JCI The Journal of Clinical Investigation

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J Clin Invest. 1972;51(2):307-313. https://doi.org/10.1172/JCI106815.

Research Article

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Coagulant Activity of Leukocytes

TISSUE FACTOR ACTIVITY

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ABSTRACT Peritoneal leukocytes harvested from rabbits which have received two spaced doses of endotoxin have significantly greater (10-fold) coagulant activity than leukocytes from control rabbits. The coagulant activity accelerates the clotting of normal plasma and activates factor X in the presence of factor VII and calcium and is therefore regarded as tissue factor. A total of 40–80 mg tissue factor activity was obtained from the peritoneal cavity of single endotoxin-treated rabbits. In leukocyte subcellular fractions, separated by centrifugation, the specific tissue factor activity sedimented mainly at 14,500 *g* and above. The procoagulant activity was destroyed after heating for 10 min at 65°C but was preserved at lower temperatures. Polymyxin B, when given with the first dose of endotoxin, reduced both the number of peritoneal leukocytes and their tissue factor activity by two-thirds. When given immediately before the second dose of endotoxin, polymyxin B had no inhibitory effect.

INTRODUCTION

Several lines of evidence suggest that white cells may play a role in blood coagulation in vivo. White cells have been shown to contain both procoagulant and anticoagulant activity (1–3), the anticoagulant activity having been more thoroughly investigated (4). Also, patients with acute leukemia may present with defibrination and thrombosis (5–10). Defibrination occurs with great frequency in acute promyelocytic leukemia and heparin may reverse the defibrination syndrome (10). A third line of evidence concerns the generalized Shwartzman phenomenon. White cells have been implicated in the Shwartzman phenomenon. White cells have been implicated in the Shwartzman phenomenon because rabbits made granulocytopenic with nitrogen mustard are protected against the endotoxin-induced phenomenon (11). Anticoagulants (12,13) also prevent the phenomenon. These findings suggest that leukocytes may mediate the intravascular coagulation of the Shwartzman phenomenon. Conflicting views have been presented on the role of white cells in the Shwartzman phenomenon because the procoagulant activity of white cells has been considered too slight to be significant (14). The purpose of this paper is to show that endotoxin injected into rabbits greatly increases the procoagulant activity of the recipients' leukocytes.

This work was presented in part at the 53rd Annual Meeting of the Federation of American Societies for Experimental Biology, and appeared in abstract form in *Fed. Proc.* 1969. **28**: 442.

Received for publication 3 June 1971 and in revised form 19 August 1971.

METHODS

Normal human plasma and human plasma deficient in factors VIII and IX were obtained from normal subjects and patients with cogenital coagulation-factor deficiencies. The blood was anticoagulated with 0.1 vol 4% trisodium citrate dihydrate and centrifuged at 2000 *g* for 15 min at 4°C. Plasma was used either fresh or stored at −60°C. Rabbit plasma was prepared in the same manner. Bovine plasma deficient in factors VII and X was prepared by the method of Bachmann (15) or obtained commercially (factor X reagent, Diagen, Oxon, England). Plastic, polycarbonated (Falcon Plastics, Oxnard, Calif.) or siliconized glassware (Siliclad, Clay-Adams, Parsippany, N. J. used as 10% solution) was utilized throughout. Endotoxin (Difco Labs, Detroit, Mich.) lipopolysaccharide B from *E. coli* 026.B6 was used within 20 hr after suspension in buffered saline. Isotonic buffered saline pH 7.3 (16), citrate saline was 1 part 4% solution of trisodium citrate dihydrate and 5 parts saline, Hanks solution (17) was supplied by Difco.

