Heterogeneity of Human Whole Blood Platelet Subpopulations. II. Use of a Subhuman Primate Model to Analyze the Relationship Between Density and Platelet Age

By Laurence Corash, Brenda Shafer, and Mark Perlow

A subhuman primate model was developed to ascertain whether or not platelet heterogeneity could be explained by aging in the peripheral circulation. Density-dependent platelet cohorts, postulated to represent cells of different ages, were isolated on isosmolar arabinogalactan gradients and labeled with radiochromium. Mean platelet lifespan was measured for the different density cohorts, and simultaneous sequential density distribution analysis was performed to follow changes in cell density during aging. The average mean lifespan of light platelets was 74.6 hr, compared to 313.6 hr for heavy platelets. After injection, labeled light platelets were recovered only in the gradient light region, in contrast to labeled heavy platelets, which were initially restricted to the dense region and progressively migrated to the light region during their lifespan. This study supports the hypothesis that platelet age in unstressed primates correlates with cell density and provides a rationale for the use of "age-dependent" markers to estimate platelet turnover rates.

The origin of platelet heterogeneity remains a controversial issue. Two hypotheses have been proposed based upon experimental and clinical observations. Karpatkin and co-workers\textsuperscript{1,2} presented data to support the theory that platelets undergo an aging process in the peripheral circulation that gives rise to heterogeneity of platelet properties. In contrast, Pennington and co-workers\textsuperscript{3,4} Paulus\textsuperscript{5} and Boneu and co-workers\textsuperscript{6,7} concluded from their observations that the major determinant of platelet heterogeneity is megakaryocyte heterogeneity. Resolution of this controversy is important to an understanding of platelet heterogeneity, since if cellular heterogeneity is the result of aging, then the use of platelet "age-dependent" markers may be of diagnostic value to estimate platelet turnover rates in a variety of disease states.

Recent methodologic developments in this laboratory have made it possible to examine the relationship between buoyant density and platelet age and to determine if aging in the peripheral circulation correlates with changes in platelet properties.\textsuperscript{8} The rhesus monkey (\textit{Macaca mulatta}) was chosen as an experimental model because of its hematologic similarity to man.\textsuperscript{9} Density-dependent platelet cohorts were isolated on isosmolar arabinogalactan gradients, labeled with radiochromium, and injected into donor animals and their survival was measured. After introduction of a labeled density cohort into a recipient, total whole blood platelets were periodically isolated and fractionated into four...
density-dependent fractions to follow the density distribution of the injected cohort during its lifespan. The goal of the study was to answer two questions: (1) Do light platelets and heavy platelets have different survivals? (2) Is platelet density modified in the peripheral circulation?

**MATERIALS AND METHODS**

**Sample acquisition.** Three adult male rhesus monkeys weighing 6-8 kg were used. Animals were housed in a primate colony under standard conditions and were fed a routine diet. The only medication used during the study was ketamine hydrochloride 0.1 mg/kg (Bristol Laboratories, Syracuse, N.Y.) for anesthesia during blood drawing. Blood samples for labeling platelets and for sequential studies were obtained by venipuncture of the large saphenous veins with a 21-gauge butterfly needle (Abbott Hospitals, North Chicago, Ill.) after application of a tourniquet at thigh level.

**Platelet counts, white cell counts, and platelet volume distribution.** Visual whole blood platelet counts were performed by a standard technique. After removal of red cells, platelet counts were performed as previously described with a Particle Data (Elmhurst, Ill.) machine. Platelet volume distribution was measured with the same machine as previously described. White cells were counted using a Particle Data counter equipped with a 70-μm orifice and settings of current \( i \), gain 17, log 10, lower threshold 16.

**Platelet survival.** Prior to study, the blood volume of each animal was measured with radiochromated autologous red cells by a standard technique. The mean blood volume for these animals was 49.6 ± 1.4 ml/kg (SEM). Platelet survival tests were performed only when animals had reached background radioactivity levels. Platelet lifespan was measured on four types of preparations: total whole blood platelets (TWBP), heavy platelets (HP), light platelets (LP), and platelet-rich plasma platelets (PRP-P). The fractions were prepared as follows:

