Heterogeneity of Human Platelets

II. FUNCTIONAL EVIDENCE SUGGESTIVE OF YOUNG AND OLD PLATELETS

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ABSTRACT In the previous communication, suggestive evidence was presented for large-heavy platelets being "young" platelets and light-small platelets being "old" platelets. Large-heavy, light-small, and total human platelet populations were compared with respect to their platelet function. After addition of adenosine diphosphate (ADP), thrombin, or epinephrine, platelet aggregation time was 3.0-, 4.5-, and 3.3-fold shorter with large-heavy platelets compared with light-small platelets, and large-heavy platelets released 3.7-, 7.6-, and 8.1-fold greater adenosine triphosphate (ATP) into the medium, respectively, than did light-small platelets. After platelet aggregation by thrombin or epinephrine, large-heavy platelets released 6.0- and 3.8-fold more ADP into the medium than did light-small platelets. After platelet aggregation by ADP, light-small platelets consumed 5.9-fold greater added extracellular ADP than did large-heavy platelets.

Large-heavy platelets aggregated by ADP, thrombin, or epinephrine released 9.1-, 8.5-, and 12.7-fold greater platelet factor 4 than light-small platelets similarly treated.

INTRODUCTION

In the previous communication (1) a method was described which separated human platelets into large-heavy and light-small platelet populations. In the present study, these platelet populations were compared with respect to platelet function: i.e., their ability to release adenosine diphosphate (ADP) after platelet aggregation induced by thrombin and epinephrine; their ability to release adenosine triphosphate (ATP) after platelet aggregation induced by thrombin, ADP, and epinephrine; and their consumption of extracellular ADP after addition of ADP. In these experiments, the aggregation time, after addition of these aggregating agents, was also monitored.

The platelet populations were also compared with respect to their ability to release an "anti-heparin" material, platelet factor 4 (PF-4), after aggregation induced by thrombin, epinephrine, and ADP.

METHODS

Human platelets were obtained, prepared, separated into extreme density populations, and incubated as described in the previous communication (1).

In incubation experiments on adenine nucleotide release and ADP utilization, additions consisted of (final concentration): ADP, 0.01 mmole/liter + $CaCl_2$, 2.5 mmoles/liter; thrombin, 1 NIH unit/ml; and epinephrine, 0.1 mmole/liter + ascorbic acid, 1 mmole/liter (control epinephrine experiments contained 1 mM ascorbic acid). Neutralized perchloric acid extracts were prepared from platelet suspension supernatants and assayed for ATP and ADP as described previously (2).

ADP consumption was measured by subtracting the final extracellular ADP concentrations from the sum of the control extracellular ADP concentration at the end of 15 min plus the added ADP at zero time.

All adenine nucleotide release and ADP utilization experiments were performed in the presence and absence of 5 mM glucose. Significant differences (with and without glucose) were not obtained with thrombin and consequently these results were averaged.

Platelet factor 4 experiments. The platelet factor 4 test employed was a minor modification of the Poplawski and Niewiarowski procedure (3). A 5% suspension of washed platelets, 0.1 ml, was preincubated with 0.1 ml of heparin, 40 μg/ml, 0.3 ml of pooled platelet-poor plasma, and 0.1 ml of test material (or 0.1 ml of human Ringer's solution as a control) for 10 min at 37°C. Final concentration of test material was: ADP, 10 mmoles/liter + $CaCl_2$, 10 mmoles/liter; thrombin, 0.03 NIH units/ml; epinephrine, 1 mmole/liter + ascorbic acid, 1 mmole/liter (epinephrine control

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contained 1 mM ascorbic acid). The concentrations of test material employed were found to be optimal for platelet factor 4 release and each resulted in platelet aggregation. After preincubation, 0.1 ml of thrombin, 16.5 NIH units/ml was added. This gave a control clotting time of 27–30 sec for the different platelet populations.

