ENDOTOXIN-INDUCED PLATELET
AGGREGATION AND SECRETION

I. MORPHOLOGICAL CHANGES AND
PHARMACOLOGICAL EFFECTS

D. EUAN MACINTYRE,* ANTHONY P. ALLEN,*
KAREEN J. I. THORNE,** AUDREY M. GLAUERT**
AND JOHN L. GORDON†

*University Department of Pathology, Tennis Court Road, Cambridge CB2 1QP,
**Strangeways Research Laboratory, Worts' Causeway, Cambridge CB1 4RN, and
†ARC Institute of Animal Physiology, Babraham, Cambridge CB2 4AT, U.K.

SUMMARY

Endotoxin lipopolysaccharide (LPS) from Acinetobacter 199A induced aggregation of blood platelets from immune adherence-positive species (rat, rabbit) but not from immune adherence-negative species such as pig and man. Aggregation occurred in 2 phases: the first was not accompanied by secretion of platelet constituents, was apparently a consequence of C3 activation, and was selectively inhibited by EGTA. The second phase of aggregation was associated with secretion of platelet granule contents, and with a lesser amount of cytoplasmic leakage. Secondary aggregation was abolished by the sulphydryl alkylating agent N-ethyl maleimide, and by agents which increased the level of cyclic AMP in platelets, such as prostaglandin E1 (a stimulator of adenylate cyclase) and methyl xanthines (inhibitors of phosphodiesterase). Secondary aggregation was partly inhibited by agents which block platelet prostaglandin biosynthesis (e.g. aspirin, indomethacin). Primary aggregation was unaffected by these inhibitors at concentrations which blocked secondary aggregation.

INTRODUCTION

Intravenous injection of bacterial endotoxin (lipopolysaccharide) in rabbits results in activation of the coagulation and fibrinolytic enzyme systems, profound thrombocytopenia, and disseminated intravascular coagulation (McKay & Shapiro, 1958; McKay, 1973; Müller-Berghaus & Lasch, 1975). The precise mechanisms involved are still uncertain but release of tissue thromboplastin, platelet aggregation and secretion, and activation of the complement system all occur (Müller-Berghaus, 1969; McKay, 1973). Previous studies of the platelet-endotoxin interaction in vitro have shown that endotoxin-induced platelet aggregation is at least partly complement dependent (Des Prez, Horowitz & Hook, 1961; Ream, Deykin, Gurewich & Wessler, 1965; Spielvogel, 1965) but it has not been established whether complement activation is involved in the initial interaction of endotoxin with platelets, in the induction of platelet secretion, in promoting platelet-platelet adhesion (i.e. aggregation), or in all these processes. It is also not known to what extent the response of platelets to endotoxin resembles that to other biological agents that induce platelet aggregation.

We have now investigated the nature and kinetics of endotoxin-induced platelet
aggregation and secretion by studying the effects of selected inhibitors, and by electron-microscopic examination of platelets exposed to endotoxin.

**METHODS**

**Measurement of platelet aggregation and release of platelet constituents**

Human blood was obtained by antecubital venepuncture from donors who had taken no medication for at least 10 days; rat blood from the inferior vena cava of animals under light ether anaesthesia; rabbit blood by venepuncture of the marginal ear vein; and pig blood from the Cambridge abattoir. Blood samples were anticoagulated with citrate or heparin, platelet-rich plasma (PRP) was prepared by differential centrifugation, and platelet aggregation was measured photometrically (Born, 1962) in 0.1-ml samples (Gordon & Drummond, 1974). Washed platelet suspensions were prepared from citrated PRP by a slight modification of the albumin density gradient centrifugation technique (Walsh, 1972).

For measurements of secretion, PRP was pre-incubated at 37 °C for 30 min with 1 µM [14C]-5-hydroxytryptamine (which is incorporated into the platelet dense bodies) and 0.1 µM [3H]adenine (which is incorporated into the cytoplasmic pool of adenine nucleotides). Endotoxin was added to the platelet samples and incubated for various times. The reaction was terminated by the addition of 4 vol. ice-cold 0.4 % (w/v) EDTA in iso-osmotic saline and immediate centrifugation (14 700 g, 30 s). Subsamples of cell-free supernatant for measurement of adenine and 5HT release were transferred into scintillation vials containing 0.33 % 5-((4-biphenylyl)-2-4-(4-butyphenyl)-1-oxa-3,4 diazole (Butyl PBD) in toluene plus 30 % (v/v) ethoxycethanol. Replicate subsamples were taken for fluorimetric measurement of release of β-N-acetyl-glucosaminidase, a platelet lysosomal enzyme (Gordon, 1975). Platelet pellets were digested in 19 M formic acid (0.5 ml, 37 °C, 30 min) before transferring to scintillation vials, or in 1 % Triton X-100 (0.3 ml, 37 °C, 30 min) for lysosomal enzyme assays.

