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MECHANISM OF ACTION OF THROMBIN ON PLATELETS *

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Thrombin, a proteolytic enzyme, is known to initiate certain reactions of platelets including aggregation (1), retraction of clots (2), and release of serotonin (3). Thrombin aggregates platelets in hypofibrinogenemic (4) or normal citrated (5) or oxalated (6) plasma, in prothrombin-poor serum (7), in buffer at pH 7.3 to 7.6 (6), and in saline containing albumin (6) or a number of compounds of low molecular weight (8). Fibrinogen,[1] a recognized substrate of thrombin, has been demonstrated in extracts of platelets (9). The present studies define some of the conditions under which platelets react with thrombin. In particular, the role of fibrinogen in these reactions was investigated.

MATERIALS AND METHODS

Glassware with which platelets had contact was coated with silicone.[2] Venous blood was obtained from human donors either through 15-gauge needles attached to plastic tubing or through 18-gauge needles attached to syringes. Blood was collected without anticoagulant (native blood) or was mixed with 0.1 vol of a 2 per cent solution of the disodium salt of EDTA, 0.1 vol of 3.8 per cent sodium citrate solution, or 0.20 vol of acid-citrate-dextrose (ACD) solution (NIH formula A).

Platelet-rich plasma was prepared by centrifugation of blood at approximately 50 G for 30 minutes at room temperature. Platelet-free plasma was obtained by centrifugation of blood or platelet-rich plasma at 22,000 G for 10 minutes at 4° C. Platelet suspensions were prepared by centrifugation of platelet-rich plasma at approximately 4,700 G for 5 minutes at 4° C, and resuspension of the sedimented platelets in a volume of buffered saline approximately one-fifth of the volume of plasma. Buffered saline consisted of equal volumes of 3.6 per cent (0.3 M) Tris solution and 0.9 per cent sodium chloride solution, the pH adjusted to 7.5 with 2 N hydrochloric acid.

Trypsinized platelets were prepared by adding 0.20 vol of trypsin in buffered saline to suspension of platelets obtained from platelet-rich plasma containing EDTA. The final concentration per ml of platelet suspension was 1,000 μg of a preparation containing trypsin and magnesium sulfate,[3] or 50 μg of salt-free trypsin [4]. Suspensions were incubated at 37° C for 10 minutes. After centrifugation at 4,700 G for 5 minutes at 4° C, sedimented platelets were suspended in about twice the volume of buffered saline containing EDTA (prepared by adding 1.0 ml of a 4.8 per cent solution of EDTA to 100 ml of buffered saline). The centrifugation was repeated, and platelets were resuspended in a volume equal to the original volume of platelet suspension, with the use of buffered saline, normal serum, or platelet-free plasma. Trypsinized platelets were used within 3 hours after the second washing.

Clottable protein from platelets (platelet fibrinogen) was obtained by a modification of the procedure of Ware, Fahey and Seegers (9). Platelet-rich plasma was prepared from 1,000 ml of normal blood or from 150 to 200 ml of blood from each of 3 patients with thrombocytosis. A diagnosis of polycythemia vera had been made in two of the patients, and the third had myelofibrosis and myeloid metaplasia. The anticoagulant used was either EDTA or ACD solution. Platelet-rich plasma was centrifuged at 1,500 G for 15 minutes at room temperature, and sedimented platelets were suspended in approximately 16.0 ml of buffered saline. The platelet suspension was divided into two equal parts, one of which was incubated at 37° C for 10 minutes with 0.20 vol of trypsin in buffered saline, and the other was incubated with buffered saline alone. The platelets were washed twice and suspended in approximately 2.0 ml of buffered saline. These suspensions were frozen and thawed five times in an alcohol-dry ice mixture and centrifuged at 22,000 G for 10 minutes at 4° C to remove platelet debris. Aliquots of the supernatant fluids were

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[1] The term fibrinogen, as used in this presentation, refers to a protein substance that clots upon the addition of thrombin.

[2] General Electric silicone SC-87, Dri-Film.

[3] Trypsin 2× crystallized (maximum 50 per cent $MgSO_4$), minimum activity 8,000 U per mg protein.

[4] Trypsin, 2× crystallized, salt-free, lyophilized, minimum activity 10,000 U per mg. Both from Worthington Biochemical Corp., Freehold, N. J.

tested for clottable protein by addition of 0.20 vol of a solution of bovine thrombin (1,000 U per ml) in saline.

Aggregation of platelets by thrombin was evaluated after adding 0.20 vol of a solution of thrombin to platelets suspended either in buffered saline or in serum. Except where specified, a preparation of bovine thrombin [5] was used in a final concentration of 200 NIH U per ml. Highly purified bovine thrombin or human thrombin [6] was employed in certain experiments that will be described. After mixing, the suspensions were incubated without agitation in 15 × 65 mm tubes at 37° C for 30 minutes. The samples were observed macroscopically for clumping of the platelets, and aliquots were examined microscopically for aggregation with a phase contrast microscope.

