The Procoagulant Activity of Granulocytes[1] (37078)

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Since the first suggestion in 1851 that leukocytes might be involved in blood coagulation at sites of vascular injury (1), various investigators have attempted to pinpoint the role these cells play in thrombus formation. Both inflammatory and noninflammatory thrombi always contain leukocytes (2), but it is unclear if this represents merely their entrapment, like red cells, as innocent bystanders. Experimentally, leukocytes have been thought to be important both in clot formation and in the physiological resolution of the thrombus (3).

Past attempts to demonstrate clot-promoting activity in leukocytes have yielded conflicting results (4–7), but Lisiewicz reported that leukocytes contained thromboplastic activity similar to that of platelet Factor III (8). Rapaport and Hjort (9) and Niemetz (10) have also found leukocytes to possess definite thromboplastic activity. Part of the difficulty in evaluating the role of leukocytes in coagulation and fibrinolysis is due to the multiple effects of these cells on these systems. Not only has procoagulant activity been demonstrated in granulocytes but also anticoagulant (11) and fibrinolytic activity (12). The fibrinolytic activity has been ascribed both to an activator of plasminogen and to a direct proteolytic activity (12).

To characterize more fully the role of leukocytes in blood coagulation, especially in view of our previous studies identifying their anticoagulant properties (11) we tested various kinds of leukocytes from rabbit and man for procoagulant activity. This paper reports results of these studies.

Materials and Methods. All glassware used to prepare glycogen and sucrose and to obtain cells and cell fractions was baked at 180° for 3 hr or soaked overnight in butanol and rinsed thoroughly with sterile, pyrogen-free saline to render it free of bacterial endotoxin (13).

Rabbit granulocytes. Polymorphonuclear leukocytes were harvested from sterile peritoneal exudates induced in normal rabbits with sterile 0.25% glycogen in endotoxin-free saline (13). Exudates from several rabbits were pooled in ice-chilled flasks. The cells were isolated by centrifugation and washed 3 times with sterile, isotonic saline and once with barbital buffer (pH 7.35). Whole leukocytes suspended in this buffer were used for clotting tests. Ninety-five percent of the cells were granulocytes.

Blood leukocytes. Rabbit and human leukocytes were also obtained from sterile, heparinized blood by dextran sedimentation (14) and hypotonic lysis of residual red cells (15). The suspensions of human blood leukocytes, predominantly neutrophils, also contained lymphocytes and monocytes in amounts that varied with different preparations.

Lymphocytes. Lymphocytes were isolated by incubating heparinized blood on sterile cotton columns, eluting with saline and removing residual red cells by hypotonic lysis as described by Walker and colleagues (15). Lymphocytes were washed and suspended in buffer for clotting tests. The cell suspensions prepared from rabbit blood contained 100% lymphocytes; those from human blood, mostly lymphocytes, also contained 8–12% neutrophils.

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The cell concentration in all these suspensions was determined in duplicate with a Model B Coulter electronic cell counter (Coulter Electronics, Hialeah, Florida). Differential counts of at least 200 leukocytes were performed on Wright's stained coverslip smears.

Granulocyte lysosomes were obtained by differential centrifugation of rabbit granulocytes disrupted in 0.34 *M* sucrose (13). The lysosomes were washed, then suspended in 0.34 *M* sucrose for clotting tests; 0.34 *M* sucrose alone was used in controls for these clotting studies.

For some studies both granulocytes and lysosomes were disrupted by freezing and thawing three times. After the last thaw, the mixture was centrifuged for 30 min at 10,000*g* and the particle-poor supernatant was separated for testing. The debris, washed with buffer and resuspended to the original volume with fresh buffer, was also tested for clotting activity.

Plasma preparations. Blood was obtained by cardiac puncture from rabbits lightly anesthetized with sodium pentobarbital (1 part 3.2% sodium citrate to 8 parts blood). Human citrated plasma was prepared as previously described (11) from normal recipients and from patients deficient in Factor V (proaccelerin), Factor VIII (AHF, antihemophilic factor), Factor IX (PTC, plasma thromboplastin component), Factor X (Stuart factor), Factor XI (PTA, plasma thromboplastin antecedent) and Factor XII (Hageman factor). Plasma deficient in Factor VII (proconvertin) was a lyophilized commercial preparation (Sera-Tec Biologicals, 525 Milltown Road, North Brunswick, N. J. 08902).

