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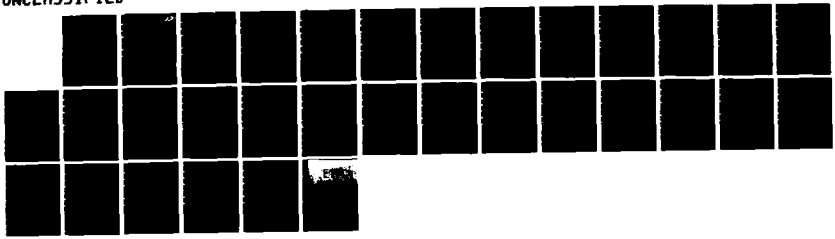

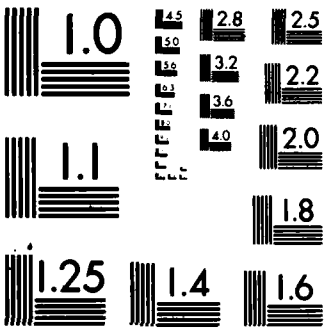

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PLATELET SIZE DOES NOT CORRELATE WITH AGE

by

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The relationship between platelet size and in vivo aging was investigated in the baboon using size-dependent platelet subpopulations separated by counter-flow centrifugation. The separation characteristics, size, LDH activity, and dense body content of the baboon platelet subpopulations were similar to those previously observed in studies of human platelets. Three independent labeling techniques were used: 1) in vivo labeling with ^{75}Se-methionine; 2) in vitro labeling with ^{51}Cr; and 3) in vivo labeling with ^{14}C-serotonin. Maximal

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incorporation of all three labels showed a close correlation between the mean platelet volume (MPV) of each fraction and the platelet radioactivity. The onset of incorporation and rate of accumulation of ^{75}Se-methionine were comparable in all fractions when corrected for differences in volume, suggesting that platelet size heterogeneity was present from the time of release of the platelets from the bone marrow. Survival studies using ^{51}Cr and ^{14}C-serotonin showed no translocation of the label from one fraction to another in the circulation over time. In vivo survival values for the three radionuclides showed a slight but significant correlation between the lifespan and the MPV of the fractions. The data suggest that large platelets were not younger platelets but rather platelets with a longer lifespan. Platelet size heterogeneity was the result of production factors in the bone marrow and not maturation in the circulation.

ABSTRACT

The relationship between platelet size and in vivo aging was investigated in the baboon using size-dependent platelet subpopulations separated by counterflow centrifugation. The separation characteristics, size, LDH activity, and dense body content of the baboon platelet subpopulations were similar to those previously observed in studies of human platelets. Three independent labeling techniques were used: 1) in vivo labeling with ^{75}Se-methionine; 2) in vitro labeling with ^{51}Cr; and 3) in vivo labeling with ^{14}C-serotonin. Maximal incorporation of all three labels showed a close correlation between the mean platelet volume (MPV) of each fraction and the platelet radioactivity. The onset of incorporation and rate of accumulation of ^{75}Se-methionine were comparable in all fractions when corrected for differences in volume, suggesting that platelet size heterogeneity was present from the time of release of the platelets from the bone marrow. Survival studies using ^{51}Cr and ^{14}C-serotonin showed no translocation of the label from one fraction to another in the circulation over time. In vivo survival values for the three radionuclides showed a slight but significant correlation between the lifespan and the MPV of the fractions. The data suggest that large platelets were not younger platelets but rather platelets with a longer lifespan. Platelet size heterogeneity was the result of production factors in the bone marrow and not maturation in the circulation.

INTRODUCTION

Studies of platelets from a variety of mammalian species have shown them to be heterogeneous with respect to size, density, age, metabolism, and function.[1-15] While this heterogeneity has been well described, the causes and importance of platelet heterogeneity remain controversial. Several authors have suggested that platelet heterogeneity is the result of platelet senescence in the circulation with large, dense platelets becoming smaller and lighter as they age in the circulation.[2,4,11] Others have proposed that platelet heterogeneity is the result of production factors in the bone marrow.[8,10]

Early experimental models of platelet aging relied upon alterations of steady state thrombopoiesis to produce populations of young and old platelets.[3,15] These studies lead to the generally accepted view that platelets produced under conditions of thrombocytopenia are significantly larger than normal. However, changes in megakaryocytopoiesis also occur during thrombocytopenia.[16] The larger platelets formed during thrombocytopenia may be the result of altered platelet production rather than a reflection of a shift in the age spectrum of circulating platelets. A number of recent studies have attempted to look at platelet aging in the steady state using density-defined platelet subpopulations.[1,2,4-6,9-12] Unfortunately, these studies have led to an array of results. At a recent conference on platelet heterogeneity, 3 groups of investigators reported new results on the relationship of platelet density to age. Rand and associates reported data supporting the theory that platelets

become less dense as they age.[11] Martin and associates concluded from their data that platelet density does not vary with platelet age.[12] Finally, the data of Mezzano and associates suggested that newly formed platelets were less dense and became more dense as they aged in the circulation.[6]

