STUDIES ON PLATELETS. XIV. HUMAN PLATELETS AS SOURCE OF ANTIFIBRINOLYSIN [1]

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Occurrence of increased plasma and serum fibrinolytic activity in patients with acute leukemia and severe thrombocytopenia (1–4) has been recently reported. It would seem that at least two mechanisms may cause this phenomenon. It is possible that profibrinolysin be activated to fibrinolysin by kinases of bacterial origin or derived from leukemic cells. It is also conceivable that thrombocytopenia may induce deficiency of circulating antifibrinolysin. The last hypothesis is supported by the findings of Johnson and Schneider (5) who found bovine platelets to contain twice as much antifibrinolytic activity as bovine plasma. Experiments to be presented in this paper discuss human platelets as source of antifibrinolytic activity and the possible role of fibrinolysis in the pathogenesis of thrombocytopenic bleeding.

MATERIALS AND METHODS

Determination of antifibrinolysin was carried out by the technic described below. The following reagents were prepared: (a) A solution of Loomis' bovine fibrinolysin,[3] containing 5 mg. per ml. in veronal buffer (6). This preparation of fibrinolysin had activity of 0.33 u. per mg.; (b) various dilutions in veronal buffer of serum or plasma under study. Determinations were made within two weeks of collection, on specimens which had been separated from the clot within one hour of venesection and kept at −20°C. until used. Since there is evidence that some factor or factors in the fibrinolytic system are extremely labile, blood was also collected in the cold and serum separated by rapid centrifugation in the cold (4°C.) and used immediately. Results were essentially identical to those obtained with stored, frozen samples; (c) solution of bovine fibrinogen in veronal buffer, containing 300 mg. per 100 ml.; (d) solution of purified bovine thrombin in saline solution, containing 100 units per ml.

Two-tenths ml. of fibrinolysin solution and 0.2 ml. of each serum or plasma dilution were transferred to chemically clean 10 × 100 mm. serology tubes and mixture incubated at 37°C. for 30 minutes. At the end of incubation, 0.3 ml. of fibrinogen solution and 0.1 ml. of thrombin solution were added in rapid succession. A clot formed within a few seconds. A stop watch was started on the addition of thrombin to the first tube and all tubes observed at frequent intervals. The end point (*clot lysis time*) was the time at which clot had completely dissolved. A test tube where veronal buffer was substituted for serum or plasma was taken as *control.*

Platelets were counted by a modification of the direct method previously described (7) using 1 per cent solution of EDTA–Na_2 in 0.7 per cent NaCl as diluting fluid and phase contrast microscope. Technics for the determination of capillary fragility, bleeding time, prothrombin utilization during clotting (prothrombin consumption) were as previously described (4). Platelet-rich and platelet-poor plasma and suspension of washed platelets in saline were also prepared as previously described (8). Sodium citrate 0.02 M (1/10 volume of 0.2 M solution) was used as anticoagulant, since preliminary experiments disclosed that it would inhibit antifibrinolytic activity to a lesser degree than sodium oxalate or EDTA–Na_2. Silicone-coated syringes and Arquad 2-C coated needles were used for collection of blood throughout all experiments.

RESULTS

(a) *Plasma and serum antifibrinolytic activity in healthy subjects and patients with various conditions*

Antifibrinolytic activity of plasma and serum was determined in 8 patients with thrombocytosis (5 with chronic bleeding and 3 with severe hypochromic anemia); 15 patients with severe thrombocytopenia (9 with idiopathic thrombocytopenic purpura and 6 with acute leukemia); 7 patients with thrombocytoasthenia (4 of the congenital va-

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riety and 3 of the acquired variety due to chronic granulocytic leukemia). In chronic granulocytic leukemia, platelets, as in the cases studied here, may appear large and bizarre, while isolated or multiple abnormalities of platelet function may be found (poor clot-retraction, prolonged bleeding time, increased capillary fragility, poor prothrombin utilization during clotting [4]). Analytical results are presented in Table I, as those found in serum. Values for plasma were essentially identical. Antifibrinolytic activity was evidently higher in patients with increased number of platelets and in healthy subjects than in patients with quantitative (idiopathic thrombocytopenic purpura, acute leukemia) or qualitative (thrombocytoasthenia) platelet deficiency.

