Regulation of Thrombopoiesis: Effects of the Degree of Thrombocytopenia on Megakaryocyte Ploidy and Platelet Volume

By Laurence Corash, Huei Yin Chen, Jack Levin, Georgiann Baker, Helen Lu, and Yuen Mok

We have established a murine model and techniques with which to serially study thrombocytopenia after induction of experimental immune thrombocytopenia of variable severity and duration. Bone marrow megakaryocyte ploidy distribution was determined by using unfraccionated bone marrow, a polyclonal megakaryocyte-specific probe, and two-color fluorescence-activated flow cytometry. With these techniques, the modal megakaryocyte ploidy class in normal murine bone marrow was 16N. Serial studies of bone marrow megakaryocyte ploidy after the induction of acute, severe thrombocytopenia (platelet count, <0.05 x 10^9/L) demonstrated no detectable change in the ploidy distribution at 12, 24, and 36 hours after the onset of thrombocytopenia. At 48 hours, the modal ploidy class shifted from 16N to 32N, and the 64N class increased significantly (P < .001). The ploidy distribution returned to normal 120 hours after the onset of thrombocytopenia. A lesser degree of thrombocytopenia (platelet count reduction to 0.100 to 0.200 x 10^9/L) delayed the modal ploidy class shift from 16N to 32N until 72 hours after the onset of thrombocytopenia. Chronic, severe thrombocytopenia (platelet count, <0.05 x 10^9/L for seven days) resulted in a modal ploidy class shift from 16N to 32N during the thrombocytopenic phase and an enhanced increase in the 64N megakaryocyte class during the recovery phase. Mean platelet volume (MPV) was simultaneously measured on isolated total platelet populations after induction of thrombocytopenia. MPV was significantly increased (P < .001) as early as eight hours after the onset of acute, severe thrombocytopenia, 40 hours before a shift in the ploidy distribution. Mild thrombocytopenia (platelet count reduction to 0.400 x 10^9/L) was not associated with a ploidy shift but did result in a significantly increased MPV (P < .001). These studies demonstrate that the temporal relationship and magnitude of the effects of thrombocytopenia upon megakaryocyte ploidy distribution are dependent upon the degree and the duration of the thrombocytopenic stimulus and that the effects of experimental thrombocytopenia on platelet volume and megakaryocyte ploidy are dissociated.

THE MECHANISMS that regulate thrombopoiesis remain incompletely understood. Previous studies suggest that experimental, acute thrombocytopenia results in alterations of platelet volume, megakaryocyte cytoplasmic volume, stages of megakaryocyte maturation, megakaryocyte ploidy distribution, and megakaryocyte colony-forming cells (Meg-CFC). While these studies indicate that thrombopoiesis is responsive to fluctuations in the circulating platelet mass, considerable controversy persists concerning many aspects of thrombopoiesis, including the normal megakaryocyte ploidy distribution, the determinants of platelet volume, and the response of Meg-CFC to thrombocytopenia. A majority of studies of thrombopoietic regulation have primarily focused on the effects of severe thrombocytopenia and, with two exceptions, have not examined the effects of lesser degrees of thrombopoietic perturbation. Furthermore, relatively few studies have extensively characterized the temporal sequence and duration of the response of megakaryocytes or their precursors to acute or chronic perturbation of the circulating platelet mass. In addition, only one study has simultaneously examined the changes in megakaryocytes and circulating platelets in a serial fashion throughout the entire period of altered thrombopoiesis.

Previous efforts have been hampered by the lack of a suitable experimental model and techniques with which to measure the effects of perturbed thrombopoiesis on all three cellular compartments of thrombopoiesis: circulating platelets, recognizable megakaryocytes, and morphologically unrecognizable precursor cells. Advances in platelet isolation, analysis of megakaryocyte DNA content, and cultures that support the growth of megakaryocyte colonies from colony-forming cells now make more feasible a unified approach to the study of thrombopoiesis. We have established a murine model and techniques to extend the study of the regulation of thrombopoiesis. Previous reports by one of us have described the extended effects of acute and chronic, severe thrombocytopenia on Meg-CFC. The present report describes our use of a murine model to serially examine the effects of immune mediated thrombocytopenia of variable severity and duration on the ploidy distribution of bone marrow megakaryocytes and on the mean platelet volume (MPV) of circulating platelets.

MATERIALS AND METHODS

General techniques. C57/BL mice, with intact spleens, were used for all studies. Thrombocytopenia was induced by the intraperitoneal injection of guinea pig antirat platelet serum (GP-PAS) that had been adsorbed against erythrocytes and leukocytes as previously described. Variable levels of thrombocytopenia were produced by adjusting the administered dose of GP-PAS; the level of thrombocytopenia was defined by the platelet count 12 or 24 hours after the onset of thrombocytopenia.
after administration of GP-PAS. Due to interindividual variation in response to GP-PAS, cohorts of animals with a uniform degree of thrombocytopenia were produced by the following procedure. A large group of animals were injected with a dose of GP-PAS calculated to produce the desired level of thrombocytopenia based upon prior experience. At 12 or 24 hours after antisera injection, platelet counts were performed on blood samples obtained from the retroorbital venous plexus, and collected into EDTA- and heparin-coated capillary tubes (Drummond Scientific Co. Broomall, PA). Platelet counts were performed by using a described technique\(^2\) with an electronic particle counter (Coulter Electronics, Hialeah, FL). The animals were grouped for further studies according to their platelet levels.