Whole blood (15 ml) was drawn into a plastic syringe (Sherwood Medical, Deland, Fla.) containing 2.5 ml sterile acid citrate dextrose (ACD) (0.085 M sodium citrate, 0.065 M citric acid, and 2% dextrose, adjusted to pH 6.5). TWBP were isolated under sterile conditions as follows: Anti-coagulated whole blood was divided into three 5.0-ml aliquots and transferred to sterile polypropylene tubes (No. 2059, Falcon Plastics, Oxnard, Calif.). Then 2 ml sterile buffered saline glucose citrate solution (BSG-citrate) pH 7.40 ± 0.05. 290 ± 1 mOsm/liter, was added to each tube. The tubes were centrifuged at 600 g for 3 min (20°C), and the supernatant PRP was removed with a plastic pipette without disturbance of the interface. The red cell pellet was restored to initial volume with sterile BSG-citrate, carefully mixed with gentle rotation, and centrifuged as before. The second supernatant fraction was removed and pooled with the first. The residual red cell pellet was restored to initial volume as before, and this washing process was repeated three more times. After the final wash, the combined supernatants were counted for platelet and leukocyte content. The final red cell pellet was restored to the initial volume, and a visual platelet count was performed. Based upon these counts, platelet recovery and the degree of leukocyte contamination were calculated. Platelet recovery averaged 98%, with less than 2% of the total platelets lost in the red cell pellet. Leukocyte contamination of the platelet fraction was minimal (less than 0.05 × 10⁷/liter). This modification of our earlier technique eliminated the problem of lymphocyte contamination, so that only platelets were labeled. Final platelet counts of the pooled fractions were approximately 60 × 10⁹/liter. This platelet preparation was designated as TWBP.

Total population platelet suspensions were adjusted to pH 6.5 with 0.15 M citric acid and incubated with 15 μCi of ⁵¹Cr sodium salt (Squibb, Princeton, N.J.) for 30 min at 20°C with occasional gentle shaking. Labeled platelets were concentrated on a 10”, 20”, two-step discontinuous arabinogalactan gradient as previously described to remove plasma proteins and non-platelet radioactivity. The labeled, protein-free platelets were resuspended in autologous platelet-poor plasma (PPP) adjusted to pH 6.5 and washed twice at 1500 gmax, 20°C, for 15 min in PPP to remove residual arabinogalactan. The washed platelets were suspended in autologous PPP for injection.

Density-dependent platelet cohorts were isolated from labeled TWBP on discontinuous arabinogalactan gradients designed to yield small cohorts containing either the lightest or heaviest
platelets. These extreme cohorts were constructed to contain 10^5-15^5, of the TWBP in order to provide adequate amounts of radioactivity to be followed in the recipient. The labeled density cohort was diluted with sterile BSG-citrate pH 6.5 and centrifuged at 1500 g for 15 min (20°C) to pellet the platelets and to remove the residual arabinogalactan. The platelets were gently resuspended in 5 ml autologous PPP that had been previously obtained from the animal and stored at -80°C.

PRP-P were prepared from 15 ml ACD-anticoagulated blood by a recommended standard technique with modification to accommodate the reduced volumes in a sterile tube system.12

Prior to injection into the donor, all labeled platelet fractions were submitted to size distribution analysis to evaluate the degree of platelet fragmentation, red and white cell contamination, and alteration of platelet modal volume. Contaminated suspensions or samples with platelet fragmentation greater than 5^5, of the area under the volume distribution curve were discarded. This was a rare occurrence. The labeled platelets were injected into a saphenous vein through a 21-gauge butterfly needle, followed by the platelet-depleted red cells obtained from the donor and a 10-ml flush of normal saline to clear the injection site. Thirty minutes after injection, a 5-ml blood sample was obtained from the opposite limb to determine recovery of the injected cohort. Each animal had platelet survival measured of the following types: PRP-P, LP, HP, and total platelet population. Animals were studied for each platelet preparation in rotation so that there was a rest period of 30 days between successive studies for each animal. During the course of the study, daily whole blood platelet counts and platelet volume distributions were measured to ensure that the subject remained in a steady state. Additionally, prior to study some animals had daily anesthesia with 0.5 ml blood sampling to determine if manipulation alone could alter the steady state. There was no evidence that this occurred.

Samples for determination of platelet radioactivity: Daily 4-ml venous blood samples were drawn into a plastic syringe containing 0.5 ml 130 mM sodium citrate and 10 mM EDTA, pH 6.76. A total platelet population free of plasma proteins, red cells, and white cells was separated as described above. The platelets were counted and the recovery computed. Prior studies had shown that high yields were routinely possible.8 The platelets were transferred to a plastic tube (No. 2063, Falcon) and centrifuged at 1500 g for 30 min to a tight pellet. The supernatant fraction was removed, leaving 1 ml above the pellet. The platelet pellets were counted in a gamma counter (Nuclear Chicago, Des Plaines, Ill.) with a wide window (base 120 MeV, window width 290 MeV) to 9^5, confidence limits. Platelet activity was expressed as dpm/10^9 platelets. Platelet survival was measured for 7 days.

