EFFECTS OF BACTERIAL ENDOTOXIN ON RABBIT PLATELETS*

III. Comparison of Platelet Injury Induced by Thrombin and by Endotoxin

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(Received for publication, March 31, 1964)

Rabbit platelets in plasma exposed to bacterial endotoxin undergo aggregation, fusion, and release of several biologically active platelet factors (1). Since a superficially similar type of platelet injury can be produced by thrombin, some investigators have proposed that platelet injury in *in vitro* systems is produced by elaboration of small quantities of thrombin which cause polymerization of fibrinogen adherent to platelet surfaces but are insufficient to produce gross clotting (2). Application of this formulation to the platelet injury induced by endotoxin requires the assumption that endotoxin interacts with components of plasma to produce thrombin and that the observed platelet changes are secondary.

Previously this laboratory has reported studies suggesting that the coagulation acceleration observed when endotoxin is added to blood *in vitro* is a result of platelet injury rather than its cause (3). The present studies were undertaken to further define and compare the nature of endotoxin-induced and thrombin-induced platelet damage.

Materials and Methods

A lipopolysaccharide endotoxin of the bovine type prepared from *Escherichia coli* 0127:B8 (Difco Laboratories Inc., Detroit) was used in a final concentration of 2 mg per ml in normal saline. Varying lots of this material were found to differ in their platelet-injuring effects and accordingly only active lots were used. Bovine topical thrombin (Parke, Davis & Co., Detroit) was freshly mixed with the provided diluent and further diluted with normal saline to the desired NIH unit strength. One NIH unit is defined as that amount of thrombin which, when mixed in 0.1 ml volume with 0.1 ml of bovine fibrinogen solution in a concentration of 3 mg per ml (Warner-Lambert-Chilcott, Morris Plains, New Jersey) will cause clot formation in 15 seconds. 3.8 per cent sodium citrate was employed in a final concentration of one part to nine

* This work was supported by grants from the United States Public Health Service (Grants HE-08399, H-3479, and AI-3285) and by The Health Research Council of The City of New York, contract U-1107.

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parts blood, and fresh solutions of heparin without preservative (Connought Laboratories, Toronto, Canada) were employed in some experiments in the concentrations indicated.

The methods of preparation of platelet-rich plasma and platelet-poor plasma by differential centrifugation, platelet enumeration, and 5-hydroxytryptamine (5 HT) assay and preparation of siliconized pyrogen-free glassware have been previously described (1).

EXPERIMENTAL

Stability of 5 HT in Various Solutions.—These experiments have utilized release of platelet 5 HT into plasma *in vitro* as an index of platelet damage. In the course of the experiments it became apparent that changes in the distribution of 5 HT between platelets and plasma are influenced not only by platelet injury but also by deterioration of 5 HT in plasma, absorption of plasma 5 HT onto normal platelets, and slow spontaneous release of 5 HT from platelets. These three processes are demonstrated in the experiments illustrated in Figs. 1 and 2.

5 HT in a final concentration of 5 μg per ml was added to platelet-poor plasma and to saline. These materials were incubated at 37°C, aliquots were removed at intervals, and the concentration of 5 HT determined (Fig. 1).

In the experiment illustrated in Fig. 2, 5 HT in a concentration of 5 μg per ml was added to platelet-rich plasma already containing endogenous 5 HT in a concentration of 9 μg per ml. This material was similarly rotated and incubated, aliquots were removed at intervals, and centrifuged at high speeds in the cold to separate platelets and plasma. 5 HT concentration was assayed directly in the plasma. Platelets were resuspended in a volume of saline equal to the plasma from which they had been separated, lysed by three cycles of freezing and thawing, and the 5 HT concentration of the platelet lysate determined.

Less than one-half of the initially added 5 HT was recovered after 120 minutes' incubation, indicating that deterioration in plasma at 37°C is fairly rapid (Fig. 1). Paired aliquots of the plasma carried through three cycles of freezing and thawing did not demonstrate any increase in 5 HT content. This indicated that virtually no platelets remained after high speed centrifugation and eliminated the possibility that disappearance of plasma 5 HT might be due to absorption onto small numbers of unsedimented platelets (*vide infra*). 5 HT in buffered saline at 37°C was considerably more stabile, over 75 per cent being recovered after 2 hours' incubation. Other experiments not documented here indicate that 5 HT is quite stabile for periods up to 8 hours in plasma kept at 4°C. Fig. 2 illustrates that 5 HT added to platelet-rich plasma becomes completely bound to platelets during incubation for 1 hour. The subsequent decrease in platelet 5 HT concentration is presumably due to slow spontaneous release of 5 HT from platelets and its subsequent destruction in plasma. All these processes influence the results of the experiments to be described.