TABLE I

Antifibrinolytic activity of serum from healthy subjects and patients with various diseases *

	Platelet count *per cu. mm.*	Bleeding time *minutes*	Tourniquet test petechiae No.	Clot retraction %	Prothrombin utilization %	Clot lysis time, *minutes* Dilution of serum 1/10	1/20	1/30	1/40	Control
1. Normal	275,000	3½	2	51	97	58	46	35	26	13
2. Normal	224,500	2	3	43	94	57	40	31	28	14
3. Normal	282,500	5½	0	47	87	55	41	34	29	12
4. Normal	279,100	4	1	52	92	58	47	37	29	14
5. Normal	239,700	2½	5	57	89	64	44	39	35	11
6. Normal	274,000	3	0	63	74	69	54	46	40	12
7. Normal	205,150	4½	4	50	79	63	52	35	27	13
8. Normal	271,350	5½	1	42	67	67	55	32	25	12
9. Normal	237,000	3	0	61	82	57	43	39	28	14
10. Normal	301,100	2½	7	45	95	58	48	38	32	14
11. Chronic bleeding	421,000	2	0	72	100	74	62	51	35	11
12. Chronic bleeding	372,500	½	0	59	82	83	68	54	37	13
13. Chronic bleeding	402,050	2	2	69	94	75	64	58	36	14
14. Chronic bleeding	393,180	½	0	73	94	71	63	57	37	15
15. Chronic bleeding	600,000	1	0	50	100	77	62	58	40	12
16. Iron deficiency anemia	420,000	2	0	54	97	87	70	60	39	14
17. Iron deficiency anemia	372,500	3	1	47	92	89	75	63	46	12
18. Iron deficiency anemia	379,000	2½	0	71	89	94	73	64	44	14
19. Idiopathic thrombocytopenic purpura	21,000	>20	70	3	4	44	28	25	22	12
20. Idiopathic thrombocytopenic purpura	27,000	>20	68	14	0	47	29	27	19	11
21. Idiopathic thrombocytopenic purpura	41,000	18	100	7	17	37	34	26	24	13
22. Idiopathic thrombocytopenic purpura	37,000	>20	100	2	5	39	27	23	17	14
23. Idiopathic thrombocytopenic purpura	<10,000	>20	100	0	9	42	35	23	21	12
24. Idiopathic thrombocytopenic purpura	15,000	>20	74	5	4	43	34	28	19	11
25. Idiopathic thrombocytopenic purpura	25,000	17	50	0	7	36	30	22	18	12
26. Idiopathic thrombocytopenic purpura	40,000	16	35	15	8	51	37	31	24	14
27. Idiopathic thrombocytopenic purpura	55,500	14	47	9	0	50	39	30	26	11
28. Ac. lymphocytic leukemia	<10,000	>20	60	0	4	29	25	18	13	11
29. Ac. myelogenous leukemia	35,000	14	54	20	9	28	27	22	18	12
30. Ac. histiocytic leukemia	31,000	>20	64	0	7	36	29	19	16	12
31. Ac. mono-myelocytic leukemia	32,000	19	57	11	6	27	22	18	17	14
32. Ac. lymphocytic leukemia	<10,000	>20	72	7	5	28	24	17	15	13
33. Ac. monocytic leukemia	47,500	12	>100	0	3	24	20	17	15	14
34. Chr. myelogenous leukemia	1,050,000	*15*	4	52	*21*	42	30	25	22	14
35. Chr. myelogenous leukemia	755,000	3	*32*	*10*	*12*	44	34	27	22	13
36. Chr. myelogenous leukemia	643,000	*7*	10	47	67	40	29	24	20	11
37. Thrombocytoasthenia	275,000	*11*	7	*9*	*10*	35	33	29	24	14
38. Thrombocytoasthenia	301,500	3	10	*6*	72	35	32	27	23	10
39. Thrombocytoasthenia	226,750	*15*	15	49	79	33	25	21	20	12
40. Thrombocytoasthenia	224,500	*15*	*>100*	57	84	37	24	22	19	12

* Cases 34–40 exhibit a partial deficiency of platelet function. Results which are abnormal are in italics. It will be noted that one or more but never all platelet functions are affected.