On days 1, 3, and 7 after injection, an additional 6-ml blood sample was obtained for isolation of density-dependent subpopulations by the described method.8 A gradient was designed for each animal to give extreme heavy and light fractions of 15^5, and two middle fractions of about 30^5, each. Gradient recoveries were computed as before.8 Platelet subfractions were processed for radioactivity as above. Platelet specific activity was expressed as the fraction of radioactivity divided by the fraction of platelets associated with a density gradient zone. Although results were analyzed for all four gradient fractions, subsequent discussion of light and dense gradient zones refers to the two extremes, the 15^5, lightest and the 15^5, heaviest platelets.

Calculation of platelet recovery and platelet survival. Platelet recovery in the recipient was calculated using the 30-min postinjection blood sample and the measured blood volume. Platelet mean survival for each preparation was derived from a weighted survival estimated from the linear and logarithmic least-squares regression analyses as recently described.12 Platelet mean lifespan was expressed in hours. Differences in platelet survival among the different preparations was evaluated by use of a computerized t test to calculate the confidence interval.

RESULTS

Packed cell volume, reticulocyte count, white cell count, platelet count, and platelet mean volume remained stable for each animal during the course of study. There was no difference in the recovery of infused labeled platelets for the different preparations. Mean platelet recovery at 30 min for all populations was 72^5, ± 8.4^5, (SD).

Mean survival for the different preparations (Table 1) showed a slightly
Table 1. Weighted Mean Platelet Survival (hr)

<table>
<thead>
<tr>
<th>Subject</th>
<th>Total</th>
<th>PRP</th>
<th>Light</th>
<th>Heavy</th>
</tr>
</thead>
<tbody>
<tr>
<td>H-822</td>
<td>200.4</td>
<td>158.6</td>
<td>98.1</td>
<td>334.0</td>
</tr>
<tr>
<td>V-777</td>
<td>180.8</td>
<td>144.9</td>
<td>46.0</td>
<td>380.7</td>
</tr>
<tr>
<td>W-595</td>
<td>188.4</td>
<td>161.2</td>
<td>79.6</td>
<td>227.8</td>
</tr>
<tr>
<td>Mean ± SEM</td>
<td>189.9 ± 5.7</td>
<td>154.8 ± 5.0</td>
<td>74.6 ± 15.3</td>
<td>313.6 ± 45.3</td>
</tr>
</tbody>
</table>

*See text for full description of platelet populations.

improved survival of TWBP compared to PRP-P ($p < 0.01$). LP that consisted of the 10\(^{-}\)\%–15\(^{-}\)\% lightest platelets had a mean survival of 74.6 hr, compared to 313.6 hr for the heavy cohort. This difference was highly significant ($p < 0.005$). PRP-P that included approximately 75\(^{-}\)\% of the TWBP also had a significantly longer lifespan than the LP cohort, $p < 0.005$. The large SE (Table 1) for the LP and HP experiments represents variability due to the differing sizes of the labeled cohorts depending on how successful we were in developing a narrow but adequate density fraction for labeling studies. When a sufficiently narrow heavy cohort could be isolated, as in V-777, the mean survival was double the mean survival of the TWBP fraction, which would be expected because the latter preparation represented a total population with an average age, while theoretically the HP were newly released platelets.

When LP were isolated, labeled, and reinfused they were recovered at 15 hr primarily in the gradient light region (Fig. 1) with a high specific activity. There

![Image](https://via.placeholder.com/150)

**Fig. 1.** Distribution of light platelet cohort from animal H-822 with serial density analyses after platelet infusion. Open symbols, gradient lightest fraction; closed symbols, heaviest fraction. Platelet specific activity, as described in Materials and Methods, plotted against time (hr). Single representative sample shown for purposes of clarity; identical experiments were performed in each subject and gave similar results.
was no radioactivity associated with the dense platelet zone. By 87 hr LP radioactivity had decreased to one-fifth the initial activity, compatible with their short lifespan, and the dense-gradient region continued to remain nonradioactive. At 160 hr all radioactivity had disappeared from the gradient. The virtual complete absence of radioactivity in the dense zone confirms that LP do not become HP and strongly suggests that there was no spontaneous elution and nonspecific transfer of label from the labeled light cohort to the dense platelets.

