Flow Cytometric Analysis of Megakaryocytes From Patients With Abnormal Platelet Counts

By Aaron Tomer, Paul Friese, Richard Conklin, Wes Bales, Linda Archer, Laurence A. Harker, and Samuel A. Burstein

Megakaryocytes (Mks) from 40 patients with quantitative platelet disorders and 19 normal volunteers were analyzed by flow cytometry for size, fine cell internal structure and granularity, membrane expression of the glycoprotein (GP) IIb/IIIa complex, and for ploidy distribution. Analysis was performed on unfractonated minimally manipulated marrows obtained from routine bone marrow aspirates. Mks were labeled with a fluorescent lineage-specific monoclonal antibody to the GPIIb/IIIa complex followed by DNA staining with propidium iodide. Eight hundred to 2,000 Mks were analyzed in each sample. The modal ploidy distribution in normals was 16N, comprising about half of the cells ≤8N and 22.0% ≥32N. Twelve thrombocytopenic patients with increased Mks on biopsy (mean platelet count [MPC] 44,600/μL) showed an increase in low ploidy cells with 53.2% ≤8N (P < .01); cell size was reduced in three patients when compared to normal cells of identical ploidy (P < .05). Eight thrombocytopenic patients with enhanced platelet destruction (with normal or increased Mks on biopsy and shortened platelet survival; MPC 41,400/μL) showed an increased proportion of high ploidy cells ≥32N to 39.2% (P < .01). Increased cell size and granularity were found in four of these patients (P < .05). Six patients with thrombocytopenia secondary to multiple mechanisms affecting both platelet production and destruction (MPC 66,700/μL) showed no shift in ploidy. Four patients with primary thrombocytosis (two with thrombocytopenia and two with polycythemia vera; MPC 822,500/μL) showed a marked shift toward high ploidy cells with 42.3% ≥32N and 7.6% ≥64N cells (P < .01). The shift was accompanied by a marked increase in cell size and granularity in the patients with thrombocytosis. Ten patients with thrombocytosis secondary to chronic blood loss, malignant or inflammatory disorders (MPC 714,000/μL), showed variable distributions with four patients exhibiting a shift in ploidy to the right similar to that found in the patients with increased platelet destruction. Based upon the present data, flow cytometric ploidy distribution may be diagnostically useful in thrombocytopenic patients by discriminating between disorders of platelet production and destruction. Additional data are required to determine the usefulness of flow cytometric ploidy measurements in thrombocytosis patients. We conclude that flow cytometry is a clinically useful method for quantitative analysis of Mks obtained from routine marrow aspirates.

ANALYSIS OF MEGAKARYOCYTE (MK) size and ploidy is a potentially useful tool for the study of mechanisms regulating megakaryocyte proliferation and ultimately, thrombopoiesis. In experimental animals it has been shown that MK size and ploidy increase in response to accelerated platelet consumption and decrease with platelet hypertransfusion. However, in man the study of Mks has proven difficult because of the rarity of these cells, which comprise only about 0.05% of the nucleated marrow cell population. Moreover, no technique has been available for the rapid and highly selective analysis of large numbers of these cells. Despite the abnormalities in the frequency, size, and ploidy distribution of Mks previously described in certain pathologic conditions, these studies have been limited by the inability to analyze quantitatively large numbers of unselected cells.

Recently, we have shown that two-color flow cytometry can be used effectively for the rapid analysis of relatively large numbers of Mks (>1,000 cells) from normal routine human bone marrow aspirates. In those studies, Mks were initially enriched, then labeled with a fluorescent lineage-specific monoclonal antibody to the membrane GPIIb/IIIa complex for cell identification and stained with propidium iodide (PI) for DNA quantitation. Cells were analyzed for membrane immunofluorescence intensity, cell size as estimated by forward light scatter (FLS), fine cell internal structure and granularity as assessed by 90° light scatter (side scatter; SSC) and relative DNA content.

