MAJOR ARTICLE

Antiplatelet Activities of Anthrax Lethal Toxin Are Associated with Suppressed p42/44 and p38 Mitogen-Activated Protein Kinase Pathways in the Platelets

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Anthrax lethal toxin (LT) is the major virulence factor produced by *Bacillus anthracis*, but the mechanism by which it induces high mortality remains unclear. We found that LT treatment could induce severe hemorrhage in mice and significantly suppress human whole-blood clotting and platelet aggregation in vitro. In addition, LT could inhibit agonist-induced platelet surface P-selectin expression, resulting in the inhibition of platelet–endothelial cell engagements. Data from Western blot analysis indicated that LT treatment resulted in the suppression of p42/44 and p38 mitogen-activated protein kinase pathways in platelets. Combined treatments with LT and antiplatelet agents such as aspirin and the RGD-containing disintegrin rhodostomin significantly increased mortality in mice. Our data suggest that platelets are a pathogenic target for anthrax LT.

The pathogenic mechanisms underlying the high mortality of systemic anthrax infections currently have no satisfactory explanation. The major virulence factor and exotoxin produced by *Bacillus anthracis*, lethal toxin (LT), is sufficient to cause animal death and reproduces manifestations similar to those induced by *B. anthracis* spore infections [1]. By contrast, LT-deficient *B. anthracis* have much less pathogenic behavior than do bacteria bearing a functional LT gene [2]. LT is composed of 2 proteins: protective antigen (PA) and lethal factor (LF) [3]. PA binds to specific cellular receptors and forms a membrane channel that mediates the entry of LF into the cell [4]. LF is a metalloprotease that can functionally inactivate the mitogen-activated protein kinase kinases (MEKs) through its protease activity [5]. When used as a treatment in vitro, LT can induce aberrant cytokine release [6] and cell death of macrophages [7–9]. These responses have been proposed to be involved in the lethal pathogenesis of *B. anthracis* infections [10]. However, recent data have resulted in continuing controversy about the potential roles of abnormal cytokine secretions and macrophage susceptibility in *B. anthracis* infections [11–13] and have indicated that the pathogenesis is far more complicated than previously suggested.

Our previous study and studies by other researchers have shown that LT can induce hemorrhage abnormalities in experimental animals [14–16]. In addition, hemorrhage symptoms have also frequently been found in patients [17–21]. Because platelet activation is associated with mitogen-activated protein kinase (MAPK) induction [22–24], these results prompted us to analyze whether LT has any antiplatelet effect. The present study shows that LT can inhibit coagulation both in

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vitro and in vivo. Using MAPK inhibitors PD98059 and SB203580 as controls, we found that the inhibition of platelet aggregation by LT treatment was correlated with the down-regulation of either p42/44 or p38 MAPK activity. In addition, we found that platelet surface P-selectin expression can also be inhibited by LT treatment, resulting in the inhibition of platelet–endothelial cell interaction. Furthermore, we found that coinjection with antiplatelet RGD-containing disintegrin and aspirin can increase mortality in LT-treated mice. These results strongly indicate that platelets are one of the pathogenic targets of LT and that LT-induced hemorrhage symptoms may play an important role in anthrax-induced pathogenesis.

MATERIALS AND METHODS

Bacterial strain and mice. The experiments in the present study were performed in accordance with guidelines of the National Science Council, Taiwan, Republic of China, for the use of experimental animals. Approval from the Animal Care and Use Committee of the Tzu-Chi University was obtained before these experiments were initiated.

The *B. anthracis* toxins PA and LF were purified from *B. anthracis* (ATCC 14186) culture supernatants [7, 25]. Anti-PA antibody that neutralizes LT-mediated cytotoxicity was purified from serum of rabbits immunized with PA (6 cycles at 3-week intervals; 100 μg each cycle). C57BL/6J mice were obtained from Jackson Laboratory; reagents were injected intravenously at the following doses: LT (LF:PA ratio, 1:5), 3.5 μg/g; aspirin, 100 μg/g [26]; and recombinant rhodostomin, 7 μg/g [27, 28]. Chemicals and proteins such as lipopolysaccharide (LPS; L4516), arachidonic acid (AA), aspirin, fibrinogen, thrombin, and bovine serum albumin (BSA) were purchased from Sigma-Aldrich.

Whole-blood clotting time, plasma clotting time, and D-dimer analyses. Anticoagulant acid citrate dextrose (ACD; 20 mmol/L citric acid, 110 mmol/L sodium citrate, and 5 mmol/L glucose; pH 7.3) were mixed with blood collected from mice treated with LT (at 1:4 vol/vol) or from healthy human donors (at 1:5 vol/vol). The LT and other inhibitors were incubated with the human blood samples at 20°C for 25 min or intravenously injected into mice 72–80 h before the experiments. The clotting of whole blood was initiated by adding a $CaCl_2$ solution to the blood sample at a final concentration of 5 mmol/L. The reactions were stopped by placing the mixture on ice, and the mixture was then centrifuged at 600 *g* at 4°C for 5 min. The efficiency of clot formation was calculated by measuring the weight percentage of solid clot mass versus the total sample, using the following equation: efficiency of clot formation = clot/total blood sample (wt/wt). The average clot formation efficiency of controls (BSA treated) within 25 min was adjusted to 100%. The plasma clotting time after calcification was measured using a method described elsewhere [29]. LT treatment was performed in vitro before the recalcification of plasma samples. Activated partial thromboplastin time (APTT) and prothrombin time (PT) analyses were performed to differentiate the intrinsic and extrinsic coagulation pathways, by use of a coagulometer (ACL Futura Plus; Instrumentation Laboratory), in accordance with the manufacturer's instructions. The control reagent—anti-fibrinogen IgG, which blocks plasma clotting—was purified from serum of rabbits immunized with human fibrinogen (6 cycles at 3-week intervals; 100 μg each cycle) utilizing a fibrinogen-conjugated affinity column. Levels of D-dimer (a degraded product of fibrin) [30] in mouse plasma were detected using ELISA kits (Imuclone; American Diagnostica). LPS was used as a positive control to induced D-dimer (5 ng/kg). Plasma was collected from mice after 48 h of BSA, LT, and LPS treatment; ~20%–25% of LT-treated mice showed weakness at this time point.

