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Functional Studies of Young Versus Old Platelets in a Patient With Chronic Thrombocytopenia

By Christine A. Johnson, Charles F. Abildgaard and Irving Schulman

Results of a comparative study of the functional capacity of young versus old platelets are presented. A girl with thrombopoietin deficiency, who predictably produced platelets in response to plasma transfusion, was used as platelet donor. Her platelets obtained 4 days after infusion were used as young test cells. Platelets obtained at 18 or 21 days after infusion were used as old cells. Young platelets were found to be associated with normal bleeding times, normal clot retraction, normal or increased platelet adhesiveness, normal aggregation to ADP and collagen, and normal platelet factor 3 availability. Old platelets were found to be associated with long bleeding times, decreased platelet adhesiveness in vivo and in vitro, and deficient platelet factor 3 availability. The clot retraction and aggregation to ADP and collagen of old platelets, however, was normal. No differences between young and old platelets were observed by electron microscopy.

THIS PAPER PRESENTS results of a study of the functional capacity of young versus old platelets, in which a patient with chronic thrombocytopenia secondary to thrombopoietin deficiency was used as the platelet donor. Because of the patient's predictable production of new platelets following plasma transfusion, she served as a unique human model for a comparative study in which the function of new and aged platelets could be repeatedly studied in vitro, and these findings further correlated with specific clinical and laboratory observations in vivo.

Case Report

The patient studied was an 18-year-old girl, first described by Schulman et al. in 1960.[1] She had a history of severe, chronic thrombocytopenia since birth which failed to respond to steroids or splenectomy. Her thrombocytopenia was due to lack of a plasma factor necessary for platelet production. As a result of this unusual deficiency, she

From the Departments of Pediatrics of the University of Illinois College of Medicine, Chicago, Ill., and the School of Medicine, University of California at Davis, Davis, Calif.

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Christine A. Johnson, M.D.: *Clinical Assistant Professor of Pediatrics, School of Medicine, University of California at Davis, Davis, Calif.* Charles F. Abildgaard, M.D.: *Professor of Pediatrics, School of Medicine, University of California at Davis, Davis, Calif.* Irving Schulman, M.D.: *Professor and Head, Department of Pediatrics, University of Illinois College of Medicine, Chicago, Ill.*

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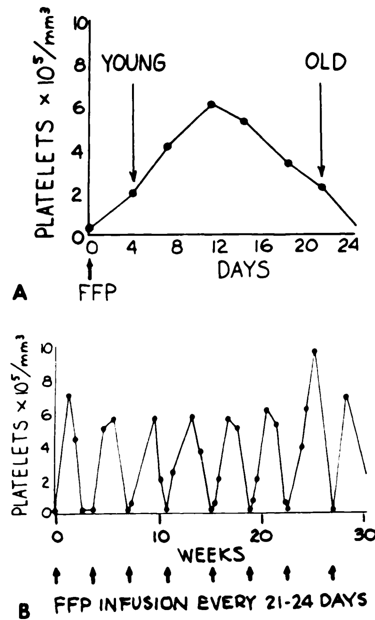

Fig. 1.—(A) Response of platelet count following administration of a single dose of fresh frozen plasma (FFP) to the patient studied. The time of sampling to obtain young and old platelets is indicated (see text). (B) Platelet response in the same patient following repeated infusions of FFP over a 5-month period.

responded to infusions of fresh frozen plasma with predictable and reproducible cycles of platelet production (Fig. 1A). Following an infusion of fresh frozen plasma, the patient's platelet count rose from her thrombocytopenic level (less than 10,000/cu. mm.) to a normal level (200–300,000/cu. mm.) by day 4. It reached a peak (500–800,000/cu. mm.) by day 9–10, then gradually fell to base line levels by day 21–23. The fall in platelet count at the end of platelet cycles was consistently linear and invariably of 9–10 days duration. Thus, it was felt to represent platelet life-span in this particular patient. Serial bone marrow examinations obtained during the cycle supported this assumption because megakaryocyte activity was seen to cease at the time that peripheral platelet counts began to fall.

