Use of Asplenic Rabbits to Demonstrate That Platelet Age and Density Are Related

By Laurence Corash and Brenda Shafer

An experimental model using asplenic rabbits injected with radioactive amino acids has been developed to examine the density distribution of newly released platelets. Platelets from asplenic rabbits had a larger mean volume and greater protein content than those from eusplenic animals. Radio-label, indicative of new platelets, is preferentially incorporated into the most dense platelets during the early postinjection period. Platelets of intermediate density also demonstrated augmented early label incorporation compared to the lightest cells. In contrast, during the late postinjection phase, there is preferential labeling of the least dense platelets. The specific radioactivity of heavy and light platelets rises to approximately equal levels, and falls abruptly, but the peak activities occur at distinctly separate times consistent with the concept of platelet density modification during platelet aging. Although platelet biologic properties are broadly distributed, these studies support the concept that on the average young platelets have an increased density and become less dense as they circulate.

The biologic significance of platelet heterogeneity remains controversial. Conflicting interpretations as to its cause have been reported by two laboratories using a similar experimental model: the incorporation of radioactive amino acids into newly released platelets. Karpatkin observed that in humans and rabbits the radioactivity was initially preferentially incorporated into heavy platelets and that subsequently there was some late enhancement of labeling in light platelets. In contrast, Pennington et al., using rats, found only minimally enriched labeling of the most dense cells and equal initial label incorporation into all other density subclasses with no apparent late transfer of label to light platelets. These conflicting observations have given rise to two theories concerning platelet heterogeneity: (1) platelet aging with loss of platelet density occurs in the peripheral circulation, or alternatively, (2) different megakaryocyte ploidy classes release platelets of varying densities that are not altered in the circulation.

Previous experience with platelet isolation in our laboratory suggested that a variety of imperfections with this model could contribute to these conflicting observations. These are as follows: (1) contamination of platelets with plasma proteins, (2) incomplete platelet recovery from whole blood, (3) cross-contamination of platelets by mononuclear leukocyte cells, (4) disturbance of thrombopoietic equilibrium, and (5) the effect of a splenic platelet pool. The last point is of particular interest as the spleen not only contains one-third of the platelet pool but may also preferentially retain the youngest and hemostatically most effective platelets.

In order to more critically evaluate the issue of platelet heterogeneity in view of these technical problems, we have modified the intrinsic label cohort model with the use of arabinogalactan platelet isolation after the administration of labeled amino acids to asplenic rabbits. This permits complete recovery of a total platelet population without plasma protein or mononuclear leukocyte contamination. The present experiments were designed to address two questions: (1) Are newly released platelets heavier platelets? (2) Is there accumulation of label in light platelets late in the time course consistent with the hypothesis that platelets become lighter with aging in the peripheral circulation?

Materials and Methods

New Zealand white rabbits weighing 2-4 kg were surgically splenectomized under general anesthesia. An equal number of rabbits acquired at the same time were anesthetized but not splenectomized and were fed and housed identically to the splenectomized rabbits. Both groups of rabbits were maintained for 1 yr after either surgery or anesthesia alone before the labeling studies were performed. To verify the effect of splenectomy, peripheral smears were examined for the presence of Howell-Jolly bodies and freshly prepared wet mounts of red cells were examined under Nomarski optics to look for surface pocks.

Blood samples from both animal groups were drawn from the central ear artery into 21-gauge or 23-gauge scalp vein needles after sedation with Ketamine and Acepromazine. Anesthesia was necessary because each animal had to be studied repeatedly and without anesthesia there was excessive rabbit morbidity and mortality. Blood samples were drawn into an anticoagulant consisting of EDTA (1%), PGE, (1.1 μg/ml), and theophylline (5.4 mg/ml). Baseline and follow-up white count, hemoglobin concentration, platelet count, and platelet mean volume were determined as previously described.

Total platelet populations and density-dependent subpopulations were isolated free from plasma proteins and other cell classes on arabinogalactan gradients.

Platelet subpopulations were isolated on discontinuous gradients at the following densities: 1.059 g/ml, 1.063 g/ml, 1.067 g/ml, and...
1.082 g/ml. Lymphocytes and monocytes were isolated from the platelet-depleted red cell pellets. After complete platelet removal, the red cells, containing all the leukocytes, were passed over a 20% arabinogalactan cushion. Total complete lymphocyte and monocyte populations were isolated at the 20% arabinogalactan interface. Residual neutrophils resedimented with the red cells.