**Collection of bone marrow cells.** At designated time points, mice were sacrificed by cervical dislocation, and the bone marrow was flushed from the femurs and humeri with a small volume of CATCH medium\(^4\) supplemented with sodium citrate (0.38%), prostaglandin \(E\)\(_2\) (PGE\(_2\)) (1.0 \(\mu\)g/mL), horse serum (5%), andDNase (30.0 Kunitz units/mL) into a larger volume of the same supplemented medium. Bone marrow was harvested and pooled from two animals for each flow cytometric analysis. A total nucleated cell count was determined by counting the diluted cell suspension with a Coulter S Plus IV particle counter (Coulter). After counting, the cells were divided into two aliquots containing 2 to 4 \(\times 10^7\) cells each in 2 to 5 mL of supplemented CATCH medium. The cells were centrifuged at either 70 or 350 \(g\) for five minutes at 23\(^\circ\)C, the supernatant fraction was aspirated, and the cells were resuspended in 100 \(\mu\)L of supplemented CATCH medium.

**Identification of megakaryocytes and measurement of megakaryocyte DNA.** Cells were defined as megakaryocytes and electronically selected from the total nucleated bone marrow cell population by a modification of the two-color fluorescence-activated flow cytometry technique originally described by Jackson and coworkers.\(^9\) Megakaryocytes were identified by labeling with a cell class-specific probe directed against multiple surface constituents. The probe used in these experiments consisted of a high-titer, polyclonal rabbit antimeplatelet serum (R-PAS) that had been adsorbed against mouse erythrocytes and leukocytes.

The potency and specificity of this reagent was evaluated by the following methods. First, the antisera was assayed in a fluorescence-activated flow assay to determine the potency of binding to isolated mouse platelets and to establish the maximum dilution sufficient to produce adequate platelet labeling. This assay technique is based on our described method for the measurement of platelet-associated immunoglobulin.\(^3\) Serial dilutions of the R-PAS were incubated with 5 \(\times 10^6\) mouse platelets, and the binding of antibody was detected by using a second-step reagent consisting of biotin-conjugated goat anti rabbit immunoglobulin Fab fragment (GAR-Fab 2') (Dako Laboratories, Santa Barbara, CA) directed against all major heavy chain classes. The fluorescence detection system consisted of avidin-conjugated fluorescein. Labeled cells were analyzed in a Becton Dickinson Research Analyzer (Becton Dickinson, Mountain View, CA) as described.\(^4\)

The specificity of the R-PAS reagent was evaluated by an immunoblot assay with solubilized murine platelet proteins as the target antigens by utilizing our recently described method for human platelets.\(^2\) The cell class specificity of the R-PAS reagent for murine megakaryocytes was also evaluated by examining the binding of R-PAS to murine bone marrow cells by light microscopy with immunocytochemical techniques.\(^2\) Using these assay systems, we determined that a 1:4,000 dilution of the R-PAS in phosphate-buffered saline (pH 7.4) produced maximal platelet surface labeling. The immunoblot analyses demonstrated that the R-PAS reagent was active against multiple murine platelet proteins ranging from less than 45 kilodaltons (kd) to more than 300 kd, the resolving limits of

the gel system. The most intense labeling occurred against the region of the gel that contained the platelet glycoprotein Ib/IIa complex. Microscopy with immunoperoxidase-labeled reagents demonstrated no reactivity of the R-PAS against nonmegakaryocytic bone marrow cells, whereas megakaryocytes of all three maturational stages were labeled by the polyclonal R-PAS.\(^4\)