The methods for measurement of platelet density are cumbersome and difficult to standardize. On the other hand, the mean platelet volume (MPV) is routinely available in most clinical laboratories.[17-19] The relationship between platelet volume and age is the subject of our study. We have recently described a quantitative method for the separation of platelets on the basis of size using counterflow centrifugation which leaves the platelet metabolically and functionally intact.[13,20,21] Using this method, we report here the relationship between platelet volume and platelet age in the baboon using 3 independent radioisotope labeling procedures.

MATERIALS AND METHODS

Six adult baboons (Papio cynocephalus) weighing 17-25 kg were used. Animals were housed in conformance with NIH Guidelines and fed a routine diet. Animals were anesthetized with ketamine hydrochloride during blood drawing. During transfusion of chromium-labeled platelets 50-100 mg of sodium pentobarbital was used as needed. All blood samples were obtained using either a 20-gauge needle or intravenous catheter by direct puncture of a femoral vein without use of a tourniquet.

Platelet Isolation

Platelets were isolated in a similar manner to that reported previously for human platelet isolation.[13] Platelet isolation was performed as follows: a 21.25 volume of blood collected in 3.75 ml of ACD, NIH, Formula A, was diluted with a 25 ml volume of a solution containing phosphate-buffered saline and albumin (PBS-A) (105.5 mM NaCl, 12.8 mM Na_2HPO_4, 2.8 mM KH_2PO_4, 2.6 mM Na_2EDTA, 15% V:V ACD, 0.5% W:V fatty acid free bovine serum albumin (Sigma Chemical Co., St. Louis, MO) 3 M NaOH to titrate pH to 6.5, osmolarity 308 mOsm/l). The diluted blood was separated into two equal volumes and transferred to two 50 ml plastic tubes; the volume in each tube was centrifuged at 160 g for 7 minutes. The platelet-rich supernatant was removed. The red cell concentrate was resuspended in 25 ml of PBS-A, after which each tube was centrifuged again and the supernatant solution removed from each tube. The platelet-rich supernatants were pooled and acidified to pH 6.5 with ACD. The acidified platelet suspension was separated into two equal volumes and

transferred to two tubes and centrifuged at 700 g for 15 minutes. The supernatant solution was removed. The platelet pellet was resuspended in 10 ml of PBS-A, and the platelet suspension was centrifuged at 700 g for 15 minutes. The supernatant was removed. Finally, the platelets were resuspended in 4.5 ml of PBS-A. The average recovery of platelets from whole blood platelets by this method was 79%. When two more washes of the red cell pellet were performed, an additional 12.8 ± 4.2% (mean ± SD, n = 5) of the platelets in the blood were recovered. The mean platelet volume (MPV) of platelets recovered from the red cell pellet showed no significant difference in MPV from those collected in the previous washes, 5.63 ± 0.43 u^3 versus 5.86 ± 0.28 u^3 respectively, and volume distribution profiles were similar. The additional washes were not routinely performed because of the additional time required.

Counterflow Centrifugation (Elutriation)

The platelets were separated into seven subpopulations using a Beckman centrifuge No. J21B with an elutriator rotor (No. JE6) and a Sanderson cell separation chamber (No. 335206) as reported previously. All separations were done in PBS-A at 20 C and 3500 rpm.

The subpopulations were separated as follows: The isolated platelets in 4.5 ml of PBS-A were loaded and the stopcock was flushed with 2 ml of buffer. The blood sample was allowed to flow into the chamber at a rate of 1 ml/minute for 4 minutes, after which the rate was increased to 2.5 ml/minute for 3 minutes, then to 3 ml/minute for 3 minutes, and then progressively to 3.5, 4.0, 5.0, 6.0, 7.0 and 8.0 ml/minute each for 2 minutes. The eluted sample at each of the last seven

flow rates was collected and labeled sequentially for a total of 7 subpopulations.

In order to obtain comparable numbers of platelets in all fractions for radioactivity studies, the platelets in fractions 1 and 2 and fractions 6 and 7 were pooled. Platelet counts, MPV, LDH activity and dense body content were determined as reported previously.[13]

Selenomethionine Incorporation and Survival

Platelet production and survival were studied using ^{75}Se-Selenomethionine (S.A. 3.3 uCi/ug, Amersham Co., Arlington Heights, IL) according to the method of Ardaillou et al.[22] Three baboons were injected intravenously on day 0 with 5.5 uCi/kg of ^{75}Se-Selenomethionine. Blood samples were taken daily from the first to the ninth days and also on the eleventh and fourteenth days post-injection. Platelets were isolated and fractioned as above. Each of the fractions was counted for radioactivity in a gamma counter and radioactivity was reported as counts per minute per 10^9 platelets. The rate of incorporation of ^{75}Se-Selenomethionine per u^3 of platelet volume in each fraction was measured. Platelet lifespan was measured as the time elapsed between the 50% point on the ascending slope and the 50% point on the descending slope of the curve after correction for utilization of methionine.

