

Relationship between the Variations in Blood Coagulation/Fibrinolytic Factors and Inflammatory Reactions Induced by LPS

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We studied the relationship between inflammatory cell profiles stimulated by a bacterial toxin, endotoxin (Lipopolysaccharide; LPS), and variations in blood coagulation/fibrinolytic system factors in mice. Since LPS stimulates coagulation activity, decrease in fibrinolytic activity and mobilization of platelets to local tissues in mice, it tends to induce thrombogenesis in tissues, and to induce inflammatory cells. The results of the present study suggest that the automated hematology analyzer for veterinary use, SFVU-1 is very useful for determining and analyzing variations of blood components in various experimental animal models such as those with stimulation by LPS.

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Key Words Automated Hematology Analyzer for Veterinary Use, SFVU-1, Endotoxin, Blood Coagulation System, Fibrinolytic System, Inflammatory Cells

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INTRODUCTION

In severe infection by gram-negative bacteria, endotoxin (Lipopolysaccharide; LPS) produced by the bacteria induces disseminated intravascular coagulation (DIC) and septic shock¹. Main causes of septic shock are microangiopathic coagulation and secondary multiple organ failure. In addition, disseminated intravascular microthrombus formation is caused by stimulation or/and disturbance of wide-range endothelial cells. Administration of LPS in animals is used as a model of sepsis. In LPS-induced inflammatory reactions, various factors such as interleukins may be released primarily from immunocompetent cells and affect the appearance of coagulation/fibrinolytic factors². It has been reported that these effects increase activation and decrease in suppression of the coagulation system, as well as decrease in activation system and the increase in suppression of the fibrinolytic system. As a result, increase in coagulation activity and decrease in fibrinolytic activity are induced, with formation of fibrins³ (*Fig. 1*).

Thus, we studied the relationship between inflammatory cell profiles stimulated by LPS and variations in blood coagulation/fibrinolytic systems in mice. We studied blood cells in mice using flow cytometry with the automated hematology analyzer for veterinary use, SFVU-1 (hereinafter, SFVU-1, Sysmex Corporation, Kobe, Japan) developed for animals.

MATERIALS AND METHODS

Male C57BL/6J mice were used at the age of 6-8 weeks. LPS (*Escherichia coli* serotype 0111: B4; Sigma) was intraperitoneally administered to mice at a dose of 50 μ g (2.0 mg/kg). Blood and tissue samples were taken at each time point. Each tissue was fixed and stored in 10% formalin for analysis of tissue sections, and stored at -80°C for analysis of gene and protein expression after being fixed with liquid nitrogen. Blood distribution was determined with blood smear specimens stained with Wright - Giemsa using the SFVU-1. After euglobulin fractions were prepared by plasma acid treatment, fibrinolytic activity and plasminogen activator (PA) activity were determined with a fibrin plate method⁴ and fibrin zymography⁵, respectively. In addition, plasma PA inhibitor-type 1 (PAI-1) activity was tested by reverse fibrin zymography⁶. The gene expression of coagulation /fibrinolytic factors [tissue factor (TF), PAI-1, urokinase-type PA (u-PA), α_2 -antiplasmin (α_2 -AP)] in the liver or kidney was tested with the Northern blot method using cDNA fragment probes for ³²P-labeled factors against total RNA extracted from each tissue. Levels of mRNA expression for each factor were determined with an Image Analyzer (LAS 1000; FUJI), and corrected for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) activity. The appearance of fibrin deposits was tested with immunostaining using fibrin (ogen) antibody.