In the present study, we have used flow cytometry for multiparameter analysis of Mks in a survey of patients with quantitative platelet disorders, with the aim of defining cytometric characteristics that might prove useful in the classification and/or diagnosis of these disorders. To obviate potential selection of cells during enrichment steps and to adapt the technique for routine clinical use, we have modified our original method to enable a convenient and relatively rapid analysis performed on unfractionated and minimally manipulated routine bone marrow aspirates.

MATERIALS AND METHODS

Patients. Bone marrow Mks were studied in 26 patients with thrombocytopenia (platelet count <100,000/μL), in 14 patients with thrombocytosis (platelet count ≥450,000/μL), and in 19 normal individuals. Before the cytometric analysis, all patients were classified into subgroups according to clinical criteria based on peripheral blood evaluation, examination of bone marrow aspirate smears and biopsy, on the response to treatment, and in some cases, on platelet

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survival studies. Routine bone marrow aspirates and platelet survival studies were performed after obtaining written informed consent according to the guidelines of the Human Subjects Committees of the participating institutions.

Twelve patients had megakaryocytic hypoplasia with normal or decreased cellularity of other marrow elements. Of this group, six were drug induced, (one chlorothiazide, one tetracycline, one atenolol, and three postchemotherapy for chronic lymphocytic leukemia, chronic myelogenous leukemia, and Wegener's granulomatosis). All of these patients had recovery of bone marrow MKs and peripheral platelet count following discontinuation of treatment. Four patients had markedly reduced MKs of unknown etiology and two patients had myelodysplasia.

Of the thrombocytopenic patients, eight had normal marrow with normal or increased MKs. In this group, seven were classified as immune (four of whom had platelet survival studies; all four had shortened platelet survival), and one patient with an infectious disease (increased MKs in marrow) was classified as nonimmune. Of the patients with intrinsic destruction, five had chronic thrombocytopenia and were receiving continuous low-dose prednisone and two had acute idiopathic thrombocytopenic purpura (ITP) with normalization of the platelet count following prednisone treatment. Six patients had thrombocytopenia secondary to multiple etiologies (eg, splenomegaly, chronic liver disease, chronic renal failure, systemic lupus erythematosus [SLE], and sepsis).

Of the group of patients with thrombocytosis, four were classified as primary (two essential thrombocythemia [ET]) and two polycythemia vera [PV] in accord with the diagnostic criteria established by the Polycythemia Vera Study Group. Ten patients had secondary thrombocytosis (three with chronic blood failure, systemic lupus erythematosus [SLE], and sepsis).

Marrow preparation

One half to 3.0 mL of bone marrow from the posterior iliac crest was collected from a single site into a 10-ml plastic syringe containing 1/10 vol acid-citrate-dextrose (ACD formula A), EDTA and protaglalinine (PGE; Sigma Chemical Co, St Louis) to a final concentration of 2.5 mmol/L and 2.2 μmol/L, respectively. The marrow was gently pipetted, passed through a 200-μm monofilament nylon filter, and diluted with cold Ca²⁺- and Mg²⁺-free phosphate buffered saline (PBS) containing 13.6 mmol/L sodium citrate, 2.2 μmol/L PGE, 1 mmol/L theophylline, 3% bovine serum albumin (BSA; fraction V, Calbiochem, La Jolla, CA), 11 mmol/L glucose, and adjusted to a pH of 7.3 and an osmolality of 290 mosm/L. Hereafter, this supplemented PBS is designated MK medium. Cells were analyzed in unfractionated marrow (three with chronic blood loss and iron deficiency and seven with malignant or inflammatory disorders).

Marrow cells were enumerated with a Baker System (Baker Instruments, Allentown, PA).

Megakaryocyte labeling

MKs were directly labeled with a fluoresceinated F(ab)₂ fragment of IgG, mouse monoclonal antibody (P4) directed to a platelet-specific GPIIb/IIIa epitope. The antibody was a gift from Dr Z. M. Ruggeri of the Research Institute of Scripps Clinic, and was fluoresceinated to an F/P ratio of 3:1 by quantitative flow cytometric measurements of the fluorescence of large MKs. An aliquot of the cell suspension was incubated under identical conditions with a fluoresceinated IgG, mouse monoclonal antibody to human thyroglobulin and used as the control cells.