Chromium-Labeled Platelet Survivals

In three baboons autologous platelets were labeled with ^{51}Cr (S.A. 101.0 uCi/ug) according to the methods of Melaragno et al,[23] and immediately reinfused. Following injection, blood samples were drawn at 1 hour and at 1, 2, 4, and 7 days. Platelets were isolated and

fractionated as previously described. Each platelet fraction was counted for radioactivity using a gamma counter and the results expressed as cpm/10^9 platelets. The blood volume of the baboon was determined from the plasma volume measured with ^{125}I-labeled albumin and the total body hematocrit. Platelet survivals were estimated using linear and logarithmic functions and the weighted mean value.[24]

^{14}C-Serotonin Incorporation and Survival

^{14}C-labeled serotonin (serotonin binoxalate, 5-(2-^{14}C), New England Nuclear Corp., Boston, MA) was used to measure platelet survival according to the method of Heyssel.[25] Four uCi of ^{14}C-serotonin was injected intravenously into each of 3 baboons. Follow-up samples were obtained at 1, 2, 4, 7, and 9 days. Platelets were isolated and fractionated. The radioactivity in each fraction was counted after dilution in Aqua-Sol (New England Nuclear Corp., Boston, MA) in a scintillation counter. The percentage uptake of injected ^{14}C-serotonin by the platelets at 24 hours was determined using the whole blood platelet count and blood volumes measured using ^{125}I-albumin.[23] Platelet survivals were estimated using the data from the first 7 days following injection.

Statistical Analyses

All data is expressed as the average of 3 separate experiments. Statistical correlations between the MPV and isotope-measured platelet production and survival were then analyzed using linear function and logarithmic function, and weighted mean estimates.[26] All statistics were performed on a T155 desk-top calculator (Texas Instruments, Dallas, TX).

RESULTS

The characteristics of the size-dependent platelet subpopulations used in the survival studies are given in Table 1. By combining fractions 1 and 2 and fractions 6 and 7, we obtained 5 subpopulations. Between 13.9 ± 5.3% (SD) and 25.5 ± 3.4% of the platelets were recovered in each fraction. The LDH activity per 10^{10} platelets and the dense body content per platelet (Table 1) showed a significant correlation ($r = 0.99$, $p < 0.001$ for both) with the MPV of the platelets in the fractions. The data represent 70 of 71 consecutive experiments performed by a single technician over a 6-month period. The single failure came on the first day of a chromium survival study when a seal in the centrifuge broke and the study was aborted. During all survival studies the hematocrit, platelet count and MPV were stable.

The incorporation of ^{75}Se-methionine in each of the subpopulations was studied in order to examine the relationship of platelet production to platelet size (Figure 1). The shapes of the incorporation curves were similar for the 5 fractions and for the unfractionated platelets. The magnitude of incorporation of ^{75}Se-methionine was significantly correlated to the MPV of the fractions ($r = 0.99$, $p < 0.001$, Table 2). When the ^{75}Se-methionine incorporation curves were corrected for differences in volume between the fractions, no differences were observed in the rate of production of platelets of different size ($r = -0.51$, $p > 0.1$, Table 3). However, when survival times were estimated from the curves, there was a significant correlation between survival time and the mean platelet

volume of the fractions ($r = 0.98$, $p < 0.001$, Table 3).

^{51}Chromium platelet survival times were measured in the original platelets and in each of the 5 fractions. The in vivo recovery of the ^{51}Cr-labeled unfractionated platelets was 72.4 ± 8.3%. There was a significant correlation between MPV and the maximum ^{51}Cr radioactivity in platelets 1 hour after transfusion ($r = 0.98$, $p < 0.001$, Table 2). No significant shifts or plateaus of ^{51}Cr radioactivity were observed (Figure 2). Survival times of the ^{51}Cr in each of the subpopulations was determined using the linear and logarithmic estimates and their weighted mean value (Table 4). As with ^{75}Se-methionine, there was a significant correlation between ^{51}Cr survival time and the MPV (linear $r = 0.98$, $p < 0.001$; exponential $r = 0.94$, $p < 0.01$; weighted mean value $r = 0.90$, $p < 0.05$).