Rabbit brain thromboplastin (Difco) was extracted with saline according to the instructions of the manufacturer, centrifuged at 500 *g* for 3 min and the supernate stored in small portions at −60°C. A single thromboplastin preparation was used as a standard for tissue factor activity. 1 ml of the standard preparation weighed 12.2 mg after

Table I

Procoagulant Activity of Peritoneal Leukocytes

	Substrate clotting times in seconds		
	E_0	E_2	CBS
Human plasma	87.6	53.6	150.6
Rabbit plasma	43.6	22.0	76.0
Celite exhausted plasma + cephalin 1/50	107.0	53.8	128.0
Human plasma + cephalin 1/50	81.0	40.0	109.0

To 0.1 ml substrate plasma, 0.1 ml suspension of leukocytes (60,000 per mm³) in citrate-buffered saline, 0.1 ml $CaCl_2$ 0.05 M is added and the clotting time recorded. E_0, leukocytes from control rabbits (no endotoxin); E_2, leukocytes from rabbits receiving two doses of endotoxin; CBS, citrate-buffered saline.

desiccation. Cephalin was prepared from rabbit brain by the method of Bell and Alton (18). A $BaSO_4$ eluate prepared from human serum exhausted with Celite was used as a source of factors VII and X. The concentration of factors VII and X equaled that present in a pool of 10 normal plasma samples (10).

Peritoneal leukocytes were obtained from white male rabbits weighing 2.2–3.2 kg by the method of Hirsh (19) as follows. Control leukocytes (E_0)[1] were obtained from rabbits which had received 250 mg starch which had been sterilized for 4 hr at 100°C and suspended in 250 ml sterile, pyrogen-free 0.15 M, saline. 18 hr later, 80 ml citrate saline was injected, through plastic perforated tubing, into the peritoneal cavity. The abdomen was kneaded briefly and 140–200 ml peritoneal fluid collected. After filtering the fluid through four thicknesses of gauze, the leukocytes were counted. They were sedimented at 250 *g* for 10 min, washed twice by gentle resuspension in citrated saline, and sedimentation at 250 *g* for 10 min after each wash, and finally suspended in citrate saline at a concentration of 60,000 per mm³. Endotoxin-treated rabbits received 30 μg/kg of endotoxin simultaneously with the starch. 16 hr later the same amount of endotoxin was given intravenously. 1 hr thereafter, the peritoneal fluid was collected and processed as described above—the resulting cells being referred to as E_2 leukocytes. E_1 leukocytes were obtained from rabbits given only the first dose of endotoxin.

The leukocytes were tested intact or after disruption. Two methods were used for disruption of leukocytes: Ultrasonic vibrations[2] for 30–45 sec; and disruption in 0.34 M sucrose, as described by Cohn and Hirsch (20). The sucrose-disrupted cells were centrifuged at 4°C, first at 400 *g*, twice (nuclear fraction) the supernate at 8200 *g* (sediment-granules), then at 14,500, 25,000, and 40,000 *g*, each time for 10 min and each sediment collected.

Cycloheximide (Nutritional Biochemicals Corp., Cleveland, Ohio) was used in doses of 1 mg/kg and polymyxin B sulfate (Sigma Chemical Co., St. Louis, Mo.) was used in doses of 5 mg/kg. Each substance being given either with the first or second dose of endotoxin to rabbits receiving two doses of endotoxin. These rabbits were paired with rabbits receiving two doses of endotoxin.

Assay methods. Factors VIII and IX were assayed by one-stage methods on the appropriate congenitally-deficient human plasma after activation for 1 min with Celite, as previously described (10). Factors II, V, VII, and X were assayed as described by Soulier and Larrieu (21). The coagulant activity of leukocytes was tested in two ways: by a one-stage method, in which 0.1 ml of leukocyte suspension and 0.1 ml of rabbit plasma, were mixed and 0.1 ml $CaCl_2$ 0.05 M was added and the clotting time recorded. Leukocyte tissue factor activity was assayed by a two-stage method as described by Nemerson (22,23) and Nemerson and Spaet (24). 0.2 ml barium sulfate eluate was incubated with 0.1 ml of leukocytes (or brain tissue factor) and 0.2 ml of 0.025 M $CaCl_2$. The clot-promoting activity of this incubation mixture was tested on 0.1 ml cephalin 1/100, 0.1 ml $CaCl_2$ 0.025, and 0.1 ml factors VII–X–deficient bovine plasma.