DNA staining. To determine the ploidy distribution, cellular DNA was stained by a modification of a method using hypotonic citrate. Briefly, the cell suspension was diluted with four volumes of 0.1% sodium citrate containing 20 μg/mL propidium iodide (PI) and 0.05% Triton X-100 (Sigma) to enhance permeabilization, and gently mixed for 20 minutes at 4°C. This resulted in highly efficient (>98%) DNA staining of the marrow cells including all cells exhibiting high membrane immunofluorescence. Cell recovery was >95% with no preferential loss of MKs compared to that of a nonpermeabilized antibody-labeled preparation. Following staining, cells were immediately analyzed by flow cytometry. The total time required to prepare the marrow cells for analysis was approximately 1.5 hours.

Flow cytometry. Cells were analyzed with either a Coulter Epics V flow cytometer (Coulter) using a 100-μm diameter nozzle or a Coulter-like experimental flow cytometer (National Laboratories, Los Alamos, CA) using a 200-μm diameter nozzle. Both cytometers are equipped with 2W argon-ion lasers tuned to deliver 200 mW at 488 nm. The optical settings for the separation of the emission spectrum of the fluorescein (green) and the PI (red), and the method for the correction of the overlap between the emitting spectra were performed as previously described. Data were collected for the measurements of the levels of the green membrane fluorescence (GF), the red PI fluorescence (RF), the FLS and the 90° SSC of each cell. MKs were selected on the basis of the specific membrane immunofluorescence by setting an electronic gate at a fluorescence level above that of the control or the major marrow cell populations. For this purpose, we used dual parameter contour plots of the GF v FLS as described earlier or plots of the GF v RF (Fig 1). Eight hundred to 3,000 MKs were analyzed in each sample. The ploidy distribution was determined by setting markers at the nadirs between peaks using the 2N and 4N peaks of the unselected marrow cells as an internal reference standard.

The levels of the FLS and the SSC were measured for each individual cell. However, to compare these parameters among the patient and the normal samples, the mean FLS and SSC of only the 16N cells was used to obviate the potential influence of an altered ploidy distribution. The 16N cells were chosen because they represent the most frequent cell class in the normals and in the majority of the patients. However, when changes in the FLS and the SSC occurred, they were detected in all cells ≥8N (data not shown). The mean levels of FLS and SSC were obtained in arbitrary units as the mean channel number and the values in the normal population were designated as normal. Values for the patient samples were considered to be increased or decreased when they differed from normal by more than 1 SD.

Since the frequency of MKs in unfractionated normal marrow is only about 0.05% of the nucleated marrow cells, acquisition of data on about 1,000 MKs requires the analysis of nearly 2 x 10⁸ cells. To obviate the acquisition and storage of such an unusually large number of events in a list mode (beyond the capacity of some flow cytometric computer programs, which are limited to analyze up to 5 x 10⁶ events per sample) and to facilitate the retrieval and analysis of the data, the following procedure was performed. First, 5 x 10⁴ un gated events derived from the total nucleated marrow cell population were obtained, then the frequency of MKs was estimated. Second, an electronic gate was set on the GF level so as to include five times the frequency of the MKs (eg, if the frequency of MKs was estimated at 1%, the gate was set to include the top 5% of the total nucleated marrow cells on the GF scale). Repeated analyses showed this margin to be sufficient because no significant differ-
ences were found in the MK frequency estimated by analysis of $5 \times 10^6$ ungated cells vs $0.25 \times 10^6$ cells of $5\%$ gated cells (data not shown).

