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Endotoxin-Induced Platelet Activation in Human Whole Blood In Vitro

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Key words

Platelet aggregation – ATP release – Whole blood – Bacterial lipopolysaccharide – Ionizing radiation

Summary

The effect of purified bacterial endotoxin was studied on human platelets *in vitro*. In adding up to 1 μg/mL of a highly purified endotoxin, we found neither aggregation nor ATP release in heparinized or citrated human platelet-rich plasma. On the other hand, endotoxin at concentrations as low as a few ng/mL (as may be found in septic patients) caused platelet aggregation in both heparinized and citrated human whole blood, as monitored by change in impedance, free platelet count, and size. Unlike collagen, the platelet aggregation with endotoxin occurred after a long lag phase, developed slowly, and was rarely coupled with measurable release of ATP. The platelet aggregating effect of endotoxin was dose-dependent and modified by exposure of the endotoxin to ionizing radiation. Thus, the activation of human platelets by "solubilized" endotoxin in plasma requires the presence of other blood cells. We propose that the platelet effect is mediated by monocytes and/or neutrophils stimulated by endotoxin.

Introduction

The incidence of Gram-negative bacteremia has increased in the past few decades and the overall mortality associated with this condition remains high (20–40%) in spite of major advancements in clinical medicine (for review see ref. 1). Gram-negative bacteria produce an endotoxin that is a lipopolysaccharide (LPS) composed of three principal regions: the O-polysaccharide, the R-core oligosaccharide, and the lipid A (2). The structure of the primary toxic component, lipid A, is strikingly similar among Gram-negative bacteria (2). The R-core is structurally more diverse and forms the basis of core typing in *Enterobacteriaceae* (2). The terminal O-polysaccharide is the most variable region of LPS; this moiety (=O-antigen) accounts for the serological specificity of the strain (2).

Sepsis due to Gram-negative bacteria in humans and animals often is complicated by disseminated intravascular coagulation and thrombocytopenia (3, 4). Low platelet counts are also seen following intravenous injection of purified endotoxin to human volunteers and experimental animals (5–9). In order to understand the mechanism of LPS-elicited thrombocytopenia, many studies have mixed isolated endotoxin and platelets *in vitro*. Endotoxin almost invariably caused aggregation of platelets, release of platelet constituents (e.g., 5-hydroxy-tryptamine; 5HT), and unmasking of platelet factor 3 (PF3) in platelet-rich plasma (PRP) and/or washed platelet suspensions of various animals such as dog, rabbit, rat, mouse, and pig (6–14). A number of mechanisms have been established for the endotoxin-platelet interaction in these species (7–9, 12, 14).

Studies with human platelets disclosed varying responses to endotoxin. A number of investigators – including us – have failed to demonstrate an aggregatory effect, activation of PF3, and/or release of 5HT with endotoxin in either human PRP or washed platelet suspensions (8, 9, 11–13, 15, 16). Other reports claim, however, that endotoxin alone produces aggregation, increase in 5HT and adenosine diphosphate (ADP) release, unmasking of PF3, and/or membrane changes of human platelets *in vitro* (10, 11, 17–19). In addition, lipid A-rich LPS or isolated lipid A component of LPS strongly potentiated the 5HT release reaction of washed human platelets with immune complexes and aggregated IgG (20, 21). *Escherichia coli* endotoxin enhanced the thrombin-induced arachidonic acid release and thromboxane B_2 production by human platelets (22). In a recent work, however, purified endotoxin inhibited the platelet aggregation and 5HT release that were induced by adenosine diphosphate, collagen, arachidonic acid or ionophore A23187 in human PRP and suspension of washed platelets (23).

Thus, despite numerous observations of thrombocytopenia with endotoxin *in vivo*, the *in vitro* effects of endotoxin on human platelets remain inconsistent and controversial. Therefore, we re-examined the effect of purified LPS on human platelets *in vitro*. The results show that activation of human platelets by endotoxin fails to take place in PRP, but occurs in the presence of other blood cells in whole blood (WB).

Materials and Methods

Source of Endotoxin

The LPS used in this work is the U.S. reference standard endotoxin (RSE), which was extracted and purified from *Escherichia coli* (Braude strain) 0113: H10: K-negative (24). The RSE was prepared at a concentration of 50 μg/mL in pyrogen-free sterile 0.9% NaCl. Some of the glass vials containing RSE in aqueous medium were exposed to γ-radiation from a ^{60}Co-source as described previously (25).