White cell tissue factor activity (the two-stage test) was compared with the activity of a standard rabbit brain tissue factor preparation, four dilutions of which were tested after 5, 10, and 15 min incubation. Results presented here are those obtained after 10 min or an average of activities after 10 and 15 min incubations. A representative set of results for 122, 61, 30.5, and 12.2 μg of tissue factor was 18.6, 22.8, 28.4, and 42.4 sec respectively. The stability of the standard brain tissue factor was checked intermittently with freshly prepared brain tissue factor. Omission of the VII–X fraction or tissue factor was also done as another control.

Cathepsin was assayed as described by Anson (25) and Cohn and Hirsh (20). The substrate was a 2% solution of denatured hemoglobin (Nutritional Biochemical Corp.) dissolved in acetate buffer and the pH adjusted to 3.8. After 30 min at 37°C, 5% trichloroacetic acid was added and the filtrate assayed for chromogen by the method of Lowry (26). Attempts to demonstrate cathepsin activity at pH 7.2 and pH 8.0 with denatured hemoglobin substrate or glycyl-L-phenylalaninamide (27) were unsuccessful.

Protein concentration was determined by the biuret method (28). Versatol[3] being used as standard.

All results given are the average of at least three separate experiments.

Preliminary experiments provided evidence that two washings of white cells obtained from peritoneal fluid were sufficient to eliminate significant amounts of plasma clotting factor activity. The number of leukocytes obtained from a single peritoneal aspirate varied considerably: mean numbers were for E_0: 1×10^9, E_1 1.5×10^9, E_2 3.3×10^9 cells.

RESULTS

Suspensions of intact leukocytes accelerated the clotting of normal plasma and of plasma lacking factors VIII, IX, XI, or XII. E_2 leukocytes had consistently significantly greater procoagulant activity than E_0 leukocytes, particularly when rabbit plasma was used as substrate (Table I). The procoagulant activity of leukocytes was shown to be tissue factor in nature by activation of

[1] *Abbreviations used in this paper:* E_0, leukocytes from control rabbits; E_1, leukocytes from rabbits receiving one dose of endotoxin; E_2, leukocytes from rabbits receiving two doses of endotoxin.

[2] Biosonic, Bronwill Scientific, Inc., Rochester, N. Y. Probe at 60.

[3] Warner-Chilcott Laboratories, Morris Plains, N. J.

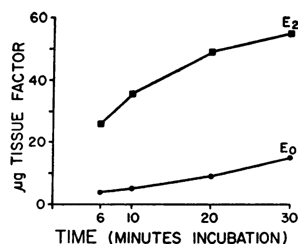

FIGURE 1 Tissue factor activity of leukocytes. E_2 leukocytes from rabbits receiving two doses of endotoxin. E_0, control leukocytes, no endotoxin was given to these rabbits. Equal numbers (6×10^6) of E_0 and E_2 leukocytes were tested.

factor X in the presence of factor VII and calcium. The leukocytes tissue factor activity showed progressive increase on incubation, E_2 leukocytes showing much more activity than E_0 leukocytes (Fig. 1). Variation in tissue factor activity of leukocytes from different animals is shown in Table II.

To determine whether the difference between E_0 and E_2 leukocytes was due to a difference in release or content, tissue factor activity was also tested after disruption of leukocytes. The tissue factor activity of disrupted E_0 leukocytes increased slightly while a greater increase was observed in disrupted E_2 leukocytes (Table III). This finding suggests that E_2 leukocytes not only release but also contain more tissue factor activity.

After sucrose disruption, leukocyte cellular fractions were separated by differential centrifugation. The specific tissue factor activities from E_1 or E_2 leukocytes were comparable and sedimented in several peaks. Most of the specific tissue factor activity was in the 14,500 g and above sediments. Disrupted E_0 leukocytes had only minimal specific tissue factor activity. Activity per 1×10^9 cells is shown in Table IV. The sum of activities of all subcellular fractions was not always equal to the activity of disrupted cells (Table IV).