Platelet kinetic studies. Platelet survival studies were performed using autologous platelets labeled with indium-111-oxine by a modification of the method of du Heyns et al. Briefly, 50 to 100 mL of citrate-anticoagulated blood was collected. Following centrifugation, platelet-rich plasma was separated and acidified with citric acid to pH 6.5. Platelets were concentrated by centrifugation, washed, and resuspended with Ringer's buffer preadjusted to pH 6.5, and incubated with $^{111}$In-oxine (1 mCi/mL, Amersham, IL). Over 90% of the radioisotope was incorporated into the platelets. An aliquot of labeled platelet suspension containing a total radioactivity of 25 $\mu$Ci was diluted with the patient's platelet-poor plasma and an aliquot of labeled platelet suspension containing a total radioactivity of 6.5, and incubated with $^{111}$In-oxine (1 mCi/mL, Amersham, IL). Over 90% of the radioisotope was incorporated into the platelets. An aliquot of labeled platelet suspension containing a total radioactivity of 25 $\mu$Ci was diluted with the patient's platelet-poor plasma and used for the in vivo study. Venous blood samples were collected two hours post injection and then twice daily. The samples were counted and the results were presented as a percentage of the initial total radioactivity. Platelet survival time was determined by computing the least-squares fit of the raw data (CPM) to a gamma-function distribution of the mean and the range of the individual ploidy classes, was determined by multinomial distribution analysis. The mean and the covariance for the normals were used as the reference distribution to determine the multivariate tolerance region against which the individual patient data was assessed. Comparisons among the cellular parameters were performed by standard least squares regression analysis as described.

Statistical analysis. To define abnormal ploidy distributions among the patients, the average ploidy distribution, as a joint distribution of the 19 normal controls with the mean and the range of the individual ploidy classes, was determined by multinomial distribution analysis. The mean and the covariance for the normals were calculated. Comparisons among the cellular parameters were performed by standard least squares regression analysis as described.

**RESULTS**

Characteristics of the patient groups. The mean and the range of the platelet counts of the various patient groups and the frequency of MKs estimated by examination of marrow biopsies are summarized in Table 1. In the thrombocytopenic patients, the mean platelet count found in the group with enhanced platelet destruction (41,400/$\mu$L) was similar to that found in the group with megakaryocytic hypoplasia (44,600/$\mu$L; P > .05). Platelet survival performed on four patients from the former group was significantly shortened compared to normal and contrasted with the survival times in three patients with megakaryocytic hypoplasia that were appropriate for the circulating platelet levels (ie, not shorter

<table>
<thead>
<tr>
<th>Table 1: Characteristics of the Patient Groups</th>
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<tbody>
<tr>
<td><strong>Group</strong></td>
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<tr>
<td>----------</td>
</tr>
<tr>
<td>Thrombocytopenia</td>
</tr>
<tr>
<td>Decreased production</td>
</tr>
<tr>
<td>Enhanced destruction</td>
</tr>
<tr>
<td>Multiple etiologies</td>
</tr>
<tr>
<td>Thrombocytosis</td>
</tr>
<tr>
<td>Primary</td>
</tr>
<tr>
<td>Secondary</td>
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</table>

Abbreviations: n.l., normal; ↑, increased; ↑↑, markedly increased; ↓, decreased.

*MK frequency as estimated by examination of bone marrow sections.*
than the values predicted by the method of Hanson and Slichter, which relates platelet survival to the level of thrombocytopenia. The mean platelet count in the group with multiple etiologies (66,600/μL) was higher than that of the former groups (P < .05) although the range was similar. In the group with thrombocytosis, the mean platelet count of the patients with primary disorders (822,500/μL) was higher than that of the patients with the secondary disorders (714,100/μL; P < .05).

General characteristics of marrow MKs. Figure 1 shows the distribution of un fractionated normal marrow cells according to the various flow cytometric measurements. Figure 1A shows the distribution according to the green membrane immunofluorescence intensity (GF) and cellular DNA content as assessed by the RF of the DNA-bound PI. The highly GF cells formed identifiable and easily distinguishable ploidy classes ranging from 2N to 32N. Figure 1B shows the distribution of cells according to GF and size as assessed by FLS; and Fig 1C shows the distribution of cells according to the GF and granularity as assessed by SSC. The bivariate display shows that the MK population in the un fractionated marrow can be identified by this method as a discrete, readily separable cell subset with high membrane immunofluorescence. MKs were selected by setting an electronic gate at the GF level above that of the major marrow cell population (Figs 1 A through C) and of the control sample incubated with fluorescent monoclonal antibody to human thyroglobulin, as described in previous studies.