Based upon the preceding observations, the following conditions were used for labeling of megakaryocytes in the two-color fluorescence-DNA assay. An aliquot containing 2 to 4 \(\times 10^7\) washed bone marrow cells was incubated in 900 \(\mu\)L of a 1:4,000 dilution of R-PAS for 60 minutes at 4\(^\circ\)C. An identical aliquot of bone marrow cells was incubated with a 1:4,000 dilution of nonimmune, normal rabbit serum (NRS) to serve as a negatively stained control sample. From this point, the NRS control and the R-PAS-labeled aliquots were treated similarly. The cells, which had been incubated with either R-PAS or NRS, were washed three times with cold CATCH medium supplemented with 0.38% citrate and PGE\(_2\) (1.0 \(\mu\)g/mL) (CATCH-PGE-citrate), suspended in 100 \(\mu\)L of CATCH-PGE-citrate medium, and then labeled with 200 \(\mu\)L of a 1:20 dilution of biotin-conjugated GAR-Fab 2' for 60 minutes at 4\(^\circ\)C. Subsequently, the cell aliquots were washed three times with cold CATCH-PGE-citrate medium, resuspended in 100 \(\mu\)L of CATCH-PGE-citrate medium, and incubated for 30 minutes at 4\(^\circ\)C with 400 \(\mu\)L of a 1:20 dilution of avidin–fluorescein isothiocyanate (FITC; 250 \(\mu\)g/mL, Becton Dickinson). After fluorescence labeling, the cells were washed three times with cold CATCH-PGE-citrate medium and resuspended in 0.67 mL of hypotonic citrate-saline buffer (100 mOsm/L) containing 1% bovine serum albumin (BSA) and propidium iodide (50 \(\mu\)g/mL). The 100-mOsm/L hypotonic solution permitted adequate penetration of the cells by propidium iodide for DNA staining with preservation of megakaryocyte cellular morphology. Cellular morphology was evaluated by microscopic examination of cytospin preparations of samples of the bone marrow cell suspensions stained with modified Romanowsky stain.\(^2\) The cells were maintained in the hypotonic propidium iodide solution at 4\(^\circ\)C for two hours to permit DNA staining by propidium iodide, treated with freshly prepared RNase (50 \(\mu\)g/mL) for 30 minutes at 23\(^\circ\)C, and filtered through nylon mesh (75-\(\mu\)m pore diameter) immediately prior to fluorescence-activated cell analysis. Cell recovery after propidium iodide staining was determined for a number of bone marrow samples (N = 6) by performing a second total nucleated cell count. Total nucleated cell recovery averaged 56.7% ± 6.3% (SD).

The fluorescein and propidium iodide channels of the flow cytometer (Becton Dickinson FACS Research Analyzer) were aligned by using 5.76-\(\mu\)m beads. Fluorescence activation was produced with the 488-nm line of a high-pressure Hg light source. The analyzer was equipped with a 75-\(\mu\)m orifice, and the following photomultiplier (PMT) conditions were used: fluorescein channel PMT = 250 V, log scale; propidium iodide channel PMT = 250 V, log scale. The data were accumulated in a single-parameter mode and stored on a hard disk for subsequent analysis. Bone marrow cells were analyzed at a flow rate of 200 to 400 cells per second without sample agitation.

Electronic selection of megakaryocytes from the unfractionated bone marrow cell population requires the positioning of an electronic fluorescein gate, or “green gate,” to discriminate the megakaryocyte subpopulation from the total bone marrow cell population. This selected subpopulation was then analyzed for DNA content by propidium iodide staining in the second analyzer channel. Instead of setting the fluorescein gate at the point where 99% of the signals from cells labeled with NRS were excluded as in the method of Jackson et al.,\(^4\) we analyzed the total cell population after labeling with both the NRS and R-PAS reagents and then selected a point that was beyond the 99% exclusion level and at the nadir of the fluorescence distribution between the unlabeled and labeled cell populations (Fig 1A). This alternative green gate, subsequently
detected by the second-step reagent in the two-color assay system. The fluorescence intensity (FL 1-logarithmic scale), and the ordinate indicates cell number (f) at full scale. The leftmost vertical marker (99%) indicates the 99% electronic exclusion gate that selects the 1% brightest cells, whereas the marker to the right (+) indicates the nadir between the unlabeled population (to the left) and the labeled population (to the right) of bone marrow cells. The latter marker is referred to as the positive green gate marker. The small fraction of cells to the right of the positive green gate marker was designated as the megakaryocyte population and was subjected to DNA analysis as shown in B. (B) The distribution of the DNA content is shown for the selected cell population, which has been electronically isolated in A on the basis of surface fluorescence. The abscissa indicates fluorescence intensity (FL 2-logarithmic scale) and the ordinate is cell number (f) at full scale. The peaks are as follows from left to right: 2N, 4N, 8N, 16N, 32N, and 64N. One thousand selected cells were analyzed to obtain the megakaryocyte ploidy distribution.

Fig 1. (A) The fluorescence distribution of unfractionated bone marrow cells is shown after labeling with the megakaryocyte-specific antisera (R-PAS) or NR5. Note that the intestinal portions of these two distributions overlap. The abscissa indicates fluorescence intensity (FL 1-logarithmic scale), and the ordinate indicates cell number (f) at full scale. The leftmost vertical marker (99%) indicates the 99% electronic exclusion gate that selects the 1% brightest cells, whereas the marker to the right (+) indicates the nadir between the unlabeled population (to the left) and the labeled population (to the right) of bone marrow cells. The latter marker is referred to as the positive green gate marker. The small fraction of cells to the right of the positive green gate marker was designated as the megakaryocyte population and was subjected to DNA analysis as shown in B. (B) The distribution of the DNA content is shown for the selected cell population, which has been electronically isolated in A on the basis of surface fluorescence. The abscissa indicates fluorescence intensity (FL 2-logarithmic scale) and the ordinate is cell number (f) at full scale. The peaks are as follows from left to right: 2N, 4N, 8N, 16N, 32N, and 64N. One thousand selected cells were analyzed to obtain the megakaryocyte ploidy distribution.