Radioactivity was measured in a Nuclear Chicago Mk 2. liquid scintillation counter under conditions giving optimal discrimination between 3H and 14C. Increases in supernatant radioactivity or lysosomal enzyme activity were expressed as percentages of the activity in a control (unstimulated) pellet.

**Electron microscopy**

Samples of PRP were fixed at room temperature for 30 min by adding 10 vol. 2.5 % (w/v) glutaraldehyde in 0.09 M cacodylate buffer (pH 7.2), containing 3 mM CaCl2. They were then centrifuged at 15 000 g for 2 min and the pellets collected and stored at 4 °C under 0.09 M cacodylate buffer pH 7.2 containing 3 mM CaCl2. The platelet pellets were then postfixed in veronal-acetate-buffered OsO4 (pH 7.2) for 1 h at room temperature, stained with 0.3 % uranyl acetate in veronal-acetate buffer for 1 h at room temperature, dehydrated in ethanol, and embedded in Araldite. Thin sections were cut with glass knives on an LKB Ultrotome III or a Cambridge Huxley ultramicrotome and stained with lead citrate. Specimens were examined in an AEI EM6B electron microscope operating at 60 kV with a 50-µm objective aperture.

**MATERIALS**

5-Hydroxy [side chain -2-14C]-tryptamine creatinine sulphate (58 mCi/mm mol) and [8-3H]-adenine (24 Ci/mm mol) were obtained from The Radiochemical Centre, Amersham, U.K.

PGE2, was kindly donated by Dr J. E. Pike, Upjohn Co., Kalamazoo, Michigan. Aspirin (acetylsalicylic acid powder) was obtained from Addenbrooke's Hospital Pharmacy, Cambridge. Indomethacin, tosyl-L-arginyl-methyl ester, butyl PBD, dibutyryl cyclic AMP, EDTA, EGTA and bovine serum albumin were obtained from Sigma Chemical Co., Kingston upon Thames, U.K. Aminophylline and N-ethylmaleimide were obtained from Koch Light Lab., Cohnbroak, U.K. Endotoxin lipopolysaccharide was obtained by phenol extraction of isolated cell walls of *Acinetobacter* 199A (Thorne, Thornley & Glauert, 1973) and was assayed by the carbocyanine method (Janda & Work, 1971).
Platelet responses to endotoxin

RESULTS

Platelet aggregation responses

Endotoxin lipopolysaccharide in concentrations up to 1 mg/ml was added to stirred samples of heparin and citrate PRP from rat, rabbit, pig and man, platelet aggregation was observed only in samples from rat and rabbit. These responses were much greater in heparin PRP than in citrate PRP, and rabbit PRP was more responsive than rat PRP; consequently, further studies were routinely performed in rabbit heparin PRP.

Fig. 1. Platelet aggregation induced by *Acinetobacter* endotoxin.

A. samples (0.1 ml) of rabbit heparin PRP were preincubated for 2 min at 37 °C before addition of endotoxin (added at the arrows). Platelet aggregation was recorded photometrically.

B. samples (0.1 ml) of washed rabbit platelets suspended in balanced salt solution were preincubated for 2 min at 37 °C before the addition of 100 μg/ml endotoxin (at the first arrow) and 15 s later (at the second arrow) 10 μl of heated (56 °C, 30 min) normal plasma, or normal plasma or C₆-deficient plasma were added.

C. heparin PRP was preincubated for 2 min at 37 °C in EGTA or saline before the addition of endotoxin (100 μg/ml).

The rate and extent of aggregation in rat and rabbit platelets was directly related to the endotoxin concentration in the range 1–300 μg/ml. All aggregation responses were characterized by a short lag followed by a transient increase in optical density (associated with the platelet shape change – see below), then a rapid and progressive decrease in optical density as platelet aggregates formed. At critical concentrations of endotoxin (usually 50–100 μg/ml) biphasic aggregation responses were observed (Fig. 1A).
Figs. 2–6. Electron micrographs of thin sections of rabbit platelets. The bar lines represent 1 μm.

Fig. 2. A, control platelets lack pseudopodia and contain dense granules (dg) and lysosomal granules (ly). x 15000.

