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The Blood Clotting Properties
of Rabbit Peritoneal Leukocytes in Vitro*

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Rabbits made granulocytopenic with nitrogen mustard are protected against the generalized Shwartzman reaction when it is provoked by endotoxin (1, 2) but not when it is provoked by thrombin (2). This raises the possibility that granulocytes are involved in the triggering of intravascular clotting by endotoxin, a step bypassed by injecting thrombin. However, Thomas and his associates (3) have postulated that white blood cells (WBC) are needed for the generalized Shwartzman reaction provoked by endotoxin because WBC supply acid mucopolysaccharides which precipitate endo-toxin-damaged fibrinogen. Recent demonstrations of acid mucopolysaccharides within rabbit leukocytes (4, 5) support this hypothesis. Conceivably, therefore, an infusion of thrombin, which depresses fibrinogen levels much more than an injection of endotoxin, could lead to the deposition of fibrin within the glomerular capillary bed without requiring a fibrinoplastic co-factor from the granulocyte. Thus, WBC could act either in the first or in the last phase of intravascular coagulation triggered by endotoxin.

As part of an evaluation of these possibilities, we have studied the *in vitro* clotting properties of rabbit peritoneal leukocytes. Rabbit WBC were found to possess weak but significant tissue thromboplastic activity. An *in vitro* effect of WBC upon the clotting or precipitation of fibrinogen could not be demonstrated. Rabbit WBC were also found to exhibit anticoagulant activity which interfered with the activation of prothrombin by prothrombinase (the final prothrombin activator generated during clotting). Which effect predominated – procoagulant or anticoagulant – depended upon the experimental conditions. These data are reported herein.

Materials

Blood was obtained with "silicone technique" from normal humans and from the aorta or inferior vena cava of rabbits anaesthetized with pentobarbital. Nine parts of blood were allowed to flow into a plastic tube containing 1 part of a citrate anticoagulant which was 0.06 M in sodium citrate and 0.04 M in citric acid. *Platelet-poor, intact normal plasma* was prepared and stored by a technique described in detail earlier (6). (Plasma is called intact if it does not contact an activating surface such as glass.) *Human plasmas deficient in factors V, VII, VIII, IX, XI, and XII* were

obtained from patients with specific hereditary deficiencies of these factors. *Exhausted plasma*, which is plasma depleted of factors XII and XI by treatment with diatomaceous earth powder, was prepared as described elsewhere (6). *A "platelet-free" human plasma substrate* for a platelet factor 3 assay was prepared according to Husom (7). *Oxalated ox plasma* was *adsorbed* with 100 mg/ml of barium sulfate powder. *Citrated human plasma* was *adsorbed* with one-tenth volume of a 1:2 dilution in distilled water of aluminum hydroxide gel (Cutter Laboratories, Berkeley, California). *Serum* for use in the thromboplastin generation test was prepared as described earlier (6).

Cephalin, an acetone-insoluble, ether-soluble fraction of human brain tissue, was prepared as previously described (8) and was diluted in barbital buffer before use. *Human brain thromboplastin* and *rabbit brain thromboplastin* were prepared as saline extracts according to Owren's method (9). *Thrombin* of bovine origin (Topostasin, Hoffmann-La Roche, Basle) was adsorbed with barium sulfate as described earlier (6) and stored either frozen at $-20°$ C or at $4°$ C in 50% glycerol. *Citrated saline* was made by adding 1 part of 0.1 M sodium citrate to 5 parts of isotonic saline. *Barbital buffer* has been described previously (6), as has diluting fluid (6), which is buffered saline 0.005 M in sodium citrate.

Adenosine diphosphate (Pabst Laboratory, Milwaukee, Wisconsin) was dissolved in saline and stored at $-20°$ C. *Endotoxin* (Lipopolysaccharide Type W, E. coli 0111: B4, Difco Laboratories, Detroit, Michigan) was dissolved in saline immediately before use. *Hirudin* (Veb Arzneimittelwerk, Dresden, Germany) was diluted in barbital buffer. *Liquoid* (sodium polyanetholsulphonate, Hoffmann-La Roche, Basle) was dissolved in saline.

Methods

1. Preparation of Suspensions and Extracts of Rabbit WBC

The technique of Hirsch (10) was followed. Adult rabbits were injected intraperitoneally with 200–450 ml of isotonic saline containing 1 mg of potato starch per ml. Sixteen hours later, 100 ml of isotonic saline without starch was injected intraperitoneally, the abdomen was kneaded briefly, and the fluid was drained from the peritoneal cavity by gravity drip (small-bore plastic tubing with multiple side-openings inserted through a large-bore needle) into plastic tubes containing 4 ml of citrate anticoagulant and marked at 40 ml. At least 2 and usually 3 such 40 ml tubes of fluid were collected each time.

The tubes were centrifuged at 1,000 rpm for 5 min at $4°$ C. The resultant WBC buttons were resuspended in 2 to 3 ml of citrated-saline, combined, and washed in a total volume of 40 ml of citrated-saline. The washed cells were packed by centrifuging as described above and suspended in an amount of diluting fluid estimated to give a WBC count of about 50,000/mm³.

A preliminary experiment (see Table 1) assured us that this single washing eliminated significant contamination with plasma clotting factors. The cells appeared intact on phase microscopy but

Table 1. Evidence that a Single Washing Eliminates Contaminating Plasma Clotting Factors from WBC Suspensions.

Assay	Activity (% of standard rabbit plasma) in:		
	Supernate of peritoneal exudate	First wash water	Second wash water
Prothrombin	7.8	<0.2	<0.2
Factor V	0.3	<0.1	<0.1
Factor VII	3.8	0.6	0.6
Factor VIII	1.9	0.5	0.5
Factor IX	6.0	1.4	1.2
Factor X	15	0.1	0.1
Clotting by thrombin[1]	+	—	—

1) 0.2 ml of thrombin (20 NIH u/ml) added to 0.4 ml of test material.

were frequently in small clumps. Staining tests for cell viability on one occasion revealed 6% of cells staining with trypan blue (11) and 9% of cells staining with eosin (12). Smears from 6 preparations were stained for acid mucopolysaccharide with aldehyde fuchsin (13); many cells with heavily staining granules were seen in 5 preparations.

The composition of 11 cell suspensions may be summarized as follows: cell count, mean 56,000/mm³ and range 36,000–72,000/mm³; per cent heterophils, mean 79% and range 55–98%; red cell contamination, mean 12 RBC per 100 WBC and range 1–41 RBC per 100 WBC. No platelets were detected on phase microscopy of wet preparations or on stained smears.

Cell suspensions were disrupted by freezing in dry ice and acetone and thawing in water at 37° C for a minimum of 3 times. This gave a preparation containing small masses of stringy or matted cellular debris, which were partly broken up in a glass hand homogenizer before the preparations were studied for clotting activity.

A granule fraction of rabbit heterophils was prepared by the technique of Cohn and Hirsch (14). A pellet of washed cells was suspended in 0.34 M sucrose and subjected to vigorous stirring and pipetting to disrupt the cells. The nuclear fraction was separated by centrifugation at 400 g and the granule fraction was then precipitated by a second centrifugation at 12,000 g for 15 min. The resultant pellet of granules was resuspended in 0.34 M sucrose in a concentration of the granules from 51,000 WBC/mm³. This granule fraction was frozen and thawed six times and exposed to ultrasonic vibration in an attempt to disrupt the granules, but this treatment failed to alter their appearance on phase microscopy.

2. Preparation of a Suspension of Human Peritoneal WBC

Two liters of fluid were obtained from a patient undergoing peritoneal dialysis for the treatment of uremia secondary to systemic lupus erythematosus. The WBC were separated by centrifugation, washed two times with 100 ml of diluting fluid, and suspended in 25 ml of diluting fluid. This suspension contained 93,500 WBC/mm³ with 48% granulocytes, 11% mononuclear cells, 39% smudged cells and 2% red cells. Platelets were not seen. The suspension stood for 60 hrs at 4° C before it was used.

3. Clotting Factor Assays

Factor V was measured in the assay with Russell's viper venom described by Borchgrevink and co-workers (15). Factor VII was assayed by the technique of Aas (16) modified by adding adsorbed ox plasma to the clotting mixture. Factor VIII and factor IX were measured in activated partial thromboplastin time assays as described elsewhere (17). Factor X was determined by a modification of Hougie's technique (18) in which cephalin supplies the lipid. Prothrombin was assayed by a modification of the method of Hjort and co-workers (8) utilizing a serum reagent ["proconvertin reagent" (19)] to supply factor X. Platelet factor 3 activity was measured in a modification of the one-stage technique described by Husom (7) in which 0.1 ml of "platelet-free" substrate plasma, 0.1 ml of Russell's viper venom 1 : 32,000 and 0.1 ml of test material are incubated together at 37° C for 3 min and clotted by adding 0.1 ml of calcium chloride.

Other clotting methods are described in the text and footnotes to the tables.

Results

1. The Absence of Adsorbed Plasma Hemostatic Factors on Rabbit Heterophils

Platelets adsorb plasma factor V and fibrinogen, and these activities can not be removed by repeated washings. In contrast, once washed suspensions of rabbit WBC (about 60,000/mm³) contained less than 0.4% of the factor V activity of rabbit plasma. Thrombin added to intact or disrupted WBC suspensions failed to "clot" or clump the cells (0.1 ml of thrombin containing 10 NIH u/ml added to 0.2 ml of a WBC preparation in the presence or absence of 0.1 ml of 20 mM calcium chloride solution). Thus, WBC do not appear to bind fibrinogen. Moreover, a WBC preparation possessed no measurable factor X activity and less than 0.6% of the prothrombin activity of rabbit plasma. The addition of adenosine diphosphate to rabbit WBC (0.2 ml of WBC

suspension, 0.2 ml of citrated "platelet-free" human plasma, and 0.2 ml of either 20 µg/ml ADP or control diluting fluid) did not cause aggregation visible on either gross or microscopic examination. We conclude, therefore, that plasma hemostatic factors are not bound to the surface of rabbit peritoneal heterophils.

2. The Failure to Demonstrate an Effect of Rabbit WBC upon Thrombin, Fibrinogen or Fibrin

The incubation of citrated rabbit plasma with suspensions of either intact or disrupted WBC for 16–20 hrs at 4° C did not precipitate fibrinogen. (Very small amounts of precipitate might not be recognizable in such mixtures because the WBC form a fine film after several hours of incubation.) The addition of traces of thrombin to the incubation mixtures, to damage or "partly-clot" the fibrinogen, did not alter these results. Thus, we could not detect a precipitate of fibrinogen in mixtures of 0.5 ml of citrated rabbit plasma, 0.1 ml of bovine thrombin containing 0.15 NIH u/ml, and 0.1 ml of a disrupted cell suspension containing 50,000 WBC/mm³ and giving a positive staining reaction for acid mucopolysaccharide with aldehyde fuchsin.

Liquoid (sodium polyanethanolsulfonate) precipitates fibrinogen from rabbit plasma, and endotoxin has been shown to sensitize rabbits to this action of Liquoid *in vivo* (3). Acid mucopolysaccharides from WBC are postulated to act like Liquoid (3) and precipitate fibrinogen *in vivo* in animals given endotoxin. Therefore, it seemed important to compare the effect of adding Liquoid and of adding disrupted rabbit WBC *in vitro* to normal rabbit plasma and to plasma from rabbits given endotoxin (2 animals given 320 and 640 µg, respectively, of endotoxin and bled 2 hrs later).

Table 2. A Comparison of the Ability of Liquoid and of Disrupted WBC to Precipitate Fibrinogen from Normal Rabbit Plasma and from Plasma from an Endotoxin-Treated Rabbit.

Material added[1]	Final conc.	Precipitate from			
		Normal plasma		Endotoxin plasma	
		37° C	4° C	37° C	4° C
Liquoid	1.0 mg/ml	++++	++++	++++	++++
	0.5	+++	+++	++++	++++
	0.25	+	+	+++	+++
	0.125	0	0	++	++
	0.063	0	0	++	++
	0.032	0	0	++	++
	0.016			+	+
	0.008			+	+
	0.002			+	+
	0.001			0	+
WBC	4,200/mm³ [2]	0	0	0	0

1) To 0.45 ml of citrated rabbit plasma was added 0.05 ml of either Liquoid or disrupted WBC to give the final concentrations of added material shown. The mixtures were placed at 37° C and at 4° C and examined for precipitate after 60 min.

2) Differential count, 98% heterophils.

As shown in Table 2, a final concentration of Liquoid exceeding 0.125 mg/ml was required to precipitate fibrinogen from normal rabbit plasma, whereas a final concentration of Liquoid of only 0.001 mg/ml still sufficed to precipitate fibrinogen from the

plasma of an animal given endotoxin. This represents more than a hundred-fold increase in the sensitivity of plasma to Liquoid *in vitro*. Disrupted WBC failed to precipitate fibrinogen from normal rabbit plasma or from the plasma of the rabbit given 640 μg of endotoxin (shown in Table 2). We conclude that endotoxin clearly and markedly sensitizes citrated rabbit plasma to the precipitation of fibrinogen by Liquoid *in vitro* but fails to sensitize citrated rabbit plasma to the precipitation of fibrinogen by disrupted rabbit peritoneal heterophils *in vitro*.

Rabbit heterophils exhibited neither fibrinoplastic nor anti-thrombin activity in thrombin time systems. Thus, similar clotting times were obtained when 0.1 ml of either diluting fluid or WBC (65,000/mm³) was added to 0.2 ml of citrated rabbit plasma and the mixture was clotted with 0.1 ml of thrombin (concentrations between 0.4 and 10 u/ml). Nor did WBC potentiate the antithrombin activity of rabbit serum. When 0.1 ml of thrombin was added to a mixture of 0.5 ml of rabbit serum with 0.4 ml of diluting fluid, or intact WBC, or disrupted WBC (31,000/mm³), subsamples after 1 min gave thrombin times of 26, 25 and 24 sec and subsamples after 6 min gave thrombin times of 237, 239, and 190 sec, respectively.

Human and dog leukocytes possess protease activity enhancing fibrinolysis but rabbit leukocytes do not (20). When we allowed the clots formed by mixing equal portions of rabbit plasma, 20 mM calcium chloride solution, and either diluting fluid or a suspension of intact or disrupted white cells (42,000/mm³) to stand overnight, the failed to lyse.

3. The Inability of Rabbit WBC to Increase Factor V Reactivity

Factor V reactivity increases on incubation with thrombin, trypsin, or Russell's viper venom in the absence of added calcium (19). Therefore, a search for ways in which WBC might enhance clotting seemed incomplete without determining if extracts of WBC could similarly activate factor V. Equal portions of citrated rabbit plasma and test material were incubated together for 4 min, diluted 1:40, and assayed for their ability to shorten the brain thromboplastin time of hereditary factor V deficiency plasma. When the test material was diluting fluid, intact WBC (61,000/mm³), disrupted WBC, or thrombin (0.2 u/ml), the clotting times were 21.7, 21.4, 21.6, and 17.2 sec, respectively. Thus, in contrast to thrombin, WBC extracts could not increase factor V reactivity.

4. The Tissue Thromboplastic Activity of Rabbit WBC

The partial thromboplastin time (PTT), which is the clotting time of plasma recalcified in the presence of free lipid with platelet factor 3-like activity, reflects the rate of formation of prothrombinase in plasma. Table 2 shows the shortening of the intact PTT (i. e., the PTT of plasma prepared and clotted without exposure to an activating surface such as glass) produced by intact WBC, by disrupted WBC, and by a WBC fraction containing granules which had been subjected to freezing and thawing and to ultrasonic vibration. These data bring out three points: first, WBC shorten the PTT significantly but certainly less than undiluted rabbit brain thromboplastin; second, rabbit WBC shorten the PTT of rabbit plasma more than they shorten the PTT of human plasma; and, third, intact WBC preparations exert more procoagulant effect than either disrupted whole WBC or disrupted granules (probably, as discussed later, because of the enhanced anticoagulant activity present in disrupted cell preparations).

Since these data were obtained in an intact clotting system, shortening could have reflected either a tissue thromboplastic activity of the WBC or an activation of

Table 3. Shortening of the Intact Partial Thromboplastin Time (PTT) of Rabbit and Human Plasma by Intact and Disrupted Rabbit WBC and by a Granule Fraction of Rabbit WBC.

Test material	Clotting times (sec)	
	Rabbit plasma	Human plasma
Diluting fluid	170	185
Rabbit brain thromboplastin	16	24
WBC suspension (42,000/mm³): Intact	46	92
Disrupted	69	120
Sucrose (0.34 M)	181	163
Granule fraction (from 50,000 WBC/mm³)	64	124

To 0.1 ml of intact plasma in a clear plastic clotting tube were added 0.1 ml of a cephalin suspension 1 : 100 in barbital buffer and 0.1 ml of the test material. After incubation for 3 min at 37° C, the mixture was recalcified with 0.1 ml of 30 mM calcium chloride solution.

intrinsic clotting, e. g., an activation of factor XII (Hageman factor) by WBC. The shortening could not be attributed to a platelet factor 3-like activity of the WBC, since the clotting mixture was already supplied with an optimal amount of this activity.

Table 4 summarizes data obtained when rabbit WBC suspensions were added to *activated* partial thromboplastin time mixtures containing plasmas deficient, respectively, in factors XII, XI, IX, and VIII. As expected, *rabbit* WBC exerted only a weak procoagulant effect upon the *human* specific dificiency plasmas, and intact cells shortened the clotting times more than disrupted cells. Yet, some shortening of each deficiency plasma was observed, a finding suggestive of tissue thromboplastic activity rather than of an activity either bypassing or activating a particular intrinsic clotting factor. Moreover, the shortening of the activated PTT of factor XII deficiency plasma rules out the possibility that the procoagulant effect observed in intact systems (see Table 3) stemmed exclusively from a direct activation of factor XII by white cells.

When *human* peritoneal leukocytes were added to partial thromboplastin time mixtures containing either *human* factor VIII deficiency plasma or *human* "exhausted plasma" (plasma depleted of factors XII and XI) a more pronounced shortening of the clotting time was found. These observations, tabulated in Table 5, reinforce the evidence for a tissue thromboplastic activity in WBC preparations.

Tissue thromboplastin not only supplies the equivalent of the combined effect of plasma factors XII, XI, IX, and VIII but also furnishes platelet factor 3-like activity.

Table 4. Shortening of the Activated PTT of Human Specific Deficiency Plasmas by Rabbit WBC

Test material	Activated PTT (sec) of plasma deficient in factor:			
	XII	XI	IX	VIII
Diluting fluid	200	111	107	122
Rabbit brain thromboplastin 1 : 200	81	78	63	81
WBC suspension (55,000/mm³): Intact	96	82	70	114
Disrupted	121	135	102	118

0.1 ml of specific deficiency plasma, 0.1 ml of cephalin 1 : 100 in kaolin 10 mg/ml, and 0.1 ml of test material were incubated together for 3 min at 37° C and clotted by adding 0.1 ml of 35 mM calcium chloride solution.

Table 5. The Ability of Human WBC to Shorten the Partial Thromboplastin Time (PTT) of Human Factor VIII Deficiency Plasma and Human "Exhausted Plasma".

Test material	PTT (sec) of:	
	Factor VIII def. pl.[1]	Exhausted pl.[2]
Diluting fluid	116	210
Human brain thromboplastin 1:100	51	43
Human WBC preparation (93,500/mm³)	51	50

1) 0.1 ml factor VIII def. pl., 0.1 ml cephalin 1:100 in kaolin 10 mg/ml, 0.1 ml test material and, after 3 min at 37° C, 0.1 ml calcium chloride solution.

2) 0.1 ml exhausted pl., 0.1 ml cephalin 1:100, 0.1 ml test material and, after 3 min at 37° C, 0.1 ml calcium chloride solution.

Thus, if WBC preparations possess tissue thromboplastic activity, they should also exhibit platelet factor 3-like activity. The following clotting times were obtained in a platelet factor 3 assay (see Methods): diluting fluid, 71 sec; frozen and thawed platelet-rich plasma (546,000/mm³) diluted 1:10, 10 sec; intact WBC suspension (55,000/mm³), 10 sec. Clearly, intact rabbit WBC possess potent platelet factor 3-like activity.

Hereditary factor VII deficiency plasma clots normally in intrinsic clotting mixtures but has a prolonged clotting time in extrinsic systems containing tissue thromboplastin. Therefore, if the procoagulant effect of WBC suspensions results from weak tissue thromboplastic activity, WBC suspensions should exert much less of a procoagulant effect upon hereditary factor VII deficiency plasma than upon plasmas deficient in those intrinsic clotting factors (see Tables 4 and 5) that tissue thromboplastin bypasses. Table 6 summarizes an experiment in which intact rabbit WBC were added to hereditary factor VII deficiency plasma with and without a 1:1000 dilution of cephalin as an added source of platelet factor 3-like activity. (This dilution of cephalin possessed the same activity in a platelet factor 3 assay as the WBC suspension we used.)

As lines 1 and 2 of Table 6 show, factor VII deficiency plasma clotted faster in the presence of cephalin than in the presence of buffer. This is expected, for the added lipid facilitated intrinsic clotting. However, when the same amount of platelet factor 3-like activity was supplied by the WBC suspension (line 3), the clotting time was much longer than with buffer. We believe that this reflects the inability of the tissue thromboplastic activity in the WBC suspension to overcome the effect of the anticoagulant activity in the suspension in the absence of factor VII. When the clotting

Table 6. Prolongation of the Clotting Time of Factor VII Deficiency Plasma by Rabbit WBC.

Source of lipid	Second reagent	Clotting time of factor VII def. plasma (sec)[1]
Buffer	Buffer	165
Cephalin 1:100	Buffer	95
WBC suspension (42,000/mm³)	Buffer	345
Cephalin 1:1000	WBC suspension (42,000/mm³)	270

1) 0.1 ml of a mixture of equal parts of factor VII def. plasma and adsorbed ox plasma (added source of factor V), 0.1 ml of the lipid source listed, and 0.1 ml of the second reagent listed were incubated together for 3 min at 37° C in a glass clotting tube. Then 0.1 ml of 35 mM calcium chloride solution was added.

mixture contained both cephalin and the suspension (line 4), its clotting time was still longer than with buffer alone. Thus, even in the presence of another source of platelet factor 3-like activity, the WBC suspension did not promote the normal intrinsic clotting of factor VII deficiency plasma. This must mean that the procoagulant effect of WBC suspensions observed with normal and other deficiency plasmas (Tables 3, 4, 5) stems from tissue thromboplastic activity.

5. The Failure to Increase the Procoagulant Activity of Rabbit WBC on Incubation with Endotoxin

Intact rabbit WBC were incubated with calcium and E. coli endotoxin in final concentrations of $10 \mu g$ and $1 \mu g/ml$. Subsamples were tested for their ability to shorten the kaolin clotting time of platelet-poor plasma, as an indication of release of platelet factor 3-like activity, and for their ability to shorten the activated PTT of factor VIII deficiency plasma, as an indication of release of tissue thromboplastic activity. Representative data are shown in Table 7. Under our conditions, endotoxin failed to release potent clotting activity from intact rabbit heterophils. In an additional experiment, endotoxin also failed to release clotting activity from rabbit WBC which had been subjected to ultrasonic vibration.

Table 7. The Failure to Demonstrate Release of Procoagulant Activity upon Incubation of Rabbit WBC with Endotoxin.

Test material	Kaolin clotting time of normal plasma[1] (sec)	Activated PTT of fact. VIII def. pl.[2] (sec)
WBC not incubated with endotoxin	44.0	53.5
WBC incubated with endotoxin[3] 10 sec	47.5	60.5
7 min	49.0	53.5
15 min	47.0	57.5
45 min	48.5	51.0
95 min	47.0	50.0

1) 0.1 ml of normal platelet-poor plasma and 0.1 ml of kaolin 10 mg/ml were incubated together for 3 min at 37° C before the addition, in rapid succession, of 0.1 ml of 30 mM calcium chloride and 0.1 ml of the test sample. Clotting time was noted from addition of the test sample.

2) 0.1 ml of factor VIII def. pl. and 0.1 ml of cephalin 1:100 in 10 mg/ml kaolin suspension were incubated together for 3 min at 37° C vefore the addition, in rapid succession, of 0.1 ml of 30 mM calcium chloride and 0.1 ml of the test sample. Clotting time was noted from addition of the test sample.

3) 1.6 ml of an intact WBC suspension containing 70,000 WBC/mm³, plus 0.2 ml of E. coli endotoxin (0111: B4, lot 463135) 100 μg/ml, plus 0.2 ml 100 mM calcium chloride solution were incubated together at 37° C and subsampled at the times listed.

6. The Characterization of the Anticoagulant Activity of Rabbit WBC

As mentioned earlier, rabbit WBC possess anticoagulant activity demonstrable in intact preparations but much more evident after the cells have been disrupted. This is illustrated in Table 8, which shows the prolongation of the activated PTT of normal rabbit plasma produced by intact and disrupted WBC.

Table 8 shows that the anticoagulant activity was also present in a fraction containing the WBC granules. These data also prove that the anticoagulant can

Table 8. Prolongation of the Activated Partial Thromboplastin Time of Normal Rabbit Plasma and of Hereditary Human Factor VII Deficiency Plasma by Rabbit WBC Preparations.

Substrate plasma	Test material	Clotting time (sec)
Rabbit plasma	Diluting fluid	40
	WBC suspension	
	$(50,000/mm^3)$:	
	Intact	45
	Disrupted	69
Human factor VII deficiency plasma	Saline	43
	Sucrose (0.34 M)	45
	WBC granule-fraction in sucrose	105

0.1 ml of substrate plasma, 0.1 ml of cephalin 1:100 in kaolin 10 mg/ml, and 0.1 ml of the test material were incubated together for 3 min at 37° C and clotted by adding 0.1 ml of 30 mM calcium chloride.

block pure intrinsic clotting, for they show that it interferes with the clotting of factor VII deficiency plasma in the presence of free lipid with platelet factor 3-like activity and of optimal contact-active surface.

The data of the next table, Table 9, indicate that the anticoagulant can also interfere with pure extrinsic clotting. The top lines of this table illustrate that disrupted WBC *lengthen* the clotting time of factor VIII deficiency plasma clotted in a plastic tube in the presence of human brain thromboplastin. However, as the bottom lines of the table illustrate, when the human brain thromboplastin was extremely dilute or replaced by saline, then the disrupted WBC preparation *shortened* the clotting time. Indeed, whenever either intrinsic clotting (see Table 3 which presents data in which intrinsic clotting was impeded by lack of an activating surface) or extrinsic clotting activity in a system was minimal, the addition of disrupted WBC to the mixture shortened its clotting time. (As already discussed, hereditary factor VII deficiency plasma is an exception because the weak tissue thromboplastic

Table 9. The Effect of Disrupted Rabbit WBC upon Factor VIII Deficiency Plasma Clotted with Increasing Dilutions of Human Brain Thromboplastin.

Dilution of thromboplastin	Clotting time (sec) of factor VIII deficiency plasma containing:	
	Diluting fluid	Disrupted WBC $(42,000/mm^3)$
Full strength	16	30
1:25	37	100
1:100	60	155
1:500	130	195
1:1000	190	200
1:2000	260	215
Saline	1,100	235

0.1 ml of factor VIII deficiency plasma, 0.1 ml of the dilutions of human brain thromboplastin shown or of saline, and 0.1 ml of either diluting fluid or of disrupted WBC suspension were incubated together for 3 min at 37° C in a plastic clotting tube and clotted by adding 0.1 ml of 30 mM calcium chloride solution.

activity of the WBC can not exert its effect.) In contrast, whenever mixtures generated stronger intrinsic (Table 8) or extrinsic (Table 9) clotting activity, then the disrupted WBC acted as an anticoagulant.

The anticoagulant was also demonstrable when plasma was clotted with a powerful Russell's viper venom-cephalin reagent (see Table 10). Since viper venom activates factor X directly, bypassing early intrinsic and extrinsic clotting reactions, these data strongly suggested that the anticoagulant interferes either with a late step in the generation of prothrombinase or with the interaction of prothrombinase and prothrombin. We have already shown (see section 2) that the anticoagulant did not interfere with the thrombin-fibrinogen reaction. Moreover, the anticoagulant did not act by binding calcium, for adding extra calcium to clotting mixtures did not diminish its effect.

Table 10. The Ability of Disrupted WBC to Inhibit Clotting Induced by Russell's Viper Venom.

Test material	Clotting time (sec)
Diluting fluid	8.0
WBC suspension (50,000/mm³): Intact	8.2
Disrupted	11.4

0.1 ml of normal human plasma, 0.1 ml of Russell's viper venom 1:32,000 in cephalin 1:100, and 0.1 ml of the test material were incubated together for 3 min at 37° C and clotted with 0.1 ml of 35 mM calcium chloride solution.

A number of additional experiments were carried out to define further the site of action of the anticoagulant. All data consistently indicated that the anticoagulant was still active in the presence of prothrombinase and, therefore, that the anticoagulant interfered with the interaction between prothrombinase and prothrombin. Consequently, for brevity, only the data from one experiment have been presented in detail in Table 11.

Table 11. The Ability of WBC to Inhibit Clotting Induced by Prothrombinase in a Modified TGT Clotting Mixture Containing Hirudin.

Incubation time (min)	Clotting time of substrate mixture (sec) containing:	
	Buffer	Disrupted WBC (61,000/mm³)
6	17	64
10	22	70
13	23	102

Incubation mixture: Equal parts of normal adsorbed plasma 1:5, normal serum 1:10, cephalin 1:100, and 30 mM calcium chloride solution.

Clotting mixture: Equal parts of normal plasma, of either buffer or WBC suspension, of hirudin (7 anti-thrombin units per ml), of 20 mM calcium chloride and of subsample from the incubation mixture. The two last reagents were added in rapid succession with the clotting time measured from the addition of the subsample from the incubation mixture.

In this experiment intrinsic prothrombinase was synthesized in a standard "thromboplastin" generation test (TGT) incubation mixture, modified only by substituting cephalin for platelets. Subsamples were added to a clotting mixture which differed

from the standard clotting mixture in containing two added reagents. First, the clotting mixture contained 0.1 ml of either barbital buffer (control) or of disrupted WBC suspension (61,000/mm³). Second, the clotting mixture contained 0.1 ml of a dilute hirudin solution. The hirudin, a specific, stoichiometric anti-thrombin (21), delayed clotting and so, as Table 11 illustrates, permitted the clear demonstration of the anticoagulant effect of the WBC in the presence of potent, fully-formed pro-thrombinase from the incubation mixture.

Similar data were obtained when the clotting of the substrate mixture was delayed by other means, e. g., by diluting the subsample from the incubation mixture 1:10, or by utilizing a mixture of bentonite-adsorbed plasma (prothrombin source) and fibrinogen in place of normal plasma as the clotting substrate. This last clotting mixture, which contained no other clotting factors except prothrombin and fibrinogen, also minimized the chance of error due to further synthesis of intrinsic prothrombinase during the interval before the substrate mixture clotted. Moreover, one could still demonstrate an effect of the anticoagulant in the absence of some mechanism for delaying clotting of the substrate, although the difference in clotting times was then less dramatic, e. g., the difference between 9 sec for a mixture containing buffer and 13 sec for a mixture containing white cell suspension.

Discussion

Procoagulant activity in white cells has been described in earlier clotting studies with leukocytes from patients with leukemia (22, 23). The data reported herein clearly establish that rabbit peritoneal leukocytes possess weak but definite tissue thromboplastic activity. The activity is probably a property of the granulocyte, since this cell predominated in our preparations (mean differential count, 79% heterophils). The thromboplastic activity of intact cells always exceeded that of disrupted cells and exceeded that of a granule fraction subjected to freezing and thawing and to ultrasonic vibration to disrupt its granules. Although this finding may reflect an effect of the anticoagulant activity liberated by cell disruption, it nevertheless indicates that rabbit granulocytes do not contain powerful coagulation activators which can be liberated by disrupting the cell or its granules. This con-clusion receives further support from our inability to increase the clotting activity of WBC suspensions on incubation with endotoxin, a material which releases intra-cellular enzymes both *in vitro* (24) and *in vivo* (25).

If the intact granulocyte has weak tissue thromboplastic activity, then this activity must be a property of the cell wall. Thus, the granulocyte differs from the platelet, whose membrane must be altered to make platelet factor 3 activity available for clotting, and from the membrane of the mature red blood cell, which is inert in clotting. Granulocyte thromboplastic activity is so weak that it can hardly play a significant role in normal hemostasis. But it could well be involved in the formation of fibrin in inflammatory exudates and in the intravascular clotting thought to be responsible for the hypofibrinogemia of acute promyelocytic leukemia (26).

Platelets adsorb clotting factors to their surface and this, undoubtedly, contributes importantly to their hemostatic properties. Serum proteins can be demonstrated on washed human leukocytes by means of fluorescent antibodies (27). However, our studies indicate that in the rabbit, at least, plasma clotting factors do not bind to the granulocyte surface.

White blood cells contain acid mucopolysaccharides (4, 5) which have been pos-tulated to precipitate endotoxin-damaged fibrinogen within the glomerular capillary

bed in the generalized Shwartzman reaction (3). Indeed, material staining like acid mucopolysaccharide has recently been described within the fibrinoid glomerular lesions of the generalized Shwartzman reaction (28). *In vitro*, we have been unable to demonstrate a fibrinoplastic effect of either intact or disrupted WBC upon normal citrated plasma, upon plasma treated with minute amounts of thrombin in an attempt to "partly-clot" its fibrinogen, or upon plasma from 2 rabbits given endotoxin. (Plasma from these 2 rabbits had a markedly increased sensivitity to precipitation by Liquoid *in vitro*.) However, our findings should not be taken as final evidence against this hypothesis, for our *in vitro* conditions could be the wrong model for the *in vivo* situation. For example, calcium ions could be required for an action mucopolysaccharide from WBC upon fibrinogen, or, possibly, peritoneal heterophils do not contain as much acid mucopolysaccharide as heterophils circulating after rabbits are given an injection of endotoxin.

Martin and Roka (22) have described an anticoagulant activity in leukocytes from patients with chronic granulocytic leukemia, and Graham and co-workers (29) mention preliminary experiments indicating the presence of anticoagulant activity in rabbit "granulocyte substance". Our data establish the existence of anticoagulant activity in rabbit peritoneal leukocytes. The anticoagulant appears to inhibit the activation of prothrombin by fully-formed prothrombinase. Its action can be readily demonstrated in intrinsic clotting systems containing optimum cephalin and contact-active surface, in clotting systems with brain thromboplastin, and in clotting systems with Russell's viper venom. Surprisingly, however, when the weak thromboplastic activity of the WBC suspension itself serves to initiate clotting (see Tables 3 and 9), the anticoagulant activity of the preparation is not very effective and its procoagulant effect predominates. Since disrupted cells consistently exhibited much more anticoagulant activity than intact cells, the anticoagulant differs from WBC thromboplastin, itself, in being located primarily within the cell. Its physiologic meaning, if any, and its chemical properties are as yet unknown.

We undertook these studies to search for evidence supporting either of two hypotheses for the role of granulocytes in the generalized Shwartzman reaction induced by endotoxin, namely, (1) that white cells are required for endotoxin to initiate intravascular clotting, and (2) that white cells are required to complete clotting by precipitating fibrinogen "partly clotted" or otherwise damaged by endotoxin. Unfortunately, our *in vitro* data support neither hypothesis. We failed to demonstrate powerful coagulant activity which could be liberated from granulocytes by either disintegration or incubation with endotoxin. We also failed to demonstrate a fibrinoplastic action of WBC. Clearly, other approaches are needed to solve the riddle of what granulocytes do in the pathogenesis of the generalized Shwartzman reaction.

Summary

A systematic *in vitro* study has been carried out of the blood clotting properties of rabbit peritoneal leukocytes. Rabbit heterophils have been shown to possess weak but definite tissue thromboplastic activity. They also contain an anticoagulant activity which is active in the intrinsic clotting system, in the extrinsic clotting system with brain thromboplastin, and in clotting systems with Russell's viper venom. When the thromboplastic activity of the white cell itself initiates clotting, the anticoagulant is much less effective and the procoagulant effect of the WBC suspension predominates. Rabbit heterophils do not bind plasma clotting factors, do not activative factor V, and, under our conditions, do not precipitate fibrinogen from

normal plasma, from plasma exposed to traces of thrombin *in vitro*, or from plasma of rabbits given endotoxin.

The relation of these findings to the role of the granulocyte in the pathogenesis of the generalized Shwartzman reaction has been discussed.

Résumé

On a étudié de façon systématique in vitro les propriétés coagulantes des leucocytes péritoneaux des lapins. On montre que les hétérophiles de lapin ont une activité de thromboplastine tissulaire faible mais distincte. Ils contiennent également une activité anticoagulante qui se manifeste dans les systèmes de coagulation intrinsèque, extrinsèque avec la thromboplastine du cerveau et également dans les systèmes de coagulation avec le venin de la vipère de Russell. Quand l'activité thromboplastique des leucocytes eux-mêmes provoque la coagulation, l'anticoagulant est beaucoup moins actif et l'effet procoagulant des suspensions de leucocytes prédomine. Les hétérophiles de lapin ne fixent pas les facteurs de coagulation plasmatiques, n'activent pas le facteur V et dans les conditions de nos essais ne précipitent pas le fibrinogène à partir du plasma normal, du plasma exposé à des traces de thrombine in vitro ou à du plasma de lapin après administration d'endotoxine.

On discute le rapport entre ces résultats et le rôle des granulocytes dans la pathogénèse de la réaction de Shwartzman généralisée.

Zusammenfassung

Es wurde eine systematische Untersuchung der Eigenschaften von Leukozyten des Peritoneums des Kaninchens im Gerinnungssystem in vitro durchgeführt. Es konnte gezeigt werden, daß die Kaninchenheterophilen eine schwache, aber eindeutige Gewebethrombokinaseaktivität aufweisen. Sie besitzen außerdem eine gerinnungshemmende Wirkung, welche im »intrinsic«-System nachweisbar ist, sowie im »extrinsic«-System in Gegenwart von Hirnthromboplastin und in Gerinnungssystemen mit Russell Viperngift. Wenn die Gerinnung durch die thromboplastische Aktivität der weißen Zellen selbst ausgelöst wird, ist das Antikoagulans viel weniger wirksam und die gerinnungsfördernde Wirkung der weißen Blutkörperchen überwiegt. Die Heterophilen des Kaninchens binden keine Gerinnungsfaktoren, sie aktivieren Faktor V nicht und fällen auch nicht Fibrinogen aus normalem Plasma unter unseren Versuchsbedingungen, sowie aus Plasma, welchem Spuren von Thrombin in vitro zugesetzt waren, oder von Kaninchen, welche Endotoxin erhalten hatten.

Die Bedeutung dieser Befunde für die Rolle der Granulozyten in der Pathogenese des generalisierten Shwartzman-Phänomens wurde diskutiert.

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