Platelet production cycles such as the one described could be repeatedly reproduced by periodic plasma transfusion every 24 days. Remarkably similar curves resulted (Fig. 1B). Closer spacing of plasma infusions, every 14 days, resulted in an overlapping of production cycles, and a continuously normal peripheral platelet count in the patient. During most of the past 9 years, she has been treated by transfusion with 250 ml. of fresh frozen plasma every 14 days and has been remarkably free of difficulties as a result.[2]

Of clinical significance is the observation that toward the end of a treatment cycle, when her platelet counts are falling but are still within the normal range, she occasionally experiences mild hemorrhagic difficulties, such as epistaxis or superficial bruising. This fact is of particular interest because at the end of a cycle the patient's platelets, though normal in number, are homogenously aging. By contrast, early in her cycle, when her circulating platelets are homogeneously young, she is entirely free of symptoms.

It occurred to us that her bleeding manifestations might be due to a functional deficiency of old platelets. We, therefore, followed her through several complete platelet cycles, studying the function of her circulating platelets early and late in the treatment cycle.

Bleeding time, platelet adhesiveness, clot retraction, platelet factor 3 availability, and aggregation in response to ADP and collagen were assessed on separate groups of young (4 days of cycle) and old (21 days of cycle) platelets. Similar platelet samples were examined by electron microscopy. The results of these studies are presented in the current report.

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Materials and Methods

In order to obtain samples of platelets uniformly young and old, our patient was followed through several complete production cycles. To accomplish this, one of her regularly scheduled infusions of plasma was omitted, and she was allowed to become thrombocytopenic. She was then treated with a unit of fresh frozen plasma. Blood specimens drawn 4 days later, when platelet counts ranged from 200–300,000/cu. mm., were taken as representative of homogeneously young platelets (Fig. 1A). Because production cycles were known to vary slightly in length, the end cycle studies (meant to reflect the function of aging platelets) were done as follows. All tests were performed on day 18. These were accepted as representative of aging platelets if by 2 or 3 days later the patient's platelet count had fallen below 50,000/cu. mm. If not, studies were repeated on day 21, and these were recorded as representative of aging platelet function. Subsequent thrombocytopenia, or end of the cycle, was always documented. As a result, both early and late platelet function studies were done at a time when peripheral platelet counts were normal (150–300,000/cu. mm). The young platelets, however, were harvested 4 days after the patient had been thrombocytopenic. The old platelets were obtained just 3 days before she became thrombocytopenic again, at the end of the platelet production cycle. Data were collected during five different such cycles. The patient was advised not to take any medication during and for 2 weeks before the testing periods.

In addition to platelet function studies, several coagulation tests were done, including prothrombin time, partial thromboplastin time, factor V, VIII, and fibrinogen. Hemoglobin, hematocrit, and reticulocyte counts were also obtained on all the blood samples drawn.

Venous blood for coagulation and platelet studies was collected through 19 gauge scalp vein needles into plastic syringes and transferred directly to plastic tubes containing buffered citrate,[3] in a ratio of nine parts of blood to one part of anticoagulant. Platelets were counted from EDTA samples by phase microscopy. Clot retraction was qualitatively observed in glass tubes at 37°C. Platelet adhesion was studied by the methods of Salzman,[4] Hume,[5] and Borchgrevink.[6] Bleeding times were done as previously described by this laboratory and consisted of a series of three puncture wounds on the volar surface of the forearm with a number 11 Bard Parker blade to a depth of 3 mm.[7] Platelet factor 3 availability to kaolin was assayed by the Hardisty and Hutton technique.[8]

Platelet aggregation was measured turbidometrically following the plan of Mustard et al.[9] with a number of modifications. Nine ml. of venous blood was collected through 19 gauge scalp vein needles into plastic syringes and then added to 1.0 cc. 3.8 per cent citrate buffer in siliconized tubes. The blood was centrifuged at room temperature for 15 minutes at 800 rpm. Four ml. of platelet-rich plasma (PRP) was removed and the blood respun at 10,000 rpm for 10 minutes to obtain platelet-poor plasma (PPP). The platelet count of the PRP was adjusted to 300,000/cu. mm. by appropriate dilution with PPP and kept at room temperature until used for testing. Three aggregating agents were made up within 1–2 hours of testing. Two molar strengths of ADP (to make final concentrations of 2 μM and 20 μM) were mixed with imidazole buffer at pH 7.3 from portions of a stock solution kept frozen at 2×10^{-3}M (ADP, Sigma). These agents were kept on ice. The collagen used was desiccated corium supplied by Armour Co., Kankakee, Ill. It was homogenized in normal saline to a final concentration of 50 μg./0.1 ml. and kept at room temperature until use. Aggregation was quantitated by use of an aggregometer (Chronolog, Philadelphia). Samples of 0.9 cc. of PRP were placed in siliconized glass cuvettes, each containing a teflon-coated magnetic stirring rod. Stirring was begun at 1200 rpm. After one minute of incubation at 37°C, 0.1 cc. of aggregating agent was inserted directly into the plasma through a micropipette. Change in optical density was continuously recorded as change in light transmission on a Bausch and Lomb VOM 5 recorder. The recorder was preadjusted so that PPP represented 100 per cent transmission and PRP was 0. Degree and speed of aggregation was then expressed as the per cent change in light transmission from the PRP base line, per unit time.

