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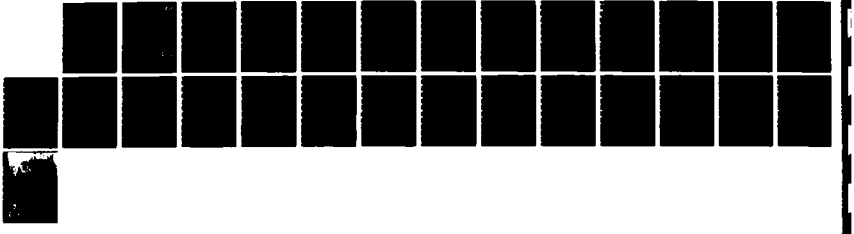

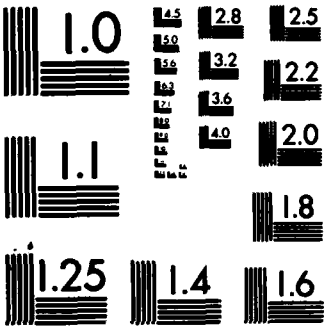

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PLATELET SIZE AS A DETERMINANT OF PLATELET FUNCTION

by

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The relationship between mean platelet volume (MPV) and platelet function was studied in human platelet subpopulations separated on the basis of size by counterflow centrifugation. The original platelet population and five size-dependent platelet fractions were suspended in buffer or autologous PPP at a platelet count of 2 X 10^8/ml. Collagen-(10 ug/ml) induced aggregation showed a significant negative correlation between MPB and onset of aggregation and positive correlations between the MPB and the rate and extent of aggregation. Thrombin stimulation (1 U/ml) demonstrated similar relationships between MPV

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and the rate and extent of aggregation. In contrast, ristocetin-induced platelet agglutination occurred at a similar rate and extent in all fractions. To quantitate further the differential response of the platelets we measured the content and release of ATP and B-thromboglobulin (B-TG). There was a significant correlation between MPV and both ATP and B-TG content and a progressive increase in the absolute release of ATP and B-TG associated with the increase in MPV after stimulation. However, the percent release of total ATP and B-TG was similar in all fractions. Our data indicate that the intrinsic function of platelets of different sizes is similar, but the absolute ability of platelets to affect their environment as measured by aggregation and total release of granular content is proportional to their size.

ABSTRACT

The relationship between mean platelet volume (MPV) and platelet function was studied in human platelet subpopulations separated on the basis of size by counterflow centrifugation. The original platelet population and five size-dependent platelet fractions were suspended in buffer or autologous PPP at a platelet count of 2×10^8/ml. Collagen-~~(10 µg/ml)~~ induced aggregation showed a significant negative correlation between MPV and onset of aggregation and positive correlations between the MPV and the rate and extent of aggregation. Thrombin stimulation ~~(1 U/ml)~~ demonstrated similar relationships between MPV and the rate and extent of aggregation. In contrast, ristocetin-induced platelet agglutination occurred at a similar rate and extent in all fractions. To quantitate further the differential response of the platelets we measured the content and release of ATP and β-thromboglobulin (β-TG). There was a significant correlation between MPV and both ATP and β-TG content and a progressive increase in the absolute release of ATP and β-TG associated with the increase in MPV after stimulation. However, the percent release of total ATP and β-TG was similar in all fractions. Our data indicate that the intrinsic function of platelets of different sizes is similar, but the absolute ability of platelets to affect their environment as measured by aggregation and total release of granular content is proportional to their size.

RUNNING TITLE: SIZE-DEPENDENT PLATELET FUNCTION

ABBREVIATIONS

MPV	Mean platelet volume
PPP	Platelet-poor plasma
ATP	Adenosine triphosphate
β-TG	β-Thromboglobulin
EGTA	Ethyleneglycol-bis-(β-aminoethyl ether) N,N'-tetraacetic acid
TCB	Tris-citrate-bicarbonate
BSA	Bovine serum albumin (fatty acid free)
% ΔT	Percent change in light transmission
EDTA	Ethylenediamine tetraacetate acid disodium salt
DMSO	Dimethyl sulfoxide
ADP	Adenosine diphosphate

