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Peptidoglycan from \textit{Staphylococcus aureus} Induces Tissue Factor Expression and Procoagulant Activity in Human Monocytes

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\textit{Staphylococcus aureus} is one of the most significant pathogens in human sepsis and endocarditis. \textit{S. aureus} can initiate blood coagulation, leading to the formation of microthrombi and multiorgan dysfunction in sepsis, whereas in endocarditis the bacterium induces fibrin clots on the inner surface of the heart, so-called endocardial vegetations. In the present study, we show that live and heat-killed \textit{S. aureus} bacteria are potent inducers of procoagulant activity in human peripheral blood mononuclear cells. Furthermore, purified peptidoglycan, the main cell wall component of \textit{S. aureus}, induced procoagulant activity in mononuclear cells in a concentration-dependent fashion. The procoagulant activity in these cells was dependent on expression of tissue factor; since antibodies to tissue factor inhibited the effect of peptidoglycan. In mononuclear cells stimulated with peptidoglycan, reverse transcription-PCR showed tissue factor gene expression, and the gene product was detected by enzyme-linked immunosorbent assay. Finally, flow cytometry identified tissue factor at the surface of CD14-positive monocytes. Peptidoglycan is known to induce proinflammatory cytokine production in monocytes. The present investigation shows that peptidoglycan also activates the extrinsic pathway of coagulation by inducing the expression of tissue factor in these cells. This mechanism helps to explain the procoagulant activity, which plays such an important role in the pathogenicity of severe \textit{S. aureus} infections.

\textit{Staphylococcus aureus} is one of the most important pathogens in gram-positive bacterial sepsis and bacterial endocarditis (5, 16). During severe sepsis and septic shock caused by \textit{S. aureus}, blood coagulation is activated, leading to disseminated intravascular coagulation (DIC) and multiorgan dysfunction syndrome (4, 11–13). It is also postulated that coagulation is activated during bacterial endocarditis, causing the formation of endocardial vegetations (2, 10, 30).

Monocytes and endothelial cells possess potential thrombogenic properties through their ability to express tissue factor (TF). TF, a single-chain transmembrane protein composed of 263 amino acid residues, is recognized as the major physiological initiator of blood coagulation (25). TF binds to plasma factor VII (FVII), forming a potent procoagulant complex, which can rapidly activate FIX and FX. Activated FX results in thrombin generation, which potentiates further FIXa generation by feedback activation of FXI (8). Endothelial cells and monocytes do not constitutively express TF but can be stimulated to do so by lipopolysaccharide (LPS), a component of the outer cell wall of gram-negative bacteria (19). Since monocytes are the only peripheral blood cells capable of expressing TF, there has been considerable interest in measuring the procoagulant activity (PCA) of these cells under physiological and pathological conditions (23). Veltrop et al. have shown that intact \textit{S. aureus} bacteria can induce TF activity in fibrin-adherent monocytes and in human vascular endothelial cells (30, 31).

Further, TF activity has been reported during experimental peritonitis and in monocytes from patients with bacterium-induced DIC (1, 11, 22).

The molecular interactions required for the induction of TF expression in monocytes by gram-positive bacteria are unclear. The cell wall of \textit{S. aureus} is composed mainly of peptidoglycan (PG) (50 to 60% by weight), teichoic acid (TA), and lipoteichoic acid (LTA). PG has a rigid structure and consists of repeating units of N-acetylgalactosamine and N-acetyl-muramic acid, to which are linked amino acid residues. TA and LTA are built of phosphorus, and ribitol or glycerol, and in LTA fatty acids are also included (15). PG, TA, and LTA can independently induce an inflammatory response in human monocytes, measured as release of tumor necrosis factor alpha (TNF-α), interleukin 1β (IL-1β), and IL-6 (17, 32). Inflammatory responses and procoagulant mechanisms play important roles in the pathogenesis of severe infectious disease. However, the molecular basis for PCA is not well understood, and the present investigation was stimulated by the hypothesis that the staphylococcal cell wall component PG could induce TF production in human monocytes. Our results show that PG has this effect, which supports the notion that inflammatory responses and PCA are intimately connected in \textit{S. aureus} infections.

