Reduction in Lipopolysaccharide-Induced Thrombocytopenia by Triflavin in a Rat Model of Septicemia

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Background—Thrombocytopenia frequently occurs early in the course of Gram-negative bacterial infections. Triflavin, an Arg-Gly-Asp–containing disintegrin, has been suggested to interfere with the interaction of fibrinogen with the glycoprotein IIb/IIIa complex. The present study was undertaken to determine whether triflavin could prevent thrombocytopenia in lipopolysaccharide (LPS)-treated rats.

Methods and Results—In this study, 51 Cr-labeled platelets were used to assess blood and tissue platelet accumulation after LPS challenge. The administration of LPS (4 mg/kg IV bolus) for 4 hours induced a reduction in radiolabeled platelets in blood and an obvious accumulation of platelets in liver. Triflavin (500 mg/kg) but not GRGDS (20 mg/kg) significantly prevented the alteration of radiolabeled platelet distribution in blood and liver when induced by LPS. Furthermore, triflavin but not GRGDS markedly suppressed the elevation in plasma thromboxane B_2 concentration within the 4-hour period of LPS administration. In LPS-treated rats, the 5-hydroxytryptamine level was lower in the blood and higher in the liver compared with levels in normal saline–treated rats. Pretreatment with triflavin (500 mg/kg) significantly reversed the 5-hydroxytryptamine concentration in blood and liver of LPS-treated rats. In histological examinations and platelet adhesion assay, triflavin markedly inhibited the adhesion of platelets to subendothelial matrixes in vivo and in vitro.

Conclusions—The results indicate that triflavin effectively prevents thrombocytopenia, possibly through the following 2 mechanisms: (1) Triflavin markedly inhibits platelet aggregation, resulting in decreased thromboxane A_2 formation. (2) It inhibits the adhesion of platelets to subendothelial matrixes, thereby leading to a reversal in the distribution of platelets in blood and liver in LPS-treated rats. (Circulation. 1999;99:3056-3062.)

Key Words: platelets • platelet aggregation inhibitors • peptides • thromboxane

Circulatory shock from Gram-negative bacterial sepsis produces a spectrum of pathophysiological alterations including cardiopulmonary, renal, hematologic, and metabolic dysfunction leading to vascular collapse. The most pronounced clinical manifestation of septic shock is disseminated intravascular coagulation (DIC). DIC is characterized by microvascular thrombosis, thrombocytopenia, and stimulation of fibrinolysis. Thrombocytopenia frequently occurs early in the course of septicemia without overt evidence of DIC in both adults and children. Bacterial infection can impair any or all of the hemostatic mechanisms including the platelets, the coagulation system, and the vessel wall. However, Gram-positive and Gram-negative bacterial infections are most frequently associated with isolated consumptive thrombocytopenia. The development of thrombocytopenia usually indicates that the infection has become systemic.

The mechanism responsible for the development of thrombocytopenia is not fully understood. There are a number of possible ways in which bacterial infections could cause platelet consumption: (1) by immune mechanisms; (2) by initiating DIC with thrombin-induced platelet consumption; (3) by directly aggregating platelets independently of thrombin action; (4) by producing vessel damage, which results in platelet interaction with subendothelial structures; and (5) by hypersplenism secondary to the bacterial infection. There is an investigation that has suggested possible roles for the participation of leukocytes in bacterially induced thrombocytopenia.

Recently, many disintegrin antiplatelet peptides have been reported. These peptides all contain RGD and bind with high affinity to integrins, a family of adhesion receptors on the cell surface. The integrins comprise a superfamily of transmem-
brane receptors that participate in cell-cell and cell-substrata interactions. Triflavin is a disintegrin purified from *Trypsurus flavoviridis* snake venom. Its primary structure consists of 70 amino acid residues including 12 cysteines with an RGD sequence at position 49-51. We previously reported that triflavin inhibits platelet aggregation by interfering with the interaction of fibrinogen with the glycoprotein (GP) IIb/IIa complex (αmβ3, integrin). Furthermore, we also demonstrated that triflavin has a more powerful influence on the antithrombotic effect and antiangiogenic activity in vivo and in vitro.