INTRODUCTION

Platelets are known to be heterogeneous with respect to size, density, function, metabolism and age (1-14). However, the causes and significance of this heterogeneity remain controversial. Recent advances in cell sizing have made one index of platelet heterogeneity, the platelet volume distribution, routinely available in most clinical laboratories (15-17). Despite the increasing availability of MPV measurements the relevance of platelet volume to platelet physiology and pathology remains to be established. A number of authors have reported an apparent correlation between the MPV and platelet aggregation (3,5,13,18-21), and several theories have been proposed to explain the positive correlation. Karpatkin (5) has suggested that different sized platelets are qualitatively different because they are of different ages. Penington and co-workers (9,10) suggest that platelet size variation arises from production in the bone marrow of platelets with different functional capacities. Alternatively, the intrinsic function of platelets of varying size may be similar and the differences in function may be quantitative ones, directly predictable from differences in size. These quantitative differences may be based on the physical properties of different sized particles as proposed recently by Holme and Murphy (22) or they may be based on metabolic capacities which correlate with differences in size. Another possibility is that the observed relationship between the MPV and in vitro tests of platelet function might reflect an artifact based on the effects of differing platelet size on the turbidometric measurement of platelet aggregation.

In this study we have examined the relationship between MPV and in vitro platelet aggregation and the release of dense body and α-granule contents.

Using human platelet subpopulations separated on the basis of size by counterflow centrifugation, we have confirmed the correlation between platelet volume and in vitro activity and attempted to define the nature of the relationship.

MATERIALS AND METHODS

Blood collection and platelet isolation

Blood (42.5 ml) was obtained from healthy male laboratory personnel who had not taken any medication known to affect platelet function nor had donated blood within the previous 10 days. The method for isolation of platelets from whole blood has been described in detail (13) and was used without modification.

Platelet subpopulations

The method for isolation of size-dependent platelet subpopulations by counterflow centrifugation has been described in detail previously (13). In brief, counterflow centrifugation opposes an outwardly-directed centrifugal force with an inwardly directed force generated by the flow of fluid through the centrifuge separation chamber. Cells of different size equilibrate at different positions in the chamber and can be removed from the centrifuge sequentially by increasing the rate of flow through the chamber. Separation is achieved on the basis of cell size and the effect of differing cell density is minimal (13).

In these experiments 4-7 x 10^9 platelets were loaded into the chamber of the counterflow centrifuge and separated into 7 sequential fractions (13). The 7 fractions and a sample of the original unfractionated platelet suspension

were placed on ice for 10 min and EGTA (Sigma Chemical Co., St. Louis, MO) was added to a final concentration of 1 mM. The platelets were then sedimented by centrifugation at 2000 x g for 10 min. The supernatant buffer was aspirated and the platelets resuspended in either Tris-citrate-bicarbonate (TCB) buffer (23) or autologous citrated PPP. In order to obtain comparable quantities of platelets in all fractions for subsequent functional studies, the platelets in fractions 1 and 2 and fractions 6 and 7 were pooled. The platelet count in all of the final suspensions was adjusted to approximately 2×10^8/ml. BSA and $CaCl_2$ were added to a final concentration of 0.05% and 3 mM respectively.

Platelet counting and sizing

Platelet counts were performed visually by phase microscopy and electronically using a Coulter ZBI Counter (Coulter Electronics, Hialeah, FL). Platelets were sized using the linear scale on a Coulter ZB Counter with an H4 channelyzer attachment (Coulter Electronics) and a 50/60 aperture electrode. Details of these procedures and calibration routines have been presented previously (13).

Platelet aggregation and ATP release

Platelet aggregation and simultaneous ATP release were measured in a Lumi-Aggregometer (Chronolog Corp., Havertown, PA). Four hundred µl of the platelet suspension to which 50 µl of luciferin/luciferase reagent was added, were stirred at 37°C in a siliconized glass cuvette for 2 min prior to stimulation. Lyophilized luciferin/luciferase reagent (Dupont Chemical Co., Wilmington, DE) was reconstituted to 50 mg/ml in 100 mM tris-HCl, pH 7.4

containing 54 mM $MgSO_4$. To control for differences in the optical density of the platelet subpopulations, each sample was adjusted to a baseline that set the optical density of the platelets in suspension at 10% light transmission. A blank of TCB buffer or PPP was set at 90% light transmission. Platelets were then stimulated with 50 µl of either human α-thrombin (Dr. John Fenton, New York State Department of Health), collagen (Hormon-Chemie, Munich, West Germany), or ristocetin A (Aggrecetin, Bio/Data Corp., Horsham, PA). Aggregation was measured as the percent change in light transmission (% ΔT) over time. ATP release was measured as maximum generation of luminescence (24).