RESULTS

Megakaryocyte DNA distribution in mice with normal platelet levels. The distribution of DNA content in murine bone marrow megakaryocytes was measured by using bone marrow harvested from normal animals. The positive green gate marker technique produced megakaryocyte DNA distributions with clear separations between the ploidy classes (Fig 1B). Under our operating conditions, the observed mg/mL. Platelet volume studies from severely thrombocytopenic animals required the collection of up to seven capillary tubes per animal. Under these conditions, three capillary tubes were added to each 330-μL volume of buffered anticoagulant solution. Samples from two to five mice, either controls or treated animals, were pooled and layered over a discontinuous arabinogalactan gradient composed of 10% (wt/wt) and 20.5% (wt/wt) layers as previously described for rabbit platelets.21 The gradients were centrifuged at 20,000 g for ten minutes at 23°C to separate the platelets from the plasma proteins, erythrocytes, and granulocytes. Lymphocytes and monocytes exhibit a density distribution that overlaps that of platelets; since these leukocytes coesinemat with platelets, they were electronically excluded from the platelet size distribution by setting an upper volume threshold.22 The MPV was measured with an electrical impedance cell analyzer (Particle Data, Inc, Chicago) equipped with a 48-μm orifice tube, a logarithmic analyzer, and a PDP 11A computer. This instrument was calibrated with 2.02-μm-diameter latex beads as previously described.23 The MPV was measured as the geometric mean of the platelet volume distribution curve and expressed in femtoliters. Contaminating erythrocytes were excluded by use of an electronic upper threshold, and a Gaussian curve-fitting program was utilized to complete the upper end of the platelet volume distribution curve.

Statistical analysis. The means and SD of the bone marrow megakaryocyte ploidy class distributions, platelet counts, and MPVs were computed by standard methods. Statistical comparisons of the MPV between the normal murine population and the experimental groups were performed with a standard t test.28 Statistical comparisons of the megakaryocyte ploidy distributions between the normal population and the experimental groups were performed with the Mann-Whitney rank test.29

Regulation of thrombopoiesis

The 1% brighter cells, whereas the marker to the right (+) indicates the nadir between the unlabeled population (to the left) and the labeled population (to the right) of bone marrow cells. The latter marker is referred to as the positive green gate marker. The small fraction of cells to the right of the positive green gate marker was designated as the megakaryocyte population and was subjected to DNA analysis as shown in B. (B) The distribution of the DNA content is shown for the selected cell population, which has been electronically isolated in A on the basis of surface fluorescence. The abscissa indicates fluorescence intensity (FL 2-logarithmic scale) and the ordinate is cell number (f) at full scale. The peaks are as follows from left to right: 2N, 4N, 8N, 16N, 32N, and 64N. One thousand selected cells were analyzed to obtain the megakaryocyte ploidy distribution.

Fig 2. The frequency distribution of megakaryocyte DNA content in the bone marrow of normal mice is shown. Ploidy classes from 2N to 256N are indicated on the abscissa. The relative frequency of each ploidy class ± 1 SD is indicated as a percentage of the total megakaryocyte population on the ordinate. The 16N cell class, the normal modal class, is identified by the solid black bar for reference. Twenty-five analyses were performed with positive green gate technique; each consisted of the pooled bone marrow from two animals. The average number of megakaryocytes analyzed in each experiment to determine the ploidy distribution was 3.413 ± 1.273 (SD).
Fig 3. The frequency distribution of megakaryocyte DNA content after the induction of various degrees of acute thrombocytopenia is shown. Each sample consisted of the pooled bone marrow from two animals. The abscissa indicates the ploidy class from 2N to 64N, and the ordinate indicates the relative frequency (in percentage) of the various ploidy classes. The time after GP-PAS administration at which bone marrow samples were obtained is indicated in hours along the lower abscissa and is the same for each experimental series. The mean platelet count ($x 10^9/\mu L$) for the animals studied at each time period is indicated above the individual histograms. The number of experiments performed at each time period is indicated in parentheses, except where only a single experiment was performed. Absence of a ploidy point is indicated above the individual histograms. The number of experiments per time period is indicated.

Bone marrow cells obtained from animals treated with GP-PAS and then exposed only to NRS in vitro did not show any evidence of in vivo megakaryocyte labeling, and labeling could only be demonstrated after in vitro exposure of the cells to R-PAS. This suggests that binding of intraperitoneally administered GP-PAS to native megakaryocytes was not present 24 hours after PAS administration, although it is possible that a small amount of binding could have occurred earlier or at a concentration that was below the detection threshold of the assay. Platelets isolated from animals 24 hours after GP-PAS administration also did not demonstrate guinea pig immunoglobulin on the surface by examination with immunoelectron microscopy.