FIG. 2

TABLE 4

One day following ^{14}C-serotonin infusion, 9.8 ± 1.3% (SD) of the radioactivity was incorporated in the platelets. The incorporation of the ^{14}C-serotonin into the platelet subpopulations showed a significant correlation with the MPV of the fractions ($r = 0.98$, $p < 0.001$, Table 2). There was no plateau of ^{14}C-serotonin radioactivity in any of the subpopulations and no shifts of radioactivity between fractions were observed during 9 days following injection (Figure 3). There was a significant correlation between the MPV and survival time measured by the linear and exponential functions ($r = 0.97$, $p < 0.01$ and $r = 0.95$, $p < 0.01$ respectively). Survival time as measured by the weighted mean value did not have a significant correlation with the MPV.

FIG. 3

DISCUSSION

Platelet kinetics have been previously studied in the baboon by several investigators.[23,27,28] The distribution, size, dense body content, and LDH activity of the baboon platelet subpopulations were similar to those values for human platelet subpopulations.[13] The intent of our study was to determine the etiology of platelet size heterogeneity during steady-state thrombopoiesis. The rate of appearance of the megakaryocyte label ^{75}Se-methionine in each of the size-dependent platelet subpopulations was studied. If young platelets are large and become smaller as they age in the circulation, then ^{75}Se would appear initially in the larger fractions and as the labeled platelets age the ^{75}Se would appear in smaller fractions in the circulation. Our results showed that ^{75}Se appeared in all of the fractions at the same time (Figure 1). The rate of incorporation per unit of platelet volume during the initial 4 days was similar in all of the fractions (Table 3) and maximum incorporation of radioactivity was directly proportional to the MPV of the fractions (Table 2). The maximum radioactivity in the platelets occurred earlier in the smaller platelets than in the larger platelets.

The survival time of the ^{75}Se isotope was progressively longer with increasing the MPV of each fraction (Table 3). However, selenomethionine does not provide a good estimate of platelet survival times because of potential reutilization of the isotope. To confirm these results unfractionated platelets were labeled with ^{51}Cr and reinfused into the

donor baboon. Daily blood samples were then fractionated into size-dependent platelet subpopulations. The rate of loss of ^{51}Cr was determined for each of the subpopulations. The incorporation of ^{51}Cr in each of the subpopulaptions was directly proportional to the MPV of each fraction. If platelets become smaller as they age, the radioactivity in the large platelet subpopulations should decrease more rapidly than the small platelet subpopulations, since as the labeled small platelets are removed they are replaced by the aging of labeled large platelets. As shown in Figure 2, radioactivity was lost from all fractions in a nearly linear manner. Recently, Blajchman et al[29] demonstrated that experimentally induced young platelets had survival curves that fit a linear function, while experimentally induced old platelets had survival curves that fit an exponential function. The observation that the survival curves of each of the subpopulations more closely approximates a linear than an exponential function as measured by the weighted mean survival times suggests that there were no major differences in the age of the size-dependent subpopulations.

In our study, when the survival times for ^{51}Cr platelets were determined for each of the subpopulations, there was a progressive increase in the survival time proportional to the MPV. To confirm our observed differences in survival time, in a recent study of 3 baboons, only the ^{51}Cr-labeled large platelets (fractions 6 and 7) or the ^{51}Cr-labeled small platelets (fractions 1 and 2) were reinfused (Thompson, C. B., unpublished data). There was no significant difference in the in vivo recovery of the transfused subpopulations. As measured by the

linear model, the survival time for the small platelets was 6.3 days and the survival time for the large platelets was 7.0 days. In this study, the transfused radioactivity was insufficient to allow analysis of platelet subpopulations.

One objection to studies involving chromium-labeled platelets is that the platelets must be isolated from the blood and labeled in vitro. If such in vitro damage altered the physical characteristics of the platelets, the chromium survival data may reflect injury during the isolation procedure. To test this possibility, we used a third method of labeling the platelets. Baboons were injected with ^{14}C-serotonin to label platelets in vivo by the selective uptake of serotonin. Heyssel has shown that approximately 10% of the serotonin will be incorporated selectively in the circulating platelets at 24 hours.[25] In this study 9.8 ± 1.3% (SD) of ^{14}C-serotonin was incorporated into baboon platelets. After 24 hours a significant correlation was observed between ^{14}C-serotonin incorporation and the MPV of the fraction (Figure 2) suggesting that in vivo uptake and storage of serotonin were dependent on platelet size. As with the ^{51}Cr data, all of the fractions lost ^{14}C-serotonin radioactivity at a similar rate. No translocation of radioactivity between fractions nor plateaus of radioactivity within a fraction were observed over time. In general, survival times showed that larger platelets had longer survival times.