Validation of turbidometric measurements of aggregation

Platelet aggregates were fixed with 50 ul of 0.1 M EDTA in 2.5% formalin (25) at 20% ΔT or after maximum % ΔT (typically 5 min). Aggregometer readings confirmed that this addition arrested aggregation and deaggregation almost instantaneously leaving the light transmission of the sample stable for at least 10 min. Aggregates were then removed by a modification of the method of Haver and Gear (21) by centrifuging the sample at 500 x g for 10 sec on a Sorvall GLC-2 centrifuge. A control platelet suspension to which 50 µl of buffer was added instead of aggregating agent, was centrifuged simultaneously. After centrifugation, 400 µl of supernate from both the aggregated sample and the control were removed and counted for the number of residual single platelet . The percent of platelets remaining unaggregated was determined by dividing the platelet count in the aggregated sample by the count in the control. Each sample was processed in duplicate and the mean used for data analysis.

Quantitation of ATP content and release

ATP content was measured by solubilization of a stirred mixture of 400 µl of platelet suspension and 50 µl of luciferin/luciferase reagent with 50 µl of 10% Triton X-100 (Sigma) in the Lumi-Aggregometer. The luminescence generated was measured and ATP concentration calculated from a standard curve obtained by adding known amounts of an ATP standard to an analogous cell-free system. The maximum release of ATP from platelets during platelet aggregation was measured as above in the Lumi-Aggregometer and converted to concentrations by use of the standard curve. In control experiments the presence of Triton X-100 was shown to have no significant effect on luminescence generated by the addition of ATP.

β-Thromboglobulin content and release

Samples prepared for ATP release as described above were removed from the Lumi-Aggregometer after 5 min and transferred to an Eppendorf microcentrifuge tube containing 50 µl of ice-cold 10% DMSO in 200 mM EDTA (26). The sample was immediately centrifuged at 12,000 x g for 4 min in an Eppendorf Model 5412 Microcentrifuge. Four hundred µl of the resulting supernate were removed, stored at -20°C and subsequently assayed for β-thromboglobulin by radioimmunoassay using a commercially available kit (Amersham Corp., Arlington Heights, IL). Total β-TG content was measured in tritonized samples, prepared as described above for total ATP content. Prior to assay the samples were diluted 100-fold with TCB buffer and the β-TG standards were reconstituted in TCB buffer. In preliminary experiments it was determined that the levels of luciferin/luciferase reagent, DMSO/EDTA, and Triton X-100 present had no effect on the standard curve. All radioimmunoassays were performed in duplicate and results calculated from the mean.

Statistics

Statistical evaluation of data was performed by linear regression analysis (27).

RESULTS

The characteristics of the size-dependent platelet subpopulations used in the functional studies are given in Table 1. Increasing fraction number corresponds closely to increasing fraction MPV and total recovery through the counterflow centrifuge was 91.8 ± 5.6% (mean ± SD, n = 10). Each of the 5 subpopulations contained between 16.8 ± 4.3% and 23.4 ± 1.2% of the recovered platelets. The ATP and β-TG content per 10^8 platelets from each subpopulation show a close correlation ($p < 0.001$, $p < 0.01$ respectively) with the MPV of the platelets (Table 1). No significant differences were seen in the platelet counts after resuspension in TCB buffer prior to aggregation studies. During all procedures the original unfractionated platelet population was also studied. The values for the unfractionated platelets were generally in the mid-range of the fractions' value.

Fig. 1 shows typical aggregation responses of the platelet subpopulations and original unfractionated platelets to stimulation with collagen (10 μg/ml) and thrombin (1 U/ml). These concentrations best illustrate the differential aggregation between fractions. However, the large platelets would routinely respond to smaller stimuli (0.25 U/ml thrombin, 2.5 μg/ml collagen) but the response of smaller platelets was undetectable (results not shown). Aggregation was quantitated by measurement of lag time (time from addition of aggregating agent to onset of aggregation), initial rate of aggregation (slope of the initial % ΔT after onset of aggregation), and maximal aggregation (maximum % ΔT after 5 min). The results of these measurements for

collagen-induced aggregation at final concentrations of 10 and 50 µg/ml are shown in Fig. 2a. For both concentrations there is a significant inverse correlation between the lag time and the MPV of the fraction ($p < 0.01$), while both the initial rate and the extent of aggregation showed significant positive correlations ($p < 0.05$, $p < 0.01$ respectively). Fig. 2b shows equivalent data for three different thrombin concentrations (0.25, 0.5, and 1.0 U/ml). As with collagen, for all three thrombin concentrations, there is a significant positive correlation between both rate ($p < 0.001$) and extent ($p < 0.05$) of aggregation and the MPV of the platelet subpopulations. The lag time for thrombin-induced aggregation was much shorter than for collagen-induced aggregation and in contrast to collagen, while there was a decrease in lag time with increasing thrombin dose, no significant differences in lag time were noted between the fractions at any given dose. The patterns observed in Fig. 2 were seen consistently in each individual experiment. Values for unfractionated platelets and their representative standard deviations are shown on the side of each figure.