Collagen

Microfibrillar collagen prepared from equine tendon (Collagenreagent Horm) was obtained from Hormon-Chemie, Munich, West Germany. Dilutions were made with SKF Horm buffer (Hormon-Chemie).

Blood Samples

After obtaining informed consent, venous blood was drawn from healthy adult volunteers of both sexes who had not ingested aspirin or other drugs for at least two weeks prior to donation. Nine volumes of WB were collected in one volume of acid-citrate-dextrose (ACD, formula A, Travenol Laboratories, Inc., Deerfield, Ill.) or saline containing sodium heparin (50 IU/mL) from porcine intestinal mucosa (Fellow Med. Div., Chromalloy Pharmaceutical, Inc, Oak Park, Mich.).

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Preparation of PRP and Platelet-Poor Plasma (PPP)

PRP and PPP were obtained by centrifuging anticoagulated WB at room temperature as described previously (16). WB, PRP and PPP were exposed only to plastic surfaces and were stored at room temperature until testing.

Platelet Counting and Sizing

A Model 810 analyzer (Baker Instruments, Allentown, Pa.) was used for counting and sizing platelets.

Platelet Aggregation and Adenosine Triphosphate (ATP) Release

Tests were carried out in a Model 500 Whole Blood Lumi-Aggregometer (Chrono-Log Corp., Havertown, Pa.), which allows for simultaneous recording of changes in electrical impedance (aggregation), luminescence (ATP release) and, in case of translucent suspensions of cells (e.g., PRP), change in transmittance (aggregation). One mL of anticoagulated WB or PRP was first warmed without stirring in the incubation well of the heater block at 37° C in a siliconized glass cuvette. The specimen was then warmed for 2 more min in the covered aggregation well while stirring (1000 rpm) with a teflon-coated stir bar. Electrodes were then inserted in the cuvette and 100 μL of luciferase-luciferin reagent (40 mg/mL) (Chrono-Lume; Chrono-Log Corp.) was added unless otherwise specified. The sample was briefly allowed to come to equilibrium and the instrument was calibrated for the impedance channel (5 Ohm change in impedance = 20% of scale) and, when appropriate, for the optical channel (90% of scale = PPP and 10% of scale = PRP). The agents to be tested for their platelet reactivity were added in a volume of 10 to 20 μL to PRP or WB that was continuously stirred at 1000 rpm at 37° C. To quantify the amount of ATP release, the luminescence channel was calibrated after completion of the test by adding a known amount of ATP (Sigma Chemical Co., St. Louis Mo.) to PRP specimens. In the case of WB, the ATP standard was added to a new WB specimen for calibration of the luminescence channel.

Results

Endotoxin (up to a final concentration of 1 μg/mL) did not effect discernible platelet aggregation (optical and impedance monitoring) or release of ATP for up to 25 min in heparinized or citrated human PRP specimens. Microfibrillar collagen (2 μg/mL) used as a positive control elicited strong platelet aggregation (optical and impedance monitoring) and ATP release reaction within 5 min in the same PRP specimens. No spontaneous platelet aggregation or ATP release was seen after addition of 0.9% NaCl (negative control) to either type of PRP.

Endotoxin produced dose-dependent platelet aggregation in every heparinized and citrated human WB specimen studied. There was no apparent difference in the results between the two anticoagulants for the WB specimens obtained from the same donors at the same time. The platelet aggregation induced by endotoxin occasionally was coupled by clotting of the WB specimen. The minimal effective concentration of RSE was between 1 and 10 ng/mL (Fig. 1). In two of eight different WB specimens tested, RSE at a concentration of 1 ng/mL resulted in a late and weak aggregation response similar to the reaction seen after addition of 0.9% NaCl (negative control) to a new WB specimen ("spontaneous aggregation") (Fig. 1). Because of a markedly delayed onset (12 to >25 min) and very slow rate of progression, "spontaneous aggregation" could be differentiated from the LPS-induced stronger aggregation response. In another study using a Chrono-Log Whole Blood Aggregometer (Model 540), "spontaneous aggregation" was observed in the citrated WB from two of 29 subjects (26).

The platelet aggregation with RSE in WB reached a maximum at final concentrations of 100–1000 ng/mL (Fig. 1). Compared to the platelet aggregations induced by collagen (2,000 ng/mL) in human WB, the aggregation response to endotoxin started after a long lag phase (mean = 8.6 min with a range of 3.7 to 13.6 min in eight heparinized WB specimens) and developed slowly in WB (Figs. 1 and 2). Although most experiments were carried out in the presence of luciferin-luciferase reagent that had been previously shown to occasionally potentiate platelet aggregation (27), RSE caused platelet aggregation in human WB in the absence of Chrono-Lume as well (not shown).