TABLE II
Tissue Factor Activity of Leukocytes

E_0	E_1	E_2
1.2	0.6	41.5
2.0	2.0	36.6
2.5	41.0	52.5
3.0	10.0	27.5
13.0	19.0	37.0
2.5	11.0	29.0

Tissue factor activity for 6×10^6 cells as assayed after 10 min incubation in the first stage of the two-stage assay, expressed in micrograms. E_1 leukocytes from rabbits receiving one dose of endotoxin.

TABLE III
Tissue Factor Activity of Disrupted Leukocytes Compared with Intact Cells

	Intact	Ultrasonically disrupted
E_0	5.3 μg	7 μg
E_2	24 μg	92 μg

Tissue factor activity of 6×10^6 cells intact and ultrasonically disrupted assayed by the two-stage assay after 10 min incubation.

Studies were then made to determine circumstances influencing release of coagulant activity. E_2 leukocytes were incubated in Hanks solution (pH 8.05) or citrate saline (pH 7.9) at +2° and 37°C, and the cell-free supernatant fluid tested. There was much more activity in the supernate of cells incubated at 37°C in Hanks solution as compared with +2°C. Suprisingly, the supernate of cells incubated in citrate saline at 37°C showed anticoagulant activity (Fig. 2).

To determine whether procoagulant activity was related to cathepsin, the acid cathepsin activities of E_0 and E_2 leukocytes were compared but showed no difference. E_0 intact liberated 21.2 μg tyrosine per mg of leukocyte protein after 30 min incubation at 37°C, E_0 disrupted 23.7 μg, E_2 intact 23.8 μg, and E_2 disrupted 27.0 μg. Cathepsin activity at neutral pH was not detectable.

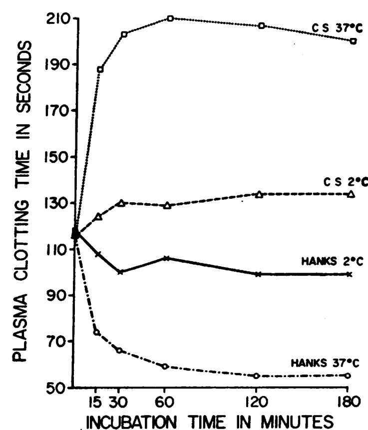

FIGURE 2 Release of coagulant activity into the ambient fluid. Undisrupted E_2 leukocytes (60,000 per mm^3) were incubated with Hanks solution or citrate saline in glass. At the end of each period of incubation the samples were centrifuged at 2200 g for 15 min. The upper half of the cell-free suspending medium was separated and kept on ice before testing. The $CaCl_2$ molarity was 0.025 when Hanks solution was used and 0.05 M for citrate saline (CS). Average clotting time for Hanks: 146.0 sec; 143.6 sec for citrate saline.

Table IV
Sedimentation of the Tissue Factor Activity

No. of cells (×10⁹)		Disrupted	400 g	8,200 g	14,500 g	25,000 g	40,000 g	40,000 g supernate
1.2	E_0	3,200	460	400	350	250	100	0
1.45	E_1	10,300	3,200	4,000	7,800	3,000	3,500	5,400
2.0	E_2	19,000	3,400	1,400	5,400	4,200	3,300	3,600

The cells were disrupted in 0.34 M sucrose and sedimented successively at the above gravities, each sediment resuspended in a predetermined volume was tested for tissue factor activity in the two-stage test. The tissue factor activity is expressed in micrograms of tissue factor activity per 10^9 cells or cell fractions.

When tested for stability at various temperatures, the tissue factor activity of disrupted cells was stable at 56°C but was destroyed at 65°C (Fig. 3).

Tests were then made of the effect of cycloheximide and polymyxin B on the development of procoagulant activity. Cycloheximide reduced the number of leukocytes about twofold in rabbits receiving two doses of endotoxin. The tissue factor activity per given number of leukocytes was not reduced, but the one-stage procoagulant effect was greater (Table V).

The number of peritoneal leukocytes was reduced when polymyxin B was given with the first dose of endotoxin. The procoagulant activity for a given number of cells was also reduced. When polymyxin B was given immediately before the second dose of endotoxin, no inhibitory effect on the procoagulant activity or the number of leukocytes was observed (Table V).

