Endotoxin enhances microvascular thrombosis in mouse cremaster venules via a TLR4-dependent, neutrophil-independent mechanism

Rolando E. Rumbaut,1,2,3 Ricardo V. Bellera,3 Jaspreet K. Randhawa,2 Corie N. Shrimpton,2 Swapan K. Dasgupta,4 Jing-Fei Dong,2 and Alan R. Burns2,3

1Medical Care Line, Michael E. DeBakey Veterans Affairs Medical Center, and Departments of Medicine, Pediatrics, and Pathology, Baylor College of Medicine, Houston, Texas
2Submitted 29 March 2005; accepted in final form 8 November 2005

Endotoxin (lipopolysaccharide, LPS) is a cell wall component of gram-negative bacteria; administration of LPS to humans reproduces many of the manifestations of gram-negative sepsis (29, 42). This condition is associated with significant morbidity and mortality and is accompanied by endothelial dysfunction and a prothrombotic state (1). Endotoxemic animal models of sepsis have demonstrated microvascular alterations consistent with the prothrombotic phenotype.

In a mouse intestinal microcirculation model, Cerwinka et al. (5) reported that endotoxemia induced stable adhesion of fluorescently labeled platelets to postcapillary venules in vivo. The LPS-induced platelet adhesion was dependent on circulating neutrophils as adhesion of platelets was attenuated in animals rendered neutropenic (6). Whether neutrophil-dependent platelet adhesion induced by endotoxemia may serve as a substrate for additional platelet recruitment and thereby enhance the rate of microvascular thrombosis in vivo remains to be determined.

We reported recently a mouse cremaster model of microvascular platelet thrombus formation (36) based on the description by Thorlacius et al. (43). In this model, a light/dye injury of microvascular endothelium induces adhesion and aggregation of platelets, with platelet thrombus formation occurring in the absence of endothelial denudation (32, 36). In the present study, we applied this microvascular thrombosis model to determine whether endotoxemia modified the rate of formation of microvascular thrombi in vivo and whether the responses were altered in mice rendered neutropenic or in mice deficient in Toll-like receptor 4 (TLR4), a primary endotoxin signaling receptor (28). Furthermore, we determined whether endotoxemia in mice resulted in enhanced platelet P-selectin expression, a marker of platelet activation. These findings support the notion that endotoxemia promotes platelet thrombus formation independent of neutrophils and without enhancement of platelet aggregation, via a TLR4-dependent mechanism.

MATERIALS AND METHODS

**Surgical preparation.** Male mice, 12–24 wk old, ~30 g were used. C57BL/6J (wild-type), C57BL/10ScNJ (TLR4-deficient), and C3H/HeJ (with a missense mutation in the TLR4 gene, see Ref. 31) were purchased from the Institute for Laboratory Animal Research. Mice were randomly assigned to each group.

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Jackson Laboratories; C3H/HeN mice (controls for C3H/HeJ) were purchased from Charles River. Animal protocols were approved by the Institutional Animal Care and Use Committee of Baylor College of Medicine. Mice were anesthetized with an intraperitoneal injection of pentobarbital sodium (50 mg/kg), with additional doses (12.5 mg/kg) as needed. The mice were then placed on a custom Plexiglas tray and maintained at 37°C with a homeothermic blanket and monitored with a rectal temperature probe (FHC, Inc.). A tracheotomy was performed to facilitate breathing, and an internal jugular vein and common carotid artery were cannulated for intravenous drug administration and blood pressure monitoring, respectively.

The cremaster muscle was exteriorized as outlined previously (36), based on the methods described by Baez (4). Briefly, a midline incision was made through the skin and fascia, and the cremaster muscle was dissected from the connective tissue. The exposed muscle was pinned gently on a Sylgard (Dow) pedestal, and subsequently a midline incision was made from the apex to the inguinal canal with care taken to minimize damage to the vessels. The cremaster muscle edges were pinned radially on the Sylgard pedestal, and the connective tissue between the cremaster and the epididymis was dissected. After that, the testis and epididymis were pushed back gently into the abdominal cavity. Throughout the experiment, the cremaster was superfused with bicarbonate-buffered saline of the following composition (in mmol/l): 127 NaCl, 4.7 KCl, 2.0 CaCl2·2H2O, 1.2 MgSO4, 28.0 NaHCO3, and 5 glucose, at the rate of 5 ml/min. The buffer was bubbled continuously with a 95% N2-5% CO2 gas mixture to maintain a pH between 7.35 and 7.45. The temperature of the buffer at the tissue interface measured with an oxygen electrode (Microelectrodes) was ~20 mmHg.