MPV was measured from 8 to 96 hours after the induction of acute, severe thrombocytopenia (reduction of platelet count to less than $0.05 \times 10^9/\mu L$). Table 1. MPV was significantly increased ($P < .001$) at eight hours after the administration of GP-PAS. The peak increment in MPV occurred at 24 hours, and MPV then slowly declined to the normal range at 96 hours. Importantly, MPV decreased continuously from the peak level, although it remained significantly elevated ($P < .001$) at each time point, as the platelet level increased dramatically during the 36- to 84-hour period after induction of acute thrombocytopenia (Table 1).

Effects of variable degrees of acute thrombocytopenia on megakaryocyte DNA distribution. The effect of the severity of thrombocytopenia on megakaryocyte DNA distri-
REGULATION OF THROMBOPOIESIS

The time course for recovery of platelet levels after the induction of experimental thrombocytopenia is shown. The platelet count (×10^6/L) is indicated on the ordinate and time (h) is indicated on the abscissa. Platelet counts during recovery from various degrees of thrombocytopenia were obtained from individual animals utilized at each time point to determine the platelet ploidy distribution. Table 1 shows the effect of acute severe thrombocytopenia on MPV.

Table 1. The Effect of Acute Severe Thrombocytopenia on MPV

<table>
<thead>
<tr>
<th>Time</th>
<th>C</th>
<th>8</th>
<th>12</th>
<th>24</th>
<th>36</th>
<th>48</th>
<th>60</th>
<th>84</th>
<th>96</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPV</td>
<td>4.6</td>
<td>6.8*</td>
<td>5.9*</td>
<td>6.2*</td>
<td>6.7*</td>
<td>5.8*</td>
<td>5.2*</td>
<td>5.1</td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>25</td>
<td>4</td>
<td>11</td>
<td>8</td>
<td>4</td>
<td>10</td>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC</td>
<td>1.62</td>
<td>.02</td>
<td>.03</td>
<td>.02</td>
<td>.10</td>
<td>.16</td>
<td>.58</td>
<td>1.42</td>
<td>2.00</td>
</tr>
<tr>
<td>SD</td>
<td>0.40</td>
<td>0.01</td>
<td>0.03</td>
<td>0.03</td>
<td>0.06</td>
<td>0.19</td>
<td>0.70</td>
<td>1.26</td>
<td></td>
</tr>
</tbody>
</table>

NOTE. C indicates a normal control population. The MPV of the normal population, 4.6 fl ± 0.5 SD, was used for statistical comparisons with the treated population. The time after GP-PAS administration is indicated in hours, MPV is expressed in femtoliters, and the platelet count (PC) × 10^9/L with the SD of the mean is shown. N represents the number of individual animals utilized at each time point to determine the platelet count. Blood from these animals was pooled to measure MPV at each time point in two separate experiments. MPV of control animals was obtained from individual animals. A separate group of animals was used for the ploidy analyses.

---

Reduction of the platelet level to 0.200 to 0.300 × 10^6 platelets/μL (mean, 0.244 ± 0.026 [SD] × 10^6 platelets/μL; range, 0.227 to 0.296 × 10^6 platelets/μL) also resulted in a delayed shift of the megakaryocyte DNA distribution similar to that observed with the preceding experimental group (Fig 3C). The 32N megakaryocyte class was not significantly increased at 48 hours after administration of GP-PAS (P > .05), and a shift in the modal ploidy class from 16N to 32N occurred at 72 hours. Additional experiments (Fig 3D) in which the platelet level was reduced to 0.300 to 0.400 × 10^6 platelets/μL (mean, 0.354 ± 0.059 [SD] × 10^6 platelets/μL; range, 0.227 to 0.441 × 10^6 platelets/μL) demonstrated no significant change in frequency of the 32N class at any time.

When very moderate thrombocytopenia (platelet count nadir of 0.400 × 10^6 platelets/μL at 16 hours after GP-PAS injection) was produced, a highly significant change in MPV occurred as early as 12 hours after administration of GP-PAS (Table 2, group B) and persisted until 144 hours after GP-PAS administration. It should be noted that the platelet count nadir was probably missed for animal group A (Table 2) due to the long sampling interval from four to 24 hours after GP-PAS injection. The increase in platelet volume during this experiment reached a peak 24 hours after administration of GP-PAS, at which time the megakaryocyte ploidy distribution was entirely within the normal range in response to slightly greater thrombocytopenia (Fig 3D). Thus, an effect on platelet volume was produced by a level of thrombocytopenia that did not alter megakaryocyte DNA distribution during the 96-hour period after moderate thrombocytopenia (Fig 3D).