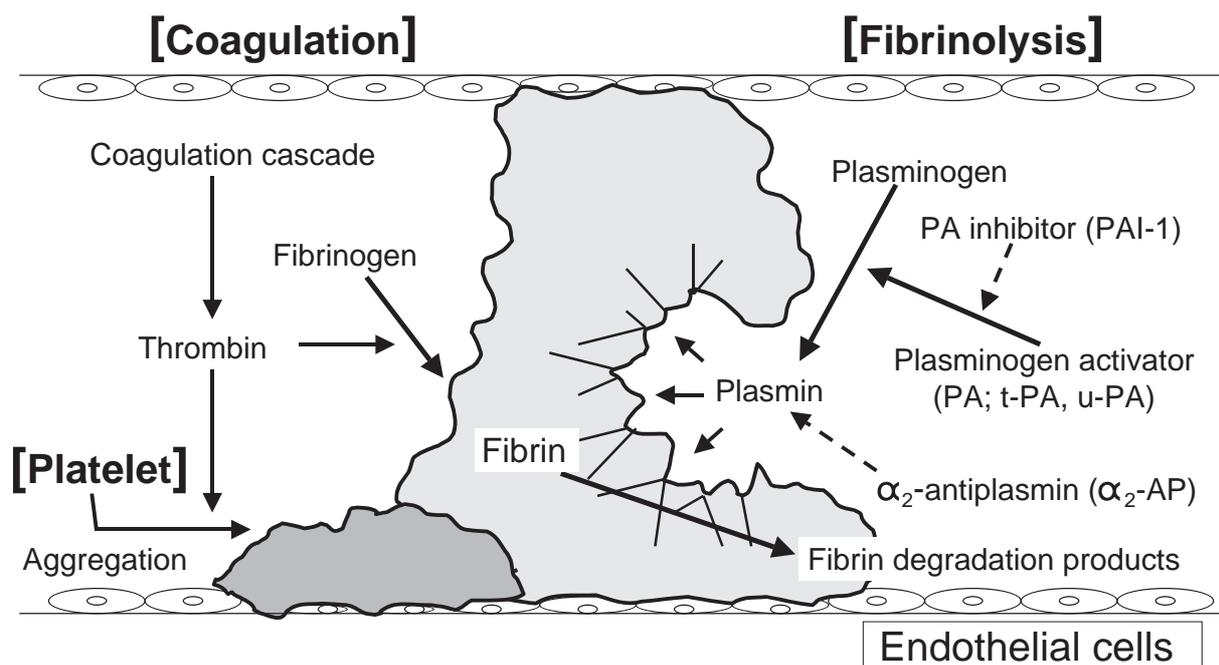


Fig.1 Mechanism of hemostasis

RESULTS

The variation in blood cell counts in mice after administration of LPS was determined with the SFVU-1. The blood scattergrams before and 3, 24 or 72 h after administration of LPS are shown in **Fig. 2**. White blood cell (WBC) counts mainly consisting of lymphocytes were markedly decreased 3 h after administration of LPS compared to those of untreated mice (0 h). Neutrophils (NEUT) were primarily increased 24 h after administration. However, the blood distribution at 72 h after administration was almost the same as that in untreated mice. The WBC count in male C57BL/6 mice (6-8 weeks old) was $5,220 \pm 710/\mu\text{L}$ before administration, but decreased to reach a minimum at 3 h after administration of LPS. Thereafter, it increased and recovered at 72 h after administration (**Fig. 3**). The percentage of NEUT among WBC was increased from 8 h after administration of LPS, and reached a maximum at 24 h ($32.1 \pm 5.35\%$ at 24 h against $9.5 \pm 2.05\%$ before administration). Thereafter, it decreased and recovered at 72 h (**Fig. 3**). The platelet count (PLT) was $821,000 \pm 132,000/\mu\text{L}$ before administration, but decreased to $332,000 \pm 73,000/\mu\text{L}$ from 3 h to 3 days after administration of LPS, and thereafter increased and recovered (**Fig. 4**). On the other hand, red blood cell (RBC) counts were not affected by administration of LPS (**Fig. 4**). Variation of WBC determined with the SFVU-1 after administration of LPS was compared with that of blood smear specimens. The differential WBC at each time point was similar to the WBC distribution on blood scattergrams determined by the SFVU-1 (**Fig. 5**).