Platelet pellets for electron microscopy were doubly fixed with 3 per cent glutaraldehyde followed by 1 per cent osmium tetroxide.[10] They were alcohol dehydrated and then were

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Table 1.—Results of Ivy Bleeding Times Performed in Triplicate Early (Day 4) and Late (Day 18 or 21) in the Patient's Platelet Cycles*

	Platelet Count (per cu. mm.)	Ivy Bleeding Time (normal ≤ 6 min.)
Studies done on day 4	190,000	3,4,4
	196,000	5,6,8
	250,000	5½,6,7
	190,000	5,5,5
Studies done on day 18 or 21	171,000	>20
	230,000	6,9,11
	340,000	5,9,16
	348,000	9,10,14
	225,000	7,8,10

* For comparison, venous platelet counts obtained at the time of each test are included.

air shipped in dry ice to Dr. James G. White, University of Minnesota, for further processing and reading.

Prothrombin time was determined by the one-stage method of Quick,[11] using rabbit brain thromboplastin (Ortho). Partial thromboplastin time was measured as described by Johnson et al.[12] Factor V assay was by the method of Shanberge et al.;[13] factor VIII assay by the method of Simone et al.[3] Fibrinogen levels were determined as described by Ratnoff and Menzie.[14]

Results

Bleeding Time

Ivy bleeding times done early in four separate cycles were all within normal limits. Prolonged bleeding times were detected at the end of 5 different cycles studies (Table 1).

Clot Retraction

Retraction was qualitatively normal both early and late in four cycles.

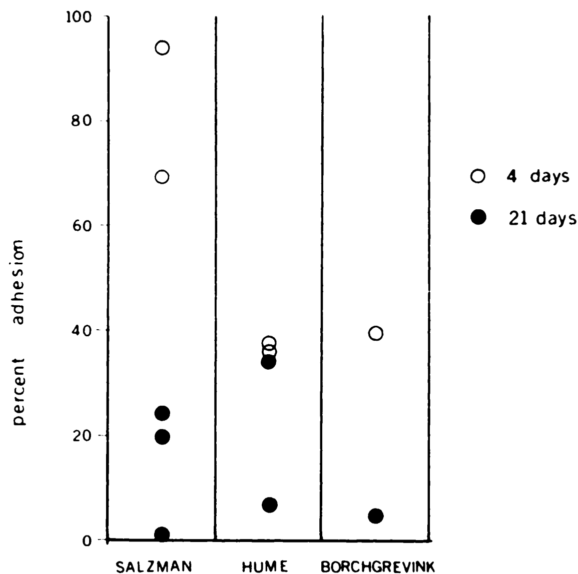

Fig. 2.—Platelet adhesiveness of young (open circles) and old (closed circles) platelets as determined by three different methods are presented. The paired studies shown represent data obtained from six different platelet cycles.

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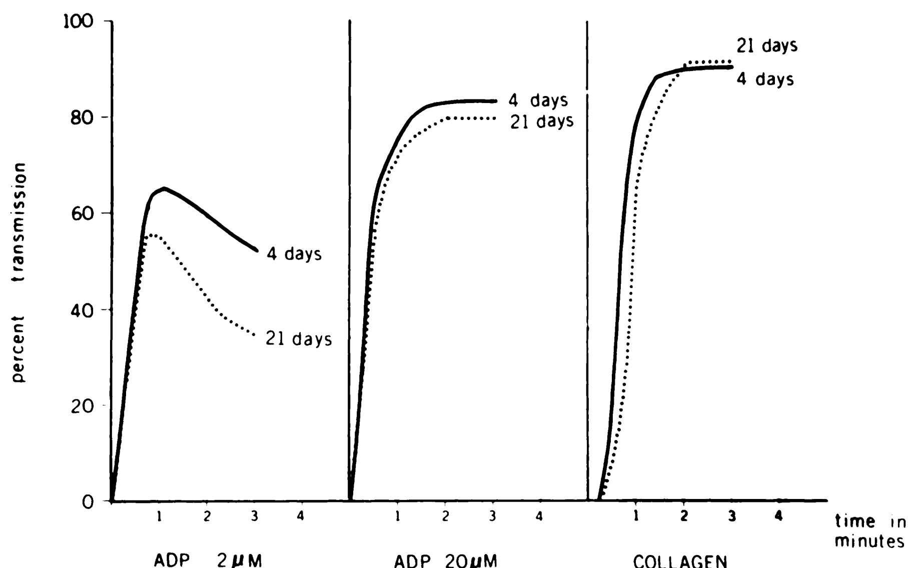