Purified bovine fibrinogen was generously provided by Dr. W. H. Seegers. Rabbit antihuman-platelet serum was prepared in this laboratory by Corn and Upshaw (10). Sections of clots were prepared by the technique of Gaintner as cited by Conley (11). Plasma clots were fixed in buffered formalin, sectioned (6 μ), and stained with eosin. Clot retraction was measured as previously described (12). Platelet counts were performed by the method of Brecher and Cronkite (13). Adsorbed serum was prepared by incubating normal blood in uncoated glass tubes at 37° C for 24 hours. Clotted blood was centrifuged at 1,000 G at room temperature for 7 minutes, and serum was adsorbed with barium sulfate as described by Quick (14); residual prothrombin could not be detected in the adsorbed serum. Serum was used immediately or stored at −20° C.

RESULTS

A. Studies of fresh platelets

1. Aggregation of platelets in plasma by thrombin. Platelet-rich plasma from normal donors was prepared with EDTA as the anticoagulant in seven experiments. Time required for macroscopically visible clumping of platelets and for clotting of plasma was measured after addition of a solution of thrombin alone or of thrombin plus calcium chloride. Data of a representative experiment are shown in Table I. Various concentrations of thrombin produced clotting of platelet-rich EDTA plasma, but macroscopic aggregation of the platelets was not observed even when the clotting time of the plasma was prolonged to > 212 seconds by decreasing the concentration of thrombin. When calcium chloride was added with thrombin, aggregation of the platelets was noted macroscopically and microscopically prior to coagulation of the plasma. As the amount of thrombin was decreased, the time of appearance of platelet aggregation remained fairly constant (16 to 22 seconds), although the clotting time of the plasma became greatly prolonged (25 to 400 seconds). Thrombin in relatively high concentration (5 U per ml) clotted plasma in less than 15 seconds, and aggregation of platelets could not be observed prior to coagulation even though calcium was present. Addition of calcium chloride solution alone produced platelet aggregation after a long delay, shortly before the plasma clotted.

TABLE I

*Time of macroscopically visible aggregation of platelets and of clotting in EDTA platelet-rich plasma after addition of thrombin or of thrombin and calcium chloride**

Thrombin	Thrombin in $CaCl_2$ sol.	Aggreg. of platelets	Plasma clotting
U/ml	*U/ml*	*sec*	*sec*
5.0		0	13
1.0		0	54
0.6		0	97
0.4		0	212
0.25		0	>300
0.1		0	>300
0.05		0	>300
	5.0	0	<15
	1.0	18	25
	0.6	16	33
	0.4	21	43
	0.25	17	66
	0.1	22	>300
	0.05	35	>300
	0	890	975

* Reaction mixture in silicone-coated tubes at 37° C consisted of 0.4 ml EDTA platelet-rich plasma (400,000 platelets per mm^3) and either 0.1 ml thrombin in buffered saline or 0.1 ml thrombin in calcium chloride solution (0.025 M). Final concentrations of thrombin are recorded; 0 = none seen.

A similar experiment was performed employing platelet-rich plasma containing sodium citrate, a less effective calcium-binding agent than EDTA. Addition of thrombin alone resulted in macroscopic aggregation of the platelets followed by coagulation of the plasma. As the amount of thrombin was decreased, the time of appearance of platelet aggregation remained relatively constant, although the clotting time of the plasma became markedly prolonged (Table II).

2. Aggregation of platelets in plasma by thrombin in the presence of heparin. Citrated platelet-

[5] Thrombin, Topical, kindly supplied by Parke, Davis and Co., Detroit, Mich.

[6] Human thrombin (5,060 U per mg tyrosine), kindly supplied by Dr. H. O. Singher, Ortho Pharmaceutical Corp., Raritan, N. J.

TABLE II

Time of macroscopically visible aggregation of platelets and of clotting in citrated platelet-rich plasma after addition of thrombin *

Thrombin	Aggreg. of platelets	Plasma clotting
U/ml	*sec*	*sec*
1.0	24	28
0.6	20	60
0.5	26	90
0.4	21	70
0.3	30	>280
0.25	27	>300
0.1	0	>300

* Reaction mixture in silicone-coated tubes at 37° C consisted of 0.4 ml citrated platelet-rich plasma and 0.1 ml thrombin in buffered saline. Final concentrations of thrombin are recorded.