Plasma PA activity was determined with fibrin zymogra-

phy (data not shown). Plasma PA in mice included t-PA (70 kDa) and u-PA (42 kDa). The plasma u-PA activity was decreased and reached a minimum at 3 h after administration of LPS, and thereafter recovered. On the other hand, plasma t-PA activity was not affected by the administration of LPS, and was unchanged. Plasma PAI activity was tested with reverse fibrin zymography (data not shown). The plasma PAI activity in mice increased to reach maximum PAI-1 (45 kDa) at 6 h after administration, and was thereafter eliminated. Overall plasma fibrinolytic activity was determined with a fibrin plate method. As a result, overall plasma fibrinolytic activity was decreased to reach a minimum at 3 h after administration of LPS, and thereafter recovered. The mRNA expression of renal coagulation/fibrinolytic system proteins determined by the Northern blot method after administration of LPS (data not shown). The mRNA levels of TF, a coagulation system activator, and PAI-1, a fibrinolytic system inhibitor, increased to maximum at 3 and 8 h after administration of LPS, respectively, and thereafter decreased. On the other hand, mRNA levels of u-PA, a fibrinolytic system activator, decreased and reached a minimum at 3 h after administration of LPS, and thereafter recovered. Administration of LPS did not influence the mRNA levels of α_2 -AP expressed in the kidney at steady-state. The appearance of fibrin deposits in mouse kidney after administration of LPS was tested with an immunostaining method. Fibrin deposits appeared to reach maximum at 4-8 h after administration of LPS, and thereafter disappeared by 24 h after administration (data not shown).

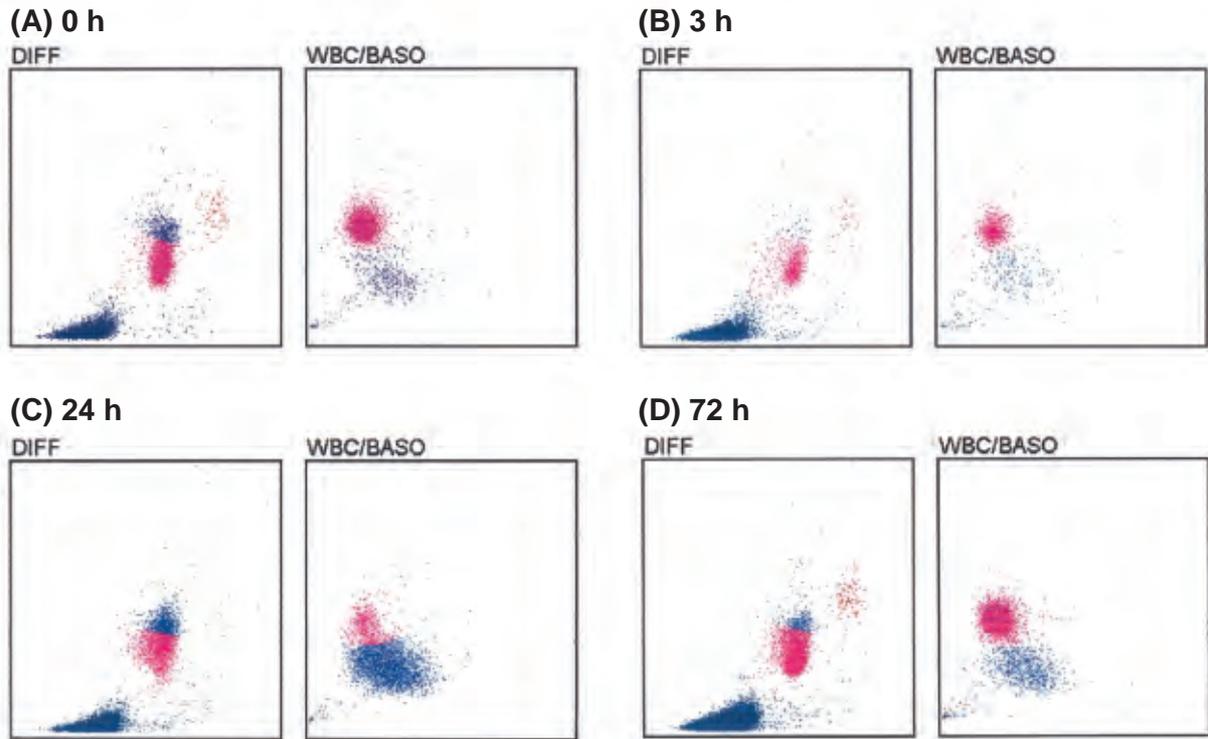


Fig. 2 Variations in hematology scattergrams in mice after administration of LPS
 The hematology scattergrams were determined with blood samples before (0 h) and after (3 h, 24 h, 72 h) administration of LPS.