Tritiated L-amino acid mixture (New England Nuclear, Boston, Mass.) was diluted in normal saline and injected via the marginal ear vein (15 μCi/kg) followed by a flush dose of normal saline. A 5-ml blood sample (designated as day 1), was obtained 12 hr later and then daily for 5–8 days. Hemoglobin, white count, and platelet count were determined daily. Density-dependent platelet cohorts were isolated daily and assayed for radioactivity and platelet protein content. Platelets for radioactivity measurement were prepared as follows: the total platelet population was passed over 2 successive 10%–20% arabinogalactan density gradients to remove plasma proteins. Density-dependent platelet subpopulations were obtained as described above and diluted with isosmolar buffered saline glucose solution. A sample was taken for platelet volume, cell count, and protein content. The remainder was sedimented at 30,000 g for 10 min into a compact pellet. The supernatant fraction was removed without disturbing the pellet, which was then allowed to dry. Deionized water (500 μl) was added to the dry pellet, which was sonicated twice with a micro probe cell disrupter (Kontes, Vineland, N.J.) for 10 sec. A second 500-μl aliquot of deionized water was added and the sonication repeated. The solubilized pellet solution (1 ml) was transferred to a scintillation vial and dissolved in 10 ml of Aquasol (New England Nuclear, Boston, Mass.). Samples were cooled overnight in the dark and counted to 99% confidence limits with correction for quench. Radioactivity was expressed as dpm per μg/mL.

Platelet mean volume was measured using a Particle Data apparatus equipped with a logarithmic amplifier. Platelet volume distributions were fitted to a Gaussian model, corrected for noise, and mean platelet volume was derived from probability plot analysis. Statistical analysis of subpopulation properties was performed by either two-tailed paired or unpaired t test as indicated.

RESULTS

Baseline Hematologic Values

There was no significant difference in hemoglobin concentration, white count, or platelet count between eusplenic and asplenic rabbits (Table 1). Inspection of peripheral smears from asplenic rabbits demonstrated the presence of Howell-Jolly bodies and visualization of wet preparations under Nomarski optics revealed increased red cell capping compared to eusplenic rabbits. There was a significant difference in total platelet population mean cell volume between the two groups (p < 0.005, unpaired t test).

Platelet density distributions were adjusted by gradient density modification to provide narrow cohorts for the lightest and the heaviest fractions. There was a tendency for asplenic rabbits to exhibit a slight shift toward a more dense platelet distribution (Table 2), but this did not achieve significance. Recovery of platelets from the gradients averaged better than 95%, as in previous studies.

Measurement of mean platelet volume and platelet protein demonstrated a consistent significant size difference between heavy (4s) and light (1s) platelets for both asplenic and eusplenic animals (p < 0.001, paired data), and the platelets from asplenic animals were consistently larger than those of eusplenic subjects for each density fraction (p < 0.001, unpaired, Table 2). This is consistent with the previous observation, independently ascertained, that the mean platelet volume of a total platelet population from asplenic rabbits is greater than that for eusplenic animals. The validity of this difference is further supported by the fact that the total platelet population volumes (Table 1) lie close to the midpoint of the gradient fraction measurements for each animal group. Platelets from asplenic animals for a given density subgroup contained significantly more protein than analogous eusplenic platelets (p < 0.001, unpaired), and similar to the differences in cell size, there were consistent differences in protein content for each subfraction in both groups (p < 0.001, paired, Table 2). When normalized for cell volume, the average protein content for all the density classes is approximately similar; thus, the increased protein is a reflection of increased platelet size.

Table 1. Baseline Hematologic Data

<table>
<thead>
<tr>
<th>Subpopulation</th>
<th>Platelet Mean Volume (μm³)</th>
<th>Platelets (10¹²/μl)</th>
<th>WBC (10³/μl)</th>
<th>Hb (g/dl)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eusplenic rabbits</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>rabbits</td>
<td>4.7 ± .2</td>
<td>446 ± 47</td>
<td>6.1 ± .8</td>
<td>12.8 ± .6</td>
<td>5</td>
</tr>
<tr>
<td>Asplenic rabbits</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rabbits</td>
<td>6.1 ± .3</td>
<td>326 ± 21</td>
<td>5.7 ± .5</td>
<td>12.5 ± .2</td>
<td>4</td>
</tr>
</tbody>
</table>

n, Number of animals.

*Mean ± SEM, each animal was sampled twice at different times and the results were averaged.
Release of Newly Formed Platelets in Asplenic Rabbits

Following injection of labeled amino acids, a newly released total platelet population rapidly appears in the circulation (Fig. 1A), rises to a well-defined peak by day 2 to day 3, and then rapidly declines. This pattern for total platelet populations is similar for asplenic and eusplenic animals.