rich plasma was prepared from a patient with thrombocytosis associated with myeloid metaplasia. Various amounts of heparin were added to aliquots of the plasma. Time of macroscopic aggregation of the platelets and of clotting of the plasma was measured after the addition of thrombin (Table III). The highest concentration of thrombin employed clotted the plasma in < 10 seconds and flocculation of platelets could not be seen prior to coagulation. Addition of a smaller amount of thrombin (1.0 U) to the citrated plasma containing heparin (0.001 mg) produced striking macroscopically visible aggregation of the platelets in 32 to 35 seconds even though coagulation of the plasma *never* occurred. In the presence of less heparin (0.0005 mg) this amount of thrombin clotted the plasma in 10 seconds,

TABLE III

Time of macroscopically visible aggregation of platelets and of clotting in citrated platelet-rich plasma after addition of heparin and thrombin *

Heparin†	Thrombin	Aggreg. of platelets	Plasma clotting
mg	*U*	*sec*	*sec*
0.1	10	0	6
0.01	10	0	8
0.001	1	34	∞
0.0005	1	0	10

* Reaction mixture in silicone-coated tubes at 37° C consisted of 0.1 ml platelet-rich plasma (8,000,000 platelets per mm³), 0.1 ml heparin solution, and 0.1 ml thrombin solution. Amounts of heparin and thrombin in the reaction mixture are recorded.

† Heparin sodium in sodium chloride solution, Upjohn Co., Kalamazoo, Mich.

and platelet aggregation prior to coagulation was not observed.

3. Recovery of platelets in serum or unclotted plasma after addition of thrombin. Platelet-rich plasma was prepared from normal donors, and thrombin alone or a mixture of thrombin and calcium chloride was added. The mixtures were incubated at 37° C for 30 minutes. Unretracting clots were twirled onto wooden applicator sticks until the serum was expressed, and the clots were discarded. Retracted clots were removed without manipulation. Platelet counts were performed on unclotted plasma and on serum expressed from clots.

Results of studies in which thrombin alone was added to EDTA platelet-rich plasma are shown in Table IV. Firm clotting of plasma occurred with 2.5 U or more of thrombin per ml. No retraction occurred in any of the tubes because of the absence of free divalent cation. The number of morphologically intact platelets in serum or unclotted plasma was inversely related to the concentration of thrombin.

When thrombin and calcium chloride were added to EDTA platelet-rich plasma (Table IV), firm clotting occurred with 1 or more U of thrombin per ml, and clots retracted with the higher thrombin concentrations. Visible clotting did not occur with low concentrations of thrombin (< 0.1 U per ml). The number of platelets in serum or unclotted plasma was inversely related to the concentration of thrombin. On microscopic examination these platelets appeared similar to those in untreated EDTA platelet-rich plasma. The platelet yield was higher when EDTA platelet-rich plasma was clotted with thrombin alone than with comparable amounts of thrombin in the presence of calcium.

In a similar experiment thrombin alone was added to citrated platelet-rich plasma. The number of platelets in serum or unclotted plasma again was inversely related to the concentration of thrombin.

The number of platelets recovered after addition of thrombin was examined in relation to plasma concentration. EDTA platelet-free plasma was diluted with various proportions of buffered saline. Platelets were added to the diluted plasma to a final concentration of 150,000 per mm³, and coagulation was induced by 0.20 vol of a solution

TABLE IV

Recovery of platelets from EDTA platelet-rich plasma after addition of thrombin or of thrombin and calcium chloride *

Tests	Thrombin	Thrombin in $CaCl_2$ sol.	Tests in which clots retracted	Descrip. of clot	Platelets in serum or unclotted plasma	
					Mean	Range
no.	*U/ml*	*U/ml*	*no.*		*×1,000/mm³*	
2	50		0	Firm	<5	3.1–5.3
2	10		0	Firm	14	13–15
2	5		0	Firm	18	16–20
2	2.5		0	Firm	28	23–34
2	1		0	Soft	45	27–63
2	0.5		0	Friable	83	46–121
2	0.1			No clots	340	310–370
2	0.01			No clots	386	354–419
7	0			No clots	335	230–450
3		50	3	Firm	<1	<1–<1
5		10	4	Firm	<1	<1– 1
5		5	3	Firm	<5	<1–<5
5		1	1	Firm	30	10–65
4		0.5	1	Soft	57	30–110
3		0.25	0	Friable	145	100–190
5		0.1		No clots	254	85–365
4		0.01		No clots	272	215–325

* Reaction mixture in silicone-coated tubes at 37° C consisted of 1.6 ml EDTA platelet-rich plasma, 0.2 ml thrombin solution, and either 0.2 ml buffered saline or 0.2 ml calcium chloride solution (0.05 M). Final concentrations of thrombin are recorded. Volume of serum or unclotted plasma was 1.9 to 2.0 ml in each test.

of calcium chloride and thrombin (final concentration 100 U per ml). Within the range tested, the number of platelets recovered was not related to the concentration of plasma. Fewer than 10,000 platelets per mm^3 were recovered in serum after coagulation of plasma ranging in concentration from 4 to 80 per cent.