In contrast, 15 hr after injection of HP the majority of cell-associated radioactivity was localized in the heavy-gradient region, while the light zone remained inactive (Fig. 2). Small amounts of radioactivity began to appear in the gradient light zone at 87 hr; by 160 hr a large part of the activity was in the light zone; and at 200 hr the dense platelets were completely nonradioactive and all activity was in the gradient light zone.

The specific activities and survivals of the two cohorts used in this experiment were different. The labeled cohort from V-777 constituted a larger part of the total platelet population and was examined for the first time at 40 hr, which explains its lower specific activity and shorter apparent survival. When platelet fractions of density intermediate between the two extreme fractions were followed, they showed the same general trend but were less dramatic because they represented a larger fraction of the total platelet population and thus the migration of label was less well defined than for the extreme heavy and light

![Graph](https://www.bloodjournal.org/content/73/3/756/F2.large.jpg)  
**Fig. 2.** Distribution of heavy platelet cohort with serial density analyses after platelet infusion in two animals, V-777 (triangular symbols) and W-595 (circles). Open symbols, lightest gradient fraction; closed symbols, heaviest gradient fraction. Platelet specific activity plotted against time (hr). Representative examples from two animals shown because first gradient analysis of V-777 was performed 39 hr after infusion.
Table 2. Platelet Density Distribution of Serial Gradients (%)

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Time After Labeled Platelet Infusion*</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>40 hr</td>
</tr>
<tr>
<td>1</td>
<td>12</td>
</tr>
<tr>
<td>2</td>
<td>40</td>
</tr>
<tr>
<td>3</td>
<td>32</td>
</tr>
<tr>
<td>4</td>
<td>16</td>
</tr>
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</table>

Percent of total platelets in a given fraction during serial density gradient analysis in animal V-777 with heavy cohort label. Fraction 1, least dense platelets; 4, most dense platelets; 2 and 3, intermediate density. This is a single representative example from one animal; other experiments gave analogous results.

*Time (hr) after labeled platelet infusion at which density distribution was performed.

fractions. The rise of radioactivity in the LP was not as great as the decline in HP activity because of distribution of labeled platelets in the two intermediate density fractions. The HP cohort, although narrow, did contain cells of differing ages; thus there was a spreading of the label among the less dense fractions, but the previously unlabeled light region became significantly radioactive as the cohort aged.

During the course of study, the animals remained in a steady state with respect to platelet mean volume and the density distribution (Table 2) of platelet subfractions. It was important to monitor this distribution, since excessive bleeding could result in a stimulation of thrombopoiesis and cause an influx of unlabeled HP. No such alteration occurred with any of the animals studied.

DISCUSSION

Earlier studies in our laboratory showed that human platelets are heterogeneous with respect to cell volume and organelle content and that these properties are density dependent.8 These studies also showed the necessity of total platelet isolation in order to avoid exclusion of platelet subsets that might result in experimental bias. The current modification of the earlier arabinogalactan method permitted the isolation of TWBP populations that are free of other cell classes as well as plasma proteins and yielded platelets structurally and functionally intact. In addition, platelet survival studies showed that arabinogalactan-purified platelets have a survival in vivo equal to or better than those of the standard PRP technique (see Table 1). Based upon these observations we felt that arabinogalactan-derived cohorts provided a suitable method to examine the relationship among platelet heterogeneity, aging, and density.

The present experiments were designed to examine the relationship between platelet density and cell age during steady-state thrombopoiesis. The rhesus monkey was chosen for study because adequate volumes of blood could be withdrawn without disturbing the steady state. This is supported by the observation that hematocrit, reticulocyte count, platelet count, and white cell count did not change during the study. We also observed that monkey LP have a smaller mean volume than HP, but the difference was not as great as for man or the rabbit. The data clearly showed that there is a difference in lifespan between LP and HP. The variability in survival among different animals for these two extreme-density fractions is due to differences in the range of plate-
let densities contained in a given cohort prepared for each experiment. Cohort size varied from approximately 8\% to 20\% of the total platelet population, depending on our ability to isolate as narrow a cohort as possible and yet to introduce sufficient label into the subject to permit serial studies. For each animal HP survival was always 2.5-3.0 times greater than LP survival. The lifespan of a total platelet population was consistently greater than that of PRP-P, probably because of the inclusion of HP usually excluded by the latter technique. Our values for rhesus monkey PRP platelet survival (6.45 ± 0.2 days) agree well with those reported by Button et al. (6.4 ± 0.70 days).9