B, platelets incubated for a short time with endotoxin have undergone a shape change and many pseudopodia have formed. These changes are associated with the initial transient increase in optical density (see Fig. 1A). The dense granules (dg) and lysosomal granules (ly) are still present. × 15000.
Fig. 3. The marginal band of microtubules (arrow) is visible in a control platelet. $dg$, dense granule; $ly$, lysosomal granule; $m$, mitochondria. ×45000.
Relationship of platelet aggregation responses to morphological changes and release of constituents

Using concentrations of endotoxin which produced biphasic aggregation responses in rabbit PRP, subsamples were taken for electron microscopic examination at the peak of the shape-change, at the end of the first phase of aggregation and at the end of the second phase (Fig. 1A). In addition, samples were taken at the same times for measurement of $[^{14}C]$-5HT (dense granule constituent), $\beta$-$N$-acetyl glucosaminidase (lysosomal granule constituent) and $[^{3}H]$adenine (a cytoplasmic marker).

Control platelets incubated in the absence of endotoxin have the shape of ellipsoidal disks (Fig. 2A) and have a peripheral band of microtubules which is clearly visible in sections in the plane of the disk (Fig. 3). The dense granules, which contain 5HT, the lysosomal granules, which contain $\beta$-$N$-acetyl-glucosaminidase, and mitochondria are distributed throughout the cell (Fig. 3).

Platelets incubated with endotoxin, and fixed during the transient increase in optical density observed at the early stages of the interaction, have undergone a change in shape and many pseudopodia have formed (Fig. 2B). The peripheral band of microtubules is disrupted (Fig. 4A), but the dense granules and lysosomal granules are still present (Fig. 2B). Curved sheets of endotoxin are attached to the surfaces of the platelets (Fig. 4A) and are sometimes observed between 2 platelets which appear to be at an early stage of aggregation (Fig. 4B). No release of constituents was detected during the shape-change, or the first phase of aggregation.

The second phase of the aggregation was associated with substantial release of 5HT, and a smaller release of $\beta$-$N$-acetyl-glucosaminidase and adenine (Table 1). Platelets fixed at this stage were observed to be in large clumps and many of them had degranulated (Fig. 5A, B).

Effects of drugs inhibiting platelet function

The second phase of platelet aggregation induced by endotoxin was abolished by agents that increase the platelet cyclic AMP concentration, such as prostaglandin $E_1$ (a potent stimulant of adenylate cyclase), aminophylline (an inhibitor of cyclic AMP phosphodiesterase) and dibutryl cyclic AMP. Secondary aggregation was partly inhibited by aspirin and indomethacin, which block platelet prostaglandin and thromboxane biosynthesis. The thio alkylating agent, $N$-ethylmaleimide, and tosyl-$L$-arginyl-methyl ester (a serine protease inhibitor) also inhibited the second phase aggregation response, but apart from this latter compound, none of the agents inhibited primary aggregation. The effects of these drugs are summarized in Table 2.

Role of plasma constituents

Rabbit platelets washed free from plasma and resuspended in balanced salt solution containing albumin (4%, w/v) did not respond to endotoxin. The addition of 10% (v/v) normal cell-free plasma restored the aggregation response, but plasma pre-viously heated at 56 °C for 30 min was inactive. When plasma from rabbits congenitally deficient in C$_6$ (the sixth component of complement) was added,
Fig. 4. Platelets incubated for a short time with endotoxin. A, the marginal band of microtubules is disrupted and pseudopodia have formed. A curved sheet of endotoxin is present (lps). B, a sheet of endotoxin (lps) is trapped between 2 platelets which appear to be at an early stage of aggregation. Both ×40000.
Fig. 5. A, B, platelets incubated for longer times with endotoxin have aggregated and fewer dense granules and lysosomal granules are visible. Curved sheets of endotoxin (lps) are seen within the aggregates. A, × 30,000; B, × 40,000.
endotoxin-induced platelet aggregation responses were increased compared to control samples receiving normal plasma (Fig. 1B). Similar results were obtained using rat platelets, except that no C6-deficient rat plasma was available. Rabbit plasma could not be used, because rat platelets resuspended in either normal or C6-deficient rabbit plasma aggregated spontaneously.