Fig. 3.—The aggregation of young and old platelets to ADP and collagen are graphically represented as relative change in light transmission per unit time after addition of aggregating agents to samples of platelet-rich plasma. The curves shown are composites, plotted from mean values of results obtained during several platelet cycles. (Concentration of aggregating agents refers to final concentration in test plasma.)

Platelet Adhesiveness

Adhesiveness occurring in vivo and in vitro was invariably increased or normal on day 4. Five of six determinations on day 21 showed a marked decrease in per cent adhesiveness over the comparable value at 4 days. Four of six determinations on day 21 were below the accepted range of normal (Fig. 2).

Platelet Aggregation

Aggregation in response to high (20 μM) and low (2 μM) molarity ADP and to collagen was assessed during several consecutive cycles. The speed and degree of aggregation of both young and old platelets always remained within normal limits for our laboratory. Although aggregation of older platelets was repeatedly slower than the corresponding response of younger platelets and disaggregation with low molarity ADP was more rapid and more pronounced in the older platelet samples, neither of these differences was considered to be significant. (Aggregation is quantitatively depicted in Fig. 3. The day 4 curve plotted represents the mean of two determinations from two different cycles while the 21-day curve represents the mean of four determinations from four different cycles.)

Platelet Factor 3 Availability

Platelet factor 3 availability of young platelets was measured during each

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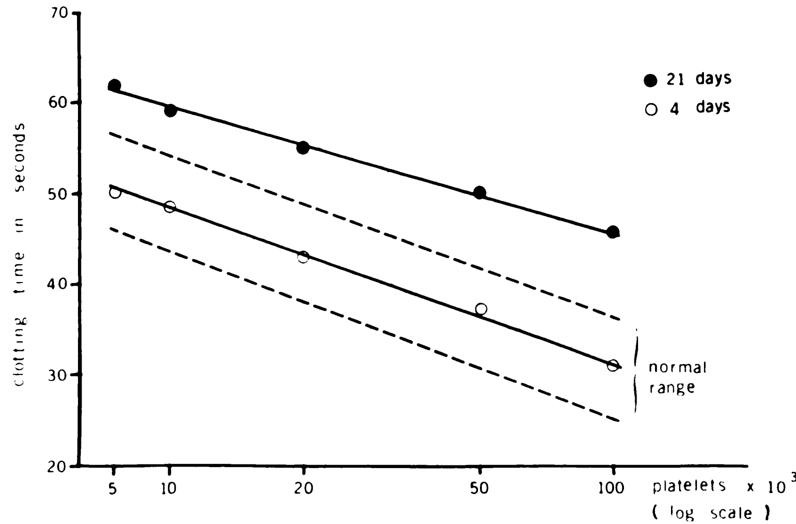

Fig. 4.—Comparative platelet factor 3 availability of young and old platelets from a representative platelet cycle is shown. The curve for young platelets (open circles) was obtained on day 4 of a platelet cycle. The corresponding curve for old platelets (closed circles) was obtained on day 21 of the same cycle.

of three cycles; all were normal. Old platelets evaluated during the same cycles showed decreased platelet factor 3 availability; all were below normal range. (Results of platelet factor 3 assays from a representative cycle are depicted in Fig. 4.)

Platelet Electron Microscopy

Electron microscopic examination of platelet pellets from days 4, 7, 11, 14, 18, and 21 of a single cycle was performed by Dr. James White. No morphological abnormalities were noted in any of the young or aging platelet samples.

Coagulation Studies

No irregularities of prothrombin time or partial thromboplastin time were observed either at the beginning or at the end of a cycle. Studies were obtained through eight separate cycles. Similarly, assays for fibrinogen, factor V, and VIII were normal throughout eight cycles. Hemoglobin and hematocrit varied only slightly during each cycle tested. Reticulocyte counts ranged between 0.8 per cent and 3.6 per cent.

