of PC, which may explain the decrease in BALF APC levels. The capacity to produce APC in the alveolar compartment may be further decreased by inflammation induced downregulation of TM expressed by respiratory epithelial cells\textsuperscript{42,43}. Cell surface-associated TM also serves as an important scavenging site for thrombin and loss of this function, in conjunction with very low levels of antithrombin, may result in an increase in the availability of free thrombin in the bronchoalveolar space. In addition, low levels of PC and APC may have been caused in part by increased consumption, increased binding to protein C inhibitor (forming APC-PCI complexes)\textsuperscript{44} and degradation of PC by the neutrophil degranulation product elastase\textsuperscript{45}, although in our investigation only LPS and not LTA elicited detectable neutrophil exocytosis in the lung.

LTA and LPS similarly impacted on bronchoalveolar fibrinolysis, whereby the LPS induced effects were strictly in accordance with our earlier studies in healthy humans, characterized by an overall suppressed fibrinolytic activity as reflected by reduced PA levels and increased PAI-1 concentrations\textsuperscript{11-13}. Although LTA and LPS reduced PA activity, tPA and uPA antigen levels were higher in BALF obtained from the challenged segments. The assays used for tPA and uPA also detect tPA and uPA complexed to their inhibitor PAI-1\textsuperscript{13}. Thus although tPA and uPA were released into the alveolar space in increased quantities after LTA or LPS administration, net PA activity was decreased due to enhanced release of PAI-1. Similar observations have been made in the alveolar space of patients with pneumonia\textsuperscript{1,3,8}. Clearly, the inhibition of fibrinolysis is expected to further contribute to enhanced fibrin deposition.

Conclusions

The present study is the first to provide insight into effects of LTA on pulmonary hemostasis in humans and to compare those with effects of LPS. We here demonstrate that pulmonary instillation of LTA results in a net procoagulant state in the bronchoalveolar space as reflected by enhanced coagulation and inhibition of anticoagulant and fibrinolytic pathways, which is comparable with the effects of LPS. Together with previous observations in patients with pulmonary disorders, these data indicate that divergent challenges such as sterile injury, bacterial infection and purified TLR agonists, induce similar changes in the hemostatic balance in the human lung.
References