The preparation and source of cephalin, inosithin, and barbital buffer have been described (11).

Clotting tests included the partial thromboplastin time (PTT) (16), one stage prothrombin time (PT) (17), and plasma recalcification time (18). In all these tests 0.1 ml of buffer or a suspension of leukocytes was added to the plasma before adding the other reagents. The recalcification time was performed similarly except that the test was performed on platelet-rich and platelet-poor plasma. All tests were done at least in duplicate at 37° together with appropriate controls.

Results. Rabbit granulocytes. The effects of various concentrations of granulocytes on the recalcification time and the PTT of normal plasma are shown in Fig. 1. In this experiment concentrations of granulocytes greater than 8000/mm^3 shortened the clotting time in the recalcification time test to the same level as the control PTT with no granulocytes (76 sec). When granulocytes

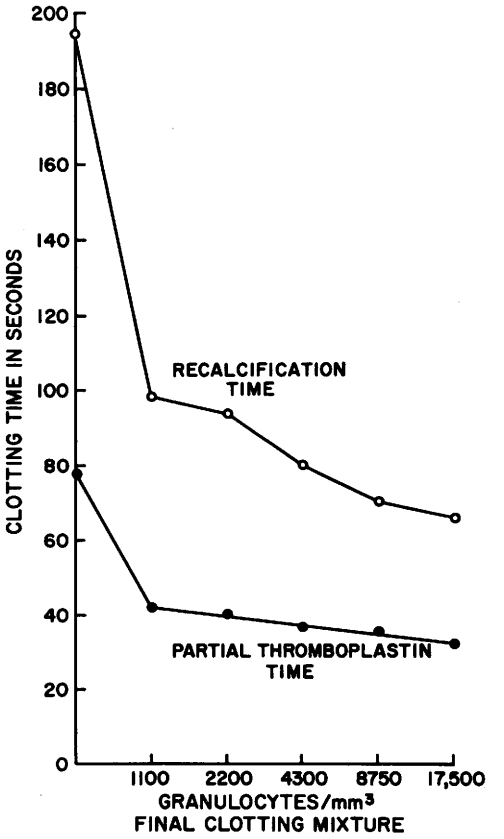

FIG. 1. The effect of whole-rabbit granulocytes on the recalcification (clotting) time and the partial thromboplastin time of normal, platelet-poor, citrated human plasma. In the recalcification time test 0.1 ml of 0.02 *M* $CaCl_2$ was added to a mixture of 0.1 ml plasma plus 0.1 ml barbital buffer or buffer containing granulocytes and the clotting time recorded. In the PTT, 0.1 ml plasma, 0.1 ml barbital buffer or buffer containing granulocytes and 0.1 ml cephalin was recalcified with 0.1 ml of 0.02 *M* $CaCl_2$ and the clotting time recorded.

TABLE I. Effect of Rabbit Granulocytes on the Recalcification Time of Rabbit and Human Plasmas.

	Plasma clotting time in seconds								
			Human deficient plasmas						
Granulocytes/mm³ clotting mixture	Normal rabbit	Normal human	Factor V	Factor VII	Factor VIII	Factor IX	Factor X	Factor XI	Factor XII
			(Platelet-rich plasma)						
A) 0—Control	64	72	200	—	369	393	343	—	>600
1,800	42	76	87	—	77	84	330	—	88
7,100	32	59	80	—	72	65	366	—	48
28,500	25	48	48	—	53	58	320	—	34
			(Platelet-poor plasma)						
B) 0—Control	77	239	>600	298	>600	>600	>600	>600	>600
1,800	44	158	122	275	136	144	562	—	107
7,100	35	100	68	250	95	86	476	—	69
28,500	26	101	65	220	70	83	357	97	38