Materials. Heparin was obtained from Connaught Medical Research Laboratories, Toronto, Canada. Platelet-poor plasma was obtained from The New York Blood Center after removal of platelets from platelet-rich plasma by centrifugation at 4000 *g* for 30 min at 4°C. Highly purified human thrombin was a gift from Dr. Kent Miller, New York State Department of Health, Albany, N. Y. Epinephrine hydrochloride was obtained from Parke, Davis & Co., Detroit, Mich. All other materials and reagents were obtained as described in the previous communication.

RESULTS

Adenine nucleotide release and ADP consumption of large-heavy, light-small, and total platelet populations

Control incubations. The presence or absence of 5 mM glucose had no effect on the relative release of ATP or ADP from large-heavy, light-small, or total platelet populations during control conditions after 15 min incubation at 37°C. Aggregation did not occur, and results were similar to those previously reported (2).

Thrombin. 15 min after addition of thrombin, large-heavy, light-small, and total platelet populations were aggregated and had released 13-, 1.7-, and 9.0-fold greater quantities, respectively, of ATP than did control platelets (Table I). Thus large-heavy platelets released 7.6-fold more ATP than light-small platelets. Similarly, the ADP release of large-heavy, light-small, and total platelet populations was 6.4-, 1.1-, and 3.5-fold greater, respectively, than control platelets. As is also apparent from Table I, large-heavy platelets aggregated 4.5 times more rapidly and released 5.8 times more ADP than did light-small platelets.

TABLE I

Effect of Thrombin on the Adenine Nucleotide Release of Separate Platelet Populations

			ATP	ADP	Aggregation time
Control*, (21)‡			0.106	0.244	
					min
Thrombin (7)	Large-heavy,	18%§	1.36	1.56	1.4
	Light-small,	12%	0.18	0.26	6.2
	Total,	100%	0.95	0.88	1.8

* Control values represent micromoles of ATP or ADP released into the media per milliliter platelets after 15 min incubation at 37°C. Values below the line represent the increment above the control value released into the media after exposure to thrombin, 1 NIH unit/ml for 15 min.

‡ Refers to number of experiments incubated in singlicate and assayed in triplicate. SEM was less than 10%.

§ Refers to the relative volume of the particular platelet population with respect to the total population.

ADP. With 0.1 mM ADP addition to the media (Table II), addition of 5 mM glucose did make a difference with respect to ATP release as well as ADP consumption. In the absence of glucose, 15 min after ADP addition, ATP release into the extracellular media for large-heavy, light-small, and total platelet populations was 9.0-, 2.6-, and 4.8-fold greater, respectively, than control nonaggregated platelets. The heavy/light platelet ATP release ratio was 3.5. In the presence of 5 mM glucose, slightly greater quantities of ATP were released into the media after aggregation with ADP (for large-heavy and total platelets). Thus, ATP release was 11.2-, 2.6-, and 5.7-fold greater than control release for large-heavy, light-small, and total platelet populations. The heavy/light platelet ATP release ratio in the presence of glucose was 4.3.

With the present techniques, ADP release into the

TABLE II

Effect of ADP Addition on ATP Release and ADP Consumption of Separate Platelet Populations

			ATP	ATP(G)§	ADP	ADP(G)	
Control,* (9)‡			0.120	0.138	2.532	2.688	Aggregation time
							min
ADP, (3)	Large-heavy,	13%‖	0.966	1.546	0.276	0.296	0.5
	Light-small,	14%	0.308	0.358	1.488	1.854	1.5
	Total,	100%	0.576	0.796	0.892	1.354	1.0

* Control values represent micromoles of ATP released or ADP present per milliliter platelets after 15 min incubation at 37°C. Values below the line represent the increment of ATP released into the media above the control value as well as the ADP consumption (disappearance from the media) after exposure to ADP, 1×10^{-4} mole/liter and $CaCl_2$, 2.5 mmoles/liter for 15 min.

‡ Refers to number of experimental incubations assayed in triplicate.

§ Presence of 5 mM glucose.

‖ Refers to the relative volume of the particular platelet population with respect to the total population.