Platelet aggregation and MAPK pathway analyses. Washed platelets were prepared as described elsewhere [27, 31–34], and agonist-induced platelet aggregation was measured using a platelet aggregometer (Chrono-Log) [27, 31–34]. Platelet counts were determined using an automated hematology analyzer (KX-21; Sysmex). Antibodies against MAPK pathway kinases (p42/44, phospho-p42/44 [pp42/44], p38, and pJNK) [35] were purchased from Cell Signaling Technology, and anti-pp38 immunoglobulin was purchased from Promega. The gel intensities were measured using Image J (version 1.32; National Institutes of Health). The "vehicle" was dimethyl sulfoxide, PBS, or both, or, in different experiments, a solvent control. The doses of reagents used were as follows: thrombin, 0.5 U/mL; collagen, 5 μg/mL; platelet-activating factor (PAF), 0.1 μmol/L; AA, 100 μmol/L; PD98059, 40 μmol/L [36, 37]; SB203580, 40 μmol/L [38, 39]; and SP600125, 3 μmol/L.

Detection of agonist-induced platelet surface P-selectin expression by flow-cytometric analysis and ELISA. Purified platelets were preincubated with platelet inhibitors for 30 min at 25°C and treated with thrombin (0.02 U/mL) for an additional 10 min in the presence of inhibitors. After fixation (in 2% paraformaldehyde and 1× PBS for 1 h), the platelets were probed by an anti–P-selectin antibody (2.5 μg/mL; Serotec) and a fluorescein isothiocyanate–labeled anti-mouse IgG (Jackson ImmunoResearch Laboratories) and then were analyzed by use of flow cytometer (FACSCalibur; Becton Dickinson). To perform ELISAs, 96-well microtiter plates were coated with 0.1 mg/mL RGD peptide (RGDSP) for 1 h and blocked with 5% BSA for 1 h; platelet adhesion was then allowed to occur for 1 h at 25°C. After washing with PBS, platelets were pretreated with the MAPK inhibitors and LT (1 μg/mL) in Tyrode's buffer for 1 h and then were activated by thrombin (0.02 U/mL) or PAF (5 nmol/L) (without inducing aggregates). Surface-expressed P-selectin and fibrinogen were detected using specific antibodies (anti-CD62P [Serotec] and affinity-purified anti-fi-

brinogen IgG). Tyrode's buffer consisted of the following (all concentrations are in millimoles per liter): NaCl, 136; KCl, 2.7; $NaHCO_3$, 12; $NaH_2PO_4 \cdot 2H_2O$, 0.42; $CaCl_2$, 1.8; $MgCl_2 \cdot 6H_2O$, 1; glucose, 5.5; and HEPES, 5 (pH 7.4).

Platelet-fibrinogen substrate and platelet–endothelial cell engagements. Washed human platelets were pretreated with various inhibitors in calcium-free Tyrode's buffer for 30 min at 25°C and then incubated for an additional 10 min with thrombin (0.02 U/mL) or PAF (5 nmol/L) in the presence of inhibitors, without stirring. The platelet suspension was washed, resuspended in cell culture medium (Dulbecco's MEM), and adjusted to 5×10^7 cells/mL. Cell culture dishes (96 well) were coated with human fibrinogen (200 μg/mL at 37°C for 2 h) [27, 40] in fibrinogen adhesion experiments or grown with a confluent live human umbilical vein endothelial cell (HUVEC) monolayer in platelet–endothelial cell engagement experiments. Dishes were washed in 1× PBS before addition of 1 mL of the above platelet suspension to each well; the suspension was then incubated for 1 h at 37°C. After removal of suspended platelets, in fibrinogen experiments, the dishes were directly subjected to ELISA; in the endothelial cell experiments, the platelet-HUVEC complexes were fixed and permeated in solution (4% paraformaldehyde, 0.1% Triton X-100, and 1× PBS) for 1 h and then subjected to ELISA. The procedures utilized in the mouse platelet-fibrinogen binding experiments were similar, but the in vitro treatments of PA, LF, and LT were modified to allow administration to mice via intravenous injection 72–80 h before platelet purification. Anti-integrin α_{IIb} immunoglobulins (Santa Cruz Biotechnology) and HRP-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories) were used in ELISAs.