Discussion

A number of probable physiological and biochemical changes in aging platelets have been reported in the past. Old platelets, as compared to young, were found by several investigators to be less adhesive to glass[15,16] and to collagen.[17] No similar decrease in adhesion to glass was demonstrated by Rolovic and Baldini.[18] Ginsburg and Aster[19] and Karpatkin[20] observed that aged platelets aggregated less rapidly in response to ADP. Aggregation to

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collagen, however, appeared to be normal;[17] and Shulman demonstrated normal platelet factor 3 availability in old platelet populations.[16] Two independent studies[21,22] indicated that old platelets were less effective in clot retraction.

In addition to these reported changes in platelet function, a number of biochemical and morphologic changes have been suggested as accompanying platelet senescence: decrease in total volume,[19,22,23] decrease in total ATP content,[19,21,24] decrease in phospholipid and in orthophosphate content,[19,24] and decrease in platelet protein incorporation.[16,19,24]

Execution and interpretation of investigations of platelet aging have been limited because of the technical difficulties involved in obtaining platelets of uniform ages from an acceptable donor. In the studies reviewed above, young platelets were obtained from splenectomized, irradiated, or cytotoxically prepared donors; often animals were used. Platelets were aged experimentally by incubation in thrombocytopenic test animals or by storage in vitro. Occasionally, relative age was only presumed with the differentiation assigned on the basis of platelet size.

Our patient, in contrast, provided a unique physiological model for the study of platelet aging. She produced a population of new platelets with normal morphology and life span in response to a biological stimulus. After platelet production ceased, her platelets aged in vivo and were later available for use as old platelets in the comparative study. Because our patient had been splenectomized, there was no question that selective splenic platelet destruction on the basis of size or age had influenced the circulating platelet population.[16,19,25]

Despite some obvious differences in methodology and in experimental models, there were no major conflicts between our findings and those of previous studies. For all the platelet functions which we investigated, with the exception of qualitative clot retraction and aggregation, the response of older platelets was significantly decreased as compared to younger ones. In some instances, as with platelet adhesion to glass, the difference was dramatic. There seemed to be a spectrum from supranormal stickiness of young platelets to deficient stickiness of old platelets. Adhesiveness appeared to correlate well not only with platelet senescence but also with our patient's bleeding times, which progressively lengthened through the platelet cycles. These findings, in addition, fit well with her hemorrhagic manifestations, the occasional end-cycle bleeding consisting primarily of superficial brusing and epistaxis.

The difference we observed between young and old platelets in aggregation to ADP and to collagen was not considered to be significant. On the other hand, aged platelets showed a marked defect in platelet factor 3 availability. It is currently felt that platelet lipoprotein is made available as a consequence of membranous changes which occur during secondary aggregation.[26] Thus, normal secondary aggregation would be expected to be accompanied by normal "release" of platelet factor 3. This apparent dichotomy in our findings is best explained by the fact that in the platelet factor 3 test used, kaolin contact, and not aggregation, was the agent triggering release

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of platelet contents. Mechanical contact of platelets in this fashion is certainly not equivalent to the physiologic release mechanism. What was found was perhaps a laboratory abnormality rather than a real defect in platelet function. It is more probable that the finding was real and that total phospholipid content of old platelets is indeed decreased. Favoring this view is a previous report of decrease in phospholipid content of aging platelets.[19,24] While total phospholipid content may become deficient on aging, the fraction necessary for aggregation may remain quite adequate.

Of interest, a similar situation may exist in regard to platelet ATP. Total platelet ATP has been found to be quantitatively reduced in old platelets.[19,21,24] Our finding of normal secondary aggregation would suggest that enough ATP for platelet aggregation is available. A number of interpretations could be offered to explain this retention of ability to supply ATP for aggregation in the face of total reduction in ATP. Holmsen,[27] in 1968, demonstrated that platelet ATP converted to ADP in the process of aggregation came from a pool of ATP distinct from that involved in metabolic functions. The results of this work would suggest that there may be a separate ATP pool, not involved in general cell metabolism, which is active in aggregation and which is not influenced by aging.