Intravital microscopy. The preparation was then transferred to the stage of an upright intravital videomicroscope (BX-50, Olympus) and allowed to equilibrate for 30 min. A x4 (numerical aperture 0.13) objective was used to survey the microvascular preparation for selective vessel were measured, photoactivation was initiated by exposure to an ultraviolet light source (365 nm) for 1 s duration. Mean blood velocity (V̇mean) was calculated based on the Newtonian definition:

\[ \gamma = \frac{V_{\text{mean}}}{\text{diameter}} \]

Photoactivation protocol. After V̇mean and diameter of the selected vessel were measured, photoactivation was initiated by exposing ~100 μm of the vessel length to epi-illumination, with a 175-W xenon lamp (Lambda LS, Sutter) and a fluorescein filter cube (HQ-405/48, Chroma). The excitation power density was measured daily (IL 1700 Radiometer, SED-033 detector, International Light) and maintained within 1% of the target of 0.6 W/cm², as described previously (36, 37). Epi-illumination was applied continuously, and the following times were recorded: 1) time of onset of platelet aggregates, and 2) time of flow cessation (≥60 s duration).

Fluorescent platelet adhesion protocol. To determine the influence of endotoxin (with and without anti-neutrophil antibodies) on platelet adhesion in the absence of light/dye injury, we used a method similar to that described by Cooper et al. (11). Briefly, ~0.9 ml of blood was withdrawn through a carotid cannula from a donor mouse, mixed with 0.1 ml of acid-citrate-dextrose buffer (Sigma), and placed in a polycarbonate tube. The blood was centrifuged at 260 g for 8 min, and then platelet-rich plasma (PRP) was obtained by a repeat centrifugation at 260 g for 3 min. Platelets were pelleted by being centrifuged at 740 g for 10 min. The platelet pellet was resuspended gently in 500 μl phosphate-buffered saline (PBS, Sigma) and allowed to rest for 30 min during which platelets and leukocytes in a 20-μl sample of the solution were stained (with cresyl blue and crystal violet, respectively) and counted on a hemocytometer. This method resulted in <0.01% leukocytes in the platelet suspensions, similar to that reported by Cooper et al. (11). Thereafter, 200 × 10⁶ platelets were suspended in 1.5 ml PBS, and 9 μl of carboxyfluorescein diacetate-N-succinimidyl ester (CFSE, final concentration 15 μM, Sigma) were added into the platelet solution. Finally, the solution was centrifuged at 740 g for 10 min, and the pellet was resuspended gently in 200 μl PBS and injected intravenously into the recipient mouse. Epi-illumination for platelet adhesion experiments was performed with the same system used for photoactivation, although the excitation power density was reduced with a neutral density filter (outer diameter 1.3, Edmund Optics) to 0.03 W/cm², and the total duration of light exposure was considerably <1 min.

To confirm that the relative adhesion of exogenous fluorescent platelets was comparable to that of endogenous platelets, we performed immunohistochemistry as shown previously (38). Briefly, after visualization of adhesion of fluorescently labeled platelets as described above, mice were euthanized by exsanguination under surgical plane of anesthesia, and 20 ml of isotonic saline were infused at 100 mmHg via a cannula placed in the right ventricle. Once blood in the major cremaster vessels had cleared, the muscle was fixed with 4% paraformaldehyde, excised, and placed in the same fixative for 30 min. The muscle was rinsed with PBS, placed in 0.1% Triton X for 20 min, and then rinsed again with PBS. Tissue samples were subsequently immersed in blocking buffer (PBS containing 0.1% Triton-X and 1% BSA) for 20 min. Samples were then incubated in blocking buffer containing primary antibody (rat anti-mouse glycophorin Ibo; 10 μg/ml; Emfret) for 60 min, and this was followed by three washes in blocking buffer before final labeling in secondary antibody (goat-anti-rat IgG conjugated to Texas Red; 10 μg/ml; Molecular Probes) for 60 min. Finally, immunolabeled samples were rinsed in PBS and mounted in Airvol 205/Celvol 205 (Celanese) beneath a no. 1.5 coverslip. Green and red fluorescence images were recorded with a 20× objective (numerical aperture 0.70) using a deconvolution restoration microscope system (Delta Vision Spectris, Applied Precision).