Collagen always produced a marked platelet secretory response (ATP release) that preceded platelet aggregation in anticoagulated WB (Figs. 1 and 2) and PRP (not shown). With RSE, however, detectable small ATP release occurred only in two of eight heparinized WB specimens and only by the highest concentration of RSE (1,000 ng/mL) (Fig. 1). The endotoxin-elicited release of ATP from platelets (when occurred) preceded the aggregation response as also observed with collagen (Fig. 1).

We have shown previously that endotoxin exposed to high doses of ionizing radiation sustains a stepwise degradation (25). The most radiosensitive region is the O-polysaccharide, followed by components of the R-core and, finally, by the lipid A. RSE specimens that received higher doses of γ-radiation, were less successful in inducing platelet aggregation in human WB *in vitro* (Figs. 3a, b, and c). The reduced platelet aggregating ability of endotoxin was characterized by progressively longer lag phases, decreasing velocities, and decreasing extents of aggregation at 25 min. Exposure of RSE to 4.32 Mrad completely eliminated its platelet aggregating activity in human WB (Fig. 2). On the other hand, as a sensitive method for detection of platelet aggregation (25), determination of the free platelet count (at 25 min after challenge) revealed that RSE exposed to low doses of γ-radiation (0.36 and 0.72 Mrad) was significantly more potent ($p < 0.05$) for inducing platelet aggregation than unexposed RSE (Fig. 3d).

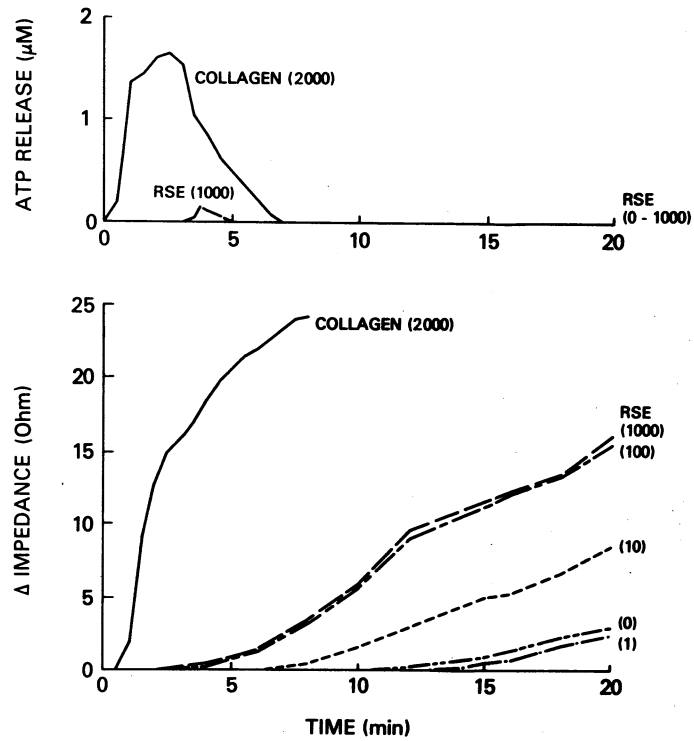

Fig. 1 Dose-dependent platelet aggregation by endotoxin (RSE) in heparinized whole blood of healthy volunteers (n = 3). For comparison, platelet activation induced by microfibrillar collagen from equine tendon is shown in the same donors. Final concentrations are given in ng/mL