4. Release of platelets from clots by lysis. Recovery of morphologically intact platelets was measured after lysis of clots induced by streptokinase; 2.4-ml aliquots of EDTA platelet-rich plasma were clotted with either 0.6 ml of buffered saline containing 300 U of thrombin, or 0.3 ml of calcium chloride solution (0.05 M) and 0.3 ml of buffered saline containing 300 U of thrombin. The plasma clotted in < 60 seconds in all of the tubes, and these were incubated at 37° C for 30 minutes. After incubation, 1 ml of buffered saline containing 500 μg of a preparation containing streptokinase [7] was added to each clot and the tubes were inverted three times and again incubated at 37° C.

Clots formed after addition of thrombin to platelet-rich plasma did not retract since divalent cation was not available, but they did lyse completely within 3 hours after addition of streptokinase. Microscopic examination of the lysed material revealed many free platelets and a few small clumps. In each of three experiments, platelet counts of the lysed material were more than 70 per cent of the initial counts of the platelet-rich plasma.

Clots that formed after addition of thrombin and calcium chloride to platelet-rich plasma retracted but were incompletely lysed after incubation with streptokinase. Complete lysis was not achieved either when the concentration of the streptokinase preparation was increased to 2,000 μg per ml or when the period of incubation after addition of streptokinase was increased to 12 hours. Microscopic examination of the partially lysed material revealed many large aggregates of platelets, strands resembling fibrin, and occasional individual platelets. Platelet counts of this material were difficult and aggregates were counted as single platelets. In each of three experiments counts indicated that less than 15 per cent of the platelets had been recovered.

The experiments were repeated, with trypsin (in buffered saline containing EDTA) instead of streptokinase as the lytic agent. Salt-free trypsin in a final concentration of 4,000 μg per ml

[7] Streptokinase-Streptodornase (Varidase), Lederle Laboratories Div., American Cyanamid Co., New York, N. Y.

completely lysed within 5 minutes clots which had not retracted because of the absence of available calcium. Retracted clots were almost wholly lysed within 30 minutes. When trypsin was added 30 minutes after clots had formed, morphologically intact platelets were recovered in large numbers. More than 60 per cent of the platelets was obtained by lysis of unretracted clots and about half this number from completely retracted clots.

Platelets obtained by lysis of clots with trypsin were as effective as fresh washed platelets in accelerating the coagulation time of recalcified plasma (Table V).

Platelets released by lysis of unretracted and retracted clots were washed once in buffered saline containing EDTA and resuspended in platelet-free plasma in a concentration of approximately 150,000 per mm^3. The plasma was then clotted by addition of 0.33 vol of a solution of calcium chloride and thrombin. In each of four experiments with trypsin, complete retraction occurred with platelets obtained by lysis of unretracted clots. In three of four experiments complete retraction was produced by platelets released by trypsin from retracted clots. In these experiments clots formed by the addition of thrombin alone did not retract since calcium was not available; there was no detectable lysis of these clots during a period of observation of 12 hours, indicating absence of residual tryptic activity. Platelets obtained by lysis of clots with streptokinase were similarly tested for their ability to produce clot retraction. Even though the platelets were washed three times before resuspension in platelet-free plasma, lysis of the subsequent clots occurred so that retraction could not be evaluated. Evidently streptokinase was adsorbed to the platelets and was not removed by washing.

TABLE V

Clotting time on recalcification of EDTA plasma containing platelets obtained by lysis of clots with trypsin *

Source of platelets	Clotting time
	min
Platelets obtained by lysis of nonretracting clots	6
Platelets obtained by lysis of retracted clots	5
Fresh untreated platelets, washed twice	6
No platelets added	15

* Clotting times were measured at 25° C in uncoated glass tubes which were tilted every 30 seconds. Mixtures contained 0.2 ml plasma and 0.2 ml calcium chloride solution (0.05 M). Platelets (approximately 150,000 per mm^3) were suspended in EDTA platelet-free plasma.

B. Studies of platelets after incubation with trypsin

1. Removal or inactivation of platelet fibrinogen. Supernatant fluid from nontrypsinized platelets that had been washed twice and disrupted by repeated freezing and thawing clotted upon addition of thrombin in each of four experiments. In contrast, the supernatant fluid from trypsinized platelets that had been similarly treated did not clot. A mixture of equal parts of supernatant fluids from trypsinized and nontrypsinized platelets was incubated for 60 minutes at 37° C. After incubation this mixture clotted upon addition of thrombin, indicating the absence of residual tryptic activity.