The present study was designed to determine the effect of triflavin on the development of lipopolysaccharide-induced thrombocytopenia in rats during lipopolysaccharide (LPS)-induced septic shock and to compare the relative activity of triflavin with that of the RGD-synthetic peptide GRGDs.

**Methods**

**Materials**

Triflavin was purified from the venom of *T flavoviridis* (Rosans, France) as previously described. Heparin, collagen (bovine tendon type I), pargyline, clorglyline, prostaglandin E (PGE), apyrase (grade III), EDTA, fibronectin (from bovine plasma), vitronectin (from human plasma), LPS (from *Escherichia coli* Serotype 0127: B8), and ammonium pyrrolidine-dithiocarbamate (PDTC) were obtained from Sigma Chemical Co. 2'-7'-Bis (2-carboxyethyl)-5 (and-6)-carboxyfluorescein acetoxymethyl (BCECF-AM) was purchased from Molecular Probes, Inc. von Willebrand factor (vWF, from human plasma) was purchased from Calbiochem. Gly-Arg-Gly-Asp-Ser (GRGDS) and Gly-Arg-Gly-Glu-Ser (GRGES) were purchased from Peninsula Laboratories. Thromboxane B, enzyme immunoassay (ELISA) kit was purchased from Cayman Co. Chromium-51 (5 mCi/mL) was purchased from Amershams.

**Animals**

Male Sprague-Dawley rats weighing 180 to 220 g were used in all studies. The animals were maintained on a 12-hour light/dark cycle under controlled temperature (20±1°C) and humidity (55±5%). Animals were given continuous access to food and water.

**Quantification of Tissue Platelet Distribution**

To quantify platelet deposition in tissues, animals were pretreated with 31Cr-labeled platelets. This technique does not alter platelet function assessed by aggregation and scanning electron microscopy.

**Histological Examination**

For transmission electron microscopy, the liver was trimmed into small blocks (~1 mm³) that were fixed in 1% osmium tetroxide (OsO4) in 0.1 mol/L cacodylate buffer solution (pH 7.4) at room temperature for 1.5 hours. The selected blocks were then dehydrated in a graded series of alcohol and embedded in Epon-812. Three micron sections were cut and mounted on 150-mesh copper grids (EMS). Sections were stained with lead citrate and borax and examined under a Hitachi H-600 electron microscope operated at 75 kV for scanning electron microscopy as described previously.

**Determination of Effect of Triflavin on Plasma Nitrate and Thromboxane A2 Concentration**

The concentration of nitric oxide (NO) and thromboxane A₂ (TxA₂) was determined in plasma as follows. Briefly, each rat was pretreated with triflavin (500 μg/kg), GRGDs (20 mg/kg), or isovolumetric normal saline 15 minutes before LPS (4 mg/kg IV bolus) challenge. Blood was drawn before starting and 4 hours after the femoral venous injection of LPS, mixed with sodium citrate (0.38% final concentration containing 250 μmol/L, clorglyline, chlorimipramine, pargyline, and 150 mmol/L NaCl), and perfused transcardially as described above. Citrated blood was immediately centrifuged and the supernatant (plasma) was collected and stored at −70°C before assay. For the estimation of tissue content of 5-hydroxytryptamine (5-HT), the respective organ was rapidly removed, weighed, and frozen in liquid nitrogen. Tissues were extracted in 0.4 mol/L HClO4 containing 2 mmol/L EDTA and 0.1% cystine-HCl in a homogenizer and sonicated. The homogenate was centrifuged at 10,000 g for 10 minutes, and then the supernatant was filtered through a Teflon membrane. The 5-HT in the resulting fluid was measured with an EIA kit (Immunotech).