Comparison of the Effects of Endotoxin and Thrombin on Citrated Platelet-Rich Plasma.—The patterns of platelet injury induced by endotoxin and by thrombin differ in several important respects.

Fig. 3 illustrates the results of an experiment in which platelet-rich plasma was mixed with endotoxin (100 μg per ml) in a siliconized flask and incubated at 37°C while undergoing standardized agitation. Aliquots were removed at intervals, platelets and plasma separated

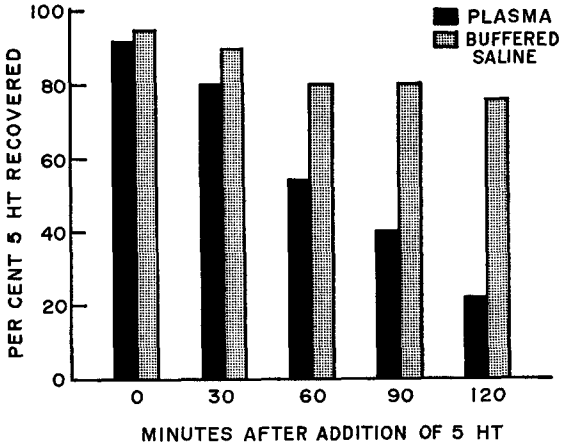

FIG. 1. Changes in 5 HT concentration in platelet-poor plasma (dark bar) and buffered saline (stippled bar) to which 5 HT had been added in a concentration of 5 μg per ml. Materials were incubated at 37°C and agitated during the course of the experiment.

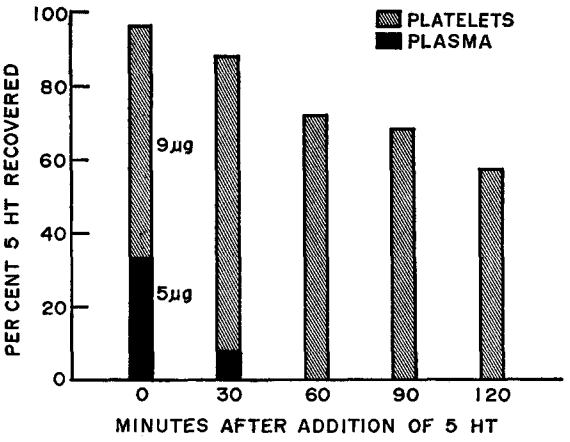

FIG. 2. Changes in 5 HT concentration in platelet-rich plasma (initial 5 HT concentration 9 μg per ml) to which exogenous 5 HT (5 μg per ml) was added. Plasma was incubated at 37°C, agitated, aliquots removed, centrifuged, and assayed for plasma and platelet 5 HT concentration.

by centrifugation in the cold, and the plasma and platelet 5 HT concentrations determined as previously described.

In a parallel experiment (Fig. 4) platelet-rich plasma was divided among 5 siliconized centrifuge tubes, thrombin was added in a concentration of 5 NIH units per ml of plasma, and all

tubes were sealed with parafilm and incubated while undergoing agitation. At the indicated intervals single tubes were removed, plasma was separated from the gel clot by expression with applicator sticks and subsequent centrifugation, and the 5 HT content of plasma and the platelet-fibrin residues was determined.

Control aliquots of platelet-rich plasma incubated with saline demonstrated no transfer of 5 HT from platelets to plasma over the period of the experiment.

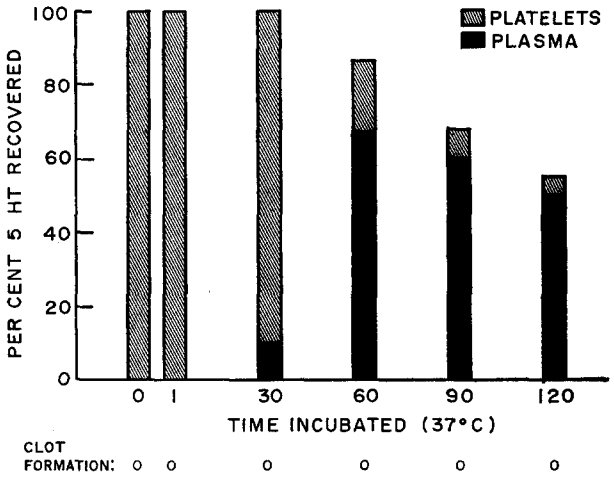

FIG. 3. Changes in plasma (dark bar) and platelet (cross-hatched bar) 5 HT concentrations over the course of incubation of rabbit platelet-rich plasma with endotoxin (100 μg per ml plasma).