2. Morphology and aggregation of trypsinized platelets. Platelets in freshly prepared suspensions remained morphologically intact after incubation with trypsin. Trypsinized and nontrypsinized platelets were uniformly dispersed when incubated in normal adsorbed serum that contained no thrombic activity and no detectable prothrombin. Nontrypsinized platelets suspended in normal adsorbed serum were grossly aggregated by addition of thrombin in 11 of 12 experiments. Aggregation was not visible macroscopically with platelet concentrations of less than 200,000 per mm^3. Marked aggregation occurred with final concentrations of thrombin ranging between 0.5 and 200 U per ml. Nontrypsinized platelets suspended in buffered saline were clumped by addition of thrombin alone in 6 of 11 experiments. In the remaining 5, aggregation occurred on addition of thrombin and serum. In contrast, trypsinized platelets suspended in normal adsorbed serum or in buffered saline were not aggregated by thrombin in 11 of 13 experiments.

Highly purified bovine or human thrombin aggregated washed platelets suspended in serum or in calcium chloride solution (final concentration 0.005 to 0.015 M). Clumping of platelets was

TABLE VI

Time of macroscopically visible aggregation of trypsinized and nontrypsinized platelets after addition of thrombin or thrombin and calcium chloride *

Substrate	Additive	Aggreg. of platelets	Plasma clotting
		sec	*sec*
TP in EDTA platelet-free plasma	Thrombin, calcium	13	24
TP in EDTA platelet-free plasma	Thrombin	None seen	180
TP in serum	Thrombin	None seen	
TP in buffered saline	Thrombin, calcium	None seen	
TP in buffered saline	Thrombin	None seen	
NTP in EDTA platelet-free plasma	Thrombin, calcium	12	27
NTP in EDTA platelet-free plasma	Thrombin	None seen	180
NTP in serum	Thrombin	14	
NTP in buffered saline	Thrombin, calcium	12	
NTP in buffered saline	Thrombin	None seen	

* TP = trypsinized platelets; NTP = nontrypsinized platelets. Substrate consisted of 0.4 ml platelet suspension (715,000 platelets per mm^3) to which was added a 0.1 ml solution of thrombin in buffered saline or in calcium chloride (0.025 M). Final concentration of thrombin was 0.5 U per ml.

produced by human thrombin in concentrations ranging between 1.0 and 100 U per ml. These preparations of thrombin did not aggregate washed platelets suspended in buffered saline, nor was aggregation produced by calcium chloride solution alone. Trypsinized platelets remained homogeneously dispersed in serum containing purified bovine or human thrombin.

In the experiments described above, platelets had been incubated with 1,000 μg of trypsin with magnesium sulfate, or 50 μg of salt-free trypsin per ml of platelet suspension. Platelets pretreated with lesser amounts of salt-free trypsin (0.1 to 1.0 μg per ml of platelet suspension), washed twice, and suspended in normal serum were aggregated by addition of thrombin.

Serum was prepared from a patient with classic hemophilia (antihemophilic factor or factor VIII deficiency) by incubating 5.0 ml of the patient's blood in uncoated glass tubes at 37° C for 2 hours. The serum contained > 50 per cent residual prothrombin. Trypsinized platelets were not aggregated on incubation in this serum, whereas nontrypsinized platelets were markedly clumped. Similar results were obtained when trypsinized and nontrypsinized platelets were incubated with normal serum that contained > 50 per cent residual prothrombin (prepared by incubating 2.0 ml native platelet-free plasma in uncoated glass tubes at 37° for 2 hours).

Aggregation of trypsinized and nontrypsinized platelets that were resuspended in EDTA platelet-free plasma was studied in four experiments. Results were similar, and data of one experiment are shown in Table VI. Macroscopically visible clumping of trypsinized and nontrypsinized platelets preceded clotting of the plasma after addition of thrombin and calcium chloride. Platelet aggregation was not observed after the addition of thrombin alone.

3. Retraction of clots by trypsinized platelets. Trypsinized or nontrypsinized platelets were suspended in EDTA platelet-free plasma. Clot retraction occurred optimally with each after addition of calcium chloride and thrombin. In four experiments trypsinized and nontrypsinized platelets were each incubated with serum and thrombin. Aggregation of nontrypsinized platelets was again noted. The platelets were washed once and

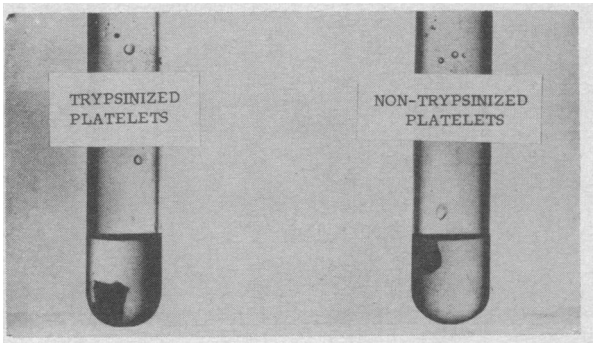

FIG. 1. RETRACTION OF CLOTS FORMED FROM PURIFIED FIBRINOGEN IN THE PRESENCE OF TRYPSINIZED AND NONTRYPSINIZED PLATELETS. Each tube contained 0.1 ml purified bovine fibrinogen (2 g per 100 ml), 0.4 ml glucose (125 mg per 100 ml), 0.5 ml buffer (pH 7.5), and 0.1 ml thrombin (50 U per ml).

