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Microparticle-associated tissue factor activity correlates with plasma levels of bacterial lipopolysaccharides in meningococcal septic shock☆,☆☆

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ABSTRACT

Introduction: The plasma level of bacterial lipopolysaccharides (LPS) is associated with activation of the coagulation system, inhibition of fibrinolysis and the nature of the clinical presentation and outcome in patients with meningococcal disease. Tissue factor (TF)-bearing microparticles (MPs) appear to contribute to the pathogenesis of disseminated intravascular coagulation (DIC). The aim of this study was to investigate the relationship between MP-associated TF activity and the level of bacterial LPS in plasma from patients with meningococcal septic shock and meningitis.

Materials and methods: MPs isolated from citrated plasmas were assessed for TF-dependent activity with both a plasma-based thrombin generation assay (CAT) and whole blood-based thromboelastometry (ROTEM). The LPS level was measured using a chromogenic Limulus amebocyte lysate assay.

Results: MPs obtained from patients with meningococcal septic shock initiated significantly more efficient and TF-dependent thrombin generation in the CAT assay compared to MPs from patients with meningococcal meningitis. Differences in MP-associated TF activity between the septic shock patients and the meningitis patients were also evident when MPs were added to whole blood using ROTEM. The level of plasma LPS in patients with septic shock (range 2–2,100 EU/mL) was correlated with thrombogram parameters in the CAT assay; lagtime ($r_s = -0.84$), time to peak ($r_s = -0.83$), peak ($r_s = 0.85$) and ETP ($r_s = 0.83$).

Conclusions: MPs obtained from patients with meningococcal septic shock displayed more efficient TF-dependent thrombin generation and clot formation compared to MPs from meningitis patients. MP-associated TF activity was closely associated with plasma LPS levels in the septic shock group.

Abbreviations: LPS, lipopolysaccharides; TF, tissue factor; MP, microparticle; DIC, disseminated intravascular coagulation; CAT, calibrated automated thrombogram; ROTEM, rotational thromboelastometry; *N. meningitidis*, *Neisseria meningitidis*; TBSA, tris-buffered saline w/0.5% v/v bovine serum albumin; PNP, pooled normal plasma; PS, phosphatidylserine; IQR, inter quartile range; EU, endotoxin units; LAL, Limulus amebocyte lysate; *E. coli*, *Escherichia coli*; TAT, thrombin-antithrombin complex; FPA, Fibrinopeptide A; CSF, cerebrospinal fluid; RT, room temperature; LT, lagtime; ETP, endogenous thrombin potential; ttPeak, time to peak.

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☆☆ **Authorship**: MH designed and performed the experiments, analyzed the data, and wrote the manuscript. RØ and BB designed and performed the experiments, analyzed the data, and critically reviewed the manuscript. JPB analyzed and interpreted data and critically reviewed the manuscript. PB collected the samples, designed the experiments, analyzed and interpreted data, and critically reviewed the manuscript. CEH designed the experiments, analyzed the data, and revised and critically reviewed the manuscript. All authors approved the final version of the manuscript to be published.

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Introduction

Meningococcal disease is caused by *Neisseria meningitidis* (*N. meningitidis*). Clinically, the majority of patients develop meningitis, which may reach epidemic proportions [1]. A minority of patients, representing as many as 30%, develop a rapidly progressing septic shock and multiple organ failure [2]. The nature of the clinical presentations is closely related to the level of lipopolysaccharides (LPS) and number of meningococci in the blood [3–5]. A level of LPS > 7 EU/mL is associated with persistent septic shock, thrombosis and disseminated intravascular coagulation (DIC) in systemic meningococcal disease [4]. A hallmark of DIC is exaggerated coagulation activation leading to fibrin deposition and subsequent consumption of blood clotting factors, with both thrombosis and bleeding as complications [1,6]. When vascular integrity is compromised, subendothelial tissue factor (TF), the primary initiator of blood coagulation, is exposed to circulating blood [7,8]. TF binds blood coagulation factors VII/VIIa (FVII/FVIIa), and the TF/FVIIa complex activates factor IX (FIX) and factor X (FX), which in turn leads to thrombin activation with subsequent platelet activation and fibrin generation [9–11]. Circulating TF on monocytes and monocyte-derived microparticles (MPs) are probably important contributors to the pathogenesis of DIC during meningococcal sepsis. Both high levels of monocyte-associated TF [12] and procoagulant TF-dependent activity caused by monocyte-derived MPs expressing TF [13] have been previously reported in this condition. Studies using heat-inactivated, LPS-containing *N. meningitidis* or purified LPS from *Escherichia coli* (*E. coli*) have illustrated the importance of LPS in the generation of TF-bearing monocytes and MPs [14–16]. Some *in vivo* studies have correlated circulating TF activity with downstream coagulation markers, implying that TF-positive MPs are associated with coagulation activation in sepsis. Woei-A-Jin *et al.* showed, in a human endotoxemia model with *E. coli* as stimuli, a concordant increase in MP-associated TF activity and thrombin-antithrombin complex (TAT) [17], whereas Wang *et al.* showed the same in a mouse model of endotoxemia [18]. Furthermore, Nieuwland *et al.* have shown that patients with meningococcal sepsis displayed an increased number of circulating procoagulant MPs and elevated levels of the coagulation marker $F_1 + F_2$ [13]. Comparing the level of LPS with downstream coagulation markers, Brandtzaeg *et al.* showed that patients with a high plasma LPS level, leading to septic shock, had a significantly higher level of fibrinopeptide A (FPA) than patients with a low or undetectable plasma LPS level (meningitis). This observation suggests that the LPS level is associated to coagulation disturbances [19]. The aim of this study was to describe the association between the activity of MP-associated TF activity and the levels of bacterial LPS in plasmas from patients with meningococcal disease. Using both the plasma-based calibrated automated thrombogram (CAT) assay and whole blood-based rotational thromboelastometry (ROTEM), we measured procoagulant activity associated with MPs in plasma from patients with meningococcal septic shock or meningitis. The ability of MPs to generate thrombin was compared to the plasma level of LPS, with the aim of contributing to the understanding of the coagulopathy observed during meningococcal septic shock.