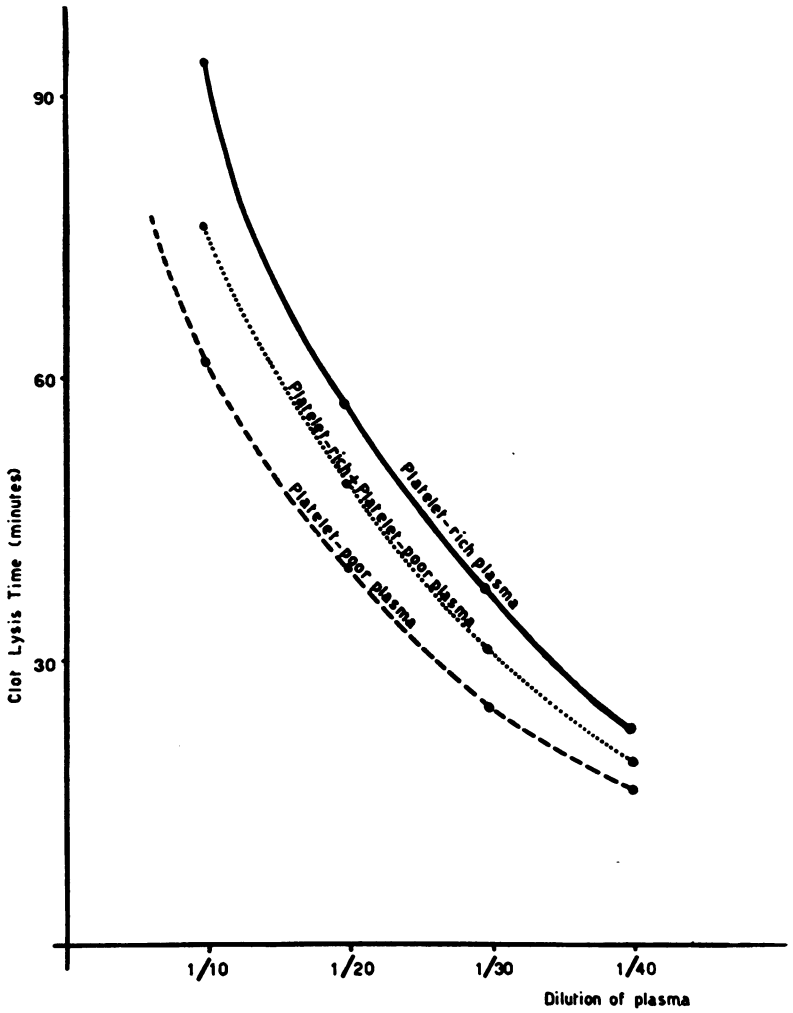

FIG. 1. ANTIFIBRINOLYTIC ACTIVITY OF PLATELET-RICH PLASMA, PLATELET-POOR PLASMA AND A MIXTURE OF EQUAL VOLUMES OF BOTH
Average results of five experiments.

(b) *Relation between number of platelets and antifibrinolytic activity of normal plasma*

Platelet-rich and platelet-poor plasma were obtained as previously described (8). Platelet-rich plasma contained an average of 300,000 platelets per cu.mm. and platelet-poor plasma of 4,000 platelets per cu.mm. A third series of samples were prepared by mixing platelet-rich and platelet-poor plasma in equal volumes. Dilutions were in veronal buffer. Results are presented in Figure 1. It is clear that platelet-rich plasma exhibited higher antifibrinolytic activity than platelet-poor plasma. Preparations containing intermediate number of platelets also exhibited intermediate antifibrinolytic activity.