To correlate the results of collagen- and thrombin-induced aggregation as measured by the aggregometer with effects on single platelets, aggregation was arrested at two different points using EDTA/formalin (Table 2). In the first experiments, aggregation was arrested when each of the platelet fractions exhibited 20% ΔT and the number of single platelets remaining was determined. At the same extent of aggregation, as measured by the aggregometer, approximately the same number of platelets were left unaggregated in each fraction ($p > 0.1$). After 5 min collagen-induced aggregation was complete (Fig. 1), at this time the number of single platelets remaining unaggregated becomes progressively less with increasing fraction size ($p < 0.05$). Both platelet counting

and aggregometry reveal the same trend of progressively greater aggregation with increasing fraction MPV. Similar data to that reported for collagen-induced aggregation were obtained with 1 U/ml thrombin (data not shown).

To test the possibility that the observed differences in aggregation reflected physical differences in size, ristocetin-induced agglutination of each of the subpopulations was measured. A dose response curve of the unfractionated platelets was performed and a dose in the mid-range of the agglutination response was chosen for study. Lack of ATP release verified that we were observing only agglutination and not the combined effects of agglutination and secondary platelet aggregation. In a series of three experiments, no difference in the rate or extent of aggregation was seen between the unfractionated platelets and any of the subpopulations. The results of a representative experiment are shown in Fig. 3. To ensure that the concentration of von Willebrand factor was not limiting, the experiments were performed on subpopulations resuspended in autologous PPP. Collagen-induced aggregation of the same plasma resuspended platelets showed the same patterns of response as those in Fig. 1.

ATP release was measured during collagen- and thrombin-induced aggregation (Fig. 4). For each dose of collagen and thrombin there was positive correlation ($p < 0.01$) between the MPV of the fraction and the absolute quantity of ATP released. However, the larger platelets had a greater initial ATP content (Fig. 1). To correct for this the data have been presented in the lower graphs as the percent of total ATP content released. When the data were expressed in this way no significant difference was seen in the ability of the platelets to release ATP in response to a given dose of either collagen or thrombin ($p > 0.1$).

The release of β-TG was also measured after thrombin- (1 U/ml) or collagen- (10 μg/ml) induced aggregation (Fig. 5). There was a positive correlation ($p < 0.05$) between the MPV and the absolute quantity of β-TG released. When release was expressed as a percent of the original β-TG content, the percent release from all the fractions was similar ($p > 0.5$).

DISCUSSION

Our results demonstrate a significant correlation between platelet volume and in vitro tests of platelet function. For both collagen- and thrombin-induced platelet aggregation increasing rate and extent of response were noted with increasing MPV of the fractions and the validity of these observations was confirmed by serial platelet counts. Holme and Murphy (22) have recently suggested that large platelets might aggregate more quickly than small ones merely because they collide with each other more frequently. However, in the present study agglutination of size-dependent platelet subpopulations by ristocetin showed no differences in the rate or extent of response between the fractions. Since in the absence of platelet activation (indicated by the absence of the release reaction during ristocetin-induced agglutination), no difference in the response patterns of the platelet subpopulations was observed, differences in aggregation patterns cannot simply reflect physical differences in size of the platelet subpopulations. Thus the differential response of the platelets represents differences in the platelets' functional ability to respond to the different aggregating agents.

One mechanism by which platelets promote aggregation is through the release reaction. By releasing aggregatory agents such as ADP and thromboxane

A_2 platelets can catalyze their own reactions since concentrations of different aggregating agents appear to be additive in their effects on platelet aggregation (28). To study the relationship between the MPV and the release reaction, ATP, a dense body constituent, and β-TG, an α-granule constituent, were measured during collagen- and thrombin-induced platelet aggregation. Large platelets contained proportionately larger amounts of ATP and β-TG than smaller ones. The differential aggregation response of the subpopulations could reflect different thresholds for the induction of the release reaction which is a qualitative difference. However, the percent release of both ATP and β-TG was the same in all fractions when tested over a range of thrombin and collagen doses. Alternatively, the larger platelets could exhibit an enhanced aggregation response by virtue of releasing greater absolute quantities of aggregatory substances, a qualitative difference. This study demonstrated an increased absolute release of both ATP and β-TG proportional to the increase in platelet size.

Platelet arachidonic acid metabolites are also important modulators of platelet activity and their role in the differential response of size-dependent platelet subpopulations is currently under investigation.

Our data indicate that the intrinsic function of size-dependent platelet subpopulations is similar but the absolute ability of platelets to affect each other and their environment as measured by aggregation and the release reaction respectively is proportional to their size.

