Flow Cytometric Analysis of Normal Human Megakaryocytes

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Megakaryocytes from normal routine human bone marrow aspirates were analyzed by flow cytometry for size, fine cell structure and granularity, membrane expression of glycoprotein (GP) IIb/IIIa and ploidy. Marrow cells were initially enriched for megakaryocytes by a Percoll density gradient and megakaryocytes were labeled with a fluoresceinated monoclonal antibody directed to the GPIIb/IIIa complex. The cells were fixed with paraformaldehyde and stained with propidium iodide (PI) for DNA quantitation. Using two-color flow cytometry, megakaryocytes were identified by their high membrane immunofluorescence and their ploidy was determined according to the relative fluorescence intensity of the PI. Forward light scatter (FSC), correlating with cell size, 90° side light scatter (SSC), reflecting primarily cell internal fine structure and granularity, and total cell membrane fluorescence were examined. To evaluate independently the relationship between size and cell membrane fluorescence obtained by flow cytometry, megakaryocytes were sorted directly on slides and analyzed by a laser-based anchored cell analyzer (ACAS). There was a strong correlation among size, SSC, and the level of membrane fluorescence. The mean diameter of megakaryocytes was 28.1 ± 12.3 µm. The modal ploidy distribution was 16N with approximately one-fifth of the cells ≤4N. The mean FSC and SSC levels increased with increasing ploidy. However, the marked overlap observed between the ranges of these parameters in adjacent ploidy classes suggested that size and SSC increase continuously rather than by discrete steps as is characteristic for ploidy. The total surface membrane fluorescence was correlated with cell size (r = 0.98) as measured by FSC or directly by the ACAS (r = 0.86), and with cell ploidy (r = 0.99) indicating an augmentation in total membrane GPIIb/IIIa expression with an increase in cell size and ploidy. However, estimated GPIIb/IIIa fluorescence density was inversely correlated with FSC suggesting that the GPIIb/IIIa surface epitope density is decreased with increasing cell maturity. We conclude that flow cytometry is a useful technique for the rapid analysis of human megakaryocytes obtained by marrow aspiration, and should be applicable to studies of pathologic states.

The cellular differentiation of human megakaryocytes has proven difficult to study because these cells comprise only 0.05% of nucleated bone marrow cells and no technique has been available for the rapid, highly selective analysis of a statistically adequate number of cells. Flow cytometry is a powerful method for the analysis of cell populations because of its rapidity, sensitivity, and ability to perform multiple quantitative measurements on large numbers of individual cells. Thus, this method is capable of evaluating rare cell populations even in complex mixtures such as bone marrow.

Recently, flow cytometry has been used to analyze the ploidy distribution of rodent and monkey megakaryocytes. Employing indirect immunofluorescence to identify megakaryocytes by antiplatelet antibody and staining the cellular DNA with propidium iodide, Jackson et al characterized the ploidy distribution of rat megakaryocytes in unfractionated marrow. Using a similar two-color flow cytometric method, Worthington et al characterized elutriated rat megakaryocytes according to their relative DNA content, surface membrane immunofluorescence intensity, and forward light scatter (FSC) as a measurement of cell size. We adapted this technique to the analysis of human megakaryocytes derived from routine bone marrow aspirates. Using two-color flow cytometry, megakaryocytes were identified by a platelet-megakaryocytic specific membrane GPIIb/IIIa epitope that was detected by a fluoresceinated monoclonal antibody. We measured membrane fluorescence intensity, relative DNA content, FSC for estimation of cell size, and side light scatter (SSC) for assessment primarily of cell internal fine structure and granularity. The relationships among these characteristics are described in the total megakaryocytic population and within the individual ploidy classes.

Materials and Methods

Marrow Preparation

Six to 8 mL of bone marrow was collected from a single site from the posterior iliac crest of normal volunteer donors. The marrow was aspirated after obtaining informed consent according to the guidelines of the Human Subjects Committee of the Research Institute of Scripps Clinic. Cells were collected into 10-mL plastic syringes containing ½ volume acid-citrate-dextrose (ACD formula A), EDTA and prostaglandin E (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.