RESULTS

Whole-blood clotting inhibition by anthrax LT treatment. To test the hypothesis that the hemorrhage abnormalities documented in anthrax-infected patients and animals might directly contribute to LT toxicity, the clotting efficiency of citric acid anticoagulant–treated human blood samples was measured after recalcification following LT treatment in vitro. Our data showed that LT—but not PA, LF, or BSA—treatment significantly suppressed clot formation (figure 1*A* and 1*B*). Data on kinetic changes in clot formation also indicated that LT treatment could prolong whole-blood clotting (figure 1*C*). To further characterize the potential anticoagulant activities of LT in vivo, intravenously injected C57BL/6J mice were also examined. Consistent with the results of human blood experiments, the efficiency of mouse whole-blood clotting was significantly reduced by LT treatment. The blood specimens collected from mice treated with a lethal dose of LT for 72 h seemed to be unable to form clots normally, even after a 25-min incubation (figure 1*D*). To examine whether LT could inhibit the coagulant factors directly, we used 3 approaches, including plasma clotting time, APTT, and PT analyses. Our data showed that LT could not significantly prolong the plasma clotting time after recalcification; by contrast, when plasma was treated with the control agent, anti-fibrinogen polyclonal antibody (25 μg/mL), to block coagulation, the clotting time was significantly prolonged (figure 1*E;* control IgG could not prolong clotting time [data not shown]). Furthermore, consistent with the results of the plasma clotting time experiments, LT also showed no significant inhibitory effect in the APTT and PT experiments (figure 1*F*). Since platelets and plasma coagulant factors are 2 major components of the coagulation system, and since our data showed that LT could block whole-blood clotting but not plasma clotting, our results might indicate that platelets are the targets of LT.

Suppression of agonist-induced platelet adhesion to fibrinogen substrates and aggregation by LT treatment, correlated with the inhibition of p42/44 and p38 MAPK. LT is known to be an MEK-specific metalloprotease that can functionally inhibit MAPK pathways, and MAPK pathways are also important for platelet functions; we therefore hypothesized that LT might be able to block platelet activities. To test the hypothesis, we performed cell adhesion experiments, using AA to stimulate the platelets to adhere to fibrinogen substrates in the presence of LT or other platelet antagonists. Our data showed that LT, MEK inhibitor PD98059, and p38 MAPK inhibitor SB203580 pretreatment—but not JNK inhibitor SP600125 pretreatment—could significantly inhibit purified human platelet adherence to fibrinogen-coated substrates, compared with the control platelets (figure 2*A*, vehicle). Aspirin and the disintegrin rhodostomin RHO(RGD) [27, 40] are well-known platelet antagonists that served as positive controls to inhibit platelet-fibrinogen interactions, and the rhodostomin mutant RHO(RGE), which is a loss-of-function mutant at the RGD motif and is unable to inhibit platelet activation, served as the negative control [27, 40] (figure 2*A*). Because rhodostomin could preferentially bind to platelet integrin $\alpha_{IIb}\beta_3$ [41] and could almost totally block fibrinogen binding (figure 2*A*), these results suggest that LT could, at least in part, suppress platelet integrin $\alpha_{IIb}\beta_3$–mediated adhesion to fibrinogen substrates. Similar inhibitory effects of LT and MAPK inhibitors were also observed in the platelet aggregation experiments, which showed that LT, PD98059, and SB203580 could significantly inhibit the platelet aggregation induced by AA treatment (figure 2*B*), and quantitative results showed that the inhibitory effects of reagent treatments are consistent with the results of the fibrinogen adhesion experiments (figure 2*A* and 2*C*). Previous studies have demonstrated that thrombin-, collagen-, and PAF-induced platelet activations are less sensitive to MAPK inhibitors [22, 42]. If LT inhibits platelet activities primarily through MAPK pathways, the inhibition should be less effective when platelets are stimulated with these agonists. Consistent

