Endotoxin-induced Changes in Human Platelet Membranes: Morphologic Evidence

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Interaction between human platelets and bacterial endotoxin was studied in vitro with transmission and scanning electron microscopy. Washed human platelets, whose aggregation was blocked with apyrase, were incubated in a plasma-free medium containing crude endotoxin that had previously been complexed with copper. Thirty minutes of incubation resulted in adherence of endotoxin particles to the platelet surface, breaks in the platelet plasma membrane with apparent attempts at repair, pseudopod formation, and centralization of platelet organelles. Copper appeared to potentiate these phenomena, since neither Cu²⁺ at low concentrations nor endotoxin alone altered the morphology of the platelet membrane. This platelet-endotoxin interaction may be an intermediary step in the detoxification and clearance of endotoxin from the plasma.

Endotoxin (lipopolysaccharide, LPS), derived from the cell wall of gram-negative organisms, produces thrombocytopenia, shock, and intravascular coagulation in both animals and man. Using the Limulus lysate test for the presence of endotoxin, endotoxinlike activity in platelets has been detected in both experimental endotoxemia and in clinical gram-negative sepsis.

Recently, Hawiger and co-workers reported the existence of a surface receptor site for LPS on the human platelet membrane. Although in nonprimates endotoxin adheres to platelets and causes aggregation along with release of procoagulant activity, morphologic evidence of a direct platelet-endotoxin interaction has not been conclusively demonstrated with primate platelets.

In assessing the role of trace metals in endotoxin activity, Lewis and Dickson were able to aggregate washed human platelets with endotoxin complexed with copper. Using a similar endotoxin-copper complex, we saw early ultrastructural changes in the human platelet membrane after interaction with LPS when platelet aggregation was blocked by apyrase.

MATERIALS AND METHODS

Platelet Isolation

Platelets were obtained from four laboratory personnel who were taking no aspirinlike drugs. Venous blood was collected in plastic tubes containing heparin (15 U/ml) and prostaglandin E₁ (PGE₁; 10 ng/ml). PGE₁ was added to improve platelet harvest from heparinized blood by preventing spontaneous platelet clumping. The blood was centrifuged at 160 g for 10 min at room temperature to obtain platelet-rich plasma (PRP). Platelet pellets were obtained by centrifuging the PRP for 20 min at 800 g; the PRP was then washed free of plasma proteins, heparin, and PGE₁ and resuspended in a buffered saline-albumin solution containing apyrase. Inclusion of apyrase (10 mg/dl) facilitated visualization of early platelet changes by blocking...
platelet aggregation. The final platelet suspension contained approximately \(7 \times 10^{12}\) platelets/liter.

**Preparation of Endotoxin**

Commercial endotoxin (*Escherichia coli* 026:B6 lipopolysaccharide, Difco, Detroit) was complexed with copper as described by Lewis and Dickson. In brief, a solution of 2.5 mg/ml endotoxin was incubated with \(10^{-3} M\) CuCl\(_2\) for 30 min at 37°C. The resultant complex was centrifuged at 28,000 g at 4°C and washed free of unbound Cu\(^{2+}\).

**Incubation of Platelets With Endotoxin**

Copper-complexed endotoxin (0.1 ml) was added to 1.0-ml aliquots of either PRP or the washed platelet suspension and incubated for 30 min at 37°C. The final concentration of endotoxin was 180 \(\mu g/ml\) platelet suspension, as determined by the quantitative Limulus lysate test; the final concentration of Cu\(^{2+}\) was \(0.3 \times 10^{-4} M\). Replicate samples were incubated with endotoxin not complexed with copper, 0.154 M NaCl, or in \(10^{-4} M\) CuCl\(_2\) alone.

With PRP, the platelets from two donors were each used in two separate experiments. Washed platelets from three subjects were used in one experiment each, while those of a fourth subject were used in five separate experiments over an 18-mo period. In one experiment, the washed platelet suspension did not contain apyrase.

**Electron Microscopy**

All platelets were fixed in suspension by addition of 9 volumes 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4). After fixation for 1 hr at room temperature, platelets were pelleted and postfixed in 1%, osmium tetroxide for 1 hr at 4°C.

For transmission microscopy, postfixed platelets were dehydrated in ethanol and embedded in Epon 812. Thin sections were stained with uranyl acetate and lead citrate and examined in a JEM 100B electron microscope.