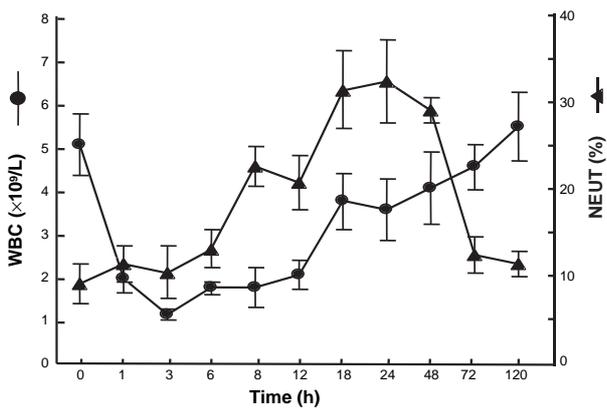


Fig. 3 Variations in WBC counts and numbers of NEUT in mice after administration of LPS
 NEUT are represented as percentages (%) of total WBC counts.
 Mean \pm SD, n=3-5

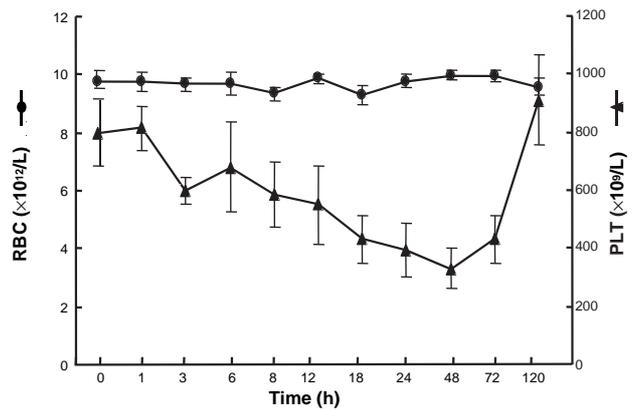


Fig. 4 Variations in RBC counts and PLT in mice after administration of LPS
 Mean \pm SD, n=3-5

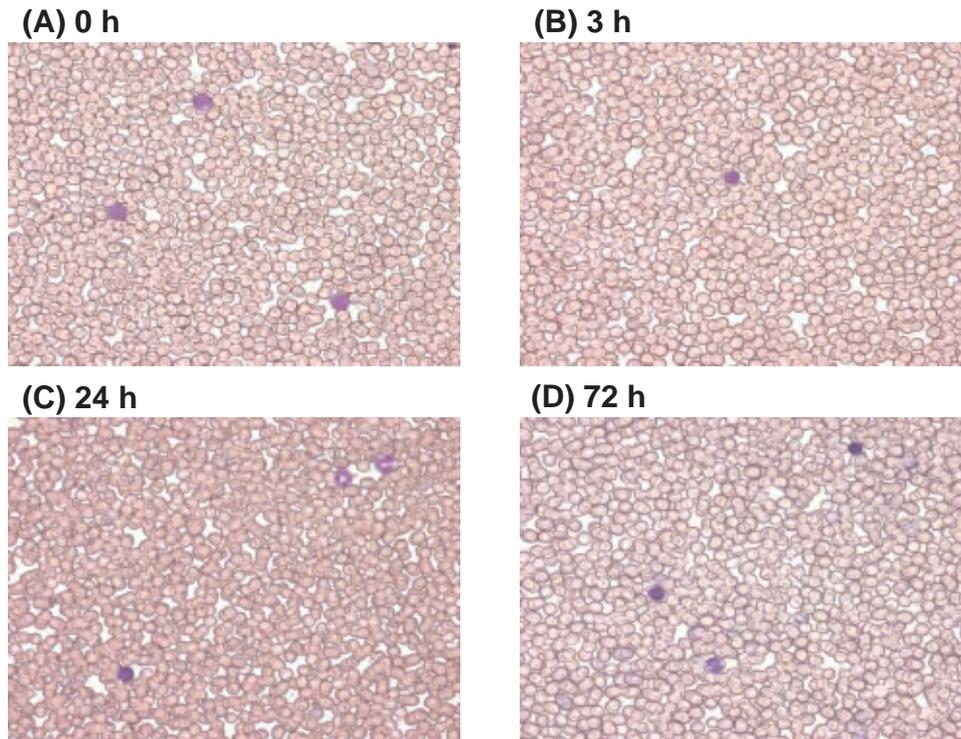


Fig. 5 Variations in Wright - Giemsa-stained blood smear specimens in mice before (0 h) and after (3 h, 24 h, 72 h) administration of LPS

DISCUSSION

We studied the relationship between inflammatory cell profiles and variations in blood coagulation/fibrinolytic system factors after administration of LPS in mice. In mice after administration of LPS, WBC counts in peripheral blood were transiently decreased, and NEUT were increased slightly after the decrease in WBC (**Fig. 2** and **Fig. 3**). Coagulation activity was transiently increased and fibrinolytic activity was decreased in plasma and kidney (data not shown). Fibrin deposits were observed in the kidney.