\textbf{MATERIALS AND METHODS}

\textbf{Materials.} Polymyxin B (PMB), LPS from \textit{Escherichia coli} strain O111:B4, LTA from \textit{S. aureus}, and FVII-deficient plasma were purchased from Sigma, St. Louis, Mo. Plasma was obtained from healthy volunteers after informed consent and stored at \(-70^\circ\text{C}\). Goat anti-human TF immunoglobulin G (IgG) was a kind gift from Marianne Kjælke, Copenhagen, Denmark, and IgG purified from a goat immunized with human β2-microglobulin was used as a control. IL-1β was from
BD Pharmingen, Stockholm, Sweden, and TNF-α was from R & D, Abingdon, England.

Strains of bacteria. The bacteria used in the study were a clinical isolate of S. aureus (strain 5120) derived from a patient with septic shock and a laboratory strain, S. aureus WOOD. The bacteria were cultured on blood agar plates. Single colonies were picked and grown in brain heart infusion (Difco, Detroit, Mich.) at 37°C overnight. Prior to incubation with peripheral blood mononuclear cells (PBMC), the bacteria were washed three times and thereafter resuspended in phosphate-buffered saline, pH 7.4. In some experiments heat-killed (80°C for 30 min) bacteria were used.

Preparation of staphylococcal PG. PG was prepared from S. aureus WOOD according to the method of Peterson et al. (24). Bacteria were grown in PYK medium (containing 0.5% [wt/vol] yeast extract, 1.3 mM KH2PO4, and 1.1 mM glucose [pH 7.2 to 7.4]) and incubated at 37°C for 2 to 4 h. Log-phase bacteria were added to 10 liters of PYK medium and incubated at 37°C for 18 h in a shaking incubator. The bacteria were harvested by centrifugation (15,000 × g, 4°C, 10 min) with bacteria washed three times and thereafter resuspended in phosphate-buffered saline, pH 7.4. In some experiments heat-killed (80°C for 30 min) bacteria were used.

Chemical characterization of PG. PG was prepared from S. aureus WOOD according to the method of Peterson et al. (24). Bacteria were grown in PYK medium (containing 0.5% [wt/vol] yeast extract, 1.3 mM KH2PO4, and 1.1 mM glucose [pH 7.2 to 7.4]) and incubated at 37°C for 2 to 4 h. Log-phase bacteria were added to 10 liters of PYK medium and incubated at 37°C for 18 h in a shaking incubator. The bacteria were harvested by centrifugation (15,000 × g, 4°C, 10 min) with bacteria washed three times and thereafter resuspended in phosphate-buffered saline, pH 7.4. In some experiments heat-killed (80°C for 30 min) bacteria were used.

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bacteria were used. Maximal induction of PCA was reached at bacterium/monocyte ratios of 1:1 and 10:1 for live and heat-killed \textit{S. aureus}, respectively. Clotting time for control cells without stimulus was $335 \pm 93$ s. Bacteria alone did not affect the clotting time (data not shown).

To study whether PG, the main cell wall component of \textit{S. aureus}, could also induce PCA, it was incubated with PBMC. Subsequently, the mixtures were added to healthy human plasma, and the clotting time was determined. LPS from \textit{E. coli} was used as a positive control. Both PG and LPS showed induction of PCA in PBMC in a concentration-dependent fashion (Fig. 2A). Compared to LPS, a thousandfold-higher amount of PG was needed to reach the same effect on clotting time.

To investigate the kinetics of the PCA induced by PG and LPS, PBMC were incubated with PG (100 ng/ml) or LPS (100 ng/ml) for different time intervals before the cell suspensions were added to human plasma and the clotting time was analyzed. After a 2-h incubation, the PCA increased in both PG- and LPS-stimulated PBMC compared to control cells (Fig. 2B). After 4 h, a plateau for the PCA was reached for both stimuli. The data show that the induction of PCA in human PBMC by PG and LPS is a rapid process, with similar kinetics for PG and LPS. The PG preparation was free of endotoxin as tested by the \textit{Limulus} amebocyte lysate assay (described in Materials and Methods). To exclude the possibility that endotoxin contamination occurred during the experimental procedure, PG was preincubated with PMB (an antibiotic that neutralizes endotoxin) before it was added to the PBMC. No difference in the induction of PCA by PG was obtained in the presence of PMB, whereas the induction of PCA by LPS was blocked following preincubation with PMB (Fig. 2C).

Expression of TF by monocytes is necessary for the PCA induced by PG in PBMC. To investigate whether TF was responsible for the PCA, IgG blocking TF activity or control IgG was added to PBMC after 4 h of incubation with PG or LPS and incubated for another 30 min. Subsequently, PCA was determined. IgG directed against TF effectively inhibited the increased PCA of LPS- and PG-stimulated cells, showing that the PCA was dependent on TF expression (Fig. 3A). Control IgG did not affect PCA.