**Determination of 5-Hydroxytryptamine**

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**Adhesion Assays**

Blood was collected from rats, and BCECF/AM-labeled platelet suspensions were prepared as described previously. In brief, the washed platelets were finally suspended in Ca2+-free Tyrode’s solution (3×10⁹ cells/mL). Fifty microliters of fibrinectin, vitronectin, vWF, and laminin (all at 1 μg/well) were added to the 96 wells (Costar) for 4 hours. After incubation, the solutions were aspirated, and the wells were filled with buffer containing BSA (0.5%). Equal volumes of BCECF-labeled platelet suspensions and peptides (triflavin and GRGDs) were mixed and collagen was added to
The administration of LPS (4 mg/kg IV bolus) for 4 hours produced a significant reduction in both radiolabeled platelets in blood (Table 1) and blood total platelet concentrations (data not shown). Less than 1% of injected radioactivity was detected in plasma and 0.002% of the total injected radioactivity was detected in the bile from either normal saline–treated rats (data not shown). On the other hand, 31Cr-labeled platelets markedly accumulated in the liver within 4 hours after LPS administration (Table 1). However, LPS did not significantly alter 31Cr-labeled platelet accumulation in the spleen, kidneys, or lungs as compared with normal saline–treated rats (Table 1). Pretreatment with triflavin (500 μg/kg) before LPS administration resulted in a significant increase in the number of radiolabeled platelets in the blood and markedly attenuated LPS-induced hepatic platelet accumulation (Table 1). The distribution of radiolabeled platelets in the spleen and kidney was not altered by pretreatment with triflavin after LPS challenge. Additionally, we found that pretreatment with triflavin followed by addition of LPS significantly reduced platelet accumulation in the lungs compared with the LPS-treated group (Table 1). However, GRGDS (20 mg/kg) had no significant effect in this reaction (Table 1), even at a higher concentration (100 mg/kg) (data not shown). These results indicate that triflavin but not GRGDS effectively reverses the platelet accumulation induced by LPS. Furthermore, triflavin (500 μg/kg IV bolus) also markedly reversed the alteration of radiolabeled-platelets in organ tissues in LPS-treated rabbits, indicating that the prevention of LPS-induced thrombocytopenia of triflavin is not limited to the rat model and may be effective in other species (ie, rabbits) (data not shown).

Administration of LPS induced a reduction in MAP detectable at 1 hour, which reached a nadir of ~75 mm Hg within 4 hours (Table 2). Heart rate increased by ~35 bpm at 2 hours and tended to remain elevated but with considerable fluctuations (data not shown). However, neither of the RGD-containing peptides significantly changed the hypotensive effect in LPS-treated rats (Table 2), even when both were administered at higher concentrations (triflavin, 1 mg/kg; GRGDS, 100 mg/kg) (data not shown). Furthermore, rats given an intravenous bolus of LPS at 4 mg/kg showed not only thrombocytopenia but also increases in hemoglobin (11.4 ± 0.8 g/dL vs 15.3 ± 0.6 g/dL, n = 4) and hematocrit (32.5 ± 2.5% vs 43.9 ± 2.2%, n = 4) as compared with normal saline–treated rats. However, triflavin (500 μg/kg) did not significantly affect the increase of hemoglobin (LPS-treated rats, 15.3 ± 0.6 g/dL vs triflavin-treated rats, 15.1 ± 0.5 g/dL, n = 4) and hematocrit (LPS-treated rats, 43.9 ± 2.2% vs triflavin-treated rats, 41.9 ± 2.8%, n = 4) induced by LPS.