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TABLE 1

Characteristics of human size-dependent platelet subpopulations

FRACTION #	MPV (u^3, n=10)	% OF RECOVERED PLATELETS (n=10)	β-TG (ug/10^8 plt., n=6)	ATP (nmoles/10^8 plt., n=7)
1/2	4.34 ± 0.39	19.5 ± 5.6	3.05 ± 0.91	1.54 ± 0.50
3	5.34 ± 0.46	19.4 ± 2.7	4.58 ± 0.77	1.89 ± 0.61
4	6.32 ± 0.50	23.4 ± 1.2	5.90 ± 1.14	2.41 ± 0.70
5	7.24 ± 0.57	20.9 ± 3.5	7.99 ± 1.73	3.11 ± 1.09
6/7	7.89 ± 0.59	16.8 ± 4.3	10.99 ± 2.07	3.79 ± 1.05
Unfractionated	6.49 ± 0.54	----	6.43 ± 1.59	2.74 ± 0.87

Results expressed as mean ± SD

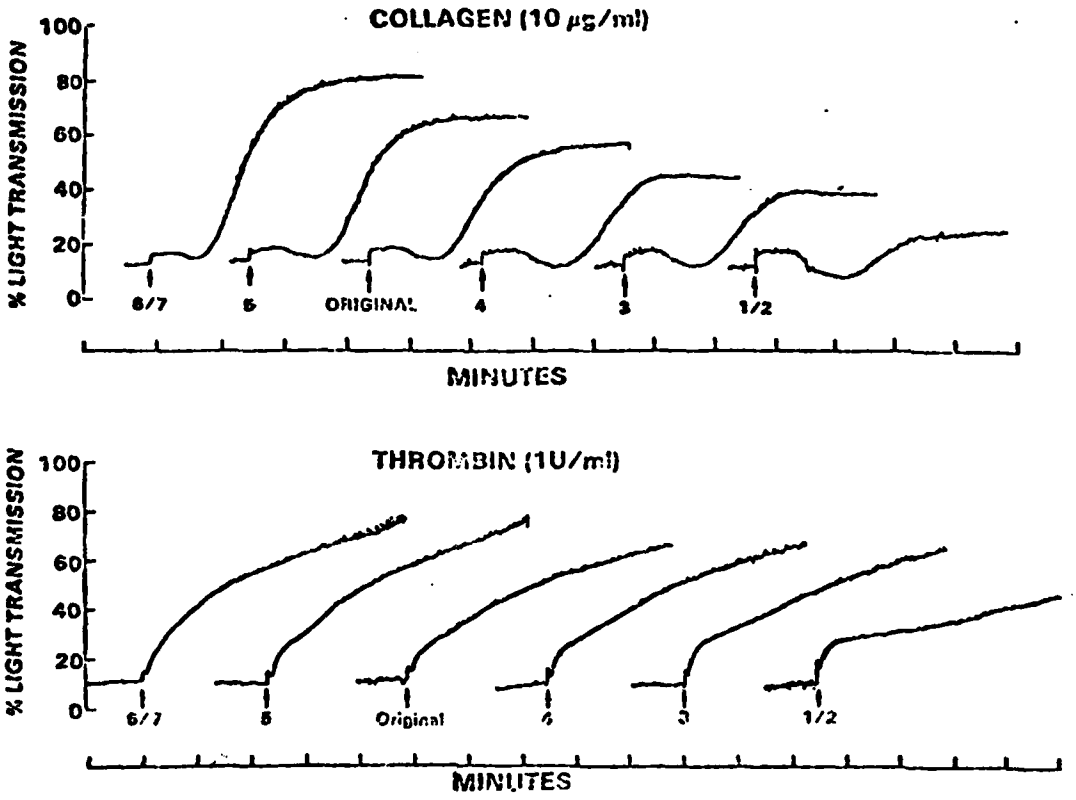
TABLE 2

Percent of single platelets remaining after partial and complete aggregation

Fraction #	Aggregation inhibited at:	
	20% ΔT	5 min
1/2	59.2 ± 6.7	23.5 ± 5.4
3	59.9 ± 5.4	14.0 ± 5.0
4	57.0 ± 8.6	12.3 ± 4.3
5	57.4 ± 5.8	10.9 ± 6.3
6/7	52.3 ± 3.6	10.4 ± 5.3
Unfractionated	62.0 ± 9.7	12.8 ± 3.0