Table 1. Release of constituents from rabbit platelets induced by Acinetobacter LPS

<table>
<thead>
<tr>
<th>Platelet constituent</th>
<th>Location</th>
<th>% release</th>
</tr>
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<tbody>
<tr>
<td>5-Hydroxytryptamine</td>
<td>Dense storage granules</td>
<td>56.1 ± 8.4</td>
</tr>
<tr>
<td>β-N-acetyl-glucosaminidase</td>
<td>Lysosomal granules</td>
<td>24.3 ± 3.6</td>
</tr>
<tr>
<td>Adenine</td>
<td>Cytoplasm</td>
<td>31.1 ± 2.5</td>
</tr>
</tbody>
</table>

Rabbit platelets in heparin PRP were stirred at 37 °C with 60 µg/ml LPS; this induced biphasic aggregation responses, and samples were taken for release measurements after 4 min (at the end of secondary aggregation). Release of 5HT and adenine was measured by an isotope prelabelling technique, and β-N-acetyl glucosaminidase release was measured fluorimetrically (see methods). Results are mean values ± S.E.; n = 4–7.

Table 2. Effect of drugs on platelet aggregation induced by Acinetobacter LPS

<table>
<thead>
<tr>
<th>Drug</th>
<th>1st phase</th>
<th>2nd phase</th>
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<tbody>
<tr>
<td>Saline</td>
<td>o</td>
<td>o</td>
</tr>
<tr>
<td>PGE1 (30 µM)</td>
<td>o</td>
<td>+ + + +</td>
</tr>
<tr>
<td>Dibutyryl cyclic AMP (0.5 mM)</td>
<td>o</td>
<td>+ + +</td>
</tr>
<tr>
<td>Aminophylline (1 mM)</td>
<td>o</td>
<td>+ +</td>
</tr>
<tr>
<td>Aminophylline (1 mM), plus dibutyryl cAMP (0.5 mM)</td>
<td>o</td>
<td>+ + + +</td>
</tr>
<tr>
<td>Aspirin</td>
<td>o</td>
<td>+ +</td>
</tr>
<tr>
<td>Indomethacin (50 µM)</td>
<td>o</td>
<td>+ + +</td>
</tr>
<tr>
<td>NEM (0.5 mM)</td>
<td>o</td>
<td>+ + +</td>
</tr>
<tr>
<td>TAMe (10 mM)</td>
<td>+ +</td>
<td>+ + + +</td>
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</table>

Heparin PRP was preincubated in drugs or saline for 5 min at 37 °C before photometric measurement of platelet aggregation induced by Acinetobacter LPS 50–100 µg/ml. Responses were measured as rate of change in light transmission.

o = no inhibition; + = 25 % inhibition; + + = 25–50 % inhibition; + + + = 50–75 % inhibition; + + + + = 75–100 % inhibition.

Role of divalent cations

At high concentrations (> 5 mM) both EDTA and EGTA abolished platelet aggregation induced by endotoxin. To investigate the nature of this inhibition, aggregation responses were measured in PRP samples containing 1–5 mM EGTA. EGTA at 1 mM greatly decreased the rate of the primary aggregation response, but once the extent of aggregation reached that of control samples, the secondary response proceeded at a rate comparable with that in controls (Fig. 1C). The minimum concentration of EGTA to block both phases of aggregation was 2 mM. In the presence of 5 mM EGTA, the addition of up to 3 mM MgCl2 did not restore the aggregation response to endotoxin. To determine whether the effect of EGTA was due to
prevention of attachment of endotoxin to platelets, or to interference with subsequent steps in the activation of platelets, electron micrographs of PRP samples which had been stirred for 5 min with endotoxin in the presence or absence of 2 mM EGTA were compared. Under conditions where platelet aggregation was abolished by EGTA, the initial binding of endotoxin to the platelet membrane was apparently unimpaired but there was no change in shape or disruption of microtubule organization (Fig. 6).

Fig. 6. The marginal band of microtubules (arrow) is intact in a platelet incubated with endotoxin in the presence of EGTA. Endotoxin (Ips) has bound to the surface of the platelet but no shape change has occurred and dense granules (dg) and lysosomal granules (ly) are still present. × 55,000.

DISCUSSION

Stimulation of platelets by Acinetobacter endotoxin was restricted to species regarded as immune adherence positive (e.g. rabbit and rat), and the platelet response required divalent cations and a heat-labile plasma factor. Our findings are consistent with several previous observations (Ream et al. 1965; Des Prez & Bryant, 1966; Des Prez, 1967; Spielvogel, 1967), but the present results identify more precisely the mechanisms involved in the platelet response to endotoxin.