To further investigate the mode of action of polymyxin B, polymyxin B was incubated at various concentrations and length of time with brain tissue factor, or E_2 leukocytes. Brain tissue factor activity was inhibited at concentrations of 30 or 70 μg/ml after 30 min incubation. Leukocyte tissue factor activity was partially inhibited only after 20 hr incubation (Table VI).

DISCUSSION

The data presented confirm that peritoneal leukocytes from rabbits have procoagulant activity, as evidenced by shortening of the plasma clotting times and assay of tissue factor activity. A new finding is that the procoagulant activity is 10-fold greater in leukocytes obtained from rabbits given two doses of endotoxin. Results confirming the development of procoagulant activity by leukocytes have been presented by Lerner, Goldstein, and Cummings (29).

The coagulant activity is thought to be due to tissue factor activity because the activity was measured by

Table V
The effect of Cycloheximide and Polymyxin B on the Endotoxin-Induced Coagulant Activity of Leukocytes

	No. of cells (×10⁹)	Tissue factor activity (μg) per 6 × 10⁶ cells		All cells		One-stage test (6 × 10⁶ cells) (Sec)	
		Intact	Disrupted	Intact	Disrupted	Intact	Disrupted
(a) E_2	2.3	100	187	38,500	71,000	35.1	31.4
	(2.1–2.7)	(90–120)	(120–240)			(31.0–37.2)	(27.4–36.4)
(b) E_2 + cycloheximide	0.92	97	199	14,800	30,400	21.5	18.0
	(0.3–1.7)	(55–135)	(155–310)			(17.0–24.3)	(13.2–22.3)
(c) E_2 + polymyxin B	0.84	32	72	4,500	10,000	61.1	73.1
	(0.42–1.2)	(14–73)	(55–100)			(47.8–86.2)	(49.0–104.8)
(d) E polymyxin B, E	3.2	85	164	45,300	87,400	35.1	33.7
	(0.4–6.8)	(36–140)	(85–190)			(31.2–55.2)	(33.2–56.0)
						citrate saline	107.8
						cephalin 1/50	61.2

Cycloheximide (1 mg/kg) (*b*) and polymyxin B (5 mg/kg) (*c*) were given intraperitoneally immediately after the first dose of endotoxin. A second injection of endotoxin was given intravenously 16 hr later. Polymyxin B 5 mg/kg (*d*) was given intravenously immediately before the second dose of endotoxin. The leukocytes were harvested, washed twice, and tested intact or disrupted. All rabbits had their peritoneum stimulated in previous experiments. Numbers in parenthesis indicate the extreme variations.

an assay which measures activated factor X, and is considered to be specific for tissue factor (Nemerson, 22, 23).

The total tissue factor activity of E_2 leukocytes obtained from one rabbit was equivalent to that present in 3–5 ml of undiluted rabbit brain extract (i.e., 36–60 mg tissue factor) (Table V).

When the tissue factor activity of leukocytes was tested, the rate of activation of factor X was slower than with brain tissue factor extracts and a maximal activation was reached sometimes only after 20–30 min of incubation (Fig. 1). A possible explanation for this slow activation is the presence of inhibitors in the leukocytes (4). When cellular fractions separated by centrifugation were tested, maximal factor X activation was reached much more rapidly consistent with the possibility that an inhibitor was removed during the fractionation procedure.

The procoagulant activity was readily available being released into the ambient fluid when leukocytes were incubated in Hanks solution at 37°C.

Cell fractions obtained by centrifugation of disrupted leukocytes showed that specific tissue factor activity was greater in the 14,500 *g* and above sediment, thus suggesting that most of the activity is insoluble and bound to particles of various sizes. It has been shown very recently that some white cell enzymes sediment at various gravitational forces and at least three types of leukocyte granules associated with these enzymatic activities have been described (30–32).