For scanning microscopy, platelets were resuspended in water after postfixation and placed in a modified “BEEM” capsule containing a Nuclepore filter (13 mm diameter, pore size 0.2 \(\mu m\)). While they were within the capsules, cells were dehydrated in acetone, passed through amyl acetate and critical-point dried from liquid CO\(_2\) in a Denton DCP-1 critical-point drier. Filters with adherent platelets were mounted on copper stubs, coated with carbon and gold-palladium in a Denton DV-502 vacuum evaporator, and examined at 20 kV with a JEM 100B electron microscope equipped with an EM-ASID-1 scanning attachment.

Suspensions of copper-complexed endotoxin were similarly prepared for both transmission and scanning microscopy.

![Fig. 1](https://example.com/fig1.png)  
Fig. 1. Transmission electron micrograph of a suspension of LPS-Cu\(^{2+}\) before addition to platelets. Endotoxin particles consist of small fragments of trilaminar membrane. \(\times 110,000\).
RESULTS

Platelets in PRP appeared not to react with the endotoxin-copper complex (LPS-Cu²⁺). Although particles of LPS-Cu²⁺ in the form of short segments of membrane with a trilaminar structure (Fig. 1) were found in the plasma surrounding the platelets, no endotoxin adhered to the platelet membranes, and cell morphology was unchanged compared to control cells. Similarly, endotoxin not complexed with copper produced no changes in platelet morphology.

Washed platelets (from all four subjects tested) exposed to endotoxin previously complexed with copper (LPS-Cu²⁺) showed four changes not present in control cells: (1) adherence of endotoxin fragments to platelet membranes, (2) discontinuities in plasma and canalicular membranes, (3) central clumping of platelet organelles, and (4) pseudopod formation. Particles of LPS-Cu²⁺ adhered to the plasma membranes (Fig. 2) of approximately 2% of the platelets observed in thin sections. The trilaminar membranes of bacterial origin were easily distinguished from platelet membranes by the greater width of their central electron-lucent layer (3.9 ± 1.2 nm in bacterial membranes, 2.7 ± 0.9 nm in platelet membranes). The adherent endotoxin particles were usually oriented perpendicular to the platelet plasma membrane (Fig. 2) but on occasion aligned parallel to the platelet surface (Fig. 3). Although endotoxin particles were scarce in the medium surrounding the platelets, they were sometimes seen within channels of the open canalicular system.

While morphologic evidence of endotoxin adherence to platelets and other ultrastructural changes were consistently detected in washed platelets at a final

Fig. 2. Transmission electron micrograph of washed human platelets 30 min after exposure to LPS complexed with copper. Segments of LPS (arrows) adhere to the platelet (P) plasma membranes. In one region endotoxin particles are oriented to reveal their trilaminar structure. × 90,000.

Fig. 3. Endotoxin particles located near a platelet (P). One LPS fragment (arrow), aligned parallel to the platelet surface, adheres to the plasma membrane. × 126,000.
Fig. 4. Control platelets 30 min after exposure to $10^{-4}$ M CuCl$_2$. Platelet organelles and surface membranes appear normal. $\times 18,000$.

Fig. 5. Outer lamina of platelet trilaminar membrane is wider and more electron dense than the inner lamina after exposure to endotoxin. In addition, the membrane shows breaks, duplications, and regions of overlap. $\times 300,000$. 
endotoxin concentration of 180 mg/ml platelet suspension, lower concentrations of endotoxin were ineffective. In addition, ultrastructural changes in platelets were not seen with LPS not complexed with copper. Similarly, copper alone did not react with the platelet surface. After incubation in 10^{-4} M CuCl_2 alone, a concentration three times the Cu^{2+} level in the mixture of LPS-Cu^{2+} and platelets, plasma membranes and the exterior coat appeared normal (Fig. 4).

Platelets treated with LPS-Cu^{2+} showed breaks in both plasma and canalicular membranes. The disjointed segments of membrane frequently overlapped, producing a shingled or imbricated appearance (Fig. 5). In many areas, plasma membranes were duplicated or multilaminar (Figs. 3, 5). Approximately 10\% of platelets observed in thin sections displayed such membrane abnormalities. In most platelets, the outer lamina of the platelet trilaminar plasma membrane and of the canalicular membranes was increased in density and width (Fig. 5).