Experimental groups. To determine the influence of endotoxemia on platelet adhesion or microvascular thrombosis, mice were injected intraperitoneally with either endotoxin (LPS) from Escherichia coli serotype 0111:B4 (purified initially by phenol extraction and purified further by ion exchange chromatography, Sigma L3024) at the specified doses in 0.5 ml of sterile, pyrogen-free isotonic saline, or 0.5 ml saline, either 2 or 4 h before the experiments. We used two lots of LPS, designated lot A (072K4093) and B (044K4119) with different endotoxin units (EU) per mg (3,000,000 vs. 1,000,000 EU/mg, respectively) and differential biological activity as indicated in the RESULTS section. The doses used ranged from 1.5 to 5.0 × 10⁶ EU/kg body wt, as detailed below. In some experiments, we repurified LPS to exclude participation of LPS-associated lipoproteins, reported to have significant biological activity, inducing nonspecific signaling via Toll-like receptor 2 (TLR2, Ref. 18). We used the phenol extraction repurification method described by Manthey et al. (25), which eliminates LPS-induced signaling via TLR2 without affecting TLR4 responses (18). Briefly, 2.5 mg of LPS were suspended in 500 μl of...
endotoxin-free water with 0.2% triethylamine (TEA). Deoxycholate was added for a final concentration of 0.5%, followed by 500 μl of water-saturated phenol. After being vortexed for 5 min, the phases were allowed to separate at room temperature for 5 min. Samples were then placed on ice for 5 min and centrifuged at 4°C for 2 min at 10,000 g. The top aqueous layer was collected, and the phenol phase was reextracted with 500 μl of 0.2% TEA-0.5% deoxycholate. The aqueous phases were pooled and reextracted with 1 ml of water-saturated phenol. The pooled aqueous phases were adjusted to 75% ethanol and 30 mM sodium acetate and were allowed to precipitate at 20°C for 1 h. The precipitates were centrifuged at 4°C for 10 min at 10,000 g, washed in 1 ml of cold 100% ethanol, and air-dried. The precipitates were resuspended in the original volume (500 μl) of 0.2% TEA.

Recovery of LPS was 33% by limulus amebocyte lysate assay (Cambrex).

To determine the influence of neutrophils on endotoxin-induced responses, mice were treated with 150 μg ip of an anti-neutrophil antibody (RB6–8C5, Purified Rat Anti-Mouse Ly-6G, azide-free/low-endotoxin, BD Pharmingen) 24 h before the experiment. At the conclusion of the experiments, ~0.5 ml blood was collected in EGTA-coated tubes, and total leukocyte and differential counts were performed with a Coulter Counter by a reference laboratory (Comparative Pathology Laboratory at Baylor College of Medicine).

In all cases, the investigator performing intravital microscopy and data analysis was blinded with regard to genotype and test agents administered to each mouse. In some experiments, cremaster tissue samples were prepared for electron microscopy as described below. The microscopist was blinded with regard to the experimental group for each individual sample.

**Platelet aggregometry.** To determine whether endotoxemia had a direct effect on mouse platelet aggregation ex vivo, we collected ~0.9 ml of blood in 0.1 ml 3.8% sodium citrate from saline- and LPS-treated mice at 2 and 4 h after injection. For these studies, PRP was obtained by centrifugation at 120 g for 5 min, repeated once. The PRP from five to six mice for each group was pooled, and samples were adjusted to equal platelet counts with HEPES-Tyrode buffer pH 7.4 (in mM: 10 HEPES, 138 NaCl, 5.5 glucose, 12 NaHCO3, 0.4 MgCl2, 2.9 KCl, 0.36 Na2HPO4, and 1.0 CaCl2). Platelet aggregation was induced with stirring (1,200 rpm) at 37°C by either 20 μM of ADP or 5 μg/ml of fibrillar type I collagen (both from Helena Laboratories) and recorded in a four-channel aggregometer (Bio/Data).