Our data suggest that during steady-state thrombopoiesis, platelet size heterogeneity was the result of production factors in the bone marrow and not maturation in the circulation. Since platelet volume distribution

was log-normal, our data are consistent with the concept that platelet production involves two independent variables, megakaryocyte growth and demarcation as proposed by Paulus.[8]

Several parameters were correlated in our study with MPV, including ^{75}Se uptake by the platelet, LDH activity, dense body content, and platelet uptake of serotonin. Because platelets are anuclear cytoplasmic fragments with limited capacity to self maintenance and repair, it is not surprising that the larger platelets have a longer lifespan than the smaller ones. The observed differences in platelet lifespans among the different subpopulations probably reflects the greater potential of the larger platelets to cope with the microcirculatory environment.

Our data suggest that large platelets are not younger platelets as previously believed but rather platelets with a longer lifespan. Platelet size heterogeneity is the result of production factors in the bone marrow and not maturation in the circulation.

TABLE 1

BABOON PLATELET SUBPOPULATIONS

The Mean Platelet Volume, % of Recovered Platelets, Dense Body Content, and LDH Activity in the Original Platelet Suspension and in Each of the Fractions. Average Recovery of the Original Platelet Suspension was 95.60 ± 7.84% (Mean ± SD)

Platelet Fraction #	Mean Volume (u^3, n = 70)	% of Recovered Platelets (n = 70)	Dense Bodies (DB/Plt. (n = 5))	LDH Activity (IU/10^{10} PLT. (n = 5))
1+2	4.28 ± 0.46	13.94 ± 5.25	6.46 ± 2.58	6.30 ± 1.34
3	4.97 ± 0.59	18.13 ± 4.56	8.65 ± 2.81	7.52 ± 1.15
4	5.73 ± 0.62	25.47 ± 3.38	10.54 ± 2.10	9.19 ± 2.12
5	6.61 ± 0.62	23.26 ± 4.32	11.87 ± 2.17	10.19 ± 1.57
6+7	7.20 ± 0.68	18.72 ± 6.10	13.21 ± 3.24	12.18 ± 2.76
Original Platelet Suspension	6.08 ± 0.46	-------	11.22 ± 2.59	9.48 ± 3.10

TABLE 2

Radioactivity Per Platelet in Size-Dependent Subpopulations Over Time

A. Days Post ^{75}Se Injection[1]

Fraction #	1	3	5	7	9	11
1+2	9.6±2.8	72.9±12.9	86.9±4.2	71.6±6.9	50.5±4.6	33.1±1.1
3	8.6±4.1	80.6±6.3	106.8±5.8	92.3±8.9	66.7±6.3	42.0±1.8
4	9.2±9.1	93.5±5.2	122.8±7.3	113.3±11.7	78.9±13.4	52.3±0.9
5	12.6±12.3	103.5±7.0	136.8±6.8	122.4±3.2	98.8±8.3	63.1±7.6
6+7	21.3±9.0	117.8±3.1	155.3±8.4	148.5±17.2	116.7±13.3	73.4±6.7
Original Platelet Suspension	17.7±3.9	101.7±16.6	125.2±13.1	116.8±11.0	84.3±12.7	56.5±11.

B. Days Post ^{51}Cr-Labeled Platelet Transfusion[1]

Fraction #	0	1	2	4	7
1+2	1746±481	1261±303	996±300	304±144	39±36
3	2175±575	1730±522	1238±407	454±165	60±43
4	2386±685	1889±518	1487±477	676±203	86±56
5	2676±815	2222±803	1737±594	776±246	98±55
6+7	2806±825	2290±750	1869±536	852±290	145±62
Original Platelet Suspension	2512±925	2053±672	1457±478	599±199	94±40

C. Days Post ^{14}C Serotonin Injection[1]

Fraction #	1	2	4	7	9
1+2	434±136	348±48	186±65	67±14	45±12
3	549±122	440±71	271±59	97±17	63±12
4	639±139	504±91	305±51	117±15	71±18
5	741±117	599±83	390±76	154±9	90±20
6+7	747±204	647±87	414±93	159±13	88±18
Original Platelet	639±82	513±77	347±74	116±41	73±12

TABLE 3

Uptake and Survival Time of ^{75}Se-Methionine in Size-Dependent Platelet Subpopulations*

Fraction #	Initial Rate of ^{75}Se Incorporation ($cpm/u^3/day$)	Survival Time (Days)
1+2	5.89	6.02
3	5.71	6.34
4	5.80	6.56
5	5.50	6.66
6+7	5.75	7.09
Original Platelet Suspension	5.85	6.45

*Calculated from the averaged data in 3 baboons

TABLE 4

A. Chromium Survival (Days) of Size-Dependent Platelet Subpopulations*

Fraction #	Linear Function	Exponential Function	Weighted Mean Value
1+2	6.13	1.72	5.06
3	6.27	1.86	5.26
4	6.46	2.01	5.75
5	6.70	1.99	6.35
6+7	6.83	2.24	6.42
Original Platelet Suspension	6.42	2.04	5.34