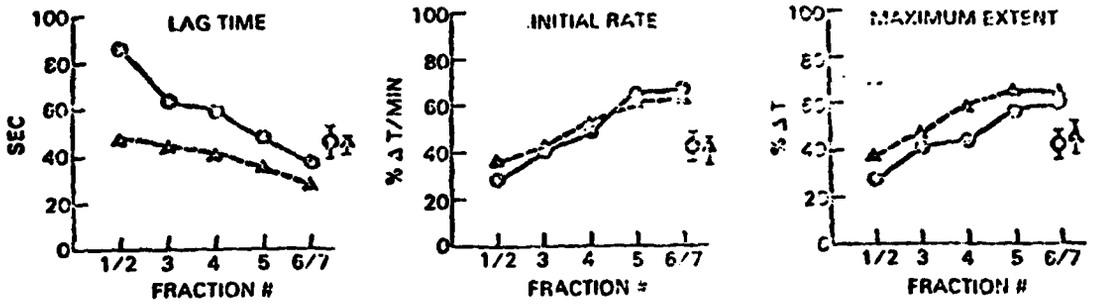
Aggregation was induced by collagen (10 μg/ml) and aggregates fixed by the addition of formalin-EDTA after 20% change in light transmission or after complete aggregation at 5 min (mean ± SD, n = 3).

FIGURE 1



Collagen- and thrombin-induced aggregation of size-dependent platelet subpopulations and original unfractionated platelets. The platelet count in each fraction was 2×10^8/ml and each figure is representative of at least four experiments.

FIGURE 2a

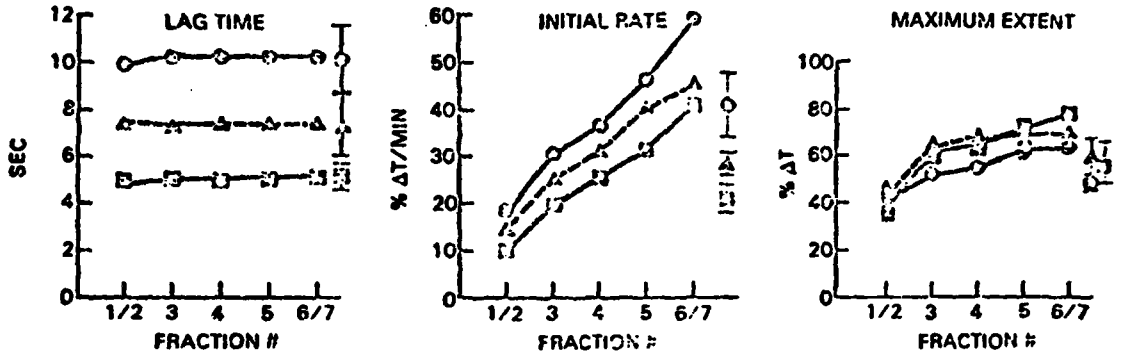


Collagen-induced aggregation of size-dependent platelet subpopulations.

—○—10 ug/ml collagen (mean, n = 4). ○ Unfractionated platelets (mean ± SD).

—△—50 ug/ml collagen (mean, n = 4). △ Unfractionated platelets (mean ± SD).

FIGURE 2b



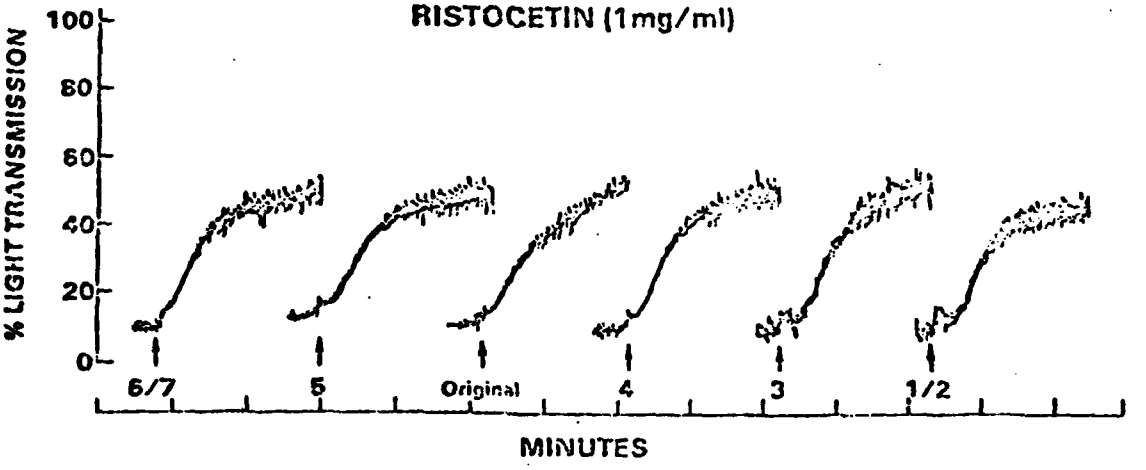
Thrombin-induced aggregation of size-dependent platelet subpopulations.