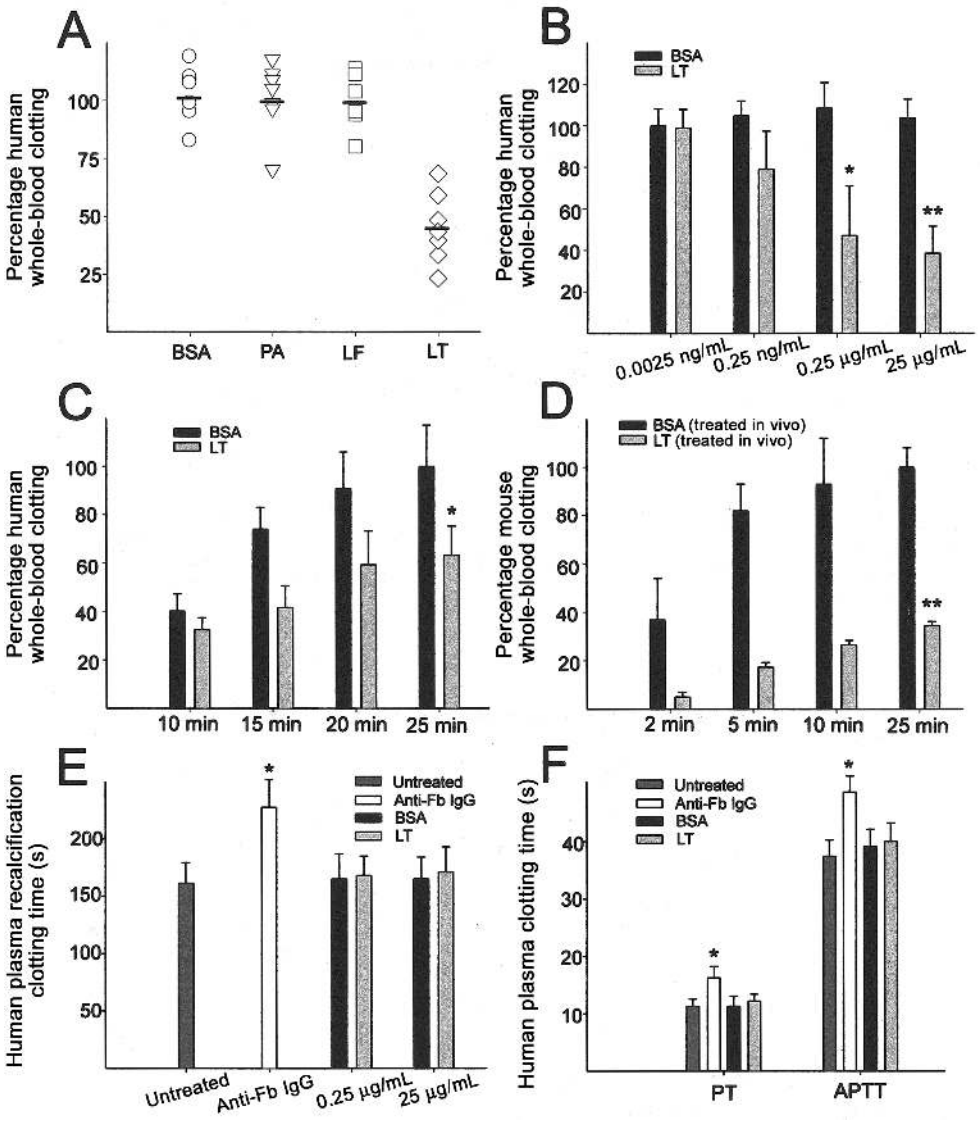

Figure 1. Anticlotting effects observed after anthrax lethal toxin (LT) treatment. Human whole-blood specimens were treated with equal concentrations (25 μg/mL) of bovine serum albumin (BSA), anthrax protective antigen (PA), lethal factor (LF), and LT, for examination of clot formation after recalcification ($n = 7$) *(A)* or dose dependence *(B)* and kinetic responses to LT and BSA treatment in suppressing clot formation in vitro ($n = 4$) *(C)*. The kinetic analysis showed the whole-blood clotting efficiency of LT- and BSA-treated mice ($n = 5$) *(D)*. Averaged data of the control groups (BSA treated) in panels A and B were adjusted to 100%; in panels C and D, the levels of BSA-treated blood clotting at 25 min were adjusted to 100%. Human plasma samples were treated with BSA and LT, for examination of clot formation after recalcification *(E)* or were subjected to activated partial thromboplastin time (APTT) and prothrombin time (PT) analysis ($n = 4$) *(F)*. The anti-fibrinogen (Fb)–treated group served as the positive control. *$P < .05$; **$P < .01$ (vs. BSA-treated control group in panels A–D; vs. untreated groups in panels E and F).

with this suggestion, our data showed that LT could not significantly block platelet aggregation through stimulation with thrombin, collagen, and PAF (figure 2*D*).

To characterize the effect of LT on platelets in vivo, platelets from LT-treated C57BL/6J mice were also examined. Consistent with results of a previous study [12], thrombocytopenia was induced after LT treatment in mice (figure 2*E*). However, adjustment of platelets purified from LT-treated mice to the same concentration as control platelets revealed that these platelets showed significantly less-efficient adherence to fibrinogen substrates (figure 2*F*). Since diseases such as sepsis and endotoxin shock, which involve disseminated intravascular coagulation (DIC), could also result in platelet disorders [30], to further determine whether the abnormalities of platelets in LT-treated