Cell Count and Size Measurement

Marrow cells were enumerated with a Hematology Series 15 Cell Counter (Baker Instruments, Allentown, PA) or a hemocytometer.
ANALYSIS OF HUMAN MEGAKARYOCYTES

The diameters of megakaryocytes freshly isolated by cell sorting were measured with an ocular micrometer as previously described.11

Fractionation of Marrow Cells

The cell suspension was adjusted to 2 x 10^6 cells/mL, mixed with Percoll (Pharmacia Fine Chemicals, Piscataway, NJ) to a final density of 1.020 g/mL (isotonic 83.1% Percoll diluted with PBS containing 13.6 mmol/L sodium citrate), layered over 12 mL of Percoll density 1.055 g/mL, and subsequently overlaid with 10 mL of MK medium. Following centrifugation at 400 g for 20 minutes at 15°C, 8 mL of the upper medium layer was removed and the cells were collected from the upper Percoll layer and the interface (±1.055 g/mL). The collected cells were then washed by mixing with 1.5 volume of cold MK medium containing 6% BSA, and centrifugated at 300 g for ten minutes at 15°C. The supernatant was decanted and the cell pellet was resuspended in the same medium and stored on ice for antibody labeling. The Percoll density gradient fractionation resulted in the depletion of 96% of total marrow cells and an increase in megakaryocytic frequency to 1.3%, representing a 26-fold enrichment. The light density fraction contained ≥96% of morphologically recognizable megakaryocytes in accord with our previous work,11 as assessed by sorting and examining by phase-contrast microscopy and hematoxylin or Wright-Giemsa staining according to the criteria described by Levine.6,12

Megakaryocyte Labeling

Megakaryocytes were directly labeled with saturating concentrations of fluoresceinated IgG, mouse monoclonal antibodies (or F(ab')2, fragment thereof) directed to a platelet-specific GP IIb/IIIa epitope. The antibodies have been shown to react only with platelets or megakaryocytes.13 These antibodies (designated P1, and P2) were a gift from Dr. Z. M. Ruggeri of the Research Institute of Scripps Clinic, and were fluoresceinated to an F/P ratio of 3:1 by standard techniques. The cell count was adjusted to 10^6 cells/mL with MK medium containing 6% BSA, and centrifuged at 300 g for 30 minutes on ice. The saturating concentration of the monoclonal antibodies was determined by quantitative flow cytometry without further delay. The total time required to prepare the marrow cells for analysis was approximately three hours.

Flow Cytometry

Cells were analyzed using a FACS-440 flow cytometer (Becton Dickenson, Mountain View, CA) equipped with a Spectraphysics 164-05 argon-ion laser, tuned to deliver 200 mW at 488 nm. A 50 μm diameter nozzle was used and the sorting rate was limited to 1,000 cells per second to optimize resolution. Small particles up to 7 μm in diameter were electronically gated out according to their FSC using standard polystyrene beads (5.3, 7.8, and 10.2 μm; Becton Dickenson), fixed human RBCs, and peripheral blood lymphocytes. A dichroic (570 nm) mirror was used to separate the emission spectrum of the fluorescein (green) and the PI (red). The green fluorescence was further filtered by a 330/30 nm band-pass filter and the red fluorescence was passed through a 625/35 nm band-pass filter before detection. Cells stained with one fluorochrome only were used to correct overlap between the emitting spectra.

Megakaryocytes were identified by their distinct green fluorescence >50-fold that of control cells stained with fluoresceinated monoclonal antibody to human thyroglobulin. Bidimensional plots of green fluorescence (FL1) vs. FSC revealed an easily distinguishable subset of the highly fluorescent large megakaryocytes and was used to establish the location of the desired cell population. Analysis of the number of cells in ploidy classes ≥8N selected at various FL1 gate levels showed this setting to be optimal since it was at the maximal FL1 level above which the number of cells in these ploidy classes decreased. To cover the entire range of fluorescence emission, green fluorescence data were collected in a four log scale, and the red fluorescence data in a three log scale, using an adjustable logarithmic photomultiplier. Data collection and analysis of the fluorescence intensities, the FSC and the SSC levels of each cell acquired in a list mode, were carried out with a Hewlett Packard computer equipped with a Consort 30 program (Becton Dickenson). The ploidy distribution was determined by setting markers at the nadirs between peaks using the 2N and 4N peaks of the FL1-ungated cells as internal reference standards. For further analysis, the total megakaryocytic population was sorted directly on poly-D-lysine coated slides. The cells were then evaluated by an anchored cell analyzer (vida infra) or stained with hematoxylin.