Comparison of the specific activity for the heaviest (4s) and the lightest (1s) platelets in asplenic animals demonstrates a consistent preferential early appearance of label in the 4s fraction with increased specific activity on the first 2 days (Fig. 1B, C, D). This activity peaks earlier and begins to decline rapidly, while that of the lightest (1s) platelets continues to rise peaking on days 3 and 4. The peak activities for these two platelet fractions are roughly equivalent in each animal but distinctly separate temporally.

This pattern is not limited to the two most extreme density classes. Comparison of the specific activity time course for platelets of intermediate density (3s, 2s) with the lightest platelets (1s) demonstrates a similar pattern to that for the heaviest platelets (4s), but as the platelet densities become closer, the activity differences become less; for example, the 2s subclass, which is very close in density to the 1s fraction, exhibits only slightly augmented early activity (Fig. 2).

Expression of the specific activities per protein content as a ratio between the heaviest and lightest platelets (4s:1s) clearly reveals the predominance of label in the 4s platelets on days 1 and 2 (Fig. 3) and predominance of label in the lightest platelets (1s) on days 4 and 5. A similar preferential pattern of label incorporation into the 4s platelets from eusplenic rabbits also occurred, but the differences were not as marked as for the asplenic animals. Of additional note is the upturn of the ratio curves late in the experiment course with a repeat predominance of heavy over light platelet activity. The total absolute amount of radioactivity was less than 10% of the initial peak activities. This pattern could be due to either a small amount of label reutilization or very late appearance of label previously incorporated into stem cells.

The necessity of complete plasma protein and mononuclear cell removal is clear from measuring the specific activity of label incorporated into these two compartments (Fig. 4). Theoretical calculation of the amount of radioactivity contained per microgram of protein in the plasma proteins and the platelet proteins from 5 ml of whole blood shows a ratio of specific activity for plasma:platelet of 750:1. Lymphocytes and monocytes have a density similar to that for platelets and if not completely removed in the present experi-
Hematopoietic Changes During the Experiment

Under ideal conditions, the hemoglobin concentration and platelet count should remain constant throughout the five days of postinjection blood sampling. Although the largest available animals were used, there was a significant decline in hemoglobin among both animal groups (Fig. 5). During the course of the study the platelet count rose in all animals, but exceeded the baseline platelet count by 2 standard deviations (data not shown) in only one animal. Because of the changes in platelet count we monitored mean platelet volume daily throughout the study (Fig. 5) to determine if a significant number of larger "stress" platelets were being released, but there was no significant change. In order to determine whether excessive blood loss with a secondary stimulation of thrombopoiesis could account for the preceding results, one eusplenic and one asplenic animal was studied only on days 1 and 4 postlabel injection to minimize the amount of blood loss. A similar pattern (Table 3) of heavy (4s) and light (1s) platelet label incorporation as found with more extensively sampling (Fig. 3) was still observed in both animal types with this limited bleeding schedule.

DISCUSSION

The intrinsic platelet labeling studies reported by Karpatkin and Pennington have led to divergent interpretations about the origins of platelet heterogeneity. Subsequently, Pennington pointed out that three major criteria necessary to support a postulated rela-

Table 3. Release of Labeled Platelets in Rabbits With Limited Sampling

<table>
<thead>
<tr>
<th></th>
<th>1H Ratio H:L (Specific Activity)</th>
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<tbody>
<tr>
<td>Eusplenic rabbit</td>
<td></td>
</tr>
<tr>
<td>Day 1</td>
<td>7.80</td>
</tr>
<tr>
<td>Day 4</td>
<td>0.70</td>
</tr>
<tr>
<td>Asplenic rabbit</td>
<td></td>
</tr>
<tr>
<td>Day 1</td>
<td>4.30</td>
</tr>
<tr>
<td>Day 4</td>
<td>0.25</td>
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H, 4s platelet fraction; L, 1s platelet fraction. One animal in each group was studied to evaluate the effect of blood loss.
tionship between platelet density and age had not been observed in their experiments: (1) delay in the incorporation of label into light platelets, (2) a rise in the proportion of labeled light platelets late in the postinjection phase, and (3) temporal separation of label incorporation into intermediate density platelets as compared to light platelets.