Mixtures containing 0.1 ml of the various citrated plasmas and 0.1 ml of either buffer or granulocytes suspended in buffer were recalcified with 0.1 ml 0.02 *M* $CaCl_2$ and the clotting time recorded. Studies A and B were performed with the same preparation of granulocytes. Different granulocytes and different plasmas gave similar results.

were added to the PTT, the clotting time of this test was also shortened. In both tests the shortening of the clotting time was proportional to the number of granulocytes added. Even though there was some variability, similar results were obtained in repeated tests using different preparations of granulocytes and different preparations of normal plasma. To further elucidate their procoagulant action, granulocytes were tested for effect on the recalcification time of normal human and rabbit plasmas as well as human plasmas deficient in a single procoagulant. These results are in Table I. Suspensions or rabbit granulocytes shortened the recalcification time of rabbit plasma and human plasma in proportion to the number of cells added; this happened with both platelet-rich and platelet-poor plasmas. Granulocytes significantly shortened the clotting time of human plasmas deficient in Factors V, VIII, IX, XI, and XII. Granulocytes had little effect on the clotting time of either platelet-rich or platelet-poor plasmas deficient in Factor X. This was documented with several preparations of rabbit granulocytes and different preparations of Factor X deficient plasma.

Although a high concentration of granulocytes shortened somewhat the recalcification time of Factor VII deficient plasma (Table I), the effect is probably insignificant because the clotting time remained quite long and granulocytes failed completely to shorten the PT of Factor VII deficient plasma (Table II).

The effect of rabbit granulocytes on the PT of various plasmas is shown in Table II. High concentrations of granulocytes prolonged the PT of normal human plasma 1–2 sec. but had no detectable effect on normal rabbit plasma. High concentrations of granulocytes slightly prolonged the PT of plasmas deficient in Factors VIII, IX, XI, and XII but the effect was minimal. The prolonged PT of plasmas deficient in Factors V, VII, and X were not corrected by the addition of granulocytes although a slight shortening of the clotting time was observed with Factor V deficient plasmas. This effect of granulocytes in the PT was equivalent to about 5% Factor V activity. In a specific Factor V assay, both granulocytes and granulocyte lysosomes demonstrated 5–15% Factor V activity.

Rabbit granulocyte lysosomes. The effects of granulocyte lysosomes on the recalcification time are shown in Fig. 2. These results parallel those observed with intact rabbit granulocytes. Lysosomes, like their parent cells, shorten the clotting time of all but Factor VII and Factor X deficient plasmas.

TABLE II. Effect of Rabbit Granulocytes on the Prothrombin Time of Rabbit and Human Plasmas.

	Plasma clotting time in seconds									
			Human deficient plasmas							
Granulocytes/mm³ clotting mixture	Normal rabbit	Normal human	Factor V	Factor V	Factor VII	Factor VIII	Factor IX	Factor X	Factor XI	Factor XII
0	7.2	13.5	32.0	37.5	36.2	13.2	14.8	57.0	17.0	18.2
2625	7.1	13.6	—	—	—	—	—	60.0	—	19.5
5250	7.1	13.7	26.2	29.2	42.0	15.1	17.2	70.0	18.3	20.4
10,500	7.1	14.0	—	—	—	—	—	—	—	—
21,000	7.1	15.1	22.3	25.8	39.3	17.2	18.6	72.7	19.3	19.3

To mixtures containing 0.1 ml of the various platelet-poor, citrated plasmas plus 0.1 ml of either barbital buffer or cells suspended in buffer, 0.2 ml simplastin was added and the clotting time recorded.

Rabbit lymphocytes had no detectable procoagulant effect on the recalcification time of either normal rabbit or normal human plasma (Fig. 3). Rather, lymphocytes tended to prolong the clotting time; 3000 lymphocytes/mm³ of final clotting mixture prolonged the clotting time from 225 sec to 432 sec. Other preparations of lymphocytes, while not prolonging the clotting time to this degree, consistently failed to show procoagulant activity like granulocytes.