Evaluation of the Correlation Between Cellular Parameters

To evaluate the correlation between two cellular parameters (eg, FL1 and FSC) the distribution curve of the megakaryocytic population according to the first parameter (the FL1) was arbitrarily divided into six equally spaced groups, each with increasing mean FL1 levels. Next, the mean level of the second parameter (the FSC) of each group was derived directly from the original data acquired in a list mode, resulting in a corresponding set of values for FSC. The correlation between FL1 and FSC was calculated using the two corresponding sets of values.

Estimation of Membrane GP IIb/IIIa Density

The membrane density of GP IIb/IIIa was estimated by calculating the cell membrane fluorescence density. The FSC distribution range of the megakaryocytic population was arbitrarily divided into six equally spaced groups, each with an increasing mean FSC level. The mean FL1 of each group was then divided by the corresponding FSC. The ratio expressing the FL1 level per unit area (hence the fluorescence density) was plotted against FSC to evaluate the relationship between these parameters.
Verification of Flow Cytometric Parameters

To verify the relationship between FLI and FSC obtained by flow cytometry, the cell size and the total surface membrane fluorescence of antibody-labeled fixed megakaryocytes sorted directly onto slides were measured with a laser-based anchored cell analyzer (ACAS 470 Workstation, Meridian Instruments Inc, Okemos, MI). A computer-controlled two-dimensional stage moves the slides in a defined manner above the objective (40 x) of an inverted epifluorescence microscope. The microscope objective focuses the argon-ion laser beam (adjusted to deliver 200 mW at 488 nm) to a 1.0 μm spot that excites fluorescence in individual cells in 1.0 μm steps. A digital signal reflecting fluorescence intensity is shown on a video monitor as a color-coded representation of the fluorescence distribution. Cell size is measured according to the fluorescence of the corresponding area. Cell size, total cell immunofluorescence, and bidimensional correlated histograms of total cell membrane fluorescence vs size were calculated and plotted with a built-in IBM-AT computer.

Statistical Analysis

Standard least squares regression analyses and correlation analyses among the cellular parameters were performed on a VAX 11/750 computer.

RESULTS

Characterization of the Total Megakaryocytic Population

Selection of cells expressing GPIIb/IIIa. Figure 1A shows the distribution of the megakaryocyte-enriched marrow cells by immunofluorescence intensity (FL1) vs FSC (reflecting primarily cell size). The plot reveals a distinct, large, highly fluorescent cell population comprising approximately 2% of the total cells (Fig 1A-1). These cells exhibit a fluorescence intensity >50-fold that of the major marrow cell population (Fig 1A-2) or the control sample incubated with fluoresceinated monoclonal antibody to human thyroglobulin (Fig 1B). To analyze the megakaryocytic cells, an electronic gate was set at a fluorescence level above that of the control or the major marrow cell population. This gate (Fig 1A, horizontal line) selected the entire discrete highly fluorescent cell population. Previous studies have shown that when the large cells in this population (arbitrarily defined as cells with a FSC level greater than that of the major cell population; channel ≥20 in Fig 1A corresponding to a cell diameter ≥20 μm) were sorted, they were morphologically pure megakaryocytes. Small megakaryocytes (channel <20 in Fig 1A corresponding to a cell diameter <20 μm) comprised approximately 25% to 30% of the selected population. Their number in the high density fraction (>1.055 g/mL) was 10% of that found in the low density (≤1.055 g/mL) fraction. To ascertain the lineage association of these small highly fluorescent cells, they were sorted and stained immunocytochemically with a monoclonal antibody to platelet membