ALTERATIONS IN THE BLOOD COAGULATION SYSTEM INDUCED BY BACTERIAL ENDOTOXINS

II. In Vitro

By DONALD G. McKAY, M.D., SANDOR S. SHAPIRO,* M.D., and JACOB N. SHANBERGE, M.D.

(From the Departments of Pathology, Obstetrics and Gynecology, Harvard Medical School, Boston, and the Pathology Laboratory, Free Hospital for Women, Brookline, Massachusetts)

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With the observation that bacterial endotoxins alter the blood coagulation system *in vivo* (1), the question was raised as to whether this is a direct or an indirect effect of the toxins on the blood. In an attempt to answer this question and to determine the mechanism by which bacterial endotoxin affects the coagulation system the following *in vitro* studies were performed.

Effect of Endotoxin on Whole Blood.—

To test the effect of bacterial endotoxins on the coagulation time of whole blood, a modified Lee-White coagulation time in silicone was used. One ml. of human blood was added to each of three tubes containing 0.2 ml. of saline for the control series, and 0.2 ml. of saline containing varying concentrations of Shear's polysaccharide[1] for the tests. Four concentrations of endotoxin were used; *i.e.*, 1, 0.1, 0.01, and 0.005 mg. per 0.2 ml. of sterile saline. The highest dilution (0.005 mg.) was calculated to be the concentration per milliliter of blood which would be achieved immediately following the intravenous injection of doses of endotoxin capable of producing the generalized Shwartzman reaction in 1 kg. rabbits. The blood of three individuals was used with all 4 concentrations of Shear's polysaccharide. The time required to achieve a solid clot in the third tube was taken as the coagulation time. The results are presented in Table I and Fig. 1.

In amounts as small as 0.005 mg. per ml., Shear's polysaccharide shortens the coagulation time of whole blood approximately 20 per cent. Within the range of amounts of endotoxin used, the shortening of the clotting time was directly proportional to the amount of endotoxin until at levels of 1 mg./ml. it was at least 50 per cent shorter than the control clotting time.

That this property of Shear's polysaccharide is shared by other bacterial endotoxins is shown in experiments illustrated in Table II using endotoxins derived from *Shigella paradysenteriae* and meningococci.[2]

* Former Josiah Macy Jr. Student Fellow in Obstetrics.

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[2] We are grateful to Dr. Walther Goebel of The Rockefeller Institute for the *Shigella* toxin and to Dr. Gregor Shwartzman of the Mt. Sinai Hospital, New York, for the meningococcus toxin.

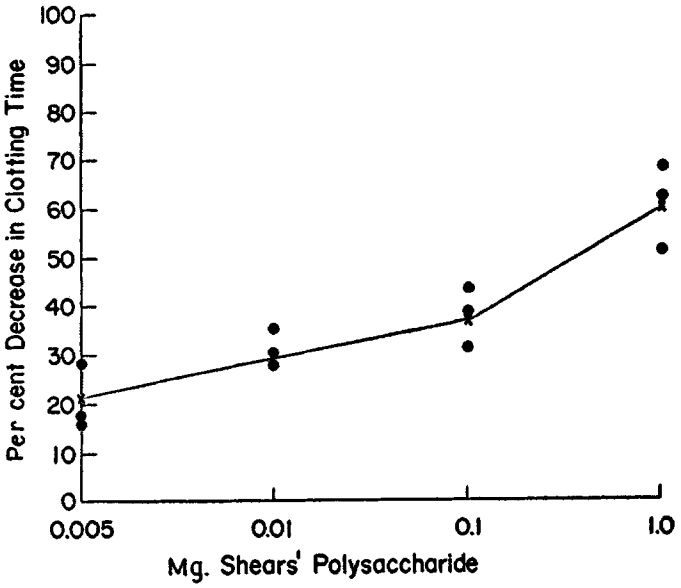

FIG. 1. Dose response curve of whole blood to bacterial endotoxin.

TABLE I

Whole Blood Coagulation Times (in Minutes in Silicone)

Amount of polysaccharide	Source of blood	Control	Test	Difference	Decrease
mg.		*min. sec.*	*min. sec.*	*min. sec.*	*per cent*
1.0	E.C.A.	32 10	11 10	21 00	65
	D.G.M.	22 10	10 15	11 55	54
	S.S.S.	40 15	11 25	28 50	72
0.1	E.C.A.	29 50	17 50	12 00	40
	D.G.M.	30 45	20 45	10 00	33
	S.S.S.	36 20	19 40	16 40	46
0.01	E.C.A.	28 00	19 55	8 05	29
	D.G.M.	36 15	23 15	13 00	36
	S.S.S.	40 00	27 50	12 10	30
0.005	E.C.A.	34 20	28 10	6 10	18
	D.G.M.	23 05	16 20	6 45	29
	S.S.S.	34 25	28 05	6 20	18

Effect of Endotoxin on Plasma.—The shortening of the whole blood coagulation time *in vitro* upon exposure to endotoxin could conceivably be caused by damage to the cellular elements of the blood with release of clot-promoting agents. Such agents have been demonstrated in red blood cells and leukocytes, as well as platelets (2). To determine whether red blood cells or leukocytes were necessary for the reaction, the effect of endotoxin on plasma clotting times was studied. Endotoxin produced no detectable shortening of the clotting time of recalcified oxalated or citrated human plasma.

TABLE II

Source of blood	Test	Coagulation time (silicone)	Difference	Per cent decrease
	Meningococcus toxin			
		min. *sec.*	*min.* *sec.*	*per cent*
S.S.S.	0.2 ml. saline	35 00		
	0.2 ml. saline containing 0.005 ml. Meningococcus toxin	24 23	10 37	30
W.A.B.	0.2 ml. saline	43 30		
	0.2 ml. saline containing 0.005 ml. Meningococcus toxin	26 30	17 00	39
D.G.M.	0.2 ml. saline	50 20		
	0.2 ml. saline containing 0.005 ml. Meningococcus toxin	33 35	16 45	33
	Shigella paradysenteriae toxin (Newcastle strain)			
S.S.S.	0.2 ml. saline	42 00		
	0.2 ml. saline containing 1.0 mgm. *Shigella* toxin	23 00	19 00	45
E.C.A.	0.2 ml. saline	16 30		
	0.2 ml. saline containing 1.0 mg. *Shigella* toxin	9 30	7 00	42
D.G.M.	0.2 ml. saline	30 30		
	0.2 ml. saline containing 1.0 mg. *Shigella* toxin	13 00	17 30	57

It was necessary to resort to native human plasma obtained in siliconized syringes and glassware by spinning whole blood for 5 minutes at 1500 R.P.M. The plasma was drawn off immediately and added to siliconized tubes for the control series and to similar tubes containing 1 mg. of Shear's polysaccharide for the test series. A second control consisted of concomitant tests on whole blood. The plasma was demonstrated to be free of red blood cells and leukocytes. The starting time was the time of addition of plasma to the tubes.

TABLE III

Effect of Endotoxins on Native Plasma

Source of blood	Test	Coagulation time in silicone	Difference	Decrease
		min. sec.	*min. sec.*	*per cent*
S.S.S.	Whole blood	68 00		
	Whole blood + 1 mg. Shear's polysaccharide	24 00	44 00	65
S.S.S.	Native plasma	50 00		
	Native plasma + 1 mg. Shear's polysaccharide	27 00	23 00	46
S.S.S.	Native plasma	60 00		
	Native plasma + 1 mg. Shear's polysaccharide	26 30	33 30	56

TABLE IV

Source of blood	Test	Coagulation time in silicone	Difference	Decrease
		min. sec.	*min. sec.*	*per cent*
S.S.S.	Heparinized platelet-poor plasma	173 30		
	Platelet-poor plasma + 1 mg. Shear's polysaccharide	134 00	39 30	23
S.S.S.	Heparinized platelet-poor plasma	170 00		
	Platelet-poor plasma + 1 mg. Shear's polysaccharide	116 00	54 00	32

The results presented in Table III demonstrate that bacterial endotoxins are capable of shortening the coagulation time of native plasma and therefore that this effect is independent of red blood cells and white blood cells.

Stetson's (3) demonstration that certain "Shwartzman-active agents"

cause platelets to clump *in vitro*, and the observation of thrombocytopenia *in vivo* following intravenous injection of toxin indicate that platelets may be involved in this reaction. Since plasma derived from blood spun at 1500 R.P.M. for 5 minutes still contains large numbers of suspended platelets, the

TABLE V

Experiments

Plasma	Platelet count	Amount used	Clotting time	Clot retraction	Prothrombin consumption time	
					15 min.	60 min.
			min.		*sec.*	*sec.*
1. A	489,000	1 ml.	5	++++	10.5	16
B	—	1 ml.	7	−	8.5	9
0.2 ml. A + 1.8 ml. B	43,000	0.9 ml. + 0.1 ml. toxin	8.5	+	9	16
		0.9 ml. + 0.1 ml. saline	16	+	9	13
2. A	307,000	1 ml.	8	+++	13.5	17
B	—	1 ml.	18	−	9	9
0.3 ml. A + 1.7 ml. B	52,000	0.9 ml. + 0.1 ml. toxin	8.5	++	8	15.5
		0.9 ml. + 0.1 ml. saline	12	±	8	13.5
3. A	599,000	1 ml.	4.5	++++	24.5	29.5
B	—	1 ml.	7	−	10	9.5
0.2 ml. A + 1.8 ml. B	61,000	0.9 ml. + 0.1 ml. SP	4.5	−	12	12
		0.9 ml. + 0.1 ml. saline	4	−	10.5	11
4. A	382,000	1 ml.	4.5	++++	19.5	23
B	—	1 ml.	7.5	−	10.5	10.5
0.6 ml. A + 2.4 ml. B	55,000	0.9 ml. + 0.1 ml. SP	9	±	14.5	14
		0.9 ml. + 0.1 ml. toxin	6.5	++	14	19
		0.9 ml. + 0.1 ml. saline	8.5	+	15	14
B	—	0.9 ml. + 0.1 ml. toxin	7	−	10	10.5

Toxin, full strength meningococcic toxin.
SP, Shear's polysaccharide—5.0 mg./ml.
Saline, 0.85 per cent NaCl.
A, slow centrifuged plasma.
B, rapid centrifuged plasma.

effect of endotoxin on platelet-poor plasma was examined to test the relative importance of these formed elements of the coagulation system.

In order to facilitate the handling of platelet-poor plasma, small amounts (1 μg./ml.) of heparin were used. The blood was handled in siliconized equipment and spun in a refrigerated centrifuge at 10,000 R.P.M. for 30 minutes. This plasma was allowed to clot spontaneously in siliconized tubes for the control series and with 1.0 mg. of Shear's polysaccharide in the test series. (Counts of platelet-rich plasma were 338,000/c.mm. and of platelet-poor plasma were 42,000/c.mm.).

The results presented in Table IV indicate that endotoxin is capable of shortening the coagulation time of (heparinized) platelet-poor plasma, although to a lesser extent than platelet-rich plasma.

Effect of Endotoxin on Prothrombin Consumption.—Since certain clot-promoting agents, such as hemolyzed red blood cells, are capable of increasing prothrombin consumption (4), it was of interest to learn whether bacterial endotoxins exert a similar effect. Native human plasma with high and low platelet counts was obtained in the following manner:—

TABLE VI

Source of blood	Test	Coagulation time	Difference	Decrease
	Hemophilic blood in uncoated glass			
				per cent
R. W. (hemophilia)	0.2 ml. saline	28 min. 00 sec.	—	—
	0.2 ml. saline + 0.005 ml. meningococcus toxin	29 min. 20 sec.	—	—
	0.2 ml. saline + 1.0 mg. of Shear's polysaccharide	31 min. 00 sec.	—	—
	Hemophilic blood in silicone			
R. W.	0.2 ml. saline	3 hrs. 55 min. 10 sec.		
	0.2 ml. saline + 0.005 ml meningococcus toxin	2 hrs. 8 min. 00 sec.	1 hr. 47 min. 10 sec.	45

All equipment was siliconized. Blood was drawn by the two-syringe technique with exchange of syringes after the initial venepuncture and flushing of the needle by a few milliliters of blood. Plasma with a high platelet count was obtained by centrifugation in a refrigerated centrifuge at 4°C. for 10 minutes at 800 R.P.M. After centrifugation plasma was transferred with a siliconized pipette to a chilled siliconized test tube and kept in an ice bath until ready for use. The platelet count on slow centrifuged plasma averaged 450,000/c.mm. Platelet-poor native plasma was obtained by centrifugation of blood at 4°C. for 20 minutes at 4200 R.P.M.; the plasma separated and kept in an ice bath until ready for use. Platelet counts on rapidly spun plasma showed only 1 or 2 platelets in the entire counting chamber. Mixtures of the two plasmas were subsequently made to obtain the desired number of platelets. The plasma clotting times were determined by transferring plasma to an uncoated glass tube and placing in a 37°C. water bath and the stop-watch started. The tubes were examined periodically but not tilted and clotting was considered complete when the plasma became homogeneously opaque.

To obtain the prothrombin consumption time, 15 minutes after the completion of clotting,

the clot was compressed with the tip of a pipette and simultaneously with the starting of the stop-watch, 0.1 ml. of serum was blown into a tube containing 0.1 ml. of rabbit brain thromboplastin (prepared according to the method of Quick (5), 0.1 ml. of 0.01 M $CaCl_2$, and 0.1 ml. of rabbit plasma deprothrombinized with tricalcium phosphate. The clotting time and end point was the appearance of fibrin strands. This procedure was repeated 45 minutes later or 60 minutes after the completion of clotting.

The results of the effect of endotoxins or prothrombin consumption are presented in Table V and show that meningococcus toxin increases prothrombin consumption but that Shear's polysaccharide does not. Shear's polysaccharide is a relatively pure substance compared with the agar washings of the meningococcus. Extraneous material in the meningococcus preparations may account for this difference.

Effect of Endotoxin on Hemophilic Blood.—In an attempt to narrow the search for the point in the coagulation system in which endotoxin exerts its effect, whole blood from a classic hemophilic patient[3] was exposed to endotoxin. This test was done in the same manner as the other whole blood coagulation studies except that uncoated as well as siliconized glassware was used. The results presented in Table VI show that endotoxin did not shorten the coagulation time of hemophilic blood in glass, but did act on the same blood in silicone.

The studies with hemophilic blood in glass indicate that bacterial endotoxins do not possess the properties of preformed thromboplastin or thrombin, since either agent would have shortened the clotting time. The effect in silicone is of interest since the percentage of shortening was the same as that seen using normal blood, even though the actual time involved was considerably longer. This suggests that under these conditions antihemophilic globulin is a limiting factor in the action of endotoxin on blood and that toxins act somewhere in the coagulation system prior to the combination of thromboplastin precursors.

Effect of Endotoxin on Fibrinogen (Fraction I).—The possibility that endotoxin shortened the coagulation time by an action similar to thrombin was further ruled out by incubating 0.2 ml. of saline containing 5 mg. of Shear's polysaccharide with 1 ml. of a solution of fraction I containing 167 mg. per cent of fibrinogen. This mixture was incubated at 37°C. for 2 days and no fibrin was formed, indicating again that bacterial endotoxin does not act in the manner of thrombin directly on fibrinogen.

SUMMARY AND CONCLUSIONS

Bacterial endotoxins *in vitro* are capable of shortening the coagulation time of normal whole blood, native platelet-rich and platelet-poor plasma, and the blood of a hemophilic patient in silicone but not in glass. The point in the

[3] We are grateful to Dr. Fred Bigelow of the Thorndike Laboratory, Boston City Hospital, for the hemophilic blood.

coagulation system at which the endotoxins act has not been found but the search has been narrowed by the demonstration that these materials act independently of leukocytes and red blood cells, and do not act as preformed thromboplastin or thrombin. The shortening of the coagulation time *in vivo* 4 hours after endotoxin injection is probably through a different mechanism than *in vitro*.

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