**Results**

**Effect of Triflavin on LPS-Induced Thrombocytopenia in Rats**

The administration of LPS (4 mg/kg IV bolus) for 4 hours produced a significant reduction in both radiolabeled platelets in blood (Table 1) and blood total platelet concentrations (data not shown). Less than 1% of injected radioactivity was detected in plasma and <0.002% of the total injected radioactivity was detected in the bile from either normal saline–treated LPS-treated animals (data not shown). On the other hand, 31Cr-labeled platelets markedly accumulated in the liver within 4 hours after LPS administration (Table 1). However, LPS did not significantly alter 31Cr-labeled platelet accumulation in the spleen, kidneys, or lungs as compared with normal saline–treated rats (Table 1). Pretreatment with triflavin (500 μg/kg) before LPS administration resulted in a significant increase in the number of radiolabeled platelets in the blood and markedly attenuated LPS-induced hepatic platelet accumulation (Table 1). The distribution of radiolabeled platelets in the spleen and kidney was not altered by pretreatment with triflavin after LPS challenge. Additionally, we found that pretreatment with triflavin followed by addition of LPS significantly reduced platelet accumulation in the lungs compared with the LPS-treated group (Table 1). However, GRGDS (20 mg/kg) had no significant effect in this reaction (Table 1), even at a higher concentration (100 mg/kg) (data not shown). These results indicate that triflavin but not GRGDS effectively reverses the platelet accumulation induced by LPS. Furthermore, triflavin (500 μg/kg IV bolus) also markedly reversed the alteration of radiolabeled-platelets in organ tissues in LPS-treated rabbits, indicating that the prevention of LPS-induced thrombocytopenia of triflavin is not limited to the rat model and may be effective in other species (ie, rabbits) (data not shown).

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was no significant difference in 5-HT content in the lungs, kidneys, and spleen between normal saline–treated and LPS-treated rats (Figure 2). Triflavin but not GRGDS significantly reversed the 5-HT content in the blood and liver compared with LPS-treated rats (Figure 2).

Transmission Electron Microscopy of Liver Sections in Normal Saline–Treated and LPS-Treated Rats

In the livers of normal saline–treated rats, Kupffer cells were evenly distributed in the lumen of the sinusoid formed by intact endothelium (Figure 3A). A notable feature of the livers removed 4 hours after LPS administration was the presence of numerous platelets, located in the Disse spaces (Figure 3, B and C) between hepatocytes and endothelial cells. The endothelium appeared to be absent or severely damaged after LPS challenge. The platelets seen within the liver still retained granules and microtubules, indicating that these cells had not undergone degranulation. Figure 3A shows an absence of platelets in the sinusoidal spaces and lack of interaction between platelets and Kupffer cells in unchallenged liver tissue. In contrast, the majority of platelets in the sinusoidal spaces of the liver in LPS-treated rats were surrounded by well-developed microvilli of hepatocytes, and there was marked interaction between platelets and cell processes of endothelial cells (Figure 3B) or Kupffer cells (Figure 3C). Furthermore, many polymorphonuclear neutrophils were observed in the sinusoidal spaces, however, there was no identifiable attachment of the platelets to the neutrophils in LPS-treated livers (data not shown). Pretreatment with triflavin (500 µg/kg) obviously attenuated the LPS-induced hepatic platelet accumulation both in sinusoidal spaces and Disse spaces (Figure 3D). This result is also reflected in Table 1, which shows that pretreatment with a similar dose of triflavin resulted in a decrease of platelet accumulation in liver of LPS-treated rats.

Scanning Electron Microscopy of Aortic Vessels in LPS-Treated Rats

Figure 4A is a scanning electron micrograph of aortic endothelium in the control group rats. The LPS-treated aortic endothelium exhibited severe damage with platelets accumulating to the subendothelium (Figure 4B). The normal discoid shape of the platelets had changed to irregular spheres, and the extension of pseudopods was observed. On the other hand, pretreatment of triflavin (500 µg/kg) markedly reduced the accumulation of platelets to the subendothelium in the LPS-treated aorta tissue; however, triflavin did not significantly prevent the endothelial damage induced by LPS (Figure 4C). In contrast, GRGDS (20 mg/kg) did not significantly inhibit the accumulation of platelets to damaged endothelium (data not shown).

Effect of RGD-Containing Peptides on Platelet Adhesion to Immobilized Extracellular Matrixes

At 1 µmol/L, triflavin effectively inhibited the adhesion of platelets to fibronectin, vitronectin, and vWF by 86±8%, 78±7%, and 72±6%, respectively (Figure 1). Furthermore, at 2 mmol/L, GRGDS significantly inhibited the adhesion of activated platelets to fibronectin, vitronectin, and vWF but was less effective than triflavin. In contrast, triflavin and GRGDS did not obviously suppress the adhesion of activated platelets to laminin (Figure 5), suggesting that the adhesion of platelets to immobilized laminin may occur through a non–RGD-dependent pathway. The control peptide GRGES (2 mmol/L) had no significant effect on cell adhesion (Figure 5), indicating that RGD-containing peptides may interrupt the adhesion of activated-platelets to subendothelial matrixes fibronectin, vitronectin, and vWF but not to laminin.