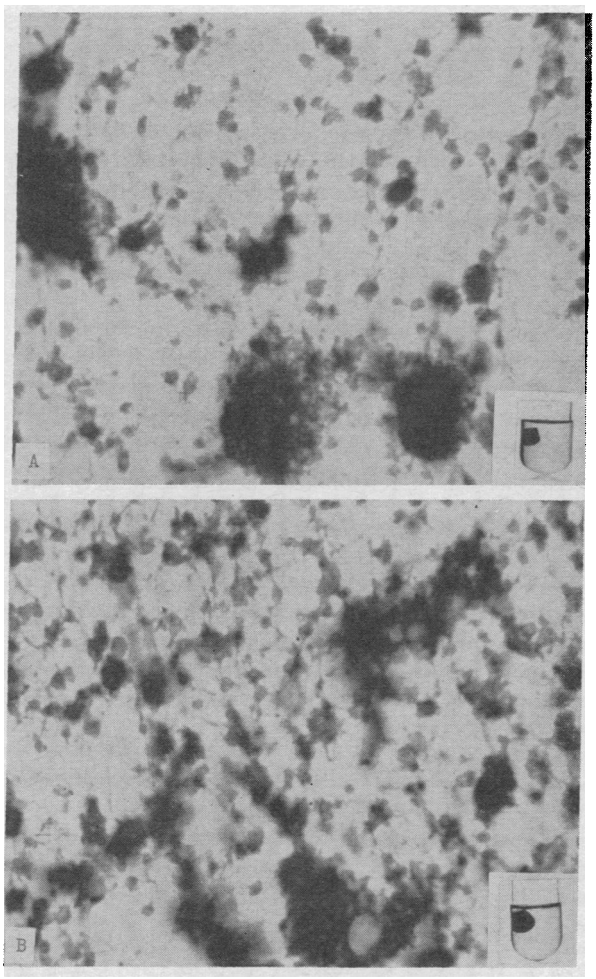

FIG. 2. SECTIONS OF CLOTS FORMED FROM PURIFIED FIBRINOGEN IN THE PRESENCE OF TRYPSINIZED PLATELETS (A) AND NONTRYPSINIZED PLATELETS (B) ×1500. Clots were fixed in formalin 1 hour after coagulation. Degree of retraction at the time of fixation is shown in the inserts. Viscous metamorphosis and aggregation of platelets are similar in the two preparations.

suspended in EDTA platelet-free plasma which was then clotted with thrombin and calcium. Retraction occurred with both trypsinized and nontrypsinized platelets that had been preincubated with serum and thrombin.

Trypsinized or nontrypsinized platelets were suspended in a buffered solution of purified bovine fibrinogen solution that contained glucose and calcium chloride. Coagulation was obtained by addition of thrombin. Retraction of clots was as rapid and complete with trypsinized platelets as with untreated platelets (Figure 1).

In the experiments described above, platelets had been incubated with 50 μg of salt-free trypsin per ml of platelet suspension. The ability of platelets to induce retraction was abolished by pretreatment with high concentrations of trypsin (1,000 μg of salt-free trypsin per ml of platelet suspension).

4. Morphologic appearance of platelets in sections of clots. Changes that occurred in association with clotting and retraction were studied by microscopic examination of fixed and sectioned clots. Photomicrographs (Figure 2) are of sections of clots formed after addition of thrombin to trypsinized or nontrypsinized platelets suspended in a buffered solution of fibrinogen, calcium chloride, and glucose. Platelets showed swelling, irregularity, and some aggregation in clots fixed 1 minute after addition of thrombin and before the onset of visible retraction. In clots fixed 1 hour after addition of thrombin and after maximal retraction had occurred, platelets showed marked swelling and aggregation. Similar observations were made on clots formed by addition of thrombin and calcium chloride solution to EDTA plasma. The morphologic changes observed in clots containing trypsinized platelets were indistinguishable from those in clots containing nontrypsinized platelets.

5. Reactions of platelets with heterologous antiserum. Platelets suspended in buffered saline were mixed with various dilutions of a potent rabbit antihuman platelet serum. The titer of serum that caused agglutination of platelets was the same with trypsinized as with nontrypsinized platelets (Table VII).