—○—0.25 U/ml thrombin (mean, n = 3). ⊖ Unfractionated platelets (mean ± SD).

—△—0.5 U/ml thrombin (mean, n = 4). ⋀ Unfractionated platelets (mean ± SD).

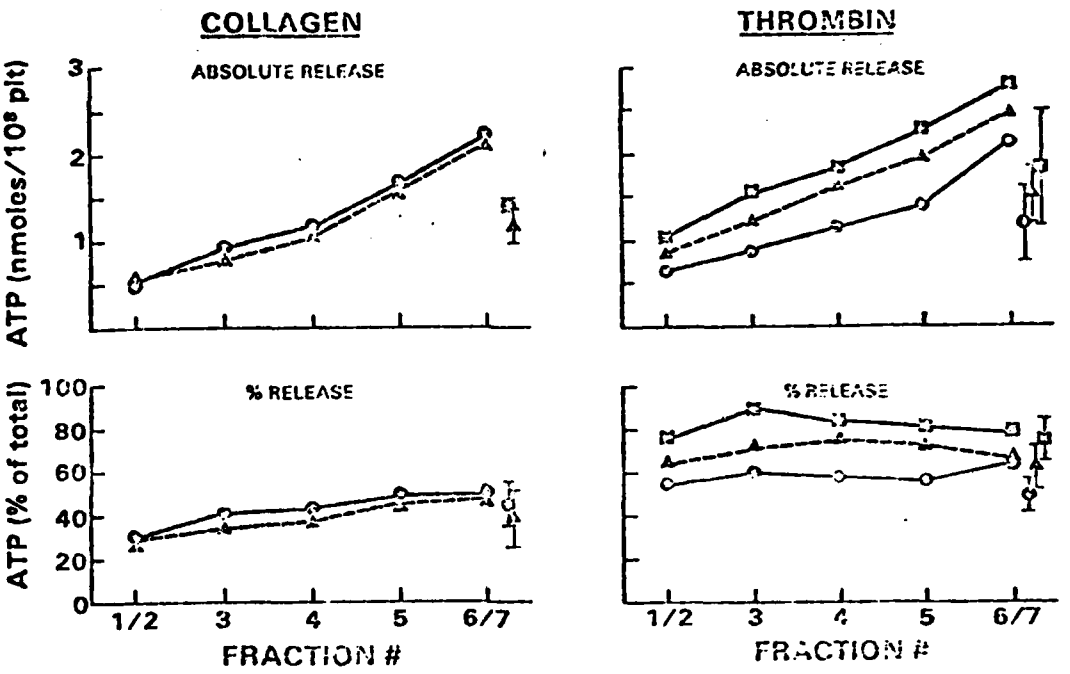
—□—1.0 U/ml thrombin (mean, n = 5). ⊟ Unfractionated platelets (mean ± SD).

FIGURE 3



Ristocetin-induced agglutination of size-dependent platelet subpopulations and original unfractionated platelets. The platelet count in each fraction was 2×10^8/ml and the figure is representative of three experiments.

FIGURE 4



Collagen-induced ATP release.

—○—10 ug/ml (mean, n = 4).

--△--50 ug/ml (mean, n = 5).

| Unfractionated platelets

(mean ± SD)

Thrombin-induced ATP release.

—○—0.25 U/ml (mean, n = 3)

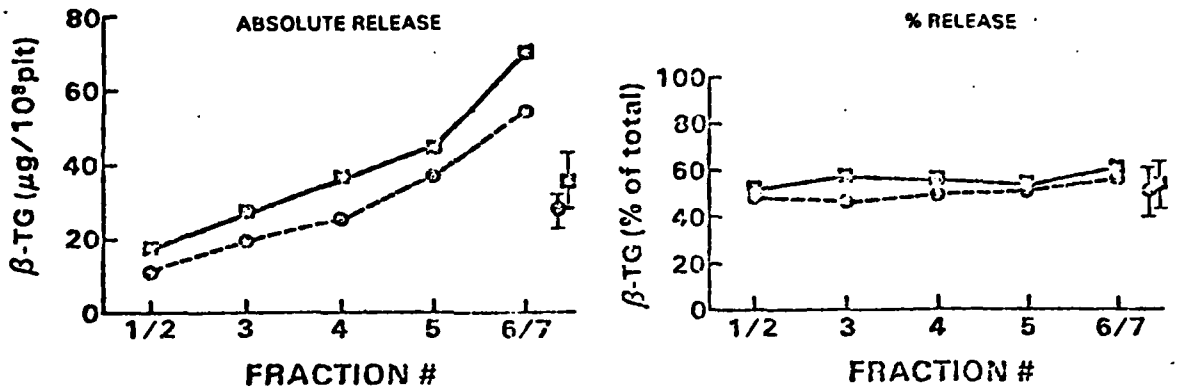
--△--0.5 U/ml (mean, n = 4).