B. ^{14}C-Serotonin Survival (Days) of Size-Dependent Platelet Subpopulations*

Fraction #	Linear Function	Expontential Function	Weighted Mean Value
1+2	6.89	2.00	5.79
3	7.16	2.17	6.55
4	7.16	2.32	5.43
5	7.42	2.51	6.75
6+7	7.56	2.56	7.37
Original Platelet Suspension	7.36	2.22	7.18

*Calculated from the averaged data of 3 baboons

FIGURE 1

^{75}Se-methionine incorporation and survival in original platelets prior to fractionation and in the 5 platelet fractions.

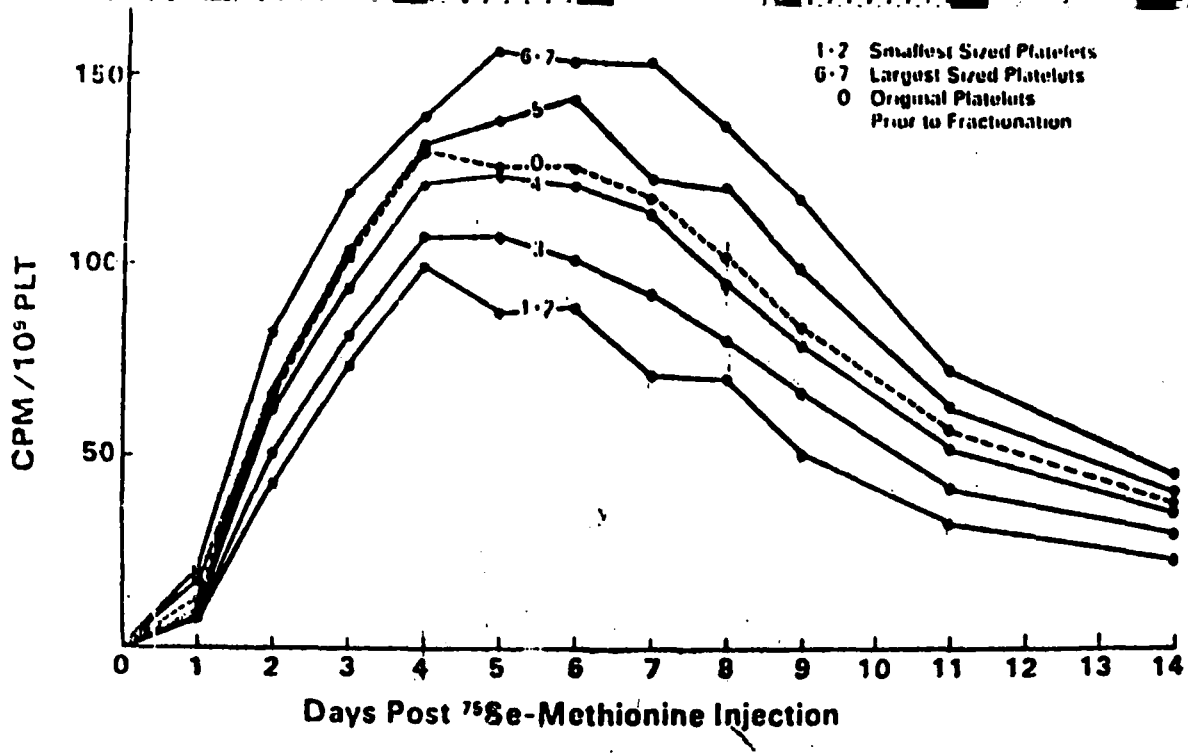

FIGURE 1 THOMPSON ET AL

FIGURE 2

Survival of ^{51}Cr-labeled platelets in the original platelet suspension and in the 5 platelet fractions.