TF initiates the coagulation cascade via activation of FVII, thereby forming a potent procoagulant, which activates the extrinsic coagulation pathway. We therefore compared FVII-depleted plasma with normal plasma in the PCA assay. Because trace amounts of FVII are present in the FVII-deficient plasma, it was diluted four times and compared with normal control plasma identically diluted. No clotting was obtained in FVII-depleted plasma by PBMC stimulated with PG or LPS, whereas a marked reduction in clotting time was obtained for normal plasma incubated with cells stimulated by 100 µg of PG/ml or 100 ng of LPS/ml (152 ± 16 or 149 ± 48 s, respectively [mean ± SD]). This further underlines the finding that TF and its activation of FVII are crucial for the activation of the extrinsic coagulation pathway by PG-stimulated PBMC.

TF RT-PCR was used to detect TF gene expression in PBMC. PG induced TF expression at levels similar to those obtained by stimulation with LPS after 4 h of incubation (Fig. 3B-I). PG and LPS induce expression of TNF-α and IL-β in monocytes, raising the possibility that PCA is induced as a
However, no TF expression was detected in cells incubated in the presence of IL-1β (5 ng/ml) or TNF-α (5 ng/ml). GRO-α, a proinflammatory CXC chemokine, expressed by stimulation with LPS, IL-1β, and TNF-α, served as a control to confirm that the cytokines included were biologically active (Fig. 3B-II) (33). Moreover, high levels of TF (1,102 ± 695 or 950 ± 384 pg/ml [mean ± SD]) were detected by ELISA in cell lysates obtained from PBMC stimulated with 100 μg of PG/ml or 100 ng of LPS/ml, respectively (the level of TF in unstimulated cells was <50 pg/ml). Using flow cytometry, it was found that PG- and LPS-stimulated cells expressed similar amounts of TF on their surface (Fig. 3C), and when CD14 was used as a cellular marker, the analysis showed that only CD14-positive cells, i.e., monocytes, expressed TF after stimulation by PG or LPS.

**PCA of adherent monocytes in the absence and presence of lymphocytes.** The PBMC preparations used here contained both monocytes and lymphocytes. Lymphocytes do not express TF, but previous work has shown that they can modulate TF expression in human monocytes (27, 29). For this reason, the PG- and LPS-induced PCA of adherent monocytes was investigated in the absence or presence of lymphocytes. The cells were incubated with medium alone, PG, or LPS for 4 h. Supernatants were removed, and human plasma was added to the wells, followed by incubation for 5 min in order to activate coagulation. Plasma supernatants were then aspirated, recalculated, and analyzed for clotting time. However, the presence or absence of lymphocytes did not significantly influence the PCA of the monocyte monolayers (Fig. 4), suggesting that lymphocytes do not contribute to the PCA in our assay.

**DISCUSSION**

The nature of the gram-positive bacterial components responsible for PCA and TF expression in monocytes is unknown. The present study demonstrates that heat-inactivated *S. aureus* bacteria were as effective as live *S. aureus* in inducing PCA, suggesting that staphylococcus-generated PCA requires only bacterial surface components. Highly purified PG, one of the main cell wall components of *S. aureus*, was investigated for its TF-inducing capacity in human PBMC. PG generated a concentration- and time-dependent PCA in PBMC. The threshold concentration of PG needed to generate PCA was 100 ng/ml, which corresponds to 10^5 CFU of *S. aureus*/ml (26).

Recently, similar concentrations of PG were detected in plasma from patients with bacterial sepsis (14). Compared to LPS, a thousandfold-larger amount of PG was required to reach similar levels of PCA. This ratio between PG and LPS is similar to their cytokine-inducing potency in PBMC in the absence of serum (17) and to the difference in LPS and PG concentrations in plasma of patients with sepsis (LPS, 5 to 500 pg/ml; PG, 10 to 190 ng/ml) (7, 14).