Disrupted rabbit granulocytes and granulocyte lysosomes were also examined in the recalcification time test to help localize the procoagulant activity (Fig. 4). A mixture containing either disrupted granulocytes or disrupted granulocyte lysosomes shortened the clotting time of normal plasma as did suspensions of intact cells or washed cell debris. Supernatant fluid from the centrifuged, disrupted cell mixture, however, slightly prolonged the clotting time; supernatant from disrupted lysosomes prolonged it slightly more than that from the whole cells. The fragments from disrupted cells and the lysosomal membranes both shortened the clotting time even more than did intact cells or lysosomes.

Tests using the method of Niemerson (19) revealed granulocytes and granulocyte lysosomes, but not lymphocytes, to possess tissue thromboplastin (Factor III) activity.

Human leukocytes. Results with one of the preparations of normal human blood leukocytes, which are representative of several experiments, are shown in Table III. This cell suspension contained 54.5% PMN, 35.5% lymphocytes and 10% monocytes. An aliquot was used to prepare a lymphocyte-rich suspension which contained 92% lymphocytes and 8% PMN. The mixed blood leukocytes shortened the recalcification (clotting) time of normal human plasma in proportion to the number added. Several different preparations

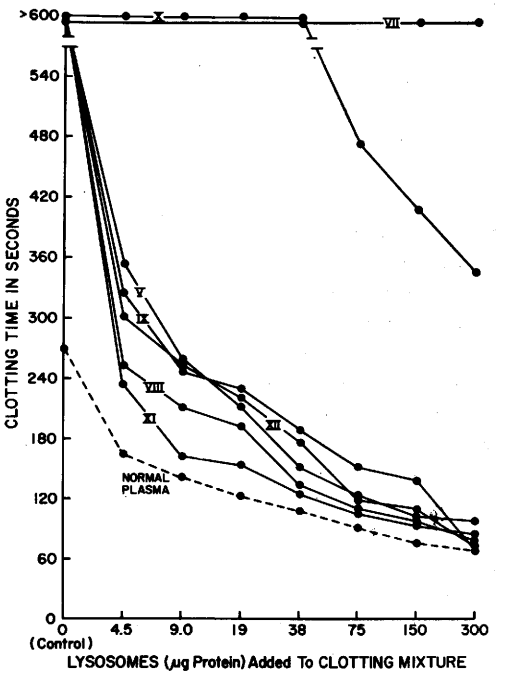

FIG. 2. The effect of rabbit granulocyte lysosomes on the recalcification clotting time of normal platelet-poor citrated human plasma and platelet-poor plasmas deficient in Factors V, VII, VIII, IX, X, XI and XII. Mixtures containing 0.1 ml of various plasmas plus 0.1 ml of 0.34 *M* sucrose or sucrose-containing lysosomes were recalcified with 0.1 ml 0.02 *M* $CaCl_2$ and the clotting time recorded.

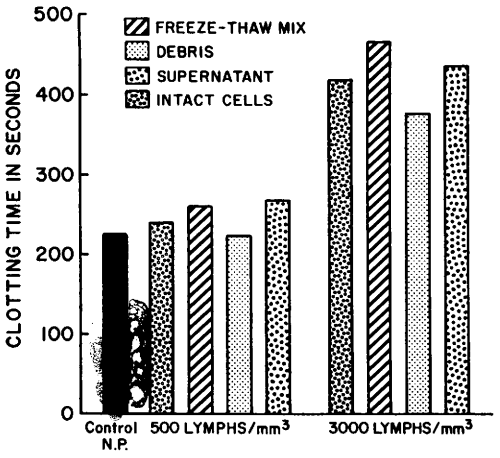

FIG. 3. Effect of various fractions of rabbit lymphocytes on the recalcification time of normal platelet-free, citrated human plasma. Two cell suspensions in buffer (3000/mm³ and 500/mm³) were disrupted by freezing and thawing three times; an aliquot of each was centrifuged for 30 min at 10,000g to separate the particulate debris from the soluble supernatant. The debris was resuspended to the original volume in fresh buffer. Mixtures containing 0.1 ml plasma plus 0.1 ml of buffer or buffer containing the various fractions were calcified with 0.1 ml 0.02 *M* $CaCl_2$ and the clotting time recorded.

of normal mixed blood leukocytes gave similar results. High concentrations of lymphocytes (10,000/mm³ contaminated with 8% neutrophils) also shortened the clotting time of normal human plasma but less than the suspension of mixed leukocytes. Lower concentrations of lymphocytes (1250/mm³) did not shorten but rather slightly prolonged the clotting time. Lymphocyte-rich preparations from other normal subjects gave similar effects.