Effect of RGD-Containing Peptides on Plasma Nitrate Formation in LPS-Treated Rats

As shown in Figure 6, nitrate production increased in LPS-treated rats. LPS caused an 8- to 28-fold rise in nitrate formation compared with that in normal saline–treated rats within a 4-hour period. Pretreatment with triflavin (500 µg/kg) did not significantly change NO production in LPS-treated rats. In contrast, pretreatment with PDTC (10 mg/kg),
an inhibitor of the nuclear factor κ-B (NFκ-B) activation, significantly inhibited LPS-induced NO formation in rats with LPS-induced septicemia (Figure 6). These results imply that the effect of triflavin prevented thrombocytopenia and that lowering the hepatic platelet accumulation was not related to the inhibition of NO production in LPS-treated rats.

**Discussion**

A number of abnormalities of blood coagulation have been described in association with septicemia, but the frequent occurrence of thrombocytopenia has also been emphasized. Thrombocytopenia occurs early in the course of septicemia and may alert the clinician to the possibility of septicemia. In this study, we used radiolabeled platelets to investigate the effect of RGD-containing peptides on LPS-induced thrombocytopenia in rats. We considered the possibility that the increase in hepatic radioactivity was due to free 51Cr that dissociated from platelets and was cleared into the bile. However, this is unlikely because bile from either normal saline–treated or LPS-treated rats contained very little radioactivity (data not shown).

The hepatic platelet accumulation and thrombocytopenia induced by LPS suggest that platelets are activated during LPS exposure. The loss of platelets in the blood was reflected in the significant and selective accumulation of platelets within the liver after LPS administration. The distribution of radiolabeled platelets in the lungs, kidneys, and spleen was not altered after exposure to LPS. Available evidence suggests that accumulation and activation of platelets within the liver of LPS-treated rats occur by indirect mechanisms. For example, injury to endothelial cells and consequent exposure of the subendothelium might activate the coagulation...
cascade and prompt platelet adherence and aggregation, thereby resulting in a decrease of platelets in circulation and an increase of platelet accumulation in the liver. However, the exact mechanisms by which LPS mediates hepatic platelet accumulation are not fully understood.

Activated platelets can release many inflammatory mediators, such as TxA₂ and 5-HT. Plasma TxA₂ concentrations are elevated in various models of septic shock. Furthermore, in the pathogenesis of septic shock, vasoactive amines such as 5-HT has been implicated as endogenous mediators contributing to liver dysfunction. The increase in 5-HT appears to be relatively specific for the liver and does not occur in other tissues. Our results provide confirmation of these findings (Figure 2). In fact, the inverse relation between hepatic and circulating 5-HT levels suggests that after LPS administration, 5-HT is mobilized from the blood into the liver. Examination of the changes of 5-HT accumulation between circulating blood and liver revealed a relation that was highly suggestive of hepatic platelet accumulation (Table 1 and Figure 2). Furthermore, examination of liver sections confirmed the presence of platelets without granulation in the sinusoidal and Disse spaces.

The series of experiments described in this report were performed to examine whether or not peptides containing the RGD sequence could effectively prevent LPS-induced thrombo-

Figure 4. Effect of triflavin on scanning electron micrograph of endothelium in normal saline-treated and LPS-treated rat thoracic aorta. Rats received (A) normal saline (control), (B) LPS (4 mg/kg IV bolus) alone, or (C) pretreatment with triflavin (500 μg/kg) followed by addition of LPS. Arrow indicates damaged endothelium; arrowhead indicates aggregating platelets. All photographs are in the same field and are a representative example of 5 similar experiments.