TABLE III

Effect of Epinephrine on the Adenine Nucleotide Release of Separate Platelet Populations

		ATP	ATP(G)§	ADP	ADP(G)	Aggregation time
Control,* (9)‡		0.160	0.088	0.412	0.344	
						min
	Large-heavy, 21%‖	0.508	1.152	1.034	1.376	3.8
Epinephrine, (3)	Light-small, 17%	0.052	0.180	0.258	0.378	12.7
	Total, 100%	0.268	0.710	0.510	0.816	8.5

* Control values represent micromoles of ATP or ADP per milliliter platelets released into the media after 15 min incubation at 37°C. Values below the line represent the increment above the control value released into the media after exposure to epinephrine, 1×10^{-4} mole/liter plus ascorbic acid, 1×10^{-3} mole/liter for 15 min. Control tubes contained ascorbic acid, 1×10^{-3} mole/liter.

‡ Refers to number of experiments assayed in triplicate.

§ Presence of 5 mM glucose.

‖ Refers to the relative volume of the particular platelet population with respect to the total population.

extracellular media after the addition of ADP could not be readily measured. However, interestingly enough, ADP consumption (ADP disappearance from the media) did show significant differences between large-heavy, light-small, and total platelet populations. In the absence of glucose, ADP consumption of large-heavy, light-small, and total population platelets was 11, 59, and 35%, respectively, of total ADP added to the media. The light/heavy ADP consumption ratio was 5.4. In the presence of 5 mM glucose, ADP consumption was slightly greater (for light-small and total platelet populations): 11, 69, and 50%, respectively, for the same platelet populations. The light/heavy ADP consumption ratio in the presence of glucose was 6.3. The aggregation time was not affected by the presence or absence of 5 mM glucose for large-heavy, light-small, or total platelet populations. Of interest was the observation that the large-heavy platelet population which consumed 5- to 6-fold less extracellular ADP than did the light-small platelet population, and a 3-fold shorter aggregation time.

Epinephrine. With 0.1 mM epinephrine + 1 mM ascorbic acid, (Table III), addition of 5 mM glucose did make a difference with respect to ATP and ADP release into the media. In the absence of glucose and the presence of epinephrine, the net release of ATP into the media from large-heavy, light-small, and total platelet populations was 3.2-, 0.32-, and 1.7-fold the release under control conditions (absence of epinephrine, presence of ascorbic acid). The heavy/light ATP release ratio was 10.0. In the presence of 5 mM glucose, ATP release was considerably greater for large-heavy, light-small, and total platelet populations after addition of epinephrine: 13-, 2.0-, and 8.1-fold greater, respectively, than under control conditions. The heavy/light ATP release ratio in the presence of glucose was 6.5. In the absence of glucose, net ADP release into the medium after the addition of epinephrine was 2.5-, 0.64-, and 1.2-fold the control release for large-heavy, light-small, and total platelet populations. The heavy/light ratio for ADP release was 4.0. In the presence of 5 mM glucose, release of ADP into the medium was again higher for large-heavy, light-small, and total platelet populations: 4.0-, 1.1-, and 2.4-fold, respectively. The heavy/light release ratio for ADP in the presence of glucose was 3.6. The aggregation time for the large-heavy platelet population, (which released 3.6- to 4.0-fold more ADP into the medium than the old platelet population) was 3.3-fold shorter than the light-small platelet population.

TABLE IV

Effect of ADP, Thrombin, and Epinephrine upon Release of Platelet Factor 4 from Separate Platelet Populations

Control, (21)*	Large-heavy 29.7‡	Light-small 26.9	Total 29.7
ADP, (7)	−10.9	−1.2	−10.9
Thrombin, (7)	−10.2	−1.2	−11.2
Epinephrine, (7)	−8.9	−0.7	−8.5

* Numbers in brackets represent number of experiments, SEM was less than 10%.