Endotoxin also produced changes in the shape of human platelets similar to those preceding aggregation. In most platelets, multiple pseudopods extended from the surface, and in a few cells (approximately 5\%) organelles were clumped in the cell center (Fig. 6). However, no platelet aggregates were present. The electron-microscopic appearance also suggested activation of the release reaction: most platelets contained fewer granules, and canalliculi were more prominent than in control cells. In one experiment, when apyrase was not included in the incubation medium, platelet aggregation did occur (Fig. 7).

Scanning electron microscopy of washed platelets confirmed the shape changes that occurred after incubation with LPS-Cu^{2+} (Fig. 8) in the presence

![Image](https://via.placeholder.com/150)

**Fig. 6.** Changes shown by platelets exposed to LPS-Cu^{2+} include pseudopod (Ps) formation, central clumping of cell organelles (arrow), and loss of granules (G). × 22,000.
of apyrase. Most platelets demonstrated long pseudopods and irregular surfaces (Fig. 9) when compared to the smooth contours of control cells (Fig. 10).

**DISCUSSION**

These data show that endotoxin complexed with copper (endotoxin concentration 180 μg/ml) can adhere to human platelets (washed free of plasma) and
Fig. 9. Scanning electron micrograph of platelets after incubation with endotoxin. Material resembling endotoxin vesicles (V) adheres to several platelets (P) that exhibit irregular surfaces and long pseudopods (Ps). x 12,500.

Fig. 10. Scanning electron micrograph of control platelets incubated with $10^{-4} \text{ M CuCl}_2$. Most platelets have a smooth discoid shape. x 12,000.
alter their surface coat and plasma membrane. The affinity of endotoxin for membranes in general has been demonstrated with artificial lipid bilayers, erythrocyte ghosts, and lysosomal membranes. In platelets, adherence of endotoxin was accompanied by shape changes and activation of the release reaction. Platelet aggregation, as observed by Lewis and Dickson, did not occur when apyrase, an antiaggregation agent, was present in our incubation medium. Detection of the surface changes induced by endotoxin was probably facilitated by elimination of the nonspecific changes associated with platelet aggregation. The biochemical studies of Hawiger and co-workers also suggest an endotoxin-induced change in the platelet membrane (endotoxin concentration 200 μg/ml) along with release of serotonin and adenine nucleotides, early indicators of platelet activation.

In fine-structural studies of a variety of nonprimate platelets, endotoxin coated the platelet surface and induces platelet aggregation. Simultaneously, morphologic evidence of the release reaction is apparent. Using endotoxin concentrations identical to the present study, we observed similar changes in rabbit and rat platelets (unpublished observations). In addition, large amounts of endotoxin were present in the medium surrounding the platelets. With human platelets, although only 2% demonstrated adherent endotoxin particles, the higher proportion of platelets showing membrane changes (10%) as well as the scarcity of free endotoxin particles in the plasma-free medium surrounding the platelets suggest that the platelet-endotoxin interaction may be more extensive than previously thought.

Although further studies are necessary, our impression is that after adherence to the platelet surface, endotoxin particles may have been rapidly integrated into the platelet membrane. Such fusion can account for regions where plasma membranes appeared duplicated, multilaminar, or where broken membranes overlapped. The low frequency of adherent endotoxin was also partly caused by a sampling problem, since adherent material was identified as endotoxin only if sectioned in a way that demonstrated the particles’ trilaminar structure. Our findings suggest that the difficulty in detecting an endotoxin interaction with primate platelets is not that the interaction is weaker than with nonprimate platelets but that the morphologic changes that occur are less destructive.

Our study suggests that Cu²⁺, besides rendering endotoxin more electron dense, also appears to potentiate the platelet-endotoxin interaction. Morphologic evidence of adherence of endotoxin to platelets and alteration of the platelet membrane was obtainable only when copper was present. The inability of even copper-complexed endotoxin to react with platelets in the presence of plasma suggests that plasma itself may interact with endotoxin to neutralize its toxicity or that inhibitors of the platelet-endotoxin reaction may be present.

Although the clinical relevance of our observations remains to be determined, serum levels of the copper-containing protein ceruloplasmin are markedly elevated in human gram-negative infection, and this rise precedes the full blown clinical disease with thrombocytopenia. The elevated copper possibly potentiates the adsorption of blood-borne endotoxin by platelets. Reduced survival of endotoxin-treated platelets after reinfusion into the circulation has been reported in the rat. Our data thus support the recently evolving
concept that platelets play a role in defense against infection, either against whole bacteria or bacterial products such as endotoxin.20

REFERENCES

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