We believe that the present experiments with the current technical modifications meet the previous criteria of Pennington. There was substantial preferential early incorporation of label into heavy platelets compared to light platelets with a distinct temporal separation in peak activities by 1–2 days in each case. The ratio of heavy to light platelet activity was consistently greater than 2 for the first 2 days of the experiment, which is highly significant, since the normal rabbit platelet lifespan is only 4–5 days. In each animal, light platelet-specific activity demonstrated a persistent late rise 1–2 days after heavy platelet peak activity, and light platelets were eventually twice as radioactive as heavy platelets. Furthermore, the difference in specific radioactivity was not limited to the most extreme density classes. Comparison of the intermediate density classes with the next most dense or the next least dense shows a sequential pattern of preferential early label appearance in all platelet classes of increased density compared to the next lightest class.

In the present study, labeled platelets within each density subclass rose sharply to peak activity and then declined sharply. This pattern is consistent with the migration of label from one density subgroup to another as opposed to the appearance and persistence of label within a given density cohort. The sharp temporal separation of the peak activities for the two extreme density classes does not appear to be consistent with a scheme of staggered platelet release from different megakaryocyte ploidy classes, as the latter would be expected to be more homogeneous due to the variation in precursor platelet release at any one time. The observation that there is some overlap between the labeling of the subclasses may be due to the wide distribution of platelet densities from 1.059 g/ml to 1.082 g/ml. The distribution of platelet densities similar to that of size is most likely the result of a series of overlapping normal density subdistributions in which the mean density of the very youngest platelets is greater than that of the very oldest, but in which there is substantial overlap in the middle ranges.

Unlike the results of Pennington et al., our specific activities were expressed as dpm per microgram of platelet protein so that differences in cell size alone would not account for the findings. The ratio of protein content per cell for heavy to light platelets was only 1.17, thus, protein content does not explain the differences in activity.

Earlier experiments from our laboratory for human platelets had shown that while the most dense platelets do contain some endoplasmic reticulum, platelets of intermediate density contain relatively infrequent rough endoplasmic reticulum. Thus, the next to most dense platelets could not account for their greater label incorporation compared to the lightest platelets by virtue of more ribosomal complexes, but must instead have incorporated label at an earlier state. Similarly, the least dense platelets, which have a late peak in radioactivity equal to that of the most dense platelets, contain little or no endoplasmic reticulum and their label accumulation must be due to migration of earlier labeled dense platelets into the light fractions. Moreover, in two of the three asplenic animals studied, light platelet peak specific radioactivity was actually greater than heavy platelet activity and in no case was it less, thus not consistent with the idea that light platelets have less active protein synthesis to account for their lower specific activity.

The present experiments also suggest an effect of the splenic platelet pool on total platelet population size distribution. One year postoperative eusplenic and asplenic animals had similar platelet counts but significant differences in mean platelet volume and platelet protein content. Comparison of the labeling pattern for an eusplenic and an asplenic animal showed that in animals with intact spleens, the temporal separation of labeling was not as distinct for heavy and light platelets. We postulate that this is due to the effect of the splenic pool and, as previously suggested, young platelets may first go to that pool and then reenter the circulation slowly. This process may possibly account for the equivalent labeling ratios observed between heavy and light platelets using animals with intact spleens.

Concern over thrombopoietic stimulation during the course of the study as a possible explanation for the results led to performance of the limited sample experiments that showed the same time sequence for density-dependent label incorporation as did the multiple sample experiments. Moreover, although the hemoglobin level fell and platelet count tended to rise during the 8-day study period, there was no significant change in mean platelet volume, thus indicating no excessive release of newly formed platelets or stress-induced platelets.

Additional supportive evidence that platelet age and density are related also comes from the extrinsic label experiments reported by our laboratory and that of Greenberg et al. In both rhesus monkeys and rabbits
it was shown that heavy platelets had a greater survival than light platelets and that in the rhesus monkey model there was in vivo label migration from the most dense platelet subclass to the least dense subclass during the course of the experiments.  

We interpret the results of the present experiments and those cited above to support the concept that under nonstressed conditions there is an aging effect on platelets in the peripheral circulation. However, this is not the sole determinant of platelet heterogeneity. Undoubtedly, events during megakaryocyte growth and platelet release also have an effect on platelet properties. Platelet characteristics are not narrowly distributed within any one density subclass, but consistent differences in platelet mean volume, mean protein content, dense body number, and serotonin content can be found among the different density classes.  

Platelet density is probably normally distributed, and the different density subclasses may represent overlapping progressive normal distributions of various platelet properties. Various pathologic states may alter platelet density and distort the usual relationship between platelet age and density, but comparison with the normal pattern could help to elucidate the pathophysiology of these disorders.

REFERENCES

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