The transient decrease in WBC counts and increase in NEUT in the peripheral blood of mice after administration of LPS may be induced by inflammatory cells in each tissue as a protective reaction against invasion of LPS. In addition, it has been reported that WBCs adhere to vascular endothelial cells in the venular region of the capillary vessels of surrounding tissue as a result of stimulation of LPS⁷. Moreover, LPS may play a role in NEUT activation in the initial stage of microcirculation injury. Platelets were decreased in peripheral blood of mice after administration of LPS, probably because platelets were consumed by thrombogenesis with platelet aggregation on the vascular endothelial cells injured by LPS stimulation. In rats after administration of LPS, uptake of platelets in tissues using the marker 5-hydroxy-

tryptamine (5-HT) has been reported⁸. 5-HT level was increased in each tissue in correlation with the decreases in PLT counts and 5-HT level in peripheral blood after administration of LPS.

The fibrin deposits in the tissue of mice after administration of LPS may be caused by increase in coagulation activity and decrease in fibrinolytic activity³. Concerning coagulation factors, expression of TF was increased in the kidney (data not shown). This expression of TF may appear on the tubular epithelial cells of the renal cortex, where TF is not normally expressed. In addition, it has been reported that LPS decreases the expression of thrombomodulin on vascular endothelial cells as a protein regulating thrombin⁹. Concerning fibrinolytic factors, increase in expression of PAI-1 and decrease in expression of u-PA were observed in the kidney. Expression of PAI-1 may appear on the vascular endothelial cells of the renal parenchyma. In addition, the expression of u-PA before administration of LPS may be decreased in the renal medulla. However, no notable fibrin deposits which were observed in the kidney, were detected in the liver after administration of LPS, probably because expression of PAI-1 was also increased in the liver by the administration of LPS, but no marked increase in the expression of TF was observed, and because u-PA, target for decrease in the expression by LPS, was not expressed

as usual. These differences in expression of coagulation/fibrinolytic factors in the kidney and liver after stimulation by LPS may be associated with the appearance of fibrin deposits. In addition, the level of expression of α_2 -AP mRNA in the kidney was not affected by administration of LPS. However, we have reported that fibrin deposits in the kidney after administration of LPS were decreased in mice with α_2 -AP gene deletion compared with control mice¹⁰. These results suggest that α_2 -AP regulates fibrinolytic activity in the kidney.

These coagulation/fibrinolytic factors changed by LPS stimulation may be influenced by both direct effects of LPS and indirect effects through other reactions. It has been reported that expression of PAI-1 was increased by direct stimulation of vascular endothelial cells by LPS, while inflammatory cells induced by LPS released TNF- α , resulting in expression of PAI-1 by the vascular endothelial cells induced by this TNF- α ². In addition, LPS is known to release IL-6 from inflammatory cells, and to increase the expression of plasminogen through IL-6-responsive elements (CTGGG/AA) present in the promoter region of the plasminogen gene¹¹. However, the IL-6-responsive elements are also present in the promoter region of the mouse α_2 -AP gene¹², but no difference in expression was observed from that after direct administration of IL-6¹³ and that administration of LPS in the present study.

Administration of LPS to mice or rats is generally used as a model of sepsis. This model is used for study of the mechanisms of sepsis or development of drugs for sepsis, and applied to analysis of effects of LPS on various cells and/or factors. When variations in blood cell components are analyzed in blood from animal models, blood cells can be determined with a small blood sample (170 μ L) with the SFVU-1 used in this study. The methodology is easy and analysis can be promptly performed. Results exhibited good correlations with those of blood smear samples, and the reproducibility of data was excellent.

CONCLUSIONS

Since LPS stimulates coagulation activity, decrease in fibrinolytic activity and mobilization of platelets to local tissues, it may tend to cause thrombogenesis and also induce inflammatory cells. The results of the present study suggest that the SFVU-1 is very useful for determining such variations in blood components in various experimental animal models.

ACKNOWLEDGEMENTS

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