**Platelet flow cytometry.** We used flow cytometry to determine whether endotoxemia induced platelet P-selectin expression, an marker of platelet activation. Fresh anticoagulated blood (0.1 ml 3.8% sodium citrate-0.9 ml blood) was diluted 1:1 with Tyrode buffer containing 1 μM PGL2, and PRP was isolated by centrifugation at 500 g for 10 min. PRP was then diluted five times with the same buffer and centrifuged at 1,300 g for 10 min. The platelet pellet thus obtained was resuspended in Tyrode buffer to a final concentration of about 2 × 10⁸ platelets/ml. Platelets were activated with thrombin (1 U/ml) for 10 min at room temperature. Phycoerythrin-labeled anti-mouse P-selectin antibody (7.5 μl, Emfret) was added to ~10⁸ platelets in 25 μl and incubated for 30 min at room temperature in the dark. The reaction was stopped by adding 400 μl PBS. Samples were analyzed on a flow cytometer (Coulter EPICS XL, Beckman-Coulter); acquisition and data analysis were performed with software EXPO32. Ten thousand events per sample were acquired to ensure adequate mean fluorescence levels; light scatter and fluorescence channels were set at a logarithmic gain. The platelet population was analyzed for fluorescence intensity, and mean fluorescence intensities were defined by comparison with unstained platelets.

**Electron microscopy.** Cremaster tissue samples were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (1 h) followed by 1% tannic acid (5 min). Postfixation in 1% osmium tetroxide (1 h) was followed by en bloc fixation in aqueous uranyl acetate. The samples were subsequently dehydrated in a graded ethanol series and embedded in LX 112 resin (Polysciences). Ultrathin sections (80 nm) were obtained using an ultramicrotome (RMC 7000, RMC) equipped with a diamond knife. Sections were stained with uranyl acetate and lead citrate before being viewed with a JEOL 200CX electron microscope.

**Statistics.** All data are expressed as means ± SE; comparisons of thrombosis times was done with one-way analysis of variance with Fisher’s post hoc test by using Statview 5.01 statistical software (SAS Institute). A P value of <0.05 was considered statistically significant.

**RESULTS**

Intravital microscopy was performed on a total of 197 mice: average weight was 29.5 ± 0.2 g, and venular diameter was 44.9 ± 0.4 μm. Electron microscopy revealed that venular thrombi in both saline- and LPS-treated mice were platelet rich with absence of fibrin; there was no discernable difference in thrombi structure or endothelial cell morphology (Fig. 1). Signs of endothelial injury were evident, including vacuole formation, but endothelial denudation was not detected. Fibrin, which has a distinct ultrastructural appearance (21, 27), was not observed in the thrombi. There was no detectable difference in relative platelet degranulation between the two groups. Leukocytes were seen occasionally in some sections, primarily neutrophils at the edges of the thrombi. However, leukocyte...
adhesion was not detected consistently by intravital microscopy during the light/dye-induced thrombosis.

**Endotoxemia enhanced microvascular platelet thrombus formation.** Figure 2 depicts the average times to platelet thrombus formation in mice treated with either saline or LPS administered 2 or 4 h before the experiments. We selected 4 h based on data published by Cerwinka et al. (5) in C57BL/6 mice, demonstrating enhanced platelet adhesion by endotoxin at that time interval. However, intraperitoneal administration of LPS to C57BL/6 mice results in peak cytokine responses (interleukin-1β, -6, and TNF-α) 2 h after injection (12). Thus we performed additional experiments to determine whether the enhanced rates of thrombosis were detected at the earlier time point. At both 2 and 4 h postinjection, LPS reduced the time required for onset of thrombus formation and flow cessation by ∼50%. Venular wall shear rate and mean arterial pressure did not differ between the saline and LPS groups at either time point (Table 1).

Because LPS was reported to induce neutrophil-dependent adhesion of platelets in mouse microvessels (6) and enhanced platelet adhesion may contribute to the accelerated rates of thrombosis, we performed additional experiments to determine whether circulating neutrophils mediated the LPS-enhanced platelet thrombus formation. For this purpose, mice were treated with the anti-neutrophil antibody RB6–8C5 24 h before administration of endotoxin. Whereas LPS alone led to an 85% reduction in neutrophil counts compared with saline, the neutrophil counts in mice treated with LPS and the antibody were 88% lower than that of mice treated with LPS alone (P < 0.05) or 98.2% lower than that of saline-treated animals. Despite the reduction in circulating neutrophil counts, antibody treatment did not influence the enhancement of thrombosis by LPS, at either 2 or 4 h, as shown in Fig. 2. LPS alone also reduced mononuclear counts by 81% compared with saline; the mean mononuclear count in mice treated with LPS and the antibody was 25% lower than that of mice treated with LPS alone, but the difference was not statistically significant (P = 0.2).