Another finding was that E_1 leukocytes, which at times (when tested intact) have very low procoagulant activity, showed when disrupted a specific tissue factor activity equal or higher to E_2 leukocytes, and the activity was localized in the same cellular fractions as

TABLE VI

Effect of Polymyxin B on the Tissue Factor Activity in Vitro

Polymyxin B	Brain extract (Incubation time)	Leukocytes (Incubation time)	
µg/ml	*0.5 hr*	*0.5 hr*	*20 hr*
0	100%	100%	100%
10	90%	100%	90%
70	30%	90%	40%

To 0.9 ml of brain thromboplastin or leukocyte suspension (60,000 per mm³ intact or disrupted), 0.1 ml of polymyxin B in saline was added. After incubation (37°C for 0.5 hr, +4°C for 19.5 hr), the tissue factor activity was measured by the two-stage assay and expressed as per cent of a control incubated with saline. Before incubation brain extract and leukocyte suspension had similar activities.

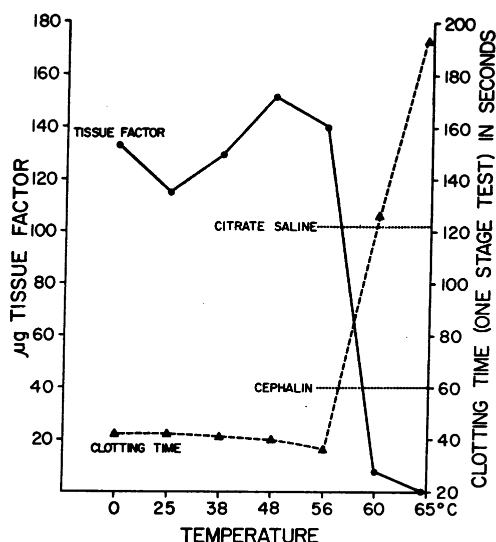

FIGURE 3 Thermostability of the coagulant activity. The 400 *g* supernates of ultrasonically disrupted E_2 leukocytes were incubated for 10 min at the indicated temperatures, then put on ice before testing the tissue factor activity and effect on the plasma clotting time. The plasma clotting times without leukocyte supernate but with cephalin or citrate saline are indicated on the figure.

for E_2 leukocytes. Thus it is proposed that the first dose of endotoxin is required for production of tissue factor activity, while the second injection of endotoxin conditions faster release of tissue factor. Endotoxin has indeed been shown to render lysosomes labile in vitro (33) without causing degranulation.

Human platelets, incubated with plasma, have been claimed to develop tissue factor activity (34). Whether the mechanism is the same (activation by endotoxin, contaminating leukocytes), remains to be determined.

Human circulating leukocytes (1) and rabbit peritoneal leukocytes are known to possess anticoagulant activity (4). Anticoagulant activity was also present in E_0 and E_2 leukocytes being released in the cell-free supernate of E_2 leukocytes incubated in citrate saline at 37°C (while procoagulant activity was released when leukocytes were incubated in Hanks solution). While it is possible that some leukocytes were disrupted during incubation (and coagulant activity was released), release of either pro or anticoagulant activities occurred mainly at 37°C suggesting an active release mechanism. Anticoagulant activity was also observed when the procoagulant activity was destroyed by heating.

The action of polymyxin B is quite complex. Polymyxin B is known to interact with lipids and phospholipids (35,36) and has been reported to prevent the Shwartzman phenomenon (37) possibly by inactivating endotoxin. Polymyxin B has been also shown to inhibit brain tissue factor activity allegedly by binding with

the phospholipid moiety of tissue factor (38). The data presented here support the latter hypothesis—tissue factor activity of leukocytes was inhibited in vitro (on prolonged incubation) and in vivo—only when polymyxin B was given with the first dose of endotoxin.

In conclusion, it has been shown that considerable amounts of procoagulant activity are readily available from leukocytes after exposure to endotoxin. It is proposed that this leukocyte-derived procoagulant activity plays an important role in intravascular coagulation and the generalized Shwartzman phenomenon (39).

ACKNOWLEDGMENTS

I wish to thank Mrs. M. Chierego, Mrs. B. Troy, and Mr. S. Panagatos for their technical assistance.

This study was supported in part by a grant from the National Institute of Health (HE-13155).

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