Stimulation of Human Leukocyte Thromboplastic Activity by Endotoxin[1] (35848)

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The experiments reported herein are an extension of earlier studies which demonstrated the need for granulocytes in endotoxin-induced intravascular coagulation (1). One purpose of these experiments was to demonstrate that leukocytes have a potent thromboplastic activity. A second purpose was to demonstrate that this activity is enhanced by endotoxin *in vitro*.

Although leukocytes are generally not included in the clotting scheme as usually depicted, there is evidence that leukocytes have a role in coagulation as it occurs in human disease, and experimental models of disease. For example, the generalized Shwartzman reaction, which is mediated by intravascular coagulation (2), requires granulocytes when produced by endotoxin (3, 4) but not when produced by thrombin (4). Additionally, intravascular coagulation which follows endotoxin injection is prevented in rabbits rendered granulocytopenic by treatment with nitrogen mustard (1). In thrombi formed in human disease, leukocytes accumulate selectively at a specific location within the thrombus (5). Some human leukemias are associated with diffuse intravascular coagulation (6), and leukocytes from such a case have been found to have potent thromboplastic activity (7). However, other reports on the coagulant activity of leukocytes have been variable; but in general suggest that they have a weak thromboplastic activity (8–10). Niemetz (11) has demonstrated that peritoneal leukocytes obtained from a rabbit treated with endotoxin have potent thromboplastic activity. Therefore, it seemed important to more accurately and unequivocally delineate this thromboplastic activity in human leukocytes.

Materials and Methods. Human leukocyte suspensions were prepared by collecting normal human blood onto glass beads (diam approx 3 mm and weighing approx 38 mg) and defibrinating by swirling for 10 min. Fifty milliliters of defibrinated blood were added to 200 ml of 3% dextran (type 200 C, av mol wt 200,000, Sigma Chemical Co., St. Louis, Mo.) in saline 9 g/liter. After a 30-min sedimentation, the leukocyte-rich supernatant was collected and centrifuged for 3 min at 1250*g*. The leukocyte button was then suspended and agitated in 2 ml of distilled water to lyse the few remaining red blood cells. After 30-sec exposure to distilled water, 1 ml of saline (27 g/liter) was added to restore isotonicity. The leukocyte suspension was then centrifuged again for 3 min at 1250*g*; and the above step of hypotonic lysis was repeated. The resultant white cell button was suspended in saline for counting and incubation. Trypan blue was excluded by 95% of these cells. Platelet-poor plasma was prepared from blood collected in 0.1 vol of anticoagulant 0.06 *M* in trisodium citrate and 0.04 *M* in citric acid by centrifuging at 6500*g* for 10 min in plastic tubes. The final concentration of leukocytes in platelet-poor plasma in the incubation mixture was approximately 50,000/mm^3. There were no contaminating red cells, and there were less than 1000 contaminating platelets/mm^3.

Platelet-rich plasma (PRP) was prepared by slow centrifugation as described by Biggs *et al.* (12). This was centrifuged for another 3 min at 1250*g* to obtain contaminating white cell counts below 10/mm^3. Leukocyte counts on platelet-rich plasma were per-

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formed by routine techniques used for cell counts in spinal fluid.

Endotoxin—Bacto lipopolysaccharide *S. marcescens* (Difco Labs., Detroit, Mich.) was dissolved in saline to a final concentration of 100 μg/ml. Incubations were performed under sterile conditions in plastic tubes at 37° under a flowing moist oxygen atmosphere. The pH remained at 7.4 throughout incubation. Penicillin (to a final conc of 100 units/ml) and streptomycin (100 μg/ml) were added to all reagents used in processing, and to the final incubation mixtures to suppress bacterial growth. Factor XI deficient substrate (exhausted plasma) was prepared by a modification of the method of Horowitz and Fujimoto (13). Factor IX and VIII congenital deficiency plasmas were purchased from Hyland Laboratories, Los Angeles, Calif. Factor VII deficiency plasma was obtained from an individual with a congenital deficiency. Factor X deficient substrate was prepared by a modification of the method of Hougie (14). Factor V deficient substrate and prothrombin deficient substrate were prepared by the methods of Goldstein *et al.* (15). Inosithin (Associated Concentrates, Woodside, N.Y.) was dissolved in Veronal buffered isotonic saline to a concentration of 50 mg/100 ml.