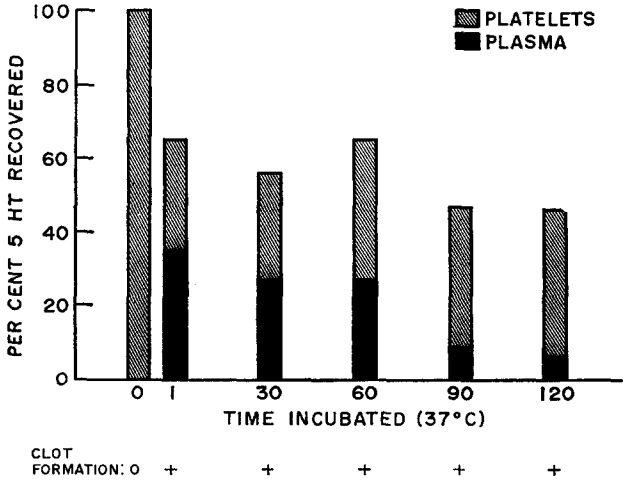

FIG. 4. Changes in plasma (dark bar) and platelet (cross-hatched bar) 5 HT concentrations over the course of incubation of rabbit platelet-rich plasma with thrombin (5 NIH units per ml plasma).

In interaction of platelets and endotoxin (Fig. 3), an appreciable lag period occurs before release of 5 HT is observed. This then progresses until virtually all of the 5 HT has been released from platelets. The concentration of 5 HT in plasma in the experiment illustrated was greatest at 60 minutes' incubation and subsequently declined somewhat due to deterioration of 5 HT in plasma even though release of platelet 5 HT continued. No clot formation was observed under these conditions. In contrast (Fig. 4) when thrombin and platelets interacted, the greatest plasma 5 HT concentration appeared immediately and the reaction was not progressive. The decrease in plasma 5 HT concentrations observed in the later specimens appeared to be due in part to deterioration of 5 HT in plasma and in part reabsorption of 5 HT onto the platelet-fibrin mesh. In all instances, a gross fibrin clot appeared immediately.

TABLE I

Effect of Graded Concentrations of Thrombin on Clot Formation and Release of Platelet 5 HT in Citrated Platelet-Rich Plasma

Thrombin added	1 min. after thrombin			60 min. after thrombin		
	Clot formation	5 HT recovered		Clot formation	5 HT recovered	
		Plasma	Platelets		Plasma	Platelets
NIH u./ml		*per cent*	*per cent*		*per cent*	*per cent*
0.5	0	1	100	0	1	97
1	+	2	87	+	1	71
2	+	2	—	+	1	75
3	+	13	61	+	2	60
4	+	55	50	+	15	50
5	+	60	35	+	28	35

Determination of the Minimum Thrombin Concentrations Required for Clot Formation and for Platelet Damage in Citrated Platelet-Rich Plasma.—The observed lag period in the endotoxin-platelet interaction might represent time required for the elaboration of thrombin in concentrations insufficient to cause gross clot but adequate to polymerize platelet fibrinogen and thereby damage platelets. However, experiments to test this point (Table I) indicated that the concentration of thrombin adequate to coagulate citrated platelet-rich plasma was appreciably less than that required to produce release of platelet 5 HT. Plasma 5 HT concentrations are greater in the 1 minute specimens than in the 1 hour specimens, for reasons indicated above.

Effects of Heparin on the Thrombin-Platelet Reactions as Compared with the Endotoxin-Platelet Reaction.—The action of thrombin on fibrinogen and on platelets is relatively insensitive to inhibition by sodium citrate, but is quite sensitive to inhibition by heparin. As illustrated in Table II, a thrombin con-

centration of 5 NIH units per ml was without effect either on platelets or fibrinogen in platelet-rich plasma containing heparin 100 μg per ml, and when

TABLE II

Effect of Heparin on Release of Platelet 5 HT by Thrombin and by Endotoxin

Additives (0.1 ml per ml plasma)				5 HT recovered in plasma	
Heparin	Thrombin	Endotoxin	Saline	Time incubated	
				1 min.	90 min.
μg/ml	*NIH u./ml*	*μg/ml*	*ml*	*per cent*	*per cent*
0	5	—	0.2	60	22
100	—	100	0.2	1	50
100	5	100	0.1	1	53
100	5	—	0.2	1	1
100	—	—	0.3	1	1
1000	—	100	0.2	1	46
1000	25	100	0.1	1	31
1000	25	—	0.2	1	2
1000	—	—	0.3	1	1

TABLE III

Effect of Graded Concentrations of Heparin on Release of Platelet 5 HT by Endotoxin