TABLE VII

Agglutination of platelets by rabbit antihuman platelet serum *

Rabbit antihuman platelet serum in buffered saline	Tryp. platelets	Nontryp. platelets
Reciprocal of dilution		
128	+++	++++
256	++++	++++
512	++++	++++
1,024	++++	++++
2,048	++++	++++
4,096	++	++
8,192	±	0
Buffer	±	0

* Three drops of diluted serum were mixed with one drop of platelet suspension and incubated at 25° C for 30 minutes. Degree of agglutination was evaluated microscopically.

DISCUSSION

During the clotting of normal blood, aggregation of platelets and formation of fibrin appear to occur at the same time. It has been shown, however, that under certain conditions platelet aggregation actually precedes fibrin formation (5, 15–17). The present experiments demonstrate that macroscopically visible aggregation of platelets preceded fibrin formation when the process of coagulation was slowed by addition of small amounts of thrombin to citrated platelet-rich plasma or by addition of calcium and small amounts of thrombin to EDTA platelet-rich plasma. It was possible to produce gross aggregation of platelets by thrombin in the absence of detectable clotting with the use of appropriate concentrations of heparin in citrated platelet-rich plasma. This observation suggests that lower concentrations of thrombin are effective in producing platelet aggregation than are required for the clotting of fibrinogen in plasma. Results obtained with EDTA platelet-rich plasma indicate that free divalent cations are necessary for the production of platelet aggregation by thrombin.

When coagulation occurs, platelets undergo a series of morphologic changes known as "viscous metamorphosis" (18, 19), a phenomenon which is associated with aggregation and apparent fusion of the platelets and with retraction of the clot (11, 20). These changes do not occur in the absence of available calcium or magnesium even though the platelets are embedded in fibrin clots formed by thrombin (11, 21). In a study of fixed and sectioned plasma clots, platelets remained discrete and unaggregated when free divalent cation was not available (11). The addition of calcium to such clots 2 hours after coagulation caused retraction of the clots (22, 23). These morphologic observations are in accord with the data of other investigators (20, 24), and with the results of the current studies, indicating that platelet aggregation, viscous metamorphosis, and clot retraction are closely related phenomena, initiated by the action of thrombin on platelets and requiring the presence of divalent cation.

Clot retraction is produced only by intact, biologically active platelets (25). Large numbers of morphologically normal and functionally active platelets were recovered by lysis of unretracted clots which had been formed in the absence of free divalent cation. Clot formation per se clearly did not destroy or obviously damage platelets. More surprising was the recovery of a smaller proportion of intact platelets by lysis of completely retracted plasma clots which had been formed by thrombin in the presence of calcium. Obviously these platelets did not undergo the progressive and irreversible changes usually associated with coagulation and retraction. That they were capable of doing so was demonstrated by their ability to produce retraction of a clot subsequently formed. The number of platelets recovered from clots varied inversely with the concentration of thrombin employed to induce coagulation. Czernobilsky and Alexander (26) have previously observed that serum expressed from clots formed by recalcification may contain platelets capable of producing clot retraction.

Platelets have been shown to contain a clottable protein similar to or identical with fibrinogen (9, 27–29). Previous investigators have debated whether platelet fibrinogen was present in the interior (30) or on the surface (28) of platelets. In the present experiments fibrinogen was not removed by repeated washing of platelets but was no longer detectable after platelets had been exposed to trypsin. Our observations favor the view that platelet fibrinogen is on the surface of platelets, since it is unlikely that trypsin could have removed or altered fibrinogen within the platelets without disrupting them. Hjort, Rapaport and Owren (31) have shown that the factor V activity of platelets can similarly be removed by trypsin.

The present experiments indicate that at least some of the effects of thrombin on platelets are mediated by fibrinogen. Washed platelets suspended in fibrinogen-free serum were grossly aggregated by thrombin, whereas platelets previously exposed to trypsin remained freely dispersed under the same conditions. Presumably the fibrinogen on the surface of the platelets was the substrate of thrombin in the production of platelet aggregation. Trypsinized platelets resuspended in a solution containing fibrinogen regained their ability to be aggregated by thrombin.

When potent preparations of thrombin were mixed with a suspension of washed platelets in serum there was pronounced clumping of the platelets, but the platelets were not destroyed.

The platelets in these clumps, when resuspended in a medium containing fibrinogen, did produce clot retraction after clotting was induced by thrombin. Thus the degree of reaction of platelets to thrombin was apparently related to the amount of fibrinogen available. Since it was necessary to add only purified fibrinogen to achieve this effect, the result must be attributable to fibrinogen alone or, conceivably, to a contaminant of the fibrinogen preparation. The latter seems unlikely, since aggregation of trypsinized platelets did not occur in whole fresh serum.