The LPS-treated mice in the 4-h experiments shown in Fig. 2 received 1.5 × 10⁶ EU/kg of lot A. For the 2-h experiments, by necessity we used a different lot of endotoxin (lot B) that demonstrated lower biological activity with regard to thrombosis at an equivalent dose based on endotoxin units. Thus we performed preliminary experiments at 4 h with lot B and found that 5.0 × 10⁶ EU/kg induced similar responses as noted previously with 1.5 × 10⁶ EU/kg of lot A (∼50% reduction in time of onset and flow cessation compared with saline, n = 6 per group, P < 0.05 for each time point). Thus we used 5.0 × 10⁶ EU/kg for the experiments shown in Fig. 2A and all further experiments unless otherwise specified. Furthermore, we performed additional experiments to exclude the possibility that LPS contaminants may be influencing the responses. Administration of 5.0 × 10⁶ EU/kg of repurified LPS (as described in MATERIALS AND METHODS) 2 h before the experiment induced comparable responses as seen with the commercial compound compared with saline (∼40% reduction in time of onset and flow cessation, n = 9 per group, P < 0.05).

**Role of neutrophils in LPS-enhanced platelet adhesion.** The data shown in Fig. 2 demonstrate that depletion of neutrophils failed to influence the enhanced microvascular thrombosis during the light/dye-induced thrombosis.

![Fig. 2. A: thrombotic responses in saline- and LPS-treated mice (n = 9 per group) and LPS-treated mice injected 24 h earlier with the anti-neutrophil antibody RB6–8C5 (n = 5). LPS or saline was injected 2 h before the experiments. B: thrombotic responses in saline- and LPS-treated mice (n = 19 per group) and LPS-treated mice injected 24 h earlier with the anti-neutrophil antibody RB6–8C5 (n = 5). Data are shown as means ± SE. *P < 0.05, †P < 0.005 for comparison with saline group.](http://ajpheart.physiology.org/)

**Table 1. MAP and wall shear rate in C57BL/6J mice treated with saline, LPS, or LPS and antineutrophil antibody RB6-8C5**

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>MAP, mmHg</th>
<th>Wall Shear Rate, s⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-h Experiments</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>9</td>
<td>94.6±8.1</td>
<td>454.6±51.4</td>
</tr>
<tr>
<td>LPS</td>
<td>9</td>
<td>85.3±4.1</td>
<td>424.3±50.3</td>
</tr>
<tr>
<td>LPS/RB6-8C5</td>
<td>5</td>
<td>83.2±7.2</td>
<td>439.6±53.9</td>
</tr>
<tr>
<td>4-h Experiments</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>19</td>
<td>80.7±2.5</td>
<td>593.4±41.0</td>
</tr>
<tr>
<td>LPS</td>
<td>19</td>
<td>89.6±3.0</td>
<td>506±32.4</td>
</tr>
<tr>
<td>LPS/RB6-8C5</td>
<td>5</td>
<td>95.0±3.0</td>
<td>426.4±71.4</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, number of mice. MAP, mean arterial pressure; LPS, lipopolysaccharide.
bosis induced by LPS. However, because neutrophils have been reported to mediate endotoxin-induced platelet adhesion in mouse intestinal venules (6), we evaluated whether the lack of effect of neutrophils in endotoxin-enhanced thrombosis could be explained by heterogeneity in responses between the cremaster and intestinal microvascular beds. For these experiments, we monitored adhesion of exogenously administered fluorescently labeled platelets in the absence of light/dye injury, as described previously (11, 38). As shown in Fig. 3, endotoxin (lots and doses as used in Fig. 2) enhanced platelet adhesion at both 2 and 4 h after injection, and the enhanced adhesion was inhibited by the anti-neutrophil antibody RB6–8C5. To confirm that LPS-induced platelet adhesion was not a phenomenon limited to exogenously labeled platelets, we performed immunofluorescence microscopy on three perfusion-fixed cremaster preparations from LPS-treated mice. Platelets were identified by using rat anti-mouse glycoprotein-Ibα antibodies (Emfret) and Texas Red-labeled anti-rat secondary antibodies (Molecular Probes). As expected, a substantially greater number of endogenous platelets (red fluorescent) localized to venules in LPS-treated mice compared with exogenous (CFSE-labeled) platelets (red and green fluorescent). In 27 venules examined, the median value of the proportion of exogenous platelets was 8.1%.