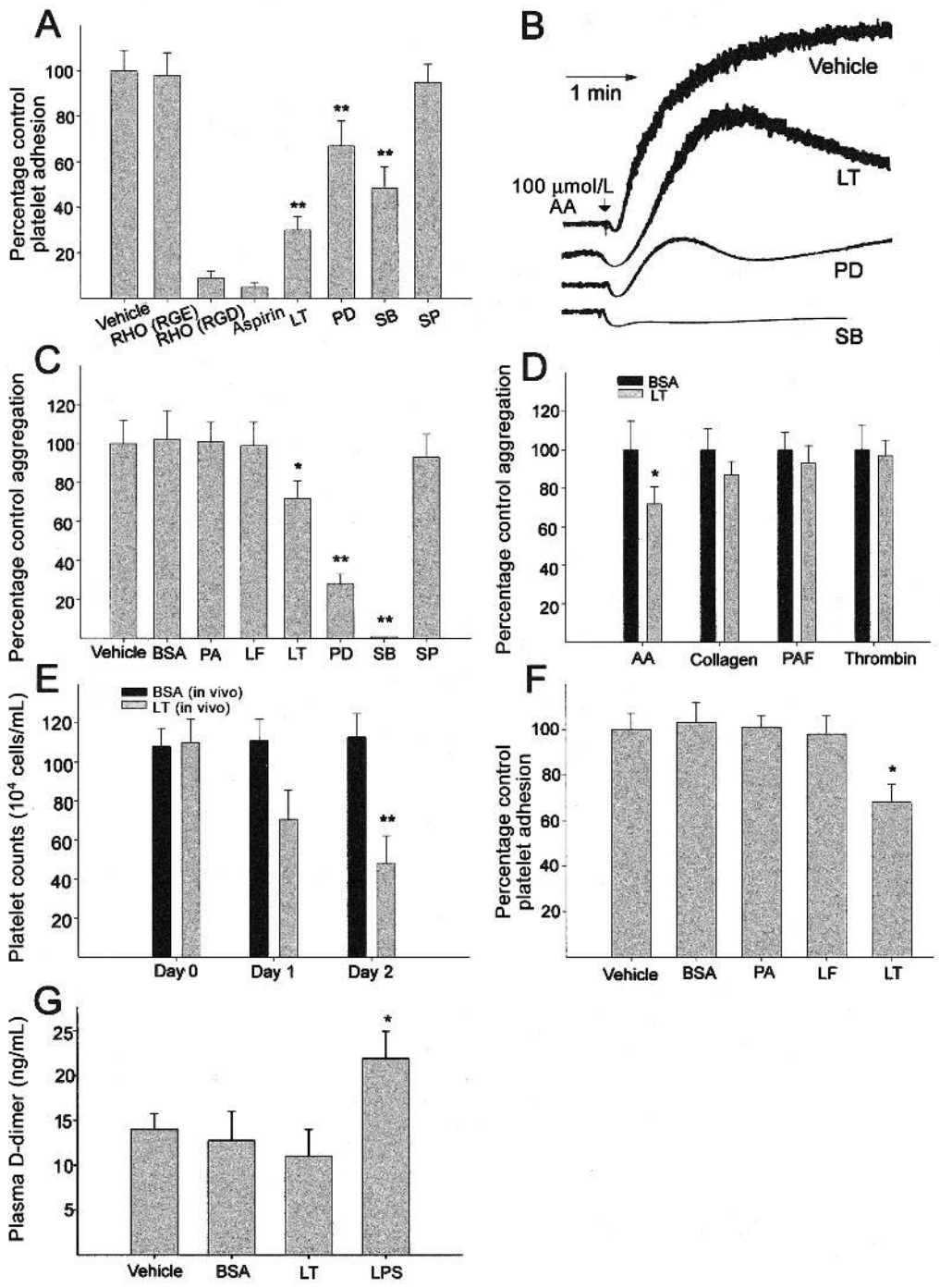

Figure 2. *A,* Antiplatelet effects observed after anthrax lethal toxin (LT) treatment. Arachidonic acid (AA; 100 μmol/L) induced human platelet adhesion to fibrinogen-coated substrates in the presence of various platelet inhibitors (rhodostomin RHO[RGD] and the D51E mutant RHO[RGE]), mitogen-activated protein kinase (MAPK) inhibitors, and LT. *B,* AA-induced platelet aggregation in the presence of LT and MAPK inhibitors PD98059 and SB203580. The *Y*-axis shows the transmission changes indicating platelet aggregation levels, and the *X*-axis shows the reaction time. The quantitative results of AA-induced platelet aggregation are also illustrated *(C),* and platelet agonists collagen, platelet-activating factor (PAF), and thrombin were also subjected to comparisons *(D).* Mouse platelet counts were recorded after LT treatment (3.5 μg/g) *(E);* platelets from these mice were purified, and their ability to adhere to fibrinogen-coated substrates was measured *(F).* Levels of mouse plasma D-dimer were also measured *(G);* lipopolysaccharide (LPS) was used as a positive control ($n = 4$). In panels A, C, and F, the averaged data of vehicle control groups were adjusted to 100%; in panels D and E, the averaged data of bovine serum albumin (BSA)–treated control groups were adjusted to 100%. $^*P < .05$; $^{**}P < .01$ (vs. vehicle control groups in panels A, C, F, and G; vs. BSA-treated groups in panels D and E). The concentrations of both SP98059 and SB203580 were 40 μmol/L, and the concentration of SP600125 was 3 μmol/L, if not indicated otherwise in the figure. LF, lethal factor; PA, protective antigen; PD, PD98059; SB, SB203580; SP, SP600125.

mice were caused by a primary bleeding disorder or a consumptive coagulopathy (e.g., DIC), we measured the plasma D-dimer levels, an indicator of DIC [30], in experimental mice. Our data showed that plasma D-dimer levels in mice were not significantly increased after treatment with LT; by contrast, LPS treatment significantly increased D-dimer levels in mouse plasma (figure 2*G*). This result indicated that, even though LT treatment can suppress platelet activity at both qualitative and quantitative levels in vivo, LT-mediated platelet disorders are not caused by a consumptive coagulopathy.

To confirm that platelet inhibition of LT is correlated with cellular MAPK activity, we performed Western blot analysis to detect the levels of pp42/44 and pp38 MAPKs, which indicate the activation status of the kinases in platelets. To obtain the internal and loading controls, we also measured the total cellular p42/44 and p38 MAPK and actin levels by use of specific antibodies on duplicated blots. We found that LT could significantly suppress AA-stimulated pp42/44 and pp38 MAPK induction to almost basal levels (figure 3*A*). Anti-PA immunoglobulin that neutralizes LT cytotoxicity was used as a control, which counteracted the suppression of p42/44 and p38 pathways by LT treatment (figure 3*A;* control immunoglobulin could not neutralize LT [data not shown]). After normalization to the loading controls, LT treatment reduced the estimated AA-induced phosphorylation of p42/44 and p38 MAPKs in platelets to ~37% and ~30%, respectively (figure 3*B*). Since p42/44 and p38 MAPK pathways were significantly blocked by LT treatment at a dosage sufficient to suppress platelet functions, our data suggested that this LT-mediated blockade was likely associated with the suppression of p42/44 and p38 MAPK pathways.