Platelets not only remained intact morphologically after incubation with trypsin but also retained their ability to support retraction of clots formed from a buffered solution of purified fibrinogen containing calcium and glucose. Sections of these clots showed that the changes of trypsinized platelets occurring during clotting and retraction were similar to those of nontrypsinized platelets. Although thrombin was apparently essential to initiate the alterations leading to viscous metamorphosis and clot retraction, the continued presence of thrombin was apparently not required for these reactions. Nonretracting clots formed by addition of thrombin to prothrombin-free EDTA plasma proceeded to retract when calcium was added more than 4 hours after clot formation, at a time when thrombin presumably had been inactivated (23). In this experiment fibrinogen solution was added at the same time as the calcium and did not clot, indicating the absence of detectable thrombic activity. These observations suggest that divalent cation, essential for clot retraction, exerts its effect at a stage later than the action of thrombin.

We conclude that fibrinogen on the surface of platelets is the substrate of thrombin in the production of platelet aggregation, viscous metamorphosis, and clot retraction. Additional experiments are in progress to determine whether fibrinogen is involved in the release of serotonin from platelets by thrombin. A relationship between fibrinogen and aggregation of platelets has been postulated by others. Lenggenhager (32) suggested that platelets might adsorb freshly formed fibrin and thus become viscous and aggregate, and Apitz (33) proposed that profibrin, an intermediate product of the conversion of fibrinogen to fibrin, might be adsorbed by platelets and produce platelet aggregation. In recent years, however, the concept of an interaction of thrombin and fibrinogen as a cause of viscous metamorphosis has been in disfavor mainly as a result of reports that viscous metamorphosis occurs in platelets from patients with congenital fibrinogen deficiency. Pinniger and Prunty (34) and Alexander and co-workers (4) showed that the platelets from such patients did undergo viscous metamorphosis when blood was exposed to glass surfaces, even though clotting did not occur. Vandenbroucke, Verstraete and Verwilghen (35) reported "pouvoir d'agglutination" of the platelets from a patient with congenital afibrinogenemia, thought on subsequent studies to have no fibrinogen on the platelets (36). However, Alexander and colleagues (4) and Setna and Rosenthal (5) described small strands resembling fibrin associated with platelets undergoing viscous metamorphosis in the blood of patients with congenital hypofibrinogenemia.

Agents possibly related to coagulation factors but other than thrombin have reportedly caused aggregation of platelets. Some of these agents include serum (19), serum rich in prothrombin (7, 37), "factor R" of Hellem (38) released from red blood cells, "product I" of Bergsagel (39), a "thrombocyte agglutinating factor" of Brinkhous and co-workers (40), EDTA plasma to which divalent cation was added (41), and an "interaction of Hageman or tissue factor with PTA or plasma platelet factor" (17). Some of the agents mentioned have been shown to contain thrombin or to produce it during their interaction with platelets (7); and in the present studies, serum containing a high concentration of prothrombin produced aggregation of nontrypsinized but not of trypsinized platelets. If there are substances in normal serum other than thrombin that cause platelet aggregation, these substances apparently do not react with trypsinized platelets. Certain chemical and other foreign agents seemingly unrelated to coagulation also can produce platelet aggregation (42, 43). In our experiments trypsinized platelets retained their ability to react with rabbit-antihuman platelet serum. Antiplatelet serum has been shown to contain several antibodies acting on platelets, including an antifibrinogen antibody (44). Our results suggest that the antifibrinogen effect of antiplatelet

serum is less than the antiplatelet effect, since removal of platelet fibrinogen by trypsin did not affect the titer. Miescher and Gorstein (45), using different techniques, have come to similar conclusions.

SUMMARY

Thrombin added to platelet-rich plasma produced platelet aggregation prior to clotting of the plasma provided free divalent cation was present. Addition of appropriate amounts of thrombin to citrated platelet-rich plasma containing small amounts of heparin produced platelet aggregation even though coagulation of the plasma never occurred.

The number of morphologically intact platelets recovered after addition of thrombin to platelet-rich plasma was inversely related to the concentration of thrombin. A large number (> 70 per cent) of morphologically intact and functionally active platelets was recovered after lysis of non-retracted clots formed in the absence of free divalent cation. A smaller number of intact platelets was recovered after lysis of retracted clots formed in the presence of free divalent cation.

Platelets incubated with trypsin remained morphologically intact. Nontrypsinized platelets in normal serum were aggregated by thrombin. Trypsinized platelets in normal serum were not aggregated by thrombin. Trypsinized platelets supported retraction of clots formed from either plasma or purified fibrinogen. The histologic changes associated with clotting and retraction were similar with trypsinized and nontrypsinized platelets. Extracts of nontrypsinized platelets contained thrombin-clottable protein (platelet fibrinogen). Extracts of trypsinized platelets did not contain platelet fibrinogen. These results suggest that the substrate for the action of thrombin in the production of platelet aggregation and clot retraction is a protein clottable by thrombin similar to or identical with fibrinogen, and situated on the surface of the platelets.

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