Figure 5. Effect of RGD-containing peptides on collagen-activated platelet adhesion to fibronectin (Fn), vitronectin (Vn), von Willebrand factor (vWF), and laminin (Ln) substrates. Platelets were washed, labeled, and activated with collagen (10 μg/mL) as described in Methods. GRGES (2 mmol/L); triflavin (1 μmol/L); and GRGDS (2 mmol/L). The extent of inhibition of adhesion is expressed as a percentage of platelets initially added to the plate. Data are presented as mean±SEM (n=6). *P<0.01; **P<0.001, significant difference compared with GRGES.

Figure 6. Effect of triflavin and PDTC in plasma nitrate concentration in LPS-treated rats at various times after the start of LPS injection. Rats received normal saline (C), LPS (4 mg/kg IV bolus; □) alone, or pretreatment with triflavin (500 μg/kg; ○) or PDTC (10 mg/kg; ▽) followed by addition of LPS. Data are presented as mean±SEM (n=6). *P<0.05; **P<0.01, significant difference compared with LPS-treated rats.
cytopenia in septic animals. The results revealed that triflavin but not GRGDS markedly reversed the alteration of radiolabeled platelets in organ tissues in LPS-treated rats. Furthermore, triflavin inhibited TxA₂ release within 4-hour periods and concurrently reversed the alteration of 5-HT accumulation in the blood and liver after LPS administration. Moreover, trigramin (500 μg/kg),²⁰ an RGD-containing disintegrin, also showed an obviously inhibitory effect at preventing LPS-induced hepatic platelet and 5-HT accumulations, but with a lower effectiveness than triflavin (data not shown).

In a previous report,²¹ triflavin was bound to the GP Ib/IIa complex of resting and activated platelets with a similar binding affinity, whereas small RGD synthetic peptides (ie, GRGDS) were bound with a much lower affinity to resting than to activated platelets.²⁰ In this study, rats were preincubated with RGD-containing peptides for 15 minutes followed by administration of LPS, indicating that triflavin had bound to the GP Ib/IIa complex of resting platelets before LPS administration. Therefore we speculated that the obvious inhibitory effect of triflavin on thrombocytopenia induced by LPS may be due at least partly to triflavin but not GRGDS, having a higher binding efficacy toward the GP Ib/IIa complex of the resting platelet membrane before administration of LPS, thereby leading to the prevention of LPS-induced platelet activation, reduced 5-HT release, and TxA₂ formation in platelets. This process eventually leads to a reduction in thrombocytopenia and hepatic platelet accumulation. Furthermore, the negative results with the small RGD peptide in this thrombocytopenia model may be related to a difference in efficacy or pharmacokinetics.

On the other hand, when endothelial continuity is disrupted, platelets rapidly adhere to the subendothelial components that are exposed. Several pieces of evidence strongly suggest that platelet GP Ib/IIa complex and vWF in plasma and/or in subendothelium mediate initial attachment. Li Platelet GP Ib/IIa complex provides binding sites for fibrinogen, fibronectin, vWF, and vitronectin. As shown in Figure 5, we found that triflavin is more effective than GRGDS at inhibiting the adhesion of platelets to immobilized matrixes (ie, fibronectin, vitronectin, and vWF), except for laminin. Taken together, these results indicate that the different abilities of triflavin compared with GRGDS to inhibit thrombocytopenia induced by LPS, at least in part, is related to its greater ability to inhibit the adhesion of platelets to extracellular matrices or subendothelium in LPS-treated aorta.

In conclusion, the most important finding in this study is that triflavin effectively prevents thrombocytopenia in LPS-treated rats. The inhibitory property of triflavin may involve the following 2 mechanisms: (1) Triflavin markedly inhibits platelet activation induced by LPS, resulting in decreased TxA₂ formation from platelets and a subsequent decrease of the TxA₂ level in plasma. (2) Triflavin inhibits the adhesion of platelets to subendothelium, thereby preventing the alteration of platelet accumulation in blood and liver in LPS-treated rats and subsequently lowering the 5-HT level in the liver and increasing the 5-HT level in the blood.

These results suggest that a combination of Arg-Gly-Asp-containing disintegrins with other therapeutic agents (ie, platelet-activating factor antagonists, antibiotics) may represent a new approach in the treatment of septicemia.

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