‡ Numbers represent clotting time in seconds at 37°C after addition of 0.1 ml of thrombin, 16.5 NIH units/ml, after preincubation for 10 min with either of the test reagents, final concentration: ADP, 10 mmoles/liter + $CaCl_2$ 10 mmoles/liter; thrombin, 0.03 NIH units/ml; or epinephrine, 1 mmole/liter + ascorbic acid, 1 mmole/liter. The preincubation mixture contained: 0.1 ml of 5% test platelet suspension, 0.1 ml of heparin 40 μg/ml, 0.3 ml of platelet-poor plasma, and 0.1 ml of test material or 0.1 ml of human Ringer (control). Numbers above the line represent control values while numbers below the line represent the decrement in clotting time from the control value preincubation with the test material.

Platelet factor 4 release from large-heavy, light-small, and total platelet populations

Platelets contain an "anti-heparin" factor which shortens the thrombin clotting time in the presence of heparin (3, 4). This factor has been purified (5, 6) and is released by the platelets into the medium after platelet aggregation by ADP, thrombin, or epinephrine (7). Platelet factor 4 also induces platelet aggregation in vivo (8) and potentiates the ADP aggregation of platelets in vitro (7).

Accordingly, the effect of platelet aggregation with ADP, thrombin, and epinephrine on the release of platelet factor 4 was examined in large-heavy, light-small, and total platelet populations, Table IV. Large-heavy and total platelet populations behaved similarly after ADP, thrombin, or epinephrine; they released platelet factor 4 with resultant 10–11 sec shortening of the thrombin clotting time. The light-small platelet population, however, was deficient in the release of platelet factor 4.

DISCUSSION

Large-heavy platelets released considerably more ADP and ATP after exposure to epinephrine and thrombin and more ATP after exposure to ADP. This was associated with a significantly shorter aggregation time compared with light-small platelets. The diminished ADP release of light-small platelets may very well be related to the longer aggregation time. It may also be related to a diminished release mechanism which in turn could affect the aggregation time. Of considerable interest was the observation that large-heavy platelets consumed considerably less added extracellular ADP than did light-small platelets. In this respect, "surface" platelet "ADPase" has been described by Spaet and Lejnieks (9) and Karpatkin and Langer (2). The latter authors also noted the inhibition of platelet "ADPase" activity during thrombin-induced platelet aggregation. Since ADP is thought to be the physiologic mediator of platelet aggregation after thrombin, epinephrine, and collagen (10) this mechanism of diminished "ADPase" activity for large-heavy platelets would be of considerable advantage in facilitating platelet aggregation. As a corollary, enhanced "ADPase" activity, which appears to be present in light-small platelets, would have the effect of impairing platelet aggregation.

It has been suggested that platelet factor 4 may be a potent and specific agent triggering platelet aggregation and blood clotting in vivo. This factor shortens the thrombin clotting time in the presence of heparin (3), precipitates fibrinogen (7), nonenzymatically clots soluble fibrin monomer complexes (7), neutralizes certain fibrinogen breakdown products (11), induces platelet aggregation in vivo (8), and potentiates ADP-induced aggregation in vitro (7). The finding that light-small platelets release negligible quantities of platelet factor 4, whereas large-heavy platelets release 10 times as much, contributes to the evidence that large-heavy platelets also have an enhanced functional capacity when compared with light-small platelets.

The above mentioned differences in platelet function provide further evidence for the thesis that large-heavy platelets represent "young" platelets whereas light-small platelets represent "old" platelets. Of interest is the recent report of Hirsch, Glynn, and Mustard (12) suggesting that young rat platelets have a greater adhesiveness to collagen than do old platelets.

Thus, the separation of large-heavy and light-small platelets (possibly young and old platelets), the establishment of their differential ability to maintain their ionic gradients and (possibly) discoid shape, and their apparent differential ability to initiate platelet-plug formation, provide a better understanding of the heterogeneity of platelet interaction.

Finally, these data make it necessary to interpret all future, as well as past, platelet metabolic or function studies with caution. Any clinical situation which increased thrombopoiesis (increased large platelet index) (13) might give results reflecting a relatively large-heavy platelet population rather than an inherent abnormality reflecting a particular clinical situation.

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