Effects of chronic, severe thrombocytopenia on megakaryocyte DNA distribution. Chronic, severe thrombocytopenia was induced by a reduction of the platelet level to less than 0.400 × 10^6 platelets/μL. The megakaryocyte DNA distribution (Figure 3B to D) and the time course of recovery of the platelet level (Fig 4) were serially measured. Reduction of the platelet level to 0.100 to 0.200 × 10^6 platelets/μL (mean, 0.154 ± 0.037 [SD] × 10^6 platelets/μL; range, 0.098 to 0.232 × 10^6 platelets/μL) did not produce a shift in the modal ploidy class at 48 hours after GP-PAS administration (Fig 3B), the time point at which a ploidy shift was consistently observed after severe, acute thrombocytopenia (Fig 3A). However, at 48 hours after administration of GP-PAS, the relative frequency of 32N megakaryocytes increased to 28%, the upper limit of the normal range for the 32N class (P < .05). More importantly, at 72 hours after GP-PAS administration, there was a shift in the modal megakaryocyte ploidy class from 16N to 32N (Fig 3B). Therefore, the shift in ploidy class in response to acute thrombocytopenia, which was always observed at 48 hours after severe thrombocytopenia (platelet level, <0.05 × 10^6 platelets/μL), was delayed for an additional 24 hours when the stimulus of thrombocytopenia was less severe. At 96 hours after the induction of moderate thrombocytopenia (0.100 to 0.200 × 10^6 platelets/μL), the modal ploidy class reverted to 16N, and a complete return to the baseline DNA distribution was observed 120 hours after GP-PAS injection. No significant increment in the 64N megakaryocyte class was observed at any time.

Fig 4. The time course for recovery of platelet levels after the induction of experimental thrombocytopenia is shown. The platelet count (×10^6/L) is indicated on the ordinate and time (h) is indicated on the abscissa. Platelet counts during recovery from various degrees of thrombocytopenia were obtained from individual animals utilized at each time point to determine the platelet ploidy distribution. Table 1 shows the effect of acute severe thrombocytopenia on MPV.

Table 1. The Effect of Acute Severe Thrombocytopenia on MPV

<table>
<thead>
<tr>
<th>Time</th>
<th>C</th>
<th>8</th>
<th>12</th>
<th>24</th>
<th>36</th>
<th>48</th>
<th>60</th>
<th>84</th>
<th>96</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPV</td>
<td>4.6</td>
<td>6.8*</td>
<td>5.9*</td>
<td>6.2*</td>
<td>6.7*</td>
<td>5.8*</td>
<td>5.2*</td>
<td>5.1</td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>25</td>
<td>4</td>
<td>11</td>
<td>8</td>
<td>4</td>
<td>10</td>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC</td>
<td>1.62</td>
<td>.02</td>
<td>.03</td>
<td>.02</td>
<td>.10</td>
<td>.16</td>
<td>.58</td>
<td>1.42</td>
<td>2.00</td>
</tr>
<tr>
<td>SD</td>
<td>0.40</td>
<td>0.01</td>
<td>0.03</td>
<td>0.03</td>
<td>0.06</td>
<td>0.19</td>
<td>0.70</td>
<td>1.26</td>
<td></td>
</tr>
</tbody>
</table>

NOTE. C indicates a normal control population. The MPV of the normal population, 4.6 fl ± 0.5 SD, was used for statistical comparisons with the treated population. The time after GP-PAS administration is indicated in hours, MPV is expressed in femtoliters, and the platelet count (PC) × 10^9/L with the SD of the mean is shown. N represents the number of individual animals utilized at each time point to determine the platelet count. Blood from these animals was pooled to measure MPV at each time point in two separate experiments. MPV of control animals was obtained from individual animals. A separate group of animals was used for the ploidy analyses.

*P < .001.
†P < .01.
Stratified an increase in rat megakaryocyte volume that reached a maximum platelet antiserum) produced a larger increase in megakaryocyte volume, cytoplasmic volume, and nuclear volume. Odell and coworkers using direct measurement of megakaryocyte DNA found the normal rat megakaryocyte cell volume than observed after a 92% reduction in the platelet level. Other studies by Harker, also using rats, extended these observations to demonstrate a parallel shift in megakaryocyte "nuclear number" as well as volume after four days and ten days of an 80% reduction in the platelet level. However, Harker did not directly determine the megakaryocyte DNA content. Studies by Penington and Olsen and Odell and coworkers using direct measurement of megakaryocyte DNA found the normal rat megakaryocyte modal ploidy class to be 16N and observed that induction of acute, severe thrombocytopenia resulted in a shift of the modal class from 16N to 32N 48 hours after reduction in the platelet level. Odell et al described an early increase in platelet size 18 hours after induction of thrombocytopenia and noted that the maximum increase in platelet size preceded the peak increase in both megakaryocyte volume and ploidy distribution by 30 hours.