Following the selection of the MKs, the relative frequency and the mean levels of the flow cytometric measurements (FLS, SSC, GF, and RF) for the individual ploidy classes were measured. The ploidy distribution derived from the MK population in Figs 1A to 1C is shown in Fig 1D.

In an attempt to shorten the flow cytometry time, MKs were also analyzed in samples fractionated over Percoll (density 1.060 g/mL). The fractionation resulted in a ten to 12-fold enrichment with a proportional decrease in the analysis time. Analysis of the ploidy distributions showed a slight increase in the frequency of cells ≥32N in the un fractionated samples relative to that in the fractionated samples in some cases. However, the differences were ≤7% of the frequency of cells ≥32N, thus did not alter the overall ploidy distribution.

Characterization of the MK population in normal and patient groups. The ploidy distribution and the mean levels of the FLS and SSC of the MKs in the normal and the patient groups are shown in Table 2. In the normal controls, the model ploidy distribution was 16N, consistent with previous results.

The analysis of the ploidy distribution of the patient groups showed different patterns. In the thrombocytopenic group, all 12 patients with megakaryocytic hypoplasia showed a significant shift to low ploidy cells (left shift) with a marked increase in cells ≤8N (53.4%; P < .01). This increment was associated with a reciprocal decrease in 16N cells primarily. An example of such a distribution pattern is shown in Fig 2A. In three patients who had a marked increase in ≤8N cells to ≥70%, the shift in ploidy was associated with a decrease in cell size compared to the FLS of normal cells in the identical ploidy classes (P < .05). The SSC level declined with the FLS. The two patients in this group with myelodysplasia showed a unique pattern with an increase in both ≤8N cells (34.5%) and ≥32N cells (35.6%).

In all patients with thrombocytopenia caused by increased platelet destruction, MK ploidy was significantly shifted toward high ploidy classes (right shift) with 39.2% of cells ≥32N (P < .01). The increase in high ploidy cells was associated with a decrease in 16N cells, but not in cells ≤8N. An example of this ploidy distribution pattern is shown in Fig 2B. The reason for this is not clear at present. The MK ploidy distribution is shown in Table 2.

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of Patients</th>
<th>2N (%)</th>
<th>4N (%)</th>
<th>8N (%)</th>
<th>16N (%)</th>
<th>32N (%)</th>
<th>64N (%)</th>
<th>128N (%)</th>
<th>Size (μm)</th>
<th>Granularity (SSC)</th>
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<tbody>
<tr>
<td>Normal</td>
<td>19</td>
<td>7.1</td>
<td>4.1</td>
<td>11.4</td>
<td>55.4</td>
<td>21.5</td>
<td>0.5</td>
<td>nI(9)</td>
<td>(2.2)</td>
<td>(0.9)</td>
</tr>
<tr>
<td>Thrombocytopenia</td>
<td>26</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Decreased production</td>
<td>12</td>
<td>16.3</td>
<td>13.7</td>
<td>23.4</td>
<td>29.1</td>
<td>15.9</td>
<td>1.6</td>
<td>nI(9)</td>
<td>(4.4)</td>
<td>(7.1)</td>
</tr>
<tr>
<td>Enhanced destruction</td>
<td>8</td>
<td>8.8</td>
<td>3.5</td>
<td>11.0</td>
<td>37.5</td>
<td>35.4</td>
<td>3.8</td>
<td>nI(9)</td>
<td>(1.9)</td>
<td>(1.4)</td>
</tr>
<tr>
<td>Multiple etiologies</td>
<td>6</td>
<td>11.9</td>
<td>3.4</td>
<td>15.0</td>
<td>44.3</td>
<td>24.8</td>
<td>0.7</td>
<td>nI(9)</td>
<td>(2.8)</td>
<td>(0.8)</td>
</tr>
<tr>
<td>Thrombocytosis</td>
<td>14</td>
<td></td>
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<td></td>
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<tr>
<td>Primary</td>
<td>4</td>
<td>10.1</td>
<td>3.7</td>
<td>9.6</td>
<td>34.3</td>
<td>34.7</td>
<td>6.3</td>
<td>1.3</td>
<td>nI(9)</td>
<td>(2.7)</td>
</tr>
<tr>
<td>Secondary</td>
<td>10</td>
<td>9.4</td>
<td>4.6</td>
<td>15.1</td>
<td>44.4</td>
<td>24.8</td>
<td>1.7</td>
<td>nI(9)</td>
<td>(2.5)</td>
<td>(1.9)</td>
</tr>
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</table>