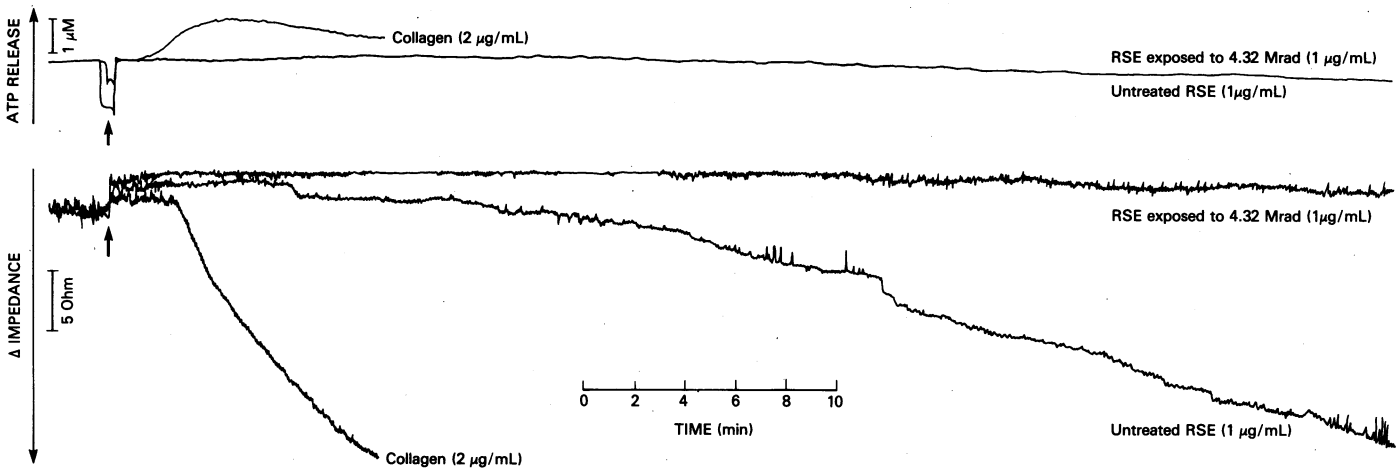

Fig. 2 Exposure of endotoxin (RSE) to a high dose of ionizing radiation abolishes the platelet aggregating activity in heparinized human whole blood. For comparison, platelet activation induced by microfibrillar collagen from equine tendon is shown in the same donor