PG-induced PCA in PBMC was dependent on TF expression by monocytes, and anti-TF IgG reduced PCA levels to background levels (Fig. 3A). Furthermore, RT-PCR could de-
FIG. 3. TF expressed by monocytes is responsible for the PCA induced by PG in PBMC. (A) PBMC were incubated with medium alone (control), PG (100 μg/ml), or LPS (100 ng/ml). After 4 h of incubation, TF-neutralizing goat IgG or control goat IgG was added to the cells, followed by another 30 min of incubation. Subsequently, the PBMC were mixed with recalciﬁed human plasma, and the clotting time was determined. Values are means ± SDs (n = 3). (B) Total RNA was isolated from PBMC incubated in medium (control) or in the presence of PG (100 μg/ml), LPS (100 ng/ml), IL-1β (5 ng/ml), or TNF-α (5 ng/ml) for 4 h. RT-PCR was performed on the isolated RNA to detect transcripts for TF, GRO-α, and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GADPH). PG and LPS both induced TF expression in PBMC (I). IL-1β and TNF-α induced expression of GRO-α but not TF (II). (C) PBMC were stimulated with PG (100 μg/ml) or LPS (100 ng/ml) for 4 h. Cells were then incubated with phycoerythrin-conjugated CD14, FITC-conjugated TF, or isotype-matched control antibodies and assayed by flow cytometry (upper panels). CD14-positive monocytes showed similar increases in TF expression after incubation with PG and with LPS (lower panel). Data represent means ± SDs (n = 4) for MFI (mean ﬂuorescence intensity), detected in the FITC channel, which is expressed on the y axis.
and at the surface of monocytes by ELISA and detection of medium alone, PG (100 ng/ml), or LPS (100 ng/ml). In wells containing lymphocytes also, PBMC were stimulated without removal of the supernatants. After 3 h of incubation, supernatants were removed, and plasma was added to the wells. Following 5 min of incubation, plasma was collected and mixed with CaCl$_2$, and the clotting times were determined. Values are means ± SDs (n = 3).

Stimulation by IL-1β or TNF-α did not induce TF gene expression in PBMC as detected by RT-PCR. Therefore, autocrine or paracrine effects of these cytokines are not responsible or sufficient for the induction of TF expression in monocytes under the conditions used. However, it cannot be ruled out that cytokines can potentiate the cellular response to PG, as in the case of whole staphylococcus bacteria in endothelial cells (31). The failure of PG- and LPS-stimulated PBMC to induce PCA in FVII-deﬁcient plasma shows that the PCA was initiated by TF expression, since FVII is required for the procoagulant effect of TF. It is interesting that TF together with FVIIa, in addition to being the major in vivo initiator of coagulation, also induces proinflammatory responses in macrophages, i.e., production of reactive and tissue-toxic oxygen species and expression of major histocompatibility complex class II antigens and cell adhesion molecules (6).

Previous work has shown that TF expressed by monocytes is crucial for the formation of endocardial vegetations in bacterial endocarditis (9). However, the role for monocytes in endocarditis is probably complex. In rabbits suffering from $S. aureus$ endocarditis, monocytopenia induced by etoposide treatment led to rapid death (30), and in these rabbits no endocardial vegetations were found. Thus, monocytes, despite promoting the formation of endocardial vegetations, appear to have an overall beneficial role during $S. aureus$ endocarditis. Other cell components of $S. aureus$ may also be of interest in the pathogenesis of endocarditis and septicemia, for example, LTA. Therefore, commercial LTA was tested in our assays and was found to be unable to induce PCA or TF expression in human monocytes. However, recently it was shown that commercial LTA is of poor quality and purity and may not be biologically relevant (20). It is, therefore, still unclear whether LTA can activate the extrinsic pathway of the coagulation system. Further, coagulase, a secreted protein from $S. aureus$, has prothrombin-activating properties converting fibrinogen to fibrin, but its contribution to bacterial virulence is uncertain (16).

TF is the main initiator of blood coagulation, and when it comes to bleeding disorders seen in cases of severe $S. aureus$ sepsis (especially DIC), the induction of TF should represent an important mechanism. The finding that PG can induce TF production in monocytes is therefore of theoretical and clinical interest. It represents a novel biological effect of PG, which connects inflammatory and procoagulant cascades, and further underlines the highly complex molecular host-microbe interplay in sepsis.

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FIG. 4. Isolated monocytes stimulated by PG express PCA in the absence of lymphocytes. PBMC were incubated in tissue culture plates for 1 h to let monocytes adhere. In wells from which lymphocytes were removed, supernatants were discarded, followed by washing and addition of medium alone, PG (100 μg/ml), or LPS (100 ng/ml). In wells containing lymphocytes also, PBMC were stimulated without removal of the supernatants. After 3 h of incubation, supernatants were removed, and human plasma was added to the wells. Following 5 min of incubation, plasma was collected and mixed with CaCl$_2$, and the clotting times were determined. Values are means ± SDs (n = 3).


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