Abbreviations: nI, normal; †, increased; ††, markedly increased; †, decreased.

*The ploidy was measured in un fractionated marrow aspirates following labeling of MKs with monoclonal antibody to GPIIb/IIIa and DNA staining with PI. Eight hundred to 3,000 MKs were analyzed in each sample.

†Size was estimated by the FLS of the 16N cells in fractionated marrow. Cell internal fine structure and granularity were assessed by SSC.

‡The mean of the relative frequency (%). The SD is listed in parenthesis below the mean.
The increase in ploidy was accompanied by an increase in cell size independent of the degree of the shift in half of the patients. The SSC level again varied directly with the FLS and the expression levels of GPIIb/IIIa rose proportionally with the ploidy and cell size (data not shown). Repeated studies in four patients (at intervals of 30 to 90 days) showed a similar shift in one patient with a stable platelet count; an increase in the rightward shift in two patients when their platelet counts were decreased, and a return to a normal ploidy distribution in one patient who recovered from acute ITP (data not shown).

The six patients with thrombocytopenia secondary to multiple mechanisms that might affect both platelet production and platelet destruction (see Materials and Methods) showed variability in the ploidy distributions. On average, there was a small increase in 8N cells with a reciprocal decrease in 16N cells (Table 2).

Among the patients with thrombocytosis, all four patients with primary disorders, (two with ET and two with PV) showed a marked right shift with 42.3% of cells ≥32N (P < .01). An example of the ploidy distribution in one patient with ET is shown in Fig 2C. The right shift in ploidy was accompanied by a marked increase in cell size (as compared to normal cells with identical ploidy) in the two patients with ET but not in the patients with PV. However, the degree of the shift was significantly higher in the former patients (50.1% v 34.0% cells ≥32N). Analysis of the deviation in the ploidy distribution showed that the most significant change was the increase in 64N and the presence of 128N cells (Fig 2C). The shift to the right was accompanied by a decrease in 16N cells only with no obvious change in the frequency of cells ≥8N.

Of the ten patients with secondary thrombocytosis, the three with chronic blood loss and iron deficiency showed a normal ploidy distribution. Seven patients with thrombocytosis secondary to malignant or inflammatory disorders showed either no recognizable shift (n = 3) or a shift to the right (n = 4) with 40.7% of cells ≥32N. The shift in ploidy was a result of an increase in the frequency of cells ≥32N with a parallel decrease in both 16N and 8N cells. An increase in cell size was detected in one patient. In all groups, the SSC varied directly with the FLS measurements and the expression levels of GPIIb/IIIa rose proportionally with the ploidy and cell size.

**DISCUSSION**

In the present study, we have used flow cytometry to define cellular characteristics of MKs obtained from patients with quantitative platelet disorders using unfractionated routine marrow aspirates. The data suggest that the measurement of the ploidy distribution may be useful in patients with thrombocytopenia by discriminating between abnormalities of production and destruction. It is also possible that the increased frequency of cells ≥64N may be used to distinguish primary from secondary thrombocytosis, but more data are required to clarify the usefulness of this determination.