Inhibition of agonist-induced platelet surface P-selectin expression by LT treatment. P-selectin is a glycoprotein stored in the α granules of platelets and is translocated to the surface on activation, which is responsible for platelet rolling and tethering to the vessel walls and important for thrombus formation [43]. Here, we employed flow-cytometric analysis to examine whether LT treatment could affect agonist-induced surface P-selectin expression. Intriguingly, although LT could not inhibit thrombin (0.5 U/mL)–induced aggregation (figure 2), our data showed that LT treatment could suppress thrombin (0.02 U/mL)–induced P-selectin expression on platelet surfaces (figure 4*A*), indicating that LT suppresses platelet functions at various levels. ELISA was employed for comparisons; it showed that both PAF- and thrombin-induced surface P-selectin and fibrinogen expression were blocked by either LT or p38 and p42/44 MAPK inhibitor treatment (figure 4*B* and 4*C*). Because LT suppression of surface P-selectin levels might reduce platelet-endothelium tethering, we further examined platelet–endothelial cell engagement. The results showed that pretreatment of platelets with the agonists PAF and thrombin before interaction with endothelial cells (HUVECs) could enhance platelet–endothelial cell binding (figure 5*A*). In addition, such cell-cell engagement was suppressed by specific blocking antibody against P-selectin (figure 5*B*), indicating the involvement of P-selectin in this process. When pretreated with LT, MEK inhibitor PD98059, and p38 MAPK inhibitor SB203580, the endothelial cell–binding activity of platelets was significantly reduced (figure 5*B*). Since LT could inhibit platelet surface P-selectin expression (figure 4), our data suggested that LT suppressed platelet–endothelial cell binding, at least in part, through the P-selectin pathway.

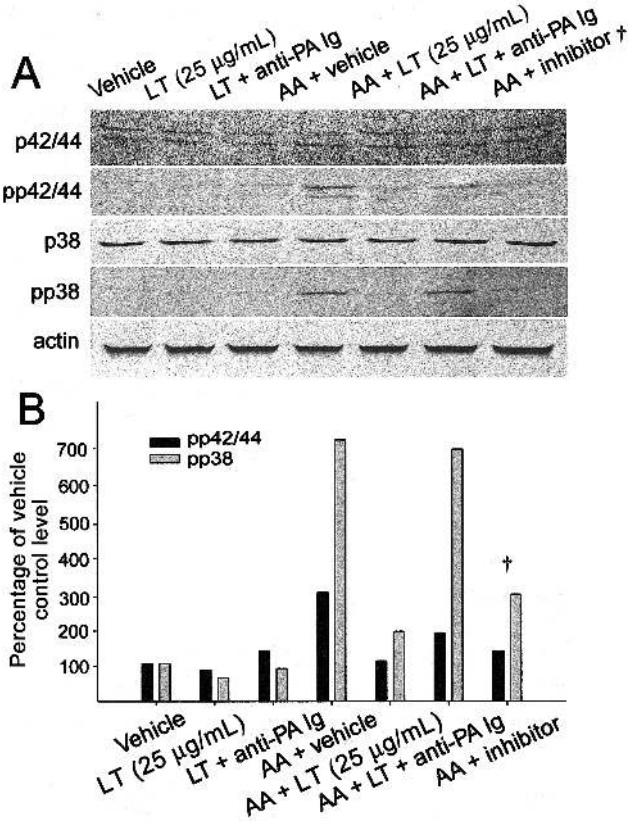

Figure 3. Western blot analysis for mitogen-activated protein kinases (MAPKs) in platelets with or without anthrax lethal toxin (LT) treatment. The data show that pp42/44 and pp38 MAPK levels were down-regulated in arachidonic acid (AA)–activated platelets after LT treatment *(A).* The levels of p42/44, p38, and actin were probed from duplicated blots. Quantitative results were derived by normalizing the blotted intensities of the phosphorylated kinase with the corresponding total kinase intensity. The levels of blotted intensities in vehicle-treated groups were adjusted to 100% *(B).* Inhibitors PD98059 and SB203580 were used in the p42/44 and p38 experiments, respectively (†). PA, protective antigen.

Increase in mortality of anthrax LT–treated mice, resulting from treatment with the platelet inhibitors aspirin and rhodostomin. After the in vitro analysis, it was important to confirm the pathogenic role of antiplatelet effects in LT-mediated mortality. If antiplatelet effects play a role in LT-mediated