**Endotoxemia failed to enhance rates of thrombosis in mice with altered TLR4 signaling.** To test the role of TLR4 in the endotoxin-induced enhancement of platelet thrombi formation, we evaluated the responses in the naturally occurring mouse strain deficient in TLR4 (C57BL/10ScNJ). These responses were compared with those of their corresponding wild-type control strain (C57BL/10J). In preliminary experiments, we found that the C57BL/10J control strain had minimally enhanced thrombotic responses to LPS at 4 h postinjection, although enhanced rates of thrombosis were evident with a twofold higher dose than used in similar experiments with C57BL/6J mice (i.e., 3.0 × 10^6 EU/kg of lot A). Thus we used the higher LPS dose for both the TLR4-deficient mice and their wild-type controls. As shown in Fig. 4, LPS enhanced the rates of platelet thrombus formation in the control strain but had no effect on thrombosis in TLR4-deficient mice, supporting a role for TLR4 in the LPS-enhanced microvascular thrombosis.

To evaluate further the role of TLR4 in LPS-enhanced thrombosis, we performed similar experiments using C3H/HeJ mice, which have a missense mutation in TLR4. As shown in Fig. 5, LPS (as used in Fig. 2A) enhanced the rates of platelet thrombus formation 2 h postinjection in the control strain (C3H/HeN) but had no effect on thrombosis in C3H/HeJ mice.
Influence of endotoxemia on platelet activation and aggregation. To determine whether the enhanced rates of microvascular thrombosis induced by LPS could be explained by a direct activation of platelets, we measured platelet P-selectin expression by flow cytometry on platelets obtained from saline- and LPS-treated mice at 2 and 4 h after injection by using LPS as used in Fig. 2A. At both 2 and 4 h after injection, platelet P-selectin was undetectable in both saline and LPS groups (%0.5% of platelets in all cases). As a positive control for the P-selectin antibody and to exclude platelet desensitization in our model of endotoxemia, we performed flow cytometry in thrombin-stimulated platelets from both saline- and LPS-treated groups at both 2 and 4 h. Thrombin stimulation resulted in P-selectin expression in >85% of platelets in all cases (Fig. 6), with no difference between saline- and LPS-treated groups. Furthermore, to determine whether endotoxemia accelerated thrombosis via enhanced platelet aggregation, we assessed platelet aggregation responses to ADP (20 μM) and collagen (5 μg/ml) as described in MATERIALS AND METHODS. As shown in Fig. 7, platelets derived from LPS-treated mice had reduced aggregation compared with those obtained from saline-treated mice at both 2 and 4 h after injection. Similar reduced aggregation was noted in platelets from LPS-treated mice in response to collagen (data not shown).

DISCUSSION

The main finding in the present paper is that endotoxemia enhanced platelet thrombus formation in mouse cremaster venules by a TLR4-dependent mechanism and without evidence of enhanced platelet aggregation or platelet P-selectin expression, a marker of platelet activation. Although endotoxemia resulted in neutrophil-dependent adhesion of platelets to microvascular endothelium, the enhancement of thrombosis was independent of neutrophils. Whereas endotoxin has been shown to enhance platelet-vessel wall interactions in several models, we are unaware of prior reports of the effects of endotoxemia on light/dye-induced models of microvascular thrombosis. Furthermore, our findings demonstrate that enhanced platelet thrombus formation occurs as early as 2 h after exposure to LPS. We selected that time point based on the temporal profile of inflammatory effects of intraperitoneal administration of LPS in mice outlined in a recent report (12). Two hours after intraperitoneal injection, C57BL/6 mice demonstrated peaks in proinflammatory cytokines, including interleukin-1β, -6, and TNF-α. Similarly, CXC chemokines such as keratinocyte-derived chemokine (KC) and macrophage inflammatory protein-2 (MIP-2) peaked at 2 h after LPS injection. The temporal correlation between pro-inflammatory and pro-thrombotic manifestations in murine endotoxemia requires further study.