After six days of variable, chronic thrombocytopenia, Martin et al observed a marked increase in total megakaryocyte cell volume, cytoplasmic volume, and nuclear volume. MPV increased by approximately 10% compared with the control during the first four days and then rapidly increased by an additional 30% at the sixth day. In a second paper, these workers reported a shift from the normal modal megakaryocyte ploidy class of 16N to 32N after 24 hours of thrombocytopenia (90% reduction in platelet level); a similar shift also was observed after six days of chronic thrombocytopenia. In a third study, Trowbridge and Martin observed an 86% reduction in the platelet level of rabbits and observed a 27% increase in MPV six hours after the induction of thrombocytopenia. However, they observed no change in the modal megakaryocyte ploidy class from their normal control value of 32N either two hours or 24 hours after the onset of thrombocytopenia, although there was an increase in the relative frequency of the 32N class. Martin et al concluded that thrombocytopenia results in an increase in megakaryocyte volume, cytoplasmic volume, and nuclear volume.

**DISCUSSION**

Previous studies have provided limited and conflicting information concerning the regulation of thrombopoiesis after experimental thrombocytopenia. Ebbe et al demonstrated an increase in rat megakaryocyte volume that reached a maximum 48 hours after a 92% reduction of the platelet level by exchange transfusion. Extreme thrombocytopenia (99.9% reduction of the platelet level induced by platelet antiserum) produced a larger increase in megakaryocyte volume than observed after a 92% reduction in the platelet level. Other studies by Harker, also using rats, extended these observations to demonstrate a parallel shift in megakaryocyte "nuclear number" as well as volume after four days and ten days of an 80% reduction in the platelet level. However, Harker did not directly determine the megakaryocyte DNA content. Studies by Penington and Olsen and Odell and coworkers using direct measurement of megakaryocyte DNA found the normal rat megakaryocyte modal ploidy class to be 16N and observed that induction of acute, severe thrombocytopenia resulted in a shift of the modal class from 16N to 32N 48 hours after reduction in the platelet level. Odell et al described an early increase in platelet size 18 hours after induction of thrombocytopenia and noted that the maximum increase in platelet size preceded the peak increase in both megakaryocyte volume and ploidy distribution by 30 hours.

After six days of variable, chronic thrombocytopenia, Martin et al observed a marked increase in total megakaryocyte cell volume, cytoplasmic volume, and nuclear volume. MPV increased by approximately 10% compared with the control during the first four days and then rapidly increased by an additional 30% at the sixth day. In a second paper, these workers reported a shift from the normal modal megakaryocyte ploidy class of 16N to 32N after 24 hours of thrombocytopenia (90% reduction in platelet level); a similar shift also was observed after six days of chronic thrombocytopenia. In a third study, Trowbridge and Martin observed an 86% reduction in the platelet level of rabbits and observed a 27% increase in MPV six hours after the induction of thrombocytopenia. However, they observed no change in the modal megakaryocyte ploidy class from their normal control value of 32N either two hours or 24 hours after the onset of thrombocytopenia, although there was an increase in the relative frequency of the 32N class. Martin et al concluded that thrombocytopenia results in an increase in megakaryocyte volume, cytoplasmic volume, and nuclear volume.
cyte ploidy and cytoplasmic volume with subsequent production of larger platelets.9

Using a modified two-color technique based on the method of Jackson et al,9 we have observed that the modal megakaryocyte ploidy class in mice with normal platelet levels is 16N. Our megakaryocyte ploidy distributions are similar to those of Jackson et al although the use of the more conservative megakaryocyte selection gate reduced the observed frequency of 2N and 4N megakaryocytes. These differences may be partially due to species variation as well. Our determinations of the normal murine megakaryocyte ploidy distribution are in general agreement with the earlier observations of Odell et al and Penington and Olsen using the Feulgen microdensitometric technique with rat bone marrow smears,6,31 those of Worthington et al32 with a single-color flow cytometric technique, and those of Mayer et al33 using immunofluorescent identification of megakaryocytes followed by Feulgen staining to measure the DNA content. In each of these studies,6,9,31,33 the 2N and 4N classes were infrequent, 16N was the modal class, and 8N cells ranged in frequency from 8.1% to 23.0%. The results from these investigations and those of the present study do not agree with the observations of other workers who have used single-color fluorescence-activated flow cytometry and found that 8N is the most common ploidy class.14,15 Jackson et al suggest that this discrepancy may be due in part to failure of the single-color flow technique to exclude cell aggregates,9 a problem that is minimized by the two-color technique.