Heparin	Additive*	Time after addition of endotoxin			
		1 min.		90 min.	
		5 HT recovered			
		Plasma	Platelets	Plasma	Platelets
μg/ml		*per cent*	*per cent*	*per cent*	*per cent*
100	Saline	1	100	1	100
100	Endotoxin*	1	100	60	26
500	"	1	100	38	38
1000	"	1	100	34	40
2500	"	1	100	12	60
5000	"	1	100	1	100

* 100 μg per ml platelet-rich plasma.

the concentration of heparin was increased to 1 mg per ml, a thrombin concentration of 25 NIH units was insufficient to produce platelet damage. In contrast (Table III), platelet damage by endotoxin is seen at both of these concentrations of heparin, and it is necessary to increase the concentration of heparin to 5 mg per ml of platelet-rich plasma to completely inhibit this property of endotoxin.

DISCUSSION

The experiments reported above indicate that the interaction of bacterial endotoxin and rabbit platelets is mediated through some mechanism other than formation of small quantities of thrombin. The results reported are in accord with notion that endotoxin-induced hypercoagulability in *in vitro* systems is a result of platelet damage rather than its cause.

The immediate release of platelet factors following exposure to thrombin has also been noted by Grette, who suggests that thrombin acts as a proteolytic substance on some component of the platelet surface other than fibrinogen (4). Grette has demonstrated that similar platelet injury and release of platelet factors can be produced by trypsin in conjunction with calcium ions.

The delayed effect of endotoxin on platelets implies an indirect action, possibly mediated through generation of proteolytic activity in plasma. Previous studies from this laboratory have suggested the participation of plasma factors in the process of platelet injury by endotoxin (1). It has been demonstrated that plasma separated from platelets and heated at 56°C centigrade for 60 minutes or absorbed at 37°C centigrade for 60 minutes with heterologous heat-killed bacterial cells is unable to produce platelet injury when remixed with platelets and endotoxin. Further, preincubation of plasma and endotoxin in the absence of platelets resulted in greatly accelerated release of 5 HT when platelets were exposed to such plasma. These observations give support to the concept that the action of endotoxin is indirect and involves generation of an injurious substance in plasma. Two facts suggest that this injurious substance is not thrombin. First, very large quantities of heparin do not inhibit the endotoxin-platelet interaction. Second, concentrations of thrombin competent to produce gross clot formation in citrated platelet-rich plasma are less than those required to cause release of platelet 5 HT.

The belief that many types of platelet injury are due to generation of small quantities of thrombin is based primarily on the observation that trypsinization of platelets render them refractory to injury. It is assumed that the principal effect of such treatment is the removal of fibrinogen absorbed to the platelet surface (2). The studies by Grette cast doubt on this formulation (4). It has been demonstrated that platelets incubated with trypsin in the absence of calcium ions are not depleted of fibrinogen. The subsequent addition of calcium ions yields release, not only of fibrinogen, but also 5 HT and adenine nucleotides, both substances regarded as intracellular platelet factors. It is of great interest that this release of intracellular platelet factors was not accompanied by aggregation of the platelets. Thus platelet aggregation and release of intracellular factors may each be distinct manifestations of platelet injury not necessarily occurring together.

Platelet injury has been observed to occur during the course of antigen-antibody reactions both *in vitro* and *in vivo* (5–10). Platelet damage occurring

during such reactions is also resistant to inhibition by heparin. Further, this system requires plasma or serum factors before platelet injury occurs. In contrast to the endotoxin effect, however, immunologically induced platelet injury requires ionized calcium and is inhibited by citrate, not only by removal of ionized calcium, but also by some mechanism independent of calcium chelation (2, 8, 9). In spite of this dissimilarity it seems possible that the endotoxin-platelet interaction may also be immunologic in nature. This notion is in keeping with recent observations attributing many of the biologic effects of endotoxin to immunologic mechanisms.

It has long been apparent that the coagulation mechanism is intimately involved in many types of tissue injury produced by immunologic reactions or endotoxin. Though the details of this relationship are still obscure, it is attractive to postulate that both systems involve proteolytic injury to platelet membranes.

SUMMARY

The platelet injury produced by bacterial endotoxin and thrombin have been compared in studies utilizing citrated rabbit platelet-rich plasma. Endotoxin-induced platelet injury is characterized by a lag period, is progressive, and does not produce gross clot formation. Thrombin-induced platelet injury is immediate, non-progressive, and is associated with clot formation. The quantity of thrombin required to produce clot formation in this citrated system is less than that required to produce release of platelet 5-hydroxytryptamine. The endotoxin-induced platelet injury required extremely large quantities of heparin for inhibition. The platelet injury induced by thrombin can be inhibited by small quantities of heparin. It is concluded that the injurious effects of endotoxin on platelets is mediated through some mechanism other than thrombin formation.

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