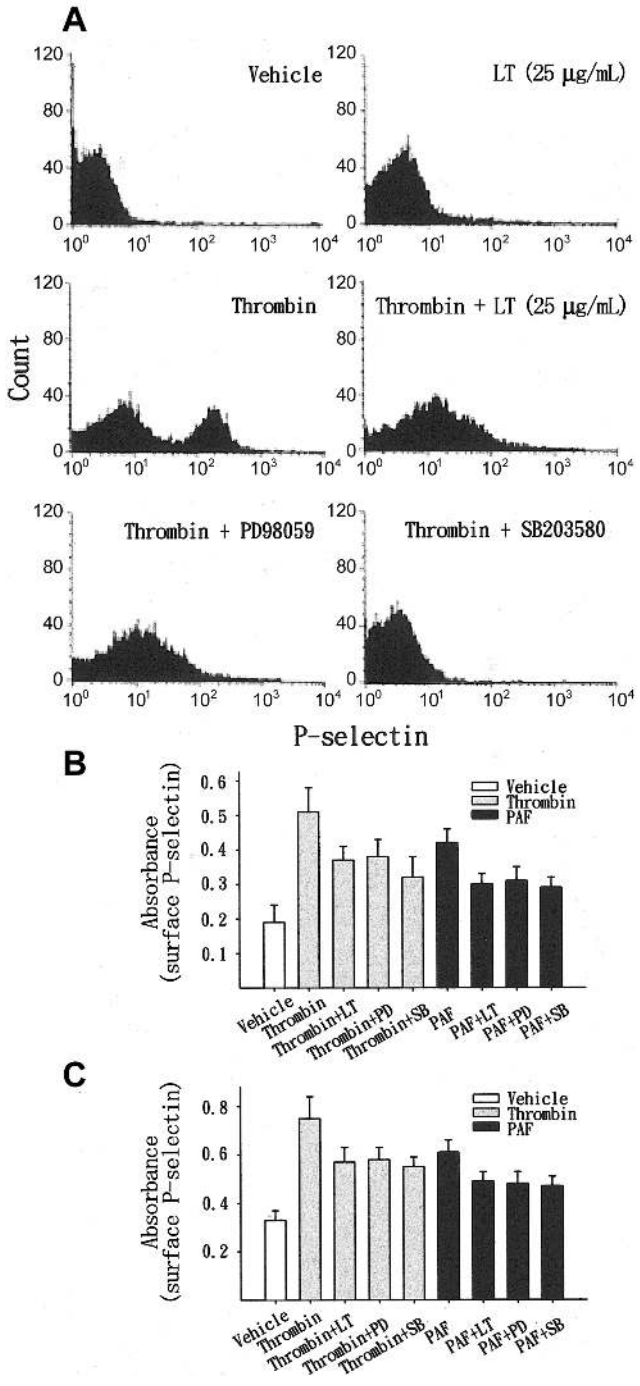

Figure 4. Surface P-selectin expression of platelets influenced by anthrax lethal toxin (LT) treatment. Flow-cytometric analysis showed that surface expression of P-selectin on platelets was affected by thrombin (0.02 U/mL), LT (25 μg/mL), mitogen-activated protein kinase kinase inhibitor PD98059, and p38 mitogen-activated protein kinase (MAPK) inhibitor SB203580 treatments *(A).* ELISA showed that surface P-selectin and surface fibrinogen was affected by thrombin, platelet-activating factor (PAF), LT (1 μg/mL), and MAPK inhibitor treatments (*B* and *C*). PD, PD98059; SB, SB203580.

pathogenesis, we might expect the mortality of LT-treated animals to increase or speed up under platelet-suppression conditions. Our strategy for testing this hypothesis was to coinject 2 potent platelet-activation inhibitors, aspirin and rhodostomin, together with a lethal dose of LT into experimental mice and monitor the effects of these agents on mortality. Our data showed that C57BL/6J mice died within 6 days after LT (3.5 μg/g) treatment. However, when coinjected with a single dose of aspirin or rhodostomin RHO(RGD), the time to mortality was decreased (figure 6). Aspirin-LT–treated mice died within 3 days after treatment, and rhodostomin-LT–treated mice died within 4 days after treatment (figure 6). By contrast, treatments utilizing LT plus RGD-motif–mutated RHO(RGE) did not speed up lethality in mice (figure 6). In contrast, control mice treated only with control protein BSA, aspirin, RHO(RGD), or RHO(RGE) showed no mortality within 5 months (data not shown). Our data indicated that platelet activity is a crucial determinant of mortality after LT treatment.

DISCUSSION

This work demonstrates the antiplatelet activities of anthrax LT. Using a platelet aggregation assay, we found that LT is able to inhibit AA-induced platelet aggregation directly; treatment with LT plus antiplatelet agents such as aspirin and rhodostomin increased mouse mortality, indicating that platelet defects play a role in LT-induced pathogenesis. Hemorrhage abnormalities are frequently found as clinical manifestations of anthrax and are usually associated with lethality [10]. For example, the stools or vomitus of patients with oropharyngeal/gastrointestinal anthrax are often blood tinged, and morbidity due to blood loss–associated fluid and electrolyte imbalances, as well as subsequent shock, has been documented [10]. In inhalation anthrax, severe hemorrhage abnormalities such as hemorrhage mediastinitis, hemorrhage pneumonitis, and submucosal hemorrhage lesions in the trachea and bronchi are often found [10]. In animal experiments, rodents (authors' unpublished data), rabbits [44], and monkeys [15, 16] have also been noted to suffer from hemorrhage symptoms after infection with anthrax. These findings indicate that hemorrhage disorders are involved in anthrax-induced mortality.

Although anthrax LT is known to be the dominant virulence factor of *B. anthracis,* its ability to induce hemorrhage abnormalities has not yet been clearly demonstrated. In current models, the hemorrhage symptoms caused by anthrax infection have been suggested to be due to bacterial propagation and aberrant immune responses. A recent study by Kirby showed that LT could induce endothelial-cell apoptosis [45]. When combined with our data, these results suggest that LT might induce hemorrhage through the creation of a vascular wound and the suppression of platelet function in vivo. The cytotoxicity found in