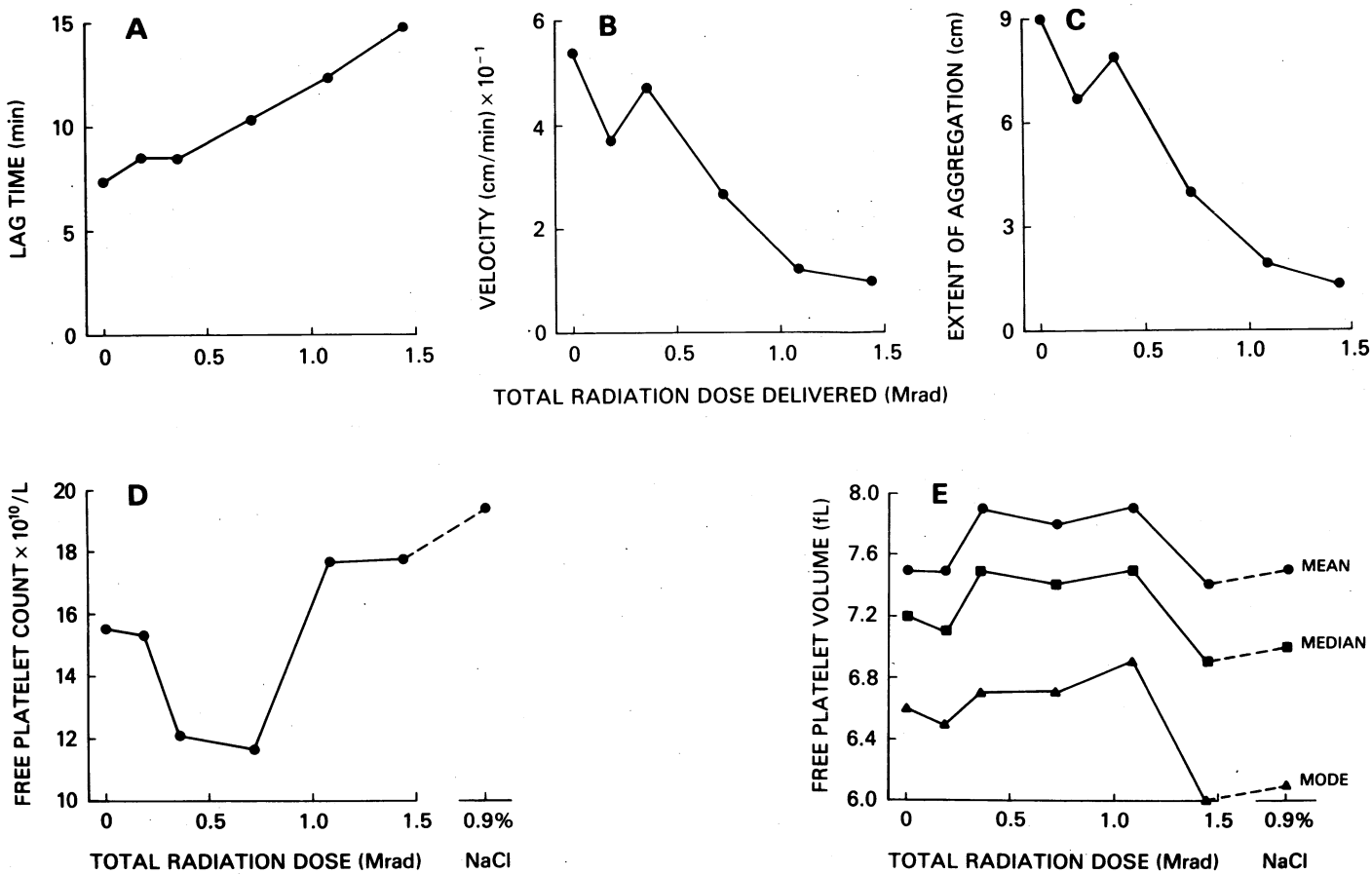

Fig. 3 Modification by ionizing radiation of the platelet aggregating activity of endotoxin (RSE) in heparinized human whole blood. The concentration of RSE in whole blood was 1 μg/mL.
Extent of aggregation, free platelet count and size distribution were measured 25 min after the addition of RSE. Calibration of impedance channel: 5 Ohm = 20% of scale = 2.28 cm. Negative control for platelet counting and sizing was prepared by replacing RSE for 0.9% NaCl for addition to whole blood in the aggregation cuvette

These doses of γ-radiation result in "mutant" LPS molecules that consist of R-core and lipid A, sometimes in combination with a single O-side chain unit (25). The platelet activation by RSE was also evident in the change of the volume of free platelets by 25 min post-challenge (Fig. 3e).

Discussion

Endotoxin causes no platelet aggregation and (ATP) release reaction in citrated or heparinized human PRP. This is in accord with a number of previous reports (8, 9, 11–13, 15, 16). On the

other hand, Timmons et al. (28) recently observed that endotoxin induces 5HT secretion from and aggregation of human platelets separated from plasma proteins. These platelets had been challenged with whole bacteria of the rough mutant Re595 of *Salmonella minnesota* that lacks O-polysaccharide and heptose phosphate in the R-core. Bacteria of the respective smooth strain possessing "complete" endotoxic LPS were ineffective. Here, we found that purified (cell-free) smooth-form endotoxin triggers a slowly developing platelet aggregation in both citrated and heparinized human WB *in vitro*. The specificity of this aggregatory activity was evidenced by several observations: the reaction was dependent on the dose and integrity of LPS, was shown to occur by monitoring the change in either the impedance or free platelet count, and was associated with a change in the size distribution of free platelets.

The lack of a strong simultaneous ATP release by endotoxin in human WB can be explained by at least three mechanisms. First, an undetectable degree of ADP (and concomitant ATP) release may be sufficient for sustaining platelet aggregation. Second, platelet aggregation may be brought about by the release of platelet aggregating factor (PAF) via the putative third pathway believed to be largely independent of ADP (and concomitant ATP) release and the cyclooxygenase pathway (29). Third, platelet aggregation may be secondary to coagulation activation (thrombin generation) induced by activation of factor X by monocytes (30, 31). According to Schwartz and Monroe (31), aggregation of human platelets by endotoxin-stimulated mononuclear cells "was not inhibited by apyrase or aspirin, indicating that neither ADP release nor cyclooxygenase activity was essential for aggregation."

Our finding that RSE exposed to low doses of γ-radiation possesses enhanced platelet aggregating activity in human WB is in accord with our former observation in dog PRP *in vitro* (25). In both systems, we found that LPS molecules with short or lacking O-polysaccharide side chain (produced by low doses of ionizing radiation) are more effective in decreasing free platelet count than those with long O-side chains. This may be due to easier insertion of smaller and less hydrophilic LPS molecules into the cell membrane, a step which is thought to precede cell activation (14). In some other studies, only incomplete LPS (lacking the O-polysaccharide side chain) showed reactivity towards human platelets (21, 28). Similar results were obtained with animal platelets (14).