Disrupted human-blood leukocytes, as well as the particle-poor supernatant from them lengthened the clotting time of normal human plasma. The washed fragments from these disrupted cells also failed to shorten the clotting time as much as the intact cells. Disrupted lymphocytes and the washed cell debris from them both slightly shortened the clotting time; their particle-poor supernatant clearly prolonged it.

Discussion. These studies performed with care to exclude an endotoxin enhancing effect (20), demonstrate that intact granulocytes from rabbit or man possess potent clot-promoting activity. Granulocytes not only accelerate the clotting of normal human plasma, they also correct the prolonged clotting time of human plasmas deficient in any one of the following Factors: V, VIII, IX, XI or XII. Granulocytes do not, however, correct the prolonged clotting time of either Factor VII or Factor X deficient plasmas. Rapaport and Hjort reported somewhat similar results and postulated that the clot-promoting effects of granulocytes are due to their tissue thromboplastic activity (9). If this were so, one would expect granulocytes to correct the clotting defect of plasma deficient in Factor VIII, IX, XI, and XII, but not those deficient in Factors V, VII, or X. The observation that granulocytes correct the recalcification time of Factor V deficient plasma seemingly contradicts this concept. It should be noted, however, that granulocytes did not completely correct the prothrombin time of Factor V deficient plasma. Thus it is possible that the Factor V activity found in granulo-

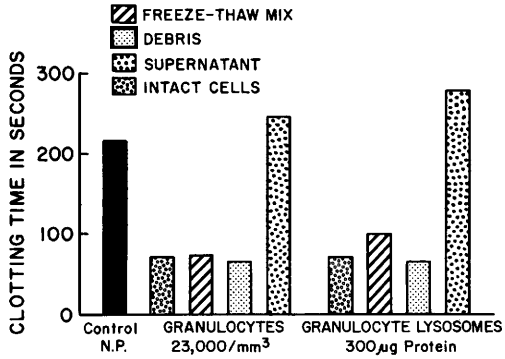

FIG. 4. Effect of various fractions of rabbit granulocytes and granulocyte lysosomes on the recalcification time of normal, platelet-poor, citrated human plasma. Cells in buffer (23,000/mm³) and lysosomes in 0.34 *M* sucrose (300 μg protein/ml) were disrupted by thrice freezing and thawing; an aliquot of each was centrifuged for 30 min at 10,000g to separate the particulate cell debris from the soluble supernatant. The debris was resuspended to the original volume in either fresh buffer or sucrose. Mixtures containing 0.1 ml plasma and 0.1 ml of the various cell and lysosome fractions were recalcified with 0.1 ml 0.02 *M* $CaCl_2$ and the clotting time recorded. Control mixtures contained 0.1 ml plasma, 0.1 ml of either buffer or 0.34 *M* sucrose, and 0.1 ml 0.02 *M* $CaCl_2$.

TABLE III. The Effect of Human-Blood Leukocytes on Human-Plasma Recalcification (Clotting) Time.

Cell suspension	Final cell concentration/mm³	Clotting time, sec
None (control)	0	138
1. Mixed blood WBC[a]	1,500	126
	3,000	96
	12,000[c]	83
Disrupted cells	12,000	188
Debris	—	126
Supernatant	—	196
2. Lymphocytes[b]	1,250	145
	2,500	119
	10,000[d]	102
Disrupted cells	10,000	111
Debris	—	114
Supernatant	—	167

Mixtures containing 0.1 ml of normal platelet-poor, citrated human plasma and 0.1 ml of either buffer or buffer containing various human blood leukocytes were recalcified with 0.1 ml 0.02 *M* $CaCl_2$ and the clotting time recorded. Cells suspended in buffer were disrupted by freezing and thawing three times; an aliquot of each was centrifuged for 30 min at 10,000*g* to separate particulate debris from the soluble supernatant. The debris was resuspended to the original volume in fresh buffer before testing.