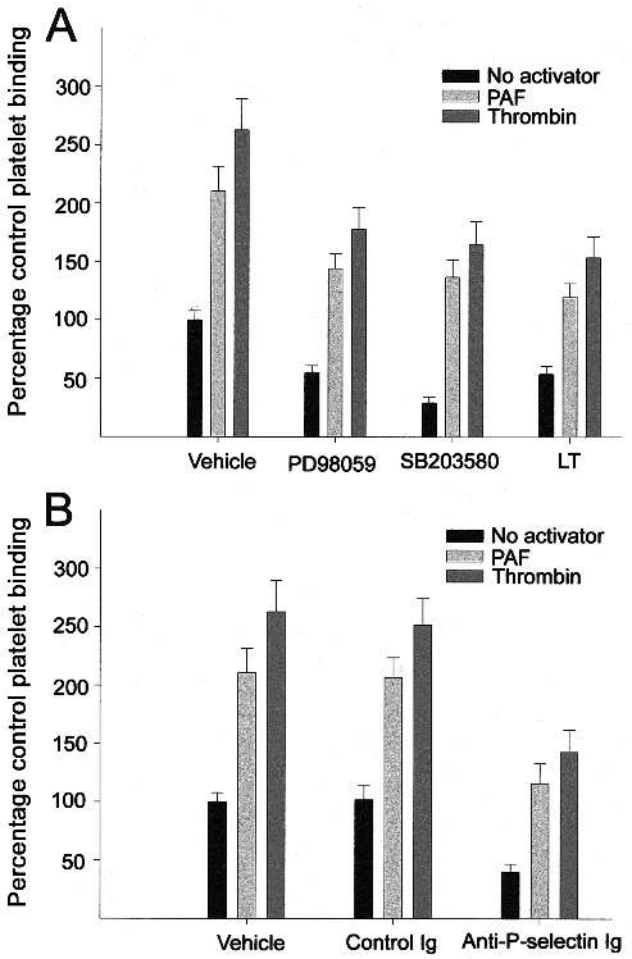

Figure 5. Treatments leading to the inhibition of agonist-induced platelet–endothelial cell engagements. Pretreatment with the anthrax lethal toxin (LT) and mitogen-activated protein kinase (MAPK) inhibitors PD98059 and SB203580 suppressed thrombin- and platelet-activating factor (PAF)–induced platelet–endothelial cell engagements *(A)*. Anti–P-selectin antibody—but not control antibody—treatment suppressed thrombin- and PAF-induced platelet–endothelial cell engagements *(B)*. The averaged data of vehicle control mice were adjusted to 100%.

macrophages and endothelial cells is mainly mediated through p38 and p42/44 MAPK pathways, respectively; our results, however, indicate that both of these pathways were blocked by LT treatment and were vital for platelet activation, which suggests that tissue-specific regulation of the MAPK pathways influenced the cytotoxicity effects.

Recent studies have shown that P-selectin expression on platelets is important for shear-induced aggregation and stable aggregate formation [46, 47]. When P-selectin was blocked by a monoclonal antibody, platelet aggregation was significantly inhibited [47]. P-selectin could also interact with P-selectin glycoprotein ligand 1 and von Willebrand factor to facilitate platelet endothelial interactions [48, 49], a process suggested to be important for recovering from hemorrhage conditions. LT might therefore, at least in part, mediate antiplatelet activities through inhibition of the P-selectin pathway. Previous studies have paid much greater attention to abnormal immune responses after LT treatment than to the coagulant system. Aberrant cytokine release by intoxicated macrophages have been proposed as a potential mechanism of LT-induced pathogenesis; however, more and more evidence has suggested that LT might have antiimmune activities. For example, LT treatment could inhibit dendritic cell function and result in suppressed adaptive immunity in mice [50] or a "silent" innate immune response without tumor necrosis factor–α induction [12, 51]. The mechanisms responsible for such inhibitory effects on innate and adaptive immunity are not clear. P-selectin plays important roles in inflammation through mediation of leukocyte-endothelium engagement [52, 53]. P-selectin expression on platelet surfaces is known to induce platelet-leukocyte interactions and might facilitate leukocyte rolling on vessel walls [54]. Although it remains to be established, suppression of platelet P-selectin expression and endothelial engagement by LT treatment might provide an explanation for suppressed inflammatory and immune response in LT-treated animals. Further characterization of the relationships between selectin pathways and innate/adaptive immune responses during LT treatment—as well as determination of whether the expression of proinflammatory molecules, such as selectins, in other cell types is also affected by LT—is warranted.

In summary, we found that LT could directly inhibit whole-blood clotting, platelet aggregation, surface P-selectin expression, and platelet–endothelial cell interactions. When mice were treated with the antiplatelet drugs aspirin and rhodostomin plus LT, their mortality significantly increased. Our data indicate that platelets are the pathogenic targets of LT and that antiplatelet activities of LT contribute to the pathogenic process.

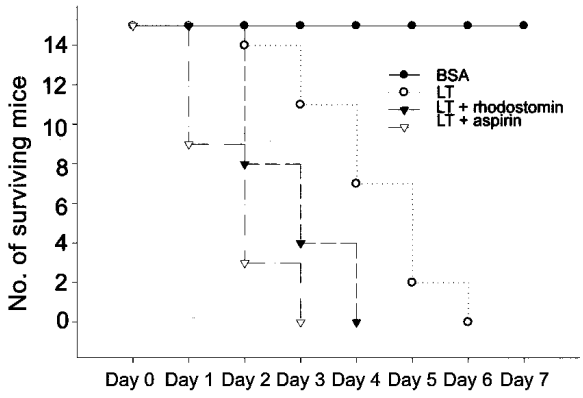

Figure 6. Increase in mortality of anthrax lethal toxin (LT)–treated mice, as a result of coinjection with a single dose of the antiplatelet agent aspirin or rhodostomin (RHO). BSA, bovine serum albumin.

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