In previous studies, we have found that flow cytometry was useful for multiparameter cytometric analysis of large numbers of MKs obtained from routine marrow aspirates. In those studies, MKs were initially enriched by density gradient centrifugation over Percoll to facilitate the selection of the MK population, a step that was necessary for establishing the method. Because enrichment steps may involve selective loss of cell subclasses, we have modified the method to permit analysis of unfractionated routine marrow aspirates. In adapting the method for routine clinical use, we have simplified the technique by omitting cell fixation and repeated washing steps usually used in the labeling procedure. We have also found that by using a directly labeled monoclonal antibody, the labeling procedure can be shortened to a single step similar to a recently described method for platelet labeling in whole blood. No treatment with RNase was used because it has been found by others and us that the treatment has only minimal effect on the resolution of the individual ploidy classes and none on the overall ploidy distribution. The omission of the RNase step also obviates incubation at 37°C that may result in cell loss. The permeabilization treatment for DNA staining was
found necessary because preliminary studies using vital staining and either Hoechst 33342 or hydroethidine resulted in variable staining efficiency when they were used without permeabilization.

Because time constraints in routine flow cytometry may be a matter for consideration, we have examined directly the effect of MK enrichment on the ploidy distribution of these cells. Fractionation of marrow over a Percoll density gradient of 1.060 g/mL results in recovery of virtually the entire recognizable MK population.\textsuperscript{16,26-28} and a ten to 12-fold enrichment. The fractionation decreased the analysis time in proportion to the degree of MK enrichment. The ploidy distribution patterns of the fractionated vs the unfractionated samples of the same marrow preparations were similar. However, in some cases, an increase in the frequency of cells $\geq 32N$ in the unfractionated preparation was noted. This may indicate that mature MKs are more susceptible to manipulation than the less mature cells. Nevertheless, the differences did not significantly affect the overall ploidy distribution. Moreover, fractionation did not affect the selection of 2N and 4N MKs, which have a relatively higher density and thus may be lost preferentially in the enrichment step.\textsuperscript{28} According to our selection criteria, only cells with membrane fluorescence levels above those of the control cells are chosen. Because even totally unlabeled cells exhibit some degree of autofluorescence, no MK with a lesser degree of membrane fluorescence can be selected and thus possible loss of such cells cannot be detected. Nevertheless, the marked increase in the frequency of 2N and 4N MKs ($\geq 50\%$ of the total MK population) found in some patients with MK hypoplasia indicated that the method used is sensitive enough to identify low ploidy cells that express relatively low levels of GPIIb/IIIa,\textsuperscript{29} and to detect alterations in their frequency.

In addition to its sensitivity, flow cytometry was found to be efficient for the analysis of large numbers of cells ($\geq 1,000$) in cases of megakaryocytic hypoplasia, even when MKs could not be identified in smears and scarcely found in marrow sections. These abilities obviate the limitations of techniques that (1) do not use lineage-specific markers and are unable to detect low ploidy MKs, or (2) have a practical limit on the number of MKs (100 to 200) that can be analyzed by microscopic examination of marrow sections or cytophotometric analysis of marrow smears.

The ploidy distribution pattern of the normal controls (modal ploidy 16N; 22.0 $\pm$ 4.7% of cells are $\geq 32N$) is consistent with our previous results.\textsuperscript{16} However, the lower frequency of the 2N and 4N cells (11.2 $\pm$ 2.3% compared to 20.9 $\pm$ 4.1% in the previous study), with the relative increase in the frequency of $\geq 16N$ cells (77.4 $\pm$ 3.6% $\pm$ 62.9 $\pm$ 6.4%), can be accounted for by the larger number of normal individuals examined in the present study (19 vs five) and the difference in the technique of cell preparation.

Analysis of the ploidy distribution patterns in the 12 thrombocytopenic patients with MK hypoplasia showed a shift toward the low ploidy cells (left shift). A left shift has been observed in experimental animals hypertransfused with platelets,\textsuperscript{3,4} reflecting a suppressive effect of the increased platelet level on MKs.\textsuperscript{1,3} In man, using primarily cytophotometric methods on stained smears, a left shift has been described in thrombocytopenic patients with acute myelogenous leukemia\textsuperscript{11,14,30} many of whom receiving chemotherapy, and in patients with chronic granulocytic leukemia.\textsuperscript{31,32} Because a left shift was observed in the present study in four patients with isolated megakaryocytic hypoplasia of unknown etiology, we conclude that left shift may be independent of treatment with marrow suppressive agents. A shift in ploidy to the left may reflect selective damage to the more mature MKs or interference with the maturation process.