Cerwinka et al. (6) demonstrated that endotoxemia induced adhesion of platelets in intestinal venules of C57BL/6J mice. Those authors reported that the LPS-induced platelet adhesion was dependent on circulating neutrophils as platelet adhesion
was attenuated significantly in animals rendered neutropenic with the anti-neutrophil antibody RB6–8C5. Because enhanced platelet adhesion might account for accelerated rates of light/dye-induced thrombosis (particularly earlier onset of responses), we tested whether neutrophils contributed to LPS-enhanced thrombosis. However, as shown in Fig. 2, neutrophil depletion did not influence the effects of LPS on thrombosis in cremaster venules at either time point. To determine whether the role for neutrophils in microvascular platelet adhesion might differ between the intestinal and cremaster microvascular beds, we performed the experiment done by Cerwinka et al. in cremaster microvessels in our model. As shown in Fig. 3, LPS enhanced platelet adhesion at both 2 and 4 h postinjection, and the enhanced adhesion was inhibited by the same anti-neutrophil antibody. These data demonstrate that the neutrophil-dependent platelet adhesion induced by LPS is not sufficient to explain the accelerated times of thrombus onset and flow cessation demonstrated in Fig. 2. This finding is consistent with the sparse neutrophil involvement in thrombi relative to the magnitude of platelet adhesion. For example, the neutrophil-dependent platelet adhesion induced by LPS shown in Fig. 3 yielded ~140 firmly adherent exogenous platelets per squared millimeter at 4 h. Based on our demonstration that 8% of adherent platelets were exogenous (similar to the expected ~10% exogenous platelets in the circulation), this corresponds to ~1,750 total adherent platelets per squared millimeter. Assuming an upper limit of platelet diameter of 4 μm, this corresponds to ~2.2% of the total endothelial surface covered by neutrophil-dependent adherent platelets (or 97.8% of endothelial surface is free of platelets). Given the dense platelet adhesion associated with light/dye endothelial injury (see Fig. 1), the potential contribution of neutrophil-dependent platelet adhesion in our model is minimal. Thus our findings support the notion that LPS enhances light/dye-induced microvascular thrombosis independent of circulating neutrophils.

The enhanced microvascular thrombosis induced by LPS was absent in mice deficient in TLR4 (C57BL/10ScNJ), whereas the corresponding control strain (C57BL/10J) demonstrated enhanced thrombosis by LPS. Similarly, mice with a missense mutation in the TLR4 gene (C3H/HeJ, see Ref. 31) failed to respond to LPS compared with the control strain (C3H/HeN). TLR4 is a primary signaling receptor for LPS (28) expressed on endothelial cells and various leukocytes, including monocytes/macrophages, neutrophils and basophils (14, 26, 39, 46, 47). The microvascular thrombi in our model consist of platelets and occasional neutrophils, associated with injured, though not denuded, endothelium (Fig. 1). The neutrophil-depletion experiments described above demonstrate that the effects of LPS were independent of neutrophils. We performed experiments to pursue whether platelets may be directly responsible for the enhanced microvascular thrombosis in response to LPS based on recent reports that TLR4 is expressed on platelets and may have a functional role (3, 9). We found that P-selectin, a marker of platelet activation, was not detected in LPS-treated mice at either time point. Furthermore, using platelet-rich plasma, we found that aggregation of platelets from LPS-treated mice was reduced relative to that of saline-treated mice (Fig. 7), similar to the findings reported by Cicala et al. (8). Those authors demonstrated that platelets from endotoxemic rats had reduced aggregation to ADP in platelet-rich plasma; however, when platelets were washed or isolated and suspended in normal plasma, the aggregation rates were similar to those of platelets from control animals. Those findings demonstrate that the reduced platelet aggregation in endotoxemia is not a result of intrinsic platelet dysfunction. The comparable P-selectin expression in response to thrombin of platelets from saline- and LPS-treated mice in our study further supports the notion that the reduced aggregation does not reflect platelet dysfunction or desensitization in our model of endotoxia.