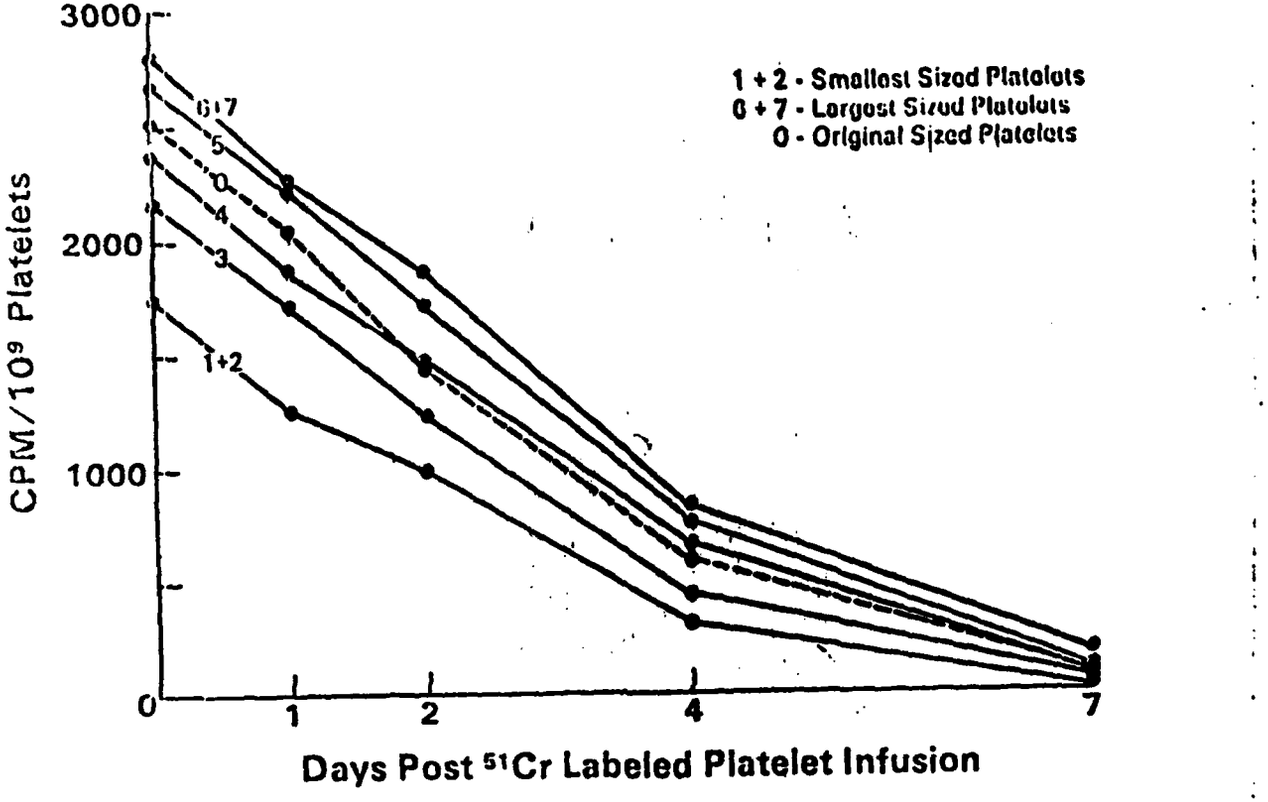

FIGURE 2 THOMPSON ET AL

FIGURE 3

^{14}C-serotonin in vivo platelet uptake and survival in the original platelet suspension prior to fractionation and in the 5 platelet fractions.