Previously, aggregation of human platelets *in vitro* was induced by enormously high LPS concentrations (often several hundred μg/mL) (10, 11, 17–19). Therefore, the results obtained under these conditions are questionable as to their relevance to situations *in vivo*. In man, measurements of endotoxin in Gram-negative sepsis show circulating LPS concentrations only up to about 5 ng/ml (32) and often <1 ng/mL (33). I.v. injection of highly purified LPS preparations to human volunteers is considered safe only up to a few ng/kg (5, 34) with resultant plasma concentrations of <1 ng LPS/mL. Our results for the *in vitro* platelet aggregating concentration of LPS in the range of >1 to 100 ng/mL in WB are consistent with clinical studies in humans. Thrombocytopenia is common in patients during Gram-negative sepsis (3) when the plasma concentrations of endotoxin may peak between 0.5 and 5 ng/mL (32). In turn, only inconsistent changes or borderline low platelet counts are seen after i.v. administration of 4 to 5 ng/kg doses of highly purified LPS to human volunteers (5, 34).

Similar to our results with platelets in WB, recent studies found that LPS at concentrations of 1–10 ng/mL "primes" human peripheral neutrophils so that they respond to chemotactic factors with an enhanced release of toxic oxygen radicals and lysosomal enzymes (35). Human neutrophils preincubated with endothelial cells in the presence of low concentrations of LPS (1–10 ng/mL) could be stimulated by chemotactic factors to produce marked endothelial injury that was not inhibited significantly by 50% human serum or plasma (35). Interestingly, like the platelet aggregation in WB, the endothelial injury reached a maximum at LPS concentrations of 100–1000 ng/mL (35). Thus, both the human platelet aggregating activity and the enhancement of human neutrophil-mediated endothelial injury provided by LPS are evident *in vitro* at very low concentrations of endotoxin (<10 ng/mL), comparable to those described in human endotoxemic states (32).

The unresponsiveness of primate platelets to LPS in PRP is thought to be due to the lack of pertinent receptors on these cells (12). The fact that we found platelet activation by endotoxin in human WB but not in human PRP indicates a need for the simultaneous presence of other blood cells. An indirect activation of human platelets by endotoxin is also supported by the comparatively long lag phases, the slow development of aggregation, and the often undetectable ATP release *in vitro*.

Two of the leukocytes react with endotoxin to produce factors that activate platelets. First, *in vitro* activation of human neutrophils by ng/mL concentrations of LPS has been demonstrated by Smedly et al. (35). It is also known that, upon stimulation, phospholipase A_2 is activated in the cell membrane of neutrophils with subsequent release of arachidonic acid and PAF (36, 37). Arachidonic acid; thromboxane A_2, a cycloxygenase product of arachidonic acid; and PAF all are potent stimulators of platelet aggregation (29, 38). Second, it is now recognized that mononuclear phagocytes are the most sensitive cells to perturbation by LPS. Stimulation of human monocytes can lead to the release of both arachidonic acid and PAF (37). In addition, Sememaro et al. (39) have shown that human platelets develop a strong procoagulant activity after simultaneous incubation with endotoxin (*E. coli*, 10 μg/mL) and unfractionated human peripheral leukocytes or pure mononuclear cells (but not with pure granulocytes) in plasma. They believed that monocytes are responsible for the platelet activation. Platelets activated this way acted on coagulation factor X independently of the intrinsic and extrinsic coagulation pathways. The endotoxin-induced, leukocyte-mediated development of platelet coagulant activity depended on the dose of endotoxin and was visible at an endotoxin concentration as low as 1 ng/mL in citrated whole blood (39). Schwartz and Monroe (31) observed that peripheral blood mononuclear cells exposed to 10 ng to 10 μg/mL of smooth-form *E. coli* 0111 LPS *in vitro* initiate aggregation of human platelets in plasma. They showed that the thrombin generated at the surface of monocytes (monocyte procoagulant activity) as a result of tissue factor expression causes both platelet aggregation and clotting of fibrinogen, giving a unified mechanism of platelet and fibrinogen consumption. Davis and Johnson (40) studied the effect of endotoxins on platelet aggregation in native (uncoagulated) human WB by impedence aggregometry. They observed that both smooth-form *E. coli* 0111 and core-deficient *S. minnesota* LPS shorten the time to clot formation, but impedence changes suggestive of accelerated platelet aggregation are minimal or absent. They felt that the changes are consistent with thrombin generation (leukocyte procoagulant activity) by endotoxin. Interestingly, they also noted that in native human WB, *E. coli* 0111 LPS blocks aggregation and delays the onset of clotting after PAF, whereas *S. minnesota* LPS usually accelerates platelet aggregation by PAF (40). Thus, though the precise mechanism might be debated, it is apparent that the endotoxin-triggered "cellular" pathways of platelet activation, described previously by the above investigators (31, 39, 40) and here by us, contribute to the activation of intravascular coagulation during Gram-negative sepsis.

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