—□—1.0 U/ml (mean, n = 5)

| Unfractionated platelets

(mean ± SD).

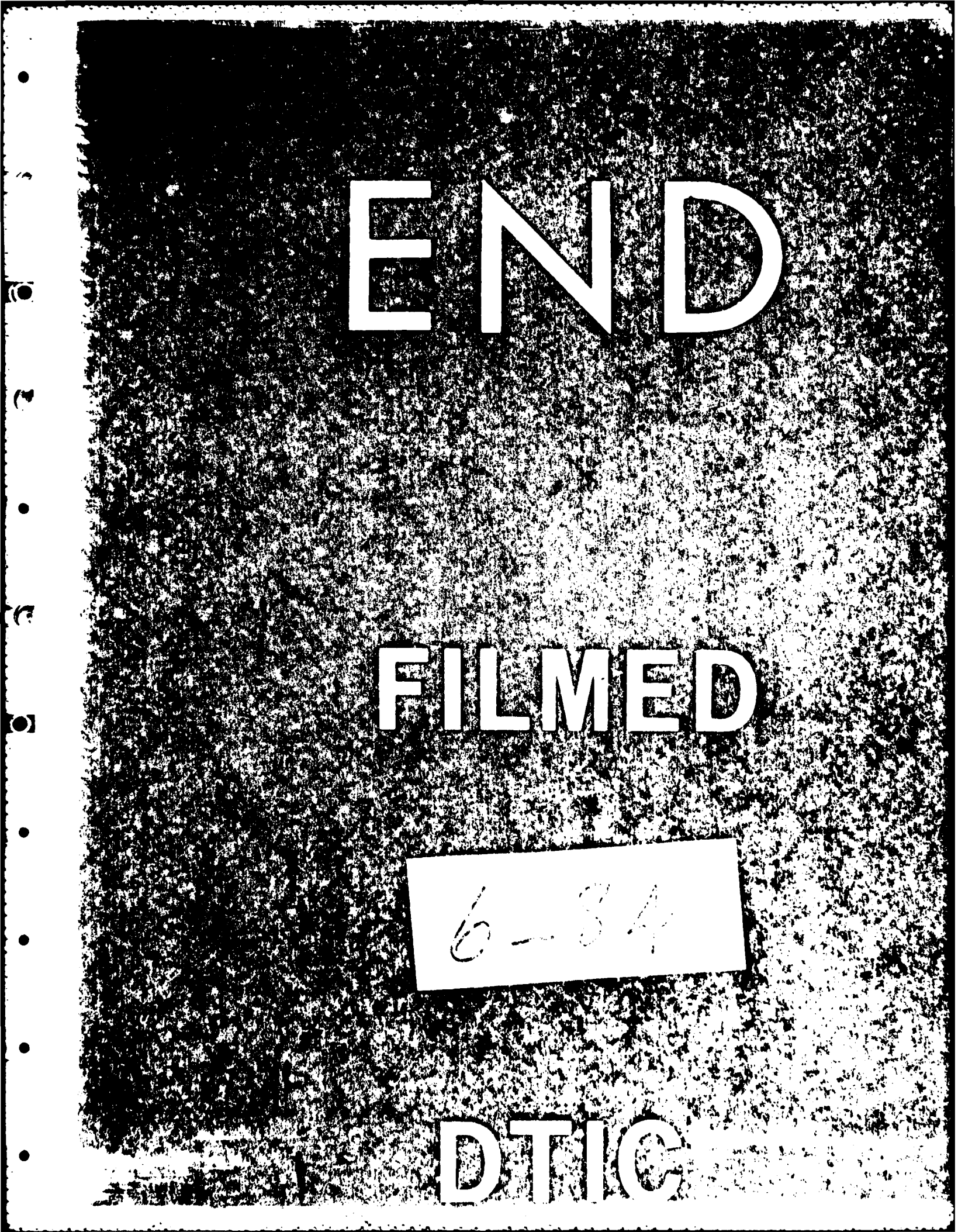
FIGURE 5



Collagen- and thrombin-induced β-TG release.

—o—10 ug/ml collagen (mean, n = 4). Unfractionated platelets (mean ± SD).

—□—1 U/ml thrombin (mean, n = 5). Unfractionated platelets (mean ± SD).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