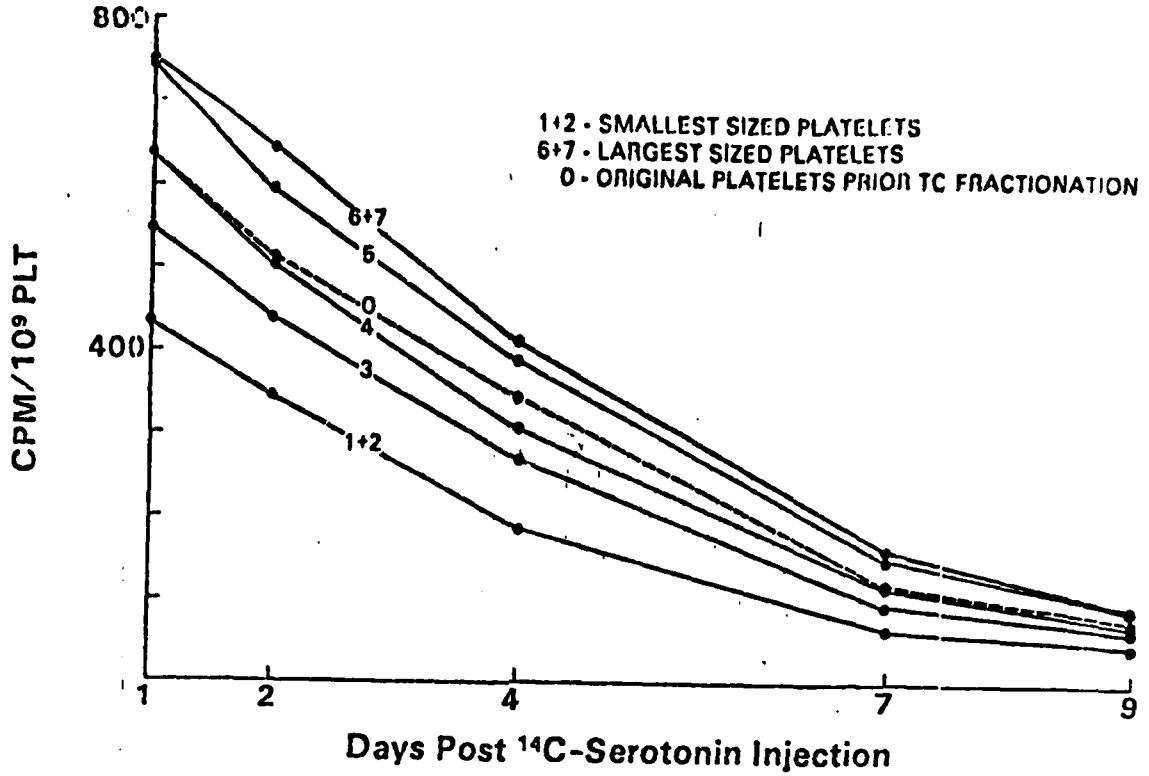

FIGURE 3 THOMPSON ET AL

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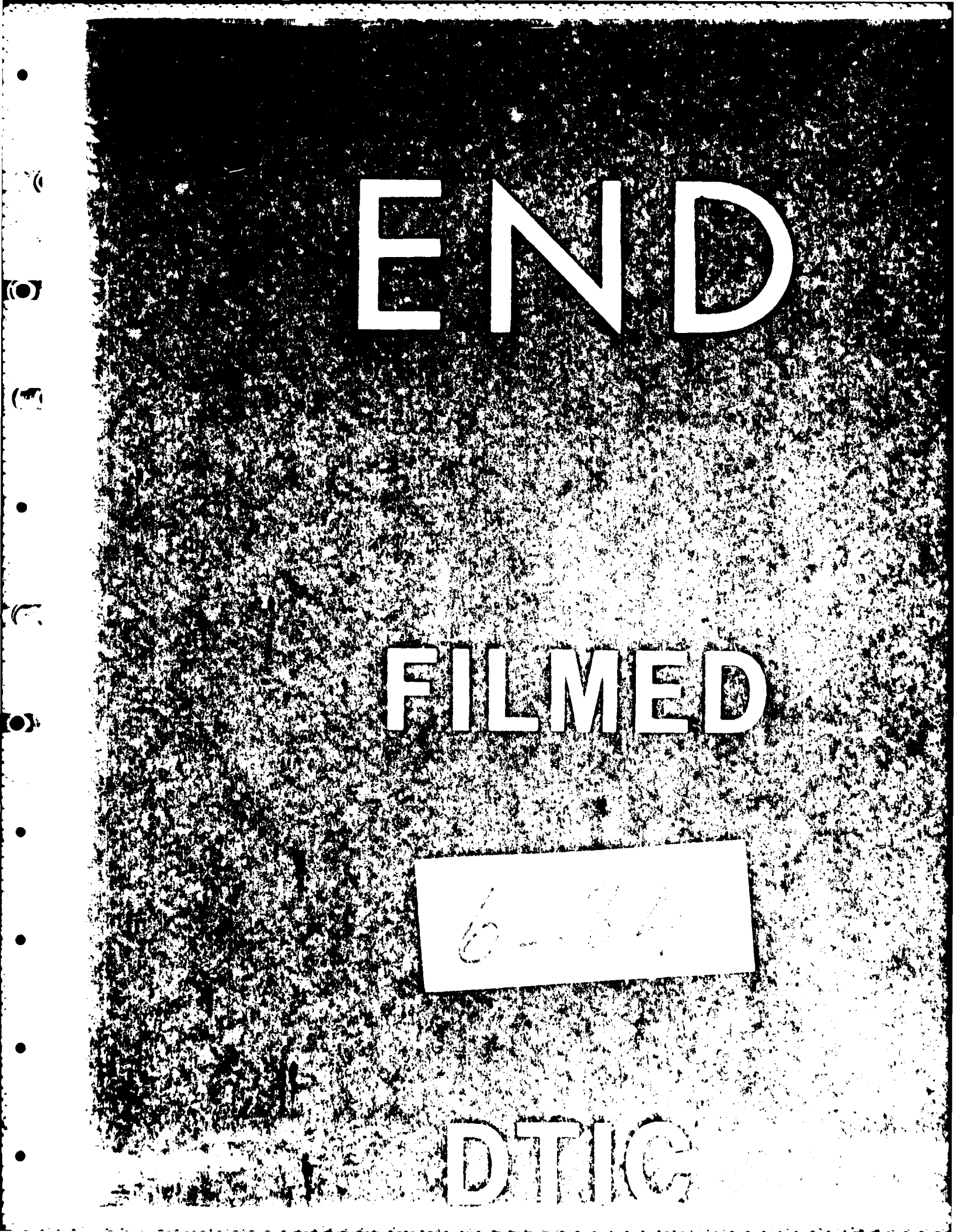
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