REFERENCES

1. Schulman, I., Pierce, M., Lukens, A., and Currimbhoy, Z.: Studies on thrombopoiesis: 1. A factor in normal plasma required for platelet production: Chronic thrombocytopenia due to its deficiency. Blood 16:943, 1960.
2. Abildgaard, C. F., Simone, J. V., Seeler, R. A., and Schulman, I.: Chronic thrombocytopenia due to "thrombopoietin" deficiency: A progress report. Blood 30:546, 1967.
3. Simone, J. V., Vanderheiden, J., and Abildgaard, C. F.: A semiautomatic one-stage factor VIII assay with a commercially prepared standard. J. Lab. Clin. Med. 69: 706, 1967.
4. Salzman, E. W.: Measurement of platelet adhesiveness. J. Lab. Clin. Med. 62:724, 1963.
5. Hume, M.: Platelet entrapment in glass spheres: Clinical and experimental application. Ann. Surg. 166:59, 1967.
6. Borchgrevink, C. F.: A method for measuring platelet adhesiveness in vivo. Acta Med. Scand. 168:157, 1960.
7. Abildgaard, C. F., Simone, J. V., Honig, G. R., Forman, E. N., Johnson, C. A., and Seeler, R. A.: Von Willebrand's disease: A comparative study of diagnostic tests. J. Pediat. 73:355, 1968.
8. Hardisty, R. M., and Hutton, R. A.: The kaolin clotting time of platelet rich plasma: a test of platelet factor 3 availability. Brit. J. Haemat. 2:258, 1965.
9. Mustard, J. F., Hegardt, B., Roswell, H. C., and MacMillan, R. L.: Effect of adenosine nucleotides on platelet aggregation and clotting time. J. Lab. Clin. Med. 64: 548, 1964.
10. Pease, D. C.: Histological Techniques for Electron Microscopy. New York, Academic Press, 1960, p. 32.
11. Quick, A. J.: Haemorrhagic Diseases. Philadelphia, Lea and Febiger, 1957, p. 379.
12. Johnson, C. A., Abildgaard, C. F., and Schulman, I.: Absence of coagulation abnormalities in children with cyanotic congenital heart disease. Lancet 2:660, 1968.
13. Shanberge, J. N., Matsuka, T., and Fukui, H.: A simple method for the preparation of a substrate for a one-stage factor V assay. Tech. Bull. Regist. Med. Techn. 37:69, 1967.
14. Ratnoff, O. D., and Menzie, C.: A new method for the determination of fibrinogen in small samples of plasma. J. Lab. Clin. Med. 37:316, 1951.
15. Wright, H. P.: Changes in the adhesiveness of blood platelets following parturition and surgical operations. J. Path. Bact. 54:461, 1942.

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16. Shulman, N. R., Watkins, S. B. J., Itsocoitz, S. B., and Students, A. B.: Evidence that the spleen retains the youngest and hemostatically most effective platelets. Trans. Ass. Amer. Physicians 81:302, 1968.

17. Hirsh, J., Glynn, M. F., and Mustard, J. F.: The effect of platelet age on platelet adherence to collagen. J. Clin. Invest. 47: 466, 1968.

18. Rolovic, Z., and Baldini, M.: Platelet adhesiveness with reference to platelet age. Scand. J. Haemat. 6:25, 1969.

19. Ginsburg, A. J., and Aster, R. H.: Changes associated with platelet aging. Amer. Fed. Clin. Res., Atlantic City Meetings, 1969, p. 325.

20. Karpatkin, S.: Heterogeneity of human platelets, II. functional evidence suggestive of young and old platelets. J. Clin. Invest. 48:1083, 1969.

21. Löhr, G. W., and Waller, H. D.: Zellstoffwechsel und zellalterung (Cellular metabolism and cellular aging). Klin. Wschr. 37:833, 1969.

22. Detwiler, T. C., Odell, T. T., Jr., and McDonald, T. P.: Platelet size, ATP contents and clot retraction in relation to platelet age. Amer. J. Physiol. 203:107, 1962.

23. McDonald, T. P., Odell, T. T., Jr., and Gosslee, D. G.: Platelet size in relation to platelet age. Proc. Soc. Exp. Biol. Med. 115:684, 1964.

24. Karpatkin, S.: Heterogeneity of human platelets: I. Metabolic and kinetic evidence suggestive of young and old platelets. J. Clin. Invest. 48:1073, 1969.

25. Murphy, E. A., Robinson, G. A., Roswell, H. C., and Mustard, J. F.: The pattern of platelet disappearance. Blood 30:26, 1967.

26. Marcus, A. J.: Platelet function. I. New Eng. J. Med. 280:1213, 1969.

27. Holmsen, H., Day, H. J., and Stormorken. H.: The blood platelet release reaction. Scand. J. Haemat., Suppl. 8:3, 1969.

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