Our data are consistent with the hypothesis that enhanced thrombosis by LPS is due to a TLR4-dependent stimulation of microvascular endothelium. This is analogous to the essential role of endothelial cell TLR4 described by Andonegui et al. (2) for LPS-induced pulmonary sequestration of neutrophils. This may occur via a direct effect on endothelial cells or an indirect...
effect of LPS induced by cytokines released via TLR4-dependent stimulation of other cells, such as monocytes/macrophages (16). Future studies with bone marrow transplant-induced chimeric mice deficient in TLR4 in either endothelial cells or monocytes/macrophages may help address this question.

One difficult aspect of these experiments is the variability in endotoxin responses at comparable endotoxin units between lots, as reported by others (17, 22). This prompted us to test whether LPS contaminants may participate in the responses. However, as shown in the results, LPS repurified by a method demonstrated to eliminate nonspecific signaling (via TLR2, Ref. 18) induced similar responses. Furthermore, the two wild-type control strains studied (C57BL/6J and C57BL/10J) differed in their responsiveness to LPS. This was not surprising, because C57BL/10J mice are known to differ from C57BL/6J mice with regard to various hematological and coagulation parameters, as well as in their inflammatory response to an infectious challenge (30, 41). The LPS- and mouse strain-dependent variability observed in this study emphasize the need for individualized dose-response evaluations with different mouse strains and LPS preparations.

The thrombosis model used in this study is based on endothelial injury due to phototoxicity induced by exposure of FITC to excitation light. Light/dye-induced phototoxicity is mediated by reactive oxygen species, particularly singlet oxygen, generated by excitation of the fluorochrome (44). Microvascular thrombosis in this model involves endothelial injury, though not widespread denudation, as shown in Fig. 1, and reported previously by others and us (32, 36). Furthermore, fibrin is either absent (as in this study) or seen rarely in models of light/dye-induced thrombosis (34). Thus the usual characteristics of this thrombosis model (absence of fibrin and of widespread endothelial denudation) differ from some thrombosis models involving extensive endothelial denudation and exposure of subendothelium (reviewed in Ref. 38). The molecular mechanisms mediating light/dye-induced microvascular thrombosis have been incompletely characterized. Two potential candidates, P-selectin and platelet endothelial cell adhesion molecule 1 (PECAM-1, CD31), are not required for platelet thrombus formation in this model because the kinetics of thrombosis in cremaster microvessels of mice lacking these molecules do not differ from their wild-type counterparts (36, 45). Some studies suggest involvement of von Willebrand factor (vWF), platelet glycoprotein Ibα (GPIbα), and the platelet integrin αIIbβ3 (7, 19, 23). A possible mediator of the enhanced thrombosis by LPS in this model is vWF, a counterligand for platelet GPIbα and an important mediator of initial platelet adhesion to injured vessels under shear (35). Because vWF is released by LPS-stimulated endothelial cells (33, 40), enhanced vWF release in LPS-treated mice may account for both the accelerated onset of thrombus formation and time to flow cessation reported in this study. Furthermore, LPS-induced stimulation of microvascular endothelial cells via TLR4 may predispose these cells to light/dye-induced injury, thereby enhancing the rates of microvascular thrombosis. These hypotheses remain to be evaluated.

In summary, we demonstrate that endotoxemia enhances formation of platelet thrombi in mouse cremaster venules by a TLR4-dependent, neutrophil-independent mechanism. The data argue against a direct effect of endotoxemia on platelets in these responses, and they are consistent with the hypothesis that enhanced microvascular thrombosis is mediated by TLR4-dependent stimulation of microvascular endothelium. The responses were evident as early as 2 h after LPS, a time that coincides with reported peaks in cytokine and chemokine responses in LPS-treated mice. The temporal association between inflammatory responses and microvascular thrombosis in murine endotoxemia is worthy of further investigation.

ACKNOWLEDGMENTS

We thank Dr. Perumal Thiagarajan for valuable input with the ex vivo platelet experiments, and Kimberly Langlois, Zhilan Zheng, and Evelyn Brown for expert technical assistance.

GRANTS

This work was supported by National Institutes of Health Grants HL-64721 (to R. A. Rambaut), AI-46773 (to R. R. Burns), HL-070537 (R. E. Rambaut and A. R. Burns), and HL-42550 (A. R. Burns).

REFERENCES