Endotoxin-induced platelet aggregation occurred in 2 phases, the first being selectively inhibited by EGTA, and the second inhibited by agents known to prevent the platelet release reaction – that is, the secretion of granule constituents and products of prostaglandin synthesis. Primary aggregation did not occur when platelets were
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suspended in heat-inactivated plasma, but was actually increased in C₆-deficient plasma. These results indicate that early complement components are necessary for this primary aggregation response—C₁₄, C₁₂, and C₂ are all heat-labile, as is factor B of the alternative pathway. Since plasma levels of C₃ are elevated in C₆-deficient rabbits (Chater, 1976), our findings with C₆-deficient plasma are consistent with the concept that C₃ activation is important in initiating the platelet aggregation response to endotoxin.

Endotoxins can activate complement by the classical or the alternative pathway—lipid-rich endotoxins mainly by the classical, and those rich in polysaccharide mainly by the alternative pathway (Morrison, Henson & Kline, 1976). The composition of endotoxin from *Acinetobacter* 199A has not been fully determined, but our results indicate that it may activate platelets primarily via the classical pathway: the alternative pathway is not calcium-dependent (and should therefore be unaffected by EGTA), and, unlike the classical pathway, is inhibited by 10-fold dilution of plasma (Lachmann, 1975). We found that the aggregation response was restored by adding 10% (v/v) cell-free plasma to platelets in balanced salt solution, and that 2 mM EGTA abolished aggregation. Responses were not restored by adding MgCl₂ to EGTA-chelated PRP. It is, however, important to emphasize that because platelet aggregation induced by virtually all stimuli requires extracellular divalent cations, the effects of chelating agents in experiments such as those presented here should not be interpreted solely in terms of an action on complement activation. Endotoxin attachment to platelets was not, however, prevented by 2 mM EGTA. These results are consistent with the concept that the primary phase of platelet aggregation induced by *Acinetobacter* endotoxin depends on activation of C₃ via the classical pathway, although this activation is not necessary for the initial attachment of endotoxin to platelets.

Secondary aggregation and the associated release of platelet granule constituents was due in part to complement-mediated lysis; release of 50% of the 5-HT from dense granules was accompanied by about 30% release of radiolabelled adenine, which acts as a cytoplasmic marker. This method overestimates the amount of lysis, because 20% of cytoplasmic adenine nucleotides (labelled by incubating platelets with [PH]adenine) is converted to hypoxanthine during the secretory response, and hypoxanthine readily diffuses out of platelets (Holmsen, 1975). Nevertheless, the amount of adenine release induced by endotoxin was greater than that in experiments with stimuli not acting via complement activation: comparable 5-HT release induced by collagen or synthetic prostaglandins is accompanied by about 10% adenine release (Holmsen, 1972; Gordon & MacIntyre, 1976). Spielvogel (1967) showed that endotoxin from *E. coli* 0127:B8 caused some platelet lysis, and Siraganian (1972) showed that *E. coli* 026:B6 endotoxin required the late complement components to induced histamine release from rabbit platelets. Our findings with agents that affect cyclic nucleotides, prostaglandin synthesis, and thio groups indicate that, although endotoxin-induced release of platelet constituents depends on complement activation, there is selective release of granule constituents and not merely cell lysis alone. Our conclusion that the late complement components are required for endotoxin-induced release, but not for the initial aggregation response, is consistent with the observation.

The results of our present study help to elucidate the various components of the platelet response to \textit{Acinetobacter} endotoxin, but we cannot assume that identical patterns will be obtained with all endotoxins. The platelet responses to different endotoxins can be at least quantitatively different (Ream et al. 1965; Spielvogel, 1967; Johansson & Nicklasson, 1976), and we recently found that some batches of endotoxin from \textit{E. coli}: 055 B₅ contained substantial amounts of ADP (D. E. MacIntyre, A. H. Drummond, J. L. Gordon, unpublished observations). This contamination can apparently arise when phenol extracts of whole cells (instead of isolated cell walls) are used, and since ADP is a potent platelet stimulant, experiments with such preparations will inevitably give a false picture of platelet activation by endotoxin.

We can, however, conclude from our present results that interaction of rabbit platelets with \textit{Acinetobacter} endotoxin can be resolved into 3 stages: (1) initial attachment of endotoxin to the cell membrane, which does not require complement activation; (2) primary aggregation, which depends on C₉ activation possibly via the classical pathway; and (3) secretion of platelet granule constituents, associated with some complement-mediated lysis and with secondary platelet aggregation. It is now important to establish the sequence of events following the interaction of endotoxin with the cell membrane, and this is the subject of a subsequent paper (Thorne, Oliver, MacIntyre & Gordon, 1977).

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