Serial density gradient analyses after injection of either HP or LP cohorts showed that it is possible to obtain a cohort with high specific activity and to subsequently reisolate it in the recipient. When a LP cohort was labeled it remained in the light region of the gradient; during the course of study there was no transfer of label to dense platelets. Conversely, when radioactive HP were infused they are gradually replaced in the dense-gradient regions by non-labeled platelets, and the light region of the gradient, which was initially non-radioactive, became radioactive as the dense zone of the gradient became nonradioactive. The consistent unidirectional pattern for the migration of labeled platelets is further evidence against nonspecific label elution with inter-platelet transfer of radioactivity. Other workers have shown before that radiochromium is firmly attached and does not significantly elute from platelets.13

In summary, these experiments showed that in an unstressed subhuman primate, LP have a short survival and do not become more dense, while in contrast HP have a longer survival in vivo and become less dense with aging in the peripheral circulation. Recent studies by Greenberg et al. showed that heavy rabbit platelets separated with our system also have longer lifespans than light rabbit platelets.14 Karpatkin also showed a difference in survival between light and heavy rabbit platelets.15

Other studies from our laboratory have explored the relationship among platelet density, structure, and function in an attempt to correlate changes in these parameters with postulated platelet aging. Heavy human platelets are larger and contain more granules but have an equal number of mitochondria per platelet compared to LP. Although the quantity of mitochondria per cell does not vary with density, monoamine oxidase activity is twofold greater in HP than LP.16,17 Friedhoff et al. also recently showed a higher platelet monoamine oxidase activity content for HP.18 Additional experiments have shown that HP contain more dense bodies and more endogenous serotonin and have more active serotonin transport than LP.16 George et al. showed that modification of platelet membrane structure also occurred with aging in the peripheral circulation.19 In light of the present study, these observations suggest that in the unstressed state primate platelets do undergo structural and functional changes in the peripheral circulation that appear to correlate with aging. Furthermore, different subcellular organelles are differently affected by this process. These age-related changes could serve as a means to estimate mean platelet age of TWBP populations.

Our observations stand in contrast to those reported by Pennington and co-workers.34 They concluded that different megakaryocyte ploidy classes gave
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rise to platelet heterogeneity and reported that cohort labeling in vivo by 75Se-methionine did not show a density-related aging effect. However, these workers used platelets separated from whole blood on colloidal silica-PVP gradients without first removing leukocytes. We have observed that lymphocytes and monocytes have a similar density distribution to platelets and that without prior removal the platelet density gradient is significantly contaminated by these cells. A representative experiment in our laboratory with an arabinogalactan gradient showed that at densities comparable to those used by Pennington and co-workers the following distribution of mononuclear leukocytes resulted: 1.061 g/ml, 11.7%; 1.065 g/ml, 18.5%; 1.070 g/ml, 33.8%; 1.074 g/ml, 27.1%; and >1.074 g/ml, 9.0%. (The percentages refer to the fraction of the total lymphocyte-monocyte population that could be recovered in high yield and that sedimented in the indicated fraction.) Use in vivo of 75Se-methionine resulted in the labeling of leukocytes as well as megakaryocytes, and thus lymphocyte contamination of a platelet gradient would seriously obscure a primary platelet effect. Moreover, Pennington and co-workers reported labeling of platelets in vitro with 75Se-methionine after 7 hr incubation, but Evatt and Levin had previously shown that PRP-P did not incorporate the isotope after up to 24 hr of incubation. It seems possible that the “platelet labeling” observed in vitro by Pennington and co-workers was also due to leukocyte contamination. In the face of these technique problems, we conclude that the data of Pennington and co-workers are not sufficient to negate the platelet aging observations of Charnatz and Karpatkin and those of the present studies. As we had noted earlier, the experiments reported by Boneu et al. were performed using PRP and sucrose gradients. In view of the loss of HP from PRP and the hyperosmolar effects of sucrose, we feel that these results are open to question. Busch and Olsen also reported a lack of platelet density change with aging, but they used PRP-P, which also may have excluded an important HP subset.

While the present studies show the aging in vivo of platelets in the peripheral circulation, they do not exclude the hypothesis that changes in thrombocytopoiesis may affect the initial distribution of platelet properties. For example, we observed increased mean volume in megaloblastic states where platelet survival is near normal. Furthermore, the current studies were performed in an unstressed system and may not hold true for the stressed state. We propose the hypothesis that both megakaryocyte growth and aging in the peripheral circulation contribute to platelet heterogeneity. Alterations in megakaryocyte maturation may affect the primary characteristics of newly released platelets, which then are further modified by aging events. The rhesus model may provide a means to study this complex interaction during stressed thrombocytopoiesis.

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