Materials and methods

Ethics statement

The project was approved by the Regional Committee for Medical Research Ethics, South-Eastern Norway Regional Health Authority, Norway.

Patients and samples

Plasma samples from patients with systemic meningococcal disease (septic shock and meningitis) were available through the Norwegian biobank register no. 948, “Studies in meningococcal disease”. The samples were originally collected prospectively in a study to elucidate the differences in the *in vivo* activation of the coagulation system in *Neisseria meningitidis*-infected patients with massive *versus* low grade bacteremia [5,19]. The present study included citrated plasma samples from 23 patients (13 septic shock and 10 meningitis) in whom plasma LPS concentrations had been measured in heparinized plasma at the same time point. 19/23 samples were from the time of admittance to the hospital, 4 samples were taken between 1–4 hours after admittance (S1, S3, S4 and S6). The median time from disease onset to hospital admission time was 12 h (range 10 – 18 h) for patients with septic shock, and 22 hours (range 6 – 72 h) for patients with meningitis. According to the Norwegian guidelines, suspected cases of meningococcal sepsis should start with antibiotic treatment i.v. or i.m. immediately if the transportation time to the hospital is more than thirty minutes. Ten patients with sepsis and 10 patients with meningitis were admitted directly to Ullevål University Hospital without prior antibiotic treatment. Three patients with sepsis (S8, S11 and S12) were primarily admitted to three different local hospitals, and antibiotic treatment was initiated on admittance to these hospitals, before transfer to Ullevål University Hospital. The patients were admitted to Ullevål University Hospital between 1985 and 2003. The majority of the samples (21/23) were collected between 1985 and 1990. Plasma was prepared from citrated whole blood (0.129 M citrate, Vacutainer®, Becton and Dickinson, Maylan Cedex, France), immediately centrifuged at 1,400 *g*, 10 min, aliquoted and stored frozen at −70 °C [19]. LPS was measured in plasma from heparinized (15 U/mL blood) vacuum tubes (Becton and Dickinson, Maylan Cedex, France) or from EndoTube ET (30 U/mL blood, Chromogenix (CoaChrom Diagnostica GmbH, Vienna, Austria)), using a chromogenic Limulus amebocyte lysate assay as previously described. The LPS-levels for some of the samples have been published earlier [4]. A LPS level of 1 EU/mL equals the activity of 100 pg/mL, as used in previous publications. Septic shock was defined as persistent hypoperfusion with an initial systolic pressure <85 mm Hg in adults (≥12 y) and ≤70 mm Hg in children (<12 y) requiring fluid and vasoactive drugs for at least 24 hours or until death, combined with minimal pleocytosis (<10^8 leukocytes/L in cerebrospinal fluid (CSF)) (n = 13). Meningococcal meningitis was the diagnosis for patients with marked pleocytosis (≥10^8 leukocytes/L in CSF), and lack of septic shock (n = 10). The samples had been stored, without thawing, at -70 ºC for 25 (23–26) years (median (IQR)) and 25 (25–26) years for the sepsis and meningitis groups, respectively.

Isolation of microparticles (MPs)

Citrated patient plasma (300 μL) was thawed for 10 min at 37 °C, and then centrifuged at 2,000 *g* for 15 min at room temperature (RT = 22-25 °C). Some of the plasma samples were thawed and refrozen once during pilot experiments. 250 μL plasma was transferred to a new tube, and the plasma was further centrifuged to pellet MPs (17,000 *g*, 30 min, RT). Subsequently, 225 μL plasma was removed, and the remaining plasma (MP-enriched) was diluted with 225 μL tris-buffered saline w/0.5% v/v bovine serum albumin (TBSA) for washing. MPs were then thoroughly resuspended and centrifuged (17,000 *g*, 30 min, RT), followed by removal of 225 μL supernatant and addition of 50 μL TBSA, giving a final volume of approximately 75 μL for use in the coagulation assays CAT or ROTEM. Due to minute amounts of plasma, sample S13 had only 80% input plasma volume in the MP preparation, and only 2 sepsis samples (S3 and S8) and 2 meningitis samples (M2 and M6) were analyzed with ROTEM.

Preparation of pooled normal plasma (PNP)

Blood was collected in blood collection tubes (0.109 M citrate, Greiner Bio-One GmbH, Kremsmünster, Austria) containing manually prefilled corn trypsin inhibitor (CTI, 18.3 μg/mL final concentration, Enzyme Research Laboratories, Indiana, USA), from 6 healthy