The shift in ploidy to the right with some increase in cell size found in the patients with accelerated platelet consumption is in agreement with previous observations both in experimental animals\textsuperscript{13} and in man.\textsuperscript{9,11,14} However, other studies have shown no change in ploidy of MKs in ITP patients.\textsuperscript{33,34} Therefore, the possibility that some ITP patients may not show right shift cannot be excluded. In the present study, the ability to analyze relatively large numbers of cells (usually $\geq 1,000$) enables a statistically adequate characterization of ploidy changes and might permit further understanding of their significance. It is of note, for example, that the shift to the right in the patients with accelerated platelet consumption was associated with a reciprocal decrease in the 16N cells without a change in the relative frequency of cells $\leq 8N$ (Table 2). This finding may indicate that factor(s) regulating MK responses to increased platelet demand might have some preferential effect on the more mature high ploidy cells. No correlation has been found between the severity of the thrombocytopenia and the degree of the shift in ploidy as was described in experimental animals.\textsuperscript{5} This may reflect the inherent variability in the patient population and in the magnitude of the individual response to similar stimuli. However, repeated studies in four patients showed a similar shift in one patient with a stable platelet count, an increase in the shift in two patients when their platelet counts decreased, and a return to a normal ploidy distribution in one patient after recovery from the acute phase. These results suggest that (1) the detection of an abnormal ploidy pattern is reproducible; (2) human MKs respond to altered platelet demand in a manner similar to that found in experimental animals; and (3) serial studies may be required to determine the magnitude of the ploidy response to the altered platelet count.

The six thrombocytopenic patients with multiple etiologies showed on average modest changes in ploidy distribution, perhaps a result of opposing mechanisms affecting both platelet production and consumption. The marked shift to the right found in two patients with ET and two patients with PV complicated by thrombocytosis is consistent with the increase in MK polyploidization found in patients with myeloproliferative disease.\textsuperscript{14,31,35} The shift in ploidy is characterized by a significant increase in cells $\geq 64N$ and involves a reciprocal decrease in 16N cells only, similar to the findings in the patients with increased platelet destruction. An increase in the very high ploidy cells is consistent with observations made by others.\textsuperscript{14,33} However, in
of response in this group may reflect differences in the variability in their ploidy distribution. Three patients with stage of PV with a shift to the left. However, this patient did not have thrombocytosis and was not included in our study.

The ten patients with secondary thrombocytosis showed variability in their ploidy distribution. Three patients with chronic blood loss and iron deficiency showed a normal ploidy distribution. Four of the patients with malignant or inflammatory disorders showed a shift to the right irrespective of the degree of the thrombocytosis. A similar shift in reactive thrombocytosis has been previously described in patients mainly with malignant diseases. The variability of response in this group may reflect differences in the mechanisms involved and in the individual capacity to respond to the underlying stimuli.

Despite the implications of some studies in experimental animals that shifts in ploidy are related to platelet demand, it should be emphasized that our results establish only correlations between MK size and ploidy distribution and the various clinical disorders. Parameters other than ploidy such as MK number or mass, and the numbers of platelets produced per MK are no doubt critical to the understanding of the mechanisms regulating platelet production.

We conclude that flow cytometry is useful for quantitative analysis of large numbers of MKs derived from unfractionated routine marrow aspirates. We believe that this technique will become increasingly valuable as new probes of MK maturation and platelet production are developed. The data from this survey suggest that this method will be of diagnostic use. Larger numbers of patients in each of the groups studied will be required to establish the usefulness of flow cytometry for routine evaluation of patients with quantitative platelet disorders.

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Flow cytometric analysis of megakaryocytes from patients with abnormal platelet counts

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