Results. 1. Evidence that freshly collected leukocytes and plasma do not have thromboplastic activity, and generate this activity over a period of hours. Freshly collected leukocytes suspended in plasma were tested for their ability to shorten the clotting time of the following mixture: 0.1 ml of normal human platelet-poor plasma and 0.1 ml of inosithin preincubated in a plastic tube for 3 min, 0.1 ml of 0.025 *M* $CaCl_2$, followed in 15 sec by a test substance, which was either saline or a leukocyte–plasma mixture. Both test materials gave the same clotting time of approximately 205 sec. When this leukocyte–plasma mixture was incubated in plastic at 37° under a moist oxygen atmosphere for a period of 6 hr, it caused a progressive shortening of the clotting time of the above mixture to a mean of 54.4 sec, as indicated in Fig. 1. When leukocytes in saline, or plasma without leukocytes were incubated as controls, no shortening of the substrate clotting time occurred over a period of 6 hr.

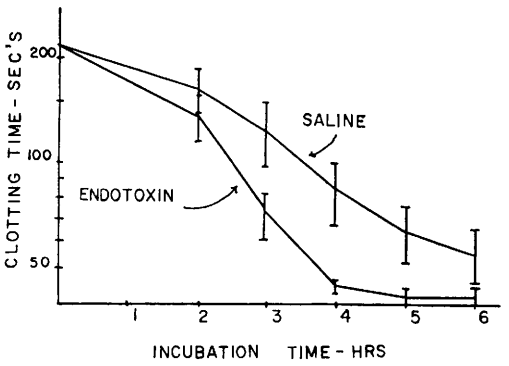

FIG. 1. Mixtures of 0.8 ml of normal platelet-poor plasma and 0.1 ml of WBC suspension, to give a final concentration of 50,000/mm^3, with either 0.1 ml of saline or 0.1 ml of endotoxin solution were incubated at 37°, under moist oxygen in plastic tubes for 6 hr. Logarithm of clotting time in assay system for procoagulant activity is plotted as the ordinate. Lines indicate two standard deviations. Each point represents the mean of 7 separate experiments for the saline controls and 9 separate endotoxin experiments.

2. Evidence that the thromboplastic activity generated by leukocytes incubated in plasma is enhanced by endotoxin. When endotoxin was added to the leukocyte–plasma mixture to a final concentration of 10 μg/ml, the shortening of the clotting time was more pronounced and occurred earlier, a mean of 45.2 sec after 4-hr incubation (Fig. 1).

3. Evidence that the activity generated is like thromboplastin and resides with the cell component of the mixture. A large batch of cells containing procoagulant activity was prepared by incubating leukocytes (final conc approx 50,000/mm^3) suspended in citrated human plastic serum. The citrated human plastic serum used for incubation was prepared by allowing human blood to clot in plastic tubes for 2 hr at 37°, and separating the serum which was then incubated another 4 hr at 37°, citrated and adsorbed with 0.1 ml of aluminum hydroxide (Cutter Laboratories, Berkeley, Calif.)/1 ml of serum for 10 min at 37° prior to use. This procedure was carried out since the cells incubated in this material were to be tested on deficiency substrates and we wished to avoid the carryover

TABLE I. Influence of Leukocyte Thromboplastic Activity on Clotting Time of Substrates with Intrinsic Deficiencies.

Substrate	5 hr WBC suspension[a] (sec)	Saline blank
Normal plasma	42	117 sec
Exhausted plasma	50	449 sec
IX deficient plasma	46	608 sec
VIII deficient plasma	61	46 min

[a] Washed leukocytes prepared as indicated in text.

of coagulation factors which might shorten the clotting times themselves. After 5 hr of incubation in 3 ml of adsorbed citrated plastic serum, the leukocytes were spun down at 1000*g* for 3 min and washed 3 times in 5 ml of citrated saline. The leukocytes were then suspended in 3 ml of saline and tested for their ability to shorten the clotting time of various substrates in plastic. It should be noted that inositihin was not present in the clotting mixtures. The data wtih inositihin present are comparable. Table I demonstrates the shortening of the clotting time using substrates deficient in factors involved in the early steps of intrinsic coagulation. Table II indicates that there was no significant shortening of the clotting time using substrates deficient in factors required for extrinsic clotting.