The two-color technique not only clearly resolves the individual ploidy classes but also is advantageous because of the large numbers of cells analyzed and the use of a megakaryocyte selection technique that is independent of morphology. Immunologic identification of megakaryocytes could potentially underestimate the number of megakaryocytes if the megakaryocyte-specific probe failed to detect a portion of the megakaryocyte population. However, the probe used in the present studies was found to be sensitive to a broad spectrum of murine platelet proteins, which indicates that there should be broad sensitivity for megakaryocyte proteins as well. The studies of Rabelino et al34 demonstrate that the small, immature human megakaryocytes express the major platelet glycoproteins Ib, IIb, and IIIa. Jackson has demonstrated good correlation between the identification of rat megakaryocytes with PAS and detection of acetylcholinesterase.35 The recent report by Ishibashi et al36 also provides evidence that immature, morphologically unrecognizable megakaryocytes are identified by a monoclonal antibody directed against the glycoprotein IIb/IIIa complex. Since it is possible that the electronic gating technique could exclude low-ploidy megakaryocytes that contain low levels of surface fluorescence, we performed additional experiments using dual-parameter analysis in which the gate was further reduced below the standard positive green gate. This less-restrictive gating technique did not significantly increase the frequency of 2N, 4N, or 8N class megakaryocytes (data not shown). Our estimate of the frequency of megakaryocytes in normal murine bone marrow (0.496%) is in approximate agreement with earlier immunofluorescent microscopic determinations using murine marrow33 and is in the range of estimates obtained by a number of workers using rodents and a variety of techniques.6-9

We have demonstrated that the modal ploidy class shifts from 16N to 32N 48 hours after the induction of acute, severe thrombocytopenia. The shift is consistently initially observed 48 hours after administration of antiserum, persists until 84 hours after GP-PAS administration, and then reverts to normal 120 hours after the onset of thrombocytopenia. Jackson et al also observed a shift in the megakaryocyte ploidy distribution of rats 48 hours after the onset of thrombocytopenia, but the more frequent sampling in the present study clearly documented that no shift in the modal ploidy class was observed until the 48-hour time point.

The degree of thrombocytopenia in the study of Ebbe et al at which a significant increase in megakaryocyte cytoplasmic volume was observed is similar to that in our study of moderate thrombocytopenia (0.300 to 0.400 x 10⁶ platelets/µL 24 hours after GP-PAS administration). Under these conditions, we observed no significant change in the megakaryocyte ploidy distribution during the entire five-day period of observation (Fig 3D). Furthermore, MPV increased significantly 12 hours after GP-PAS administration in mice, with even a lesser reduction of platelet levels (Table 2). After acute severe thrombocytopenia (platelet count reduction to less than 0.05 x 10⁶ platelets/µL after administration of GP-PAS), we observed an increase in MPV 40 hours prior to a significant effect on the megakaryocyte ploidy distribution and 28 hours before the initial increase in the proportion of 32N megakaryocytes. Our observations indicate that the increases in platelet volume after acute thrombocytopenia clearly precede and therefore are dissociated from major changes in the megakaryocyte ploidy distribution; these increases constitute the initial response to reduced levels of circulating platelets, even when the degree of diminution of the circulating platelet mass is insufficient to produce a detectable alteration in megakaryocyte DNA content.

Moderate degrees of thrombocytopenia (reduction in platelet levels to approximately 10% to 20% of the normal level) resulted in a more delayed shift of the ploidy distribution. Very moderate thrombocytopenia (reduction in platelet level to 40% of normal) did not produce a shift in the modal ploidy class even as late as 96 hours after GP-PAS administration, although platelet volume was increased. These results suggest that the effect of thrombocytopenia on nuclear endoreduplication is modulated by the magnitude of the stimulus. Pertinently, Odell and Shelton40 reported that endomitosis was not significantly increased in megakaryocytes unless platelet levels were reduced to less than 50% of normal.

The present studies of chronic, severe thrombocytopenia indicated that the megakaryocyte ploidy distribution remained significantly shifted during persistent thrombocytopenia and that the recovery phase was associated with a more marked increase in the 64N cell class than was observed during recovery from acute thrombocytopenia (Fig 5). This increased proportion of 64N class megakaryocytes may also explain the more rapid rate of platelet recovery observed in response to chronic thrombocytopenia as com-
pared with that after acute, severe thrombocytopenia (Fig 4). It is of interest that the megakaryocyte DNA distribution returned to normal 120 hours after discontinuation of platelet antiserum regardless of the magnitude or duration of the shift in the megakaryocyte DNA distribution. The present studies are consistent with the earlier results of Penington and Olsen. The lesser increase in the proportion of 32N megakaryocytes observed by Penington and Olsen is likely due to their less frequent schedule of platelet antiserum administration, which probably did not produce as severe and consistent a period of prolonged thrombocytopenia as did our administration of antiserum at 48-hour intervals (Fig 4). Overall, our data demonstrate that the magnitude and temporal sequence of alterations that follow the induction of thrombocytopenia depend upon the magnitude and duration of the thrombocytopenic stimulus.

REFERENCES

21. Corash L, Shafer B: Use of asplenic rabbits to demonstrate that platelet age and density are related. Blood 60:166, 1982
36. Ishibashi T, Ruggeri ZM, Harker LA, Burstein SA: Separation of human megakaryocytes by state of differentiation on continu-


Regulation of thrombopoiesis: effects of the degree of thrombocytopenia on megakaryocyte ploidy and platelet volume

L Corash, HY Chen, J Levin, G Baker, H Lu and Y Mok