[a] Suspension contained 54.5% PMN, 35.5% Lymphs, 10% Monocytes.

[b] Suspension contained 92% Lymphocytes, 8% PMN.

[c] This concentration of cells shortened the clotting time of rabbit plasma to 35 sec from a control of 78 sec.

[d] This concentration of cells shortened the clotting time of rabbit plasma to 60 sec from a control of 78 sec.

cytes and lysosomes suffices to correct the recalcification time, sensitive to even small amounts of Factor V, but not the prothrombin time, which is less sensitive to low levels of Factor V. The best evidence that leukocytes contain tissue thromboplastic activity is the demonstration that they act in concert with Factor VII to activate Factor X (10). We confirm such activity in granulocytes and granulocyte lysosomes. Intact granulocytes and granulocyte lysosomes exhibit more potent procoagulant activity than disrupted cells and lysosomes. We have previously demonstrated that cationic proteins isolated from rabbit granulocyte lysosomes possess strong anticoagulant activity. This anticoagulant prevents the formation of intrinsic prothrombin activator. Thus it is possible that disruption of lysosomes (and intact granulocytes) by freezing and thawing liberates enough anticoagulant activity to mask the procoagulant properties.

It is unlikely that the granulocyte or lysosomal membranes account for the total clot promoting effect. One would expect membranes to mimic platelet factor 3 and therefore to have little or no corrective effect on plasma deficient in Factors VIII, IX, XI, or XII. Since granulocytes do correct these deficient plasmas, it is likely that most of the effect is due to tissue thromboplastin although additional platelet factor 3 activity cannot be excluded.

Interestingly, rabbit lymphocytes have no detectable procoagulant activity. Why they differ from rabbit granulocytes is unclear but it may be related to their different lysosomal constituents or a different surface membrane reactivity (21). In specific assay, lymphocytes exhibit little or no tissue thromboplastin activity.

Human lymphocytes were slightly different. Several preparations showed slight procoagulant activity but much less than the homologous blood leukocyte suspension containing predominantly granulocytes. Perhaps human lymphocytes differed from rabbit lymphocytes partly because human-cell preparations contained small numbers of granulocytes; slight procoagulant activity in human lymphocyte preparations was apparent only with the higher cell concentrations. Further study is necessary, however, to establish this with certainty.

Normal human blood leukocytes, predominantly granulocytes, have procoagulant activity much like rabbit granulocytes. Even though the procoagulant effect *in vitro* with both human and rabbit granulocytes occurred with cell concentrations comparable to those found in normal circulating blood, it remains a speculation that this occurs *in vivo*. The results should, however, reopen the question in view of the known role of these cells in the Arthus and Schwartzman reactions (22–

24), the intravascular coagulation produced experimentally by injecting endotoxin (25) or leukocytes from endotoxin-treated rabbits (24), and the defibrination syndrome observed to complicate some cases of leukemia (26, 27).

Summary. Rabbit granulocytes and human-blood leukocytes (mostly granulocytes) shortened the recalcification time of normal rabbit and human plasmas and human plasmas deficient in Factors VIII, IX, XI and XII but not those deficient in Factors VII and X. While these cells shortened the recalcification time of Factor V deficient plasma they failed to shorten the prothrombin time. Neither did granulocytes shorten the prothrombin time of plasmas deficient in Factors VII and X. Rabbit granulocyte lysosomes resembled the intact parent cells in their effect on normal and deficient plasmas. Rabbit lymphocytes had no detectable procoagulant activity. The minimal procoagulant activity observed with the human lymphocyte suspension may have resulted in part from contaminating granulocytes.

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