(c) *Antifibrinolytic activity of serum obtained by recalcification of platelet-rich or platelet-poor plasma*

Two samples of blood were collected with "Silicone technic" (9) using 1/10 volume of 0.2 M sodium citrate as anticoagulant. Platelet-rich and platelet-poor plasma were then obtained and recalcified by addition of 1/10 volume 0.2 M Ca Cl_2. After incubation at 37°C. for one hour, serum was separated and tested for antifibrinolytic activity. Serum obtained from platelet-rich plasma

TABLE II

Antifibrinolytic activity of serum obtained by recalcification of platelet-rich or platelet-poor plasma (ten experiments) *

Dilution of serum	1/10	1/20	1/30	1/40	Control
	min.	*min.*	*min.*	*min.*	*min.*
Serum from platelet-rich plasma	101±12	76±10	54±8	30±5	12±3.5
Serum from platelet-poor plasma	68±7	37±5	28±4	17±4	12±3.2

* Clot lysis time—Average value and standard deviation given (figures approximated to one-minute intervals).

exhibited higher antifibrinolytic activity (Table II).

(d) *Liberation of antifibrinolysin from platelet-rich plasma*

Platelet-rich plasma was repeatedly frozen and thawed. Observation of plasma after a number of these steps showed many platelet "ghosts" but also considerable clumping of platelets, preventing actual determination of the number of platelets either intact or lyzed.

The effect of repeated freezing and thawing, as shown in Table III, apparently resulted in increased antifibrinolytic activity. The same table shows, however, that such procedure, if continued for a long time, eventually resulted in loss of this effect. This was confirmed in a parallel experiment, when the fibrinolytic activity of serum left at 20°C. for three days was compared to that of another sample thawed and refrozen daily for three days. The second sample had negligible antifibrinolytic activity, which was, on the other hand, intact in the first sample which had been kept continuously frozen.

Another technic consisted in adding to platelet-rich plasma 2/10 volume of either normal inactivated serum or of serum from a patient with idiopathic thrombocytopenic purpura exhibiting a high titer platelet agglutinin. After one hour of incubation at 37°C., many platelets appeared clumped and there were several platelet "ghosts," possibly indicating platelet lysis. Liberation of antifibrinolytic activity by such serum is shown in Table IV. The results of Table V indicated that liberation of antifibrinolysin through the action of serum was also a function of the number of platelets available.

(e) *Liberation of antifibrinolysin from washed platelets*

Good antifibrinolytic activity was found after resuspending in equal volume of saline solution without washing, platelets separated from citrated plasma by centrifugation at 3,000 r.p.m. for 15 minutes. On the other hand, Table IV indicated the very low antifibrinolytic activity of isolated human platelets washed three times with saline solution. Equally negative results were obtained after treating washed human platelets with repeated freezing and thawing either in the absence or in the presence of a small volume of normal human plasma or serum, addition of saponin (0.007 per cent), thrombin, acetone. The addition of serum from patients with idiopathic thrombocytopenic purpura was also ineffective. None of these agents, which clumped platelets heavily, were able to liberate antifibrinolysin from washed platelets.

TABLE III

The effect of repeated freezing and thawing on antifibrinolytic activity of platelet-rich plasma (ten experiments) *

Dilution of plasma	1/10	1/20	1/30	1/40	Control
	min.	*min.*	*min.*	*min.*	*min.*
Platelet-rich plasma†	87±9	58±7	38±5	21±4	12±3
Platelet-rich plasma, 3× frozen and thawed	158±20	87±11	63±7	42±4	12±2
Platelet-rich plasma, 12× frozen and thawed	34±4	25±4	18±3	14±2	12±3

* Clot lysis time—Average value and standard deviation given (figures approximated to one-minute intervals).
† Containing 300,000 platelets±20,000 per cu. mm.

TABLE IV

Liberation of antifibrinolysin from platelet-rich plasma on addition of inactivated serum from patients with idiopathic thrombocytopenic purpura *

Dilution of plasma	1/10	1/20	1/30	1/40	Control
	min.	*min.*	*min.*	*min.*	*min.*
Platelet-rich plasma†	86±8	62±7	36±6	24±3	12±4
Platelet-rich plasma + normal serum‡	101±16	79±8	48±5	31±4	13±2
Platelet-rich plasma + serum of patient with idiopathic thrombocytopenic purpura‡	153±18	98±12	73±7	53±7	11±4
Platelets in saline†	18±4	15±3	13±3	13±2	12±3
Platelets in saline + serum of patient with idiopathic thrombocytopenic purpura‡	22±4	18±5	15±4	15±4	11±3

* Clot lysis time—Average value and standard deviation given (figures approximated to one-minute intervals).
† Containing 300,000 platelets±35,000 per cu. mm.
‡ Two-tenths ml. of serum from patient with chronic idiopathic thrombocytopenic purpura (containing platelet agglutinin with titer 1/128) or normal donor added to 0.8 ml. of platelet-rich plasma or platelet suspension in saline.

DISCUSSION

The experiments reported should be interpreted with caution. The mixed use of reagents of human and bovine origin and the necessity of using anticoagulants, which inhibit to a certain degree the activity of antifibrinolysin, may have, at least in part, vitiated the findings. For these reasons, it was felt necessary to overcome some of these difficulties by a multiple experimental approach to the problem.

Even so, conclusions suggested by the preceding experiments are only tentative. Antifibrinolytic activity of plasma and serum, however, appears related to platelets and to their number. Thus, serum of patients with thrombocytosis exhibits higher antifibrinolytic activity than normal serum, and this, in turn, is more active than serum from patients with quantitative (thrombocytopenia) or qualitative (thrombocytoasthenia) platelet deficiency. It is significant to note that serum from patients with idiopathic thrombocytopenic purpura exhibits perhaps higher antifibrinolytic activity than that from patients with the secondary type of thrombocytopenia (acute leukemia or aplastic anemia), a finding for which we have no explanation at this time.

Experiments *in vitro* have also confirmed the relationship of number of platelets to antifibrinolytic activity. This is higher in platelet-rich than in platelet-poor plasma and in serum from platelet-rich than in serum from platelet-poor plasma. Yield of antifibrinolytic activity, possibly through the lytic effect of serum from patients with idiopathic thrombocytopenic purpura, is largest in plasma containing the highest number of platelets. There seems to be sufficient evidence to state that antifibrinolysin in human blood is, at least in part, related to platelets, or to platelet products or represents material bound to or carried by platelets. The experiments, however, do not answer definitely the question whether antifibrinolysin constitutes an intimate component of platelets or is simply carried by them. The enhancement of antifibrinolysin activity in platelet-rich human plasma by repeated freezing and thaw-

TABLE V

Liberation of antifibrinolysin from platelet-rich plasma on addition of inactivated serum from patient with idiopathic thrombocytopenic purpura (six experiments) *

Dilution of plasma		1/10	1/20	1/30	1/40	Control
		min.	*min.*	*min.*	*min.*	*min.*
Platelet number	800,000/cu. mm.	102±12	72±11	43±7	27±4	13±4
	600,000/cu. mm.	89±10	60±9	41±7	24±4	13±3
	300,000/cu. mm.	78±9	54±7	34±5	20±3	13±2
	150,000/cu. mm.	72±8	49±7	27±6	19±4	13±4
	20,000/cu. mm.	61±9	43±6	24±5	15±2	13±3

Two-tenths ml. of serum from patient with chronic idiopathic thrombocytopenic purpura (containing platelet agglutinin with titer 1/128) added to 0.8 ml. of platelet-rich plasma.

* Clot lysis time—Average value and standard deviation given (figures approximated to one-minute intervals).

ing and by addition of serum from patients with idiopathic thrombocytopenic purpura seems to indicate that antifibrinolytic activity is liberated as platelets are destroyed. On the other hand, no antifibrinolysis can be obtained from human platelets after repeated washing. It is still present, however, if platelets are only separated from plasma by single centrifugation and resuspended in saline solution. This last experiment could be interpreted as evidence that antifibrinolysin may be washed away from platelets.

The discrepancy between the various experimental results may best be explained by a number of considerations. It appears, first of all, difficult to lyze platelets which have been washed free of plasma or serum. Also, antifibrinolysin might have been lost during the process of separation and washing of platelets. It appears unlikely that a factor or factors present in serum or plasma may be necessary for the release of antifibrinolysin from platelets since addition of serum or plasma to washed human platelets fails to increase antifibrinolytic activity of the mixture, beyond the antifibrinolytic activity supplied by serum or plasma themselves. Whether platelets contain or carry antifibrinolysin is not clearly demonstrated by these experiments. These studies, nevertheless, raise the question whether platelets may not only contain but also carry factors with important biological function and other circulating chemicals.

Recent work has shown the presence in platelets of a number of factors which are specifically active in various phases of the hemostatic process (10). It appears that at least some of them may represent agents carried by or absorbed into the platelets rather than intimate constituents of platelets. Thus it is thought by some investigators (11) that serotonin, adrenalin (11), perhaps histamine are possibly absorbed on platelets. Administered Dextran may coat the platelet surface (12). None of the experimental results presented contradict the conclusion that antifibrinolysin also may be carried by or absorbed into platelets.

Finally, the observations reported here may have clinical significance. As mentioned in the introduction, increased fibrinolysis may be encountered in patients with severe secondary thrombocytopenia (aplastic anemia, acute leukemia) and be accompanied by severe purpura. We have recently encountered one such case. Transfusion of platelets as platelet-rich plasma, but not of separated, washed human platelets induced considerable decrease in the bleeding manifestations which was accompanied by reduction of fibrinolysis and increased antifibrinolytic activity of serum (Table VI).

SUMMARY

1. Antifibrinolytic activity of serum is higher in patients with thrombocytosis than in patients with quantitative or qualitative platelet deficiency. *In vitro,* antifibrinolytic activity of plasma is directly proportional to the number of platelets available and, in platelet-rich plasma, is enhanced by procedures leading to lysis of platelets (repeated freezing and thawing, addition of platelet-lytic serum). No antifibrinolysin, however, may be obtained from human platelets washed repeatedly. Addition of plasma or serum to washed platelets also fails to release antifibrinolysin. Thus, whether platelets contain, carry or absorb antifibrinolysin requires further investigation. The available evi-

TABLE VI

Effect of platelet transfusion (platelet-rich plasma) on serum antifibrinolytic activity in a patient with acute histiocytic leukemia *

Dilution of serum		1/10	1/20	1/30	1/40	Control	Platelet count (Direct method)/ cu.mm.
		Clot lysis time, *minutes*					
Before transfusion		32	27	21	17	13	12,000
After transfusion	5 min.	60	52	43	30	13	125,000
	2 hrs.	43	40	38	27	13	105,000
	24 hrs.	39	38	31	25	13	65,000
	48 hrs.	39	29	24	22	13	39,000
	72 hrs.	37	29	24	18	13	18,000

* Patient, 29 year-old male, weight 110 pounds, received 250 ml. of platelet-rich polycythemic plasma containing 1,100,000 platelets per cu.mm.

dence seems to suggest that platelets are carriers of antifibrinolysin.

2. Elevated fibrinolytic activity may be seen in patients with severe thrombocytopenia. The finding may be explained in some instances by low blood antifibrinolytic activity, which, in such cases, may become an important pathogenetic factor of bleeding.

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