4. Evidence that thromboplastic activity is not due to platelets. PRP with 1631 leukocytes/mm^3 incubated at 37° for 18 hr in glass tubes generated thromboplastic activity as described earlier by Biggs *et al.* (12). A sample of the same PRP was rendered leukocyte poor and similarly incubated. No thromboplastic activity developed (Table III). Because thromboplastic activity was generated by only 1631 leukocytes/mm^3, a white cell preparation was serially diluted prior to a 5-hr incubation in platelet-poor plasma. As shown in Table IV, clearly discernible activity was generated by a concentration of leukocytes as low as 49/mm^3 when there was less than 0.1 platelets/mm^3.

TABLE II. Influence of Leukocyte Thromboplastic Activity on Clotting Time of Substrates with Extrinsic Deficiencies.

Substrate	5 hr WBC suspension[a] (sec)	Saline blank
Normal plasma	42	117 sec
VII deficient plasma	130	144 sec
X deficient plasma	449	3 hr
V deficient plasma	254	241 sec
II deficient plasma	251	319 sec

[a] Washed leukocytes prepared as indicated in text.

TABLE III. Influence of Number of Contaminating Leukocytes on Ability of Incubated PRP to Generate Thromboplastic Activity.

Platelets (per mm^3)	Leukocytes (per mm^3)	Substrate clotting time (sec) 0 hr	18 hr
749,000	1631	297	43.7
600,000	6	297	388

TABLE IV. Influence of Leukocyte Concentration in Incubation Mixture on Thromboplastic Activity Generated.

Leukocytes (per mm^3)	Substrate clotting time (sec) after 5 hr	Contaminating platelets (per mm^3)
49,000	40.2	100
4900	34.8	10
490	43.0	1
49	68.0	0.1
4.9	162	0.01
0.49	201	0.001
0.0	278	0.0

Discussion. The data presented above extends prior observations on the coagulant activity of leukocytes. In this system, freshly isolated live leukocytes have no significant thromboplastic activity. Some earlier reports of thromboplastic activity in normal human cells have relied upon extracts of cell contents (9, 10). However, intact cells from a patient with acute promyelocytic leukemia and intravascular coagulation have been reported to have significant thromboplastic activity (7).

The present data indicate that normal human leukocytes, incubated in either plasma or citrated human plastic serum, develop a striking procoagulant activity. The fact that this activity remains with the cells after multiple washings suggests that it is not a plasma factor. This activity fails to shorten the clotting times of those substrates deficient in clotting factors necessary for extrinsic clotting, while dramatically shortening the clotting time of those substrates deficient in factors required only for intrinsic clotting. This defines this procoagulant activity as similar to that of tissue thromboplastin. The data reported herein indicate that the thromboplastic activity is due to leukocytes and not to platelets. Platelets devoid of leukocytes do not generate this activity.

The significance of a procoagulant activity which requires several hours to be generated *in vitro* might be questioned. However, if one compares the time of appearance of the procoagulant activity in endotoxin–leukocyte mixtures reported herein with the observed delayed appearance of intravascular coagulation following endotoxin injection into an intact animal (1, 2), one notes a striking similarity. In addition, other leukocyte mediators of the inflammatory response also require a time lag prior to their release (16).

One must consider why this procoagulant activity is not present in freshly collected leukocytes from normal individuals. Certainly, an *in vivo* situation could be considered quite comparable to an incubation under physiological conditions *in vitro* over a period of 6 hr. Why, therefore, is there no procoagulant activity in freshly isolated leukocytes? We believe that the activity we find in leukocytes incubated without endotoxin is also triggered, but by something other than endotoxin. In the experiments reported herein, that trigger is probably the trauma of preparation of the leukocyte suspension.

Summary. Leukocytes incubated *in vitro* in plasma or serum, but not in saline, develop a potent procoagulant activity like tissue factor. This activity is not present in freshly collected leukocytes from normal individuals. The generation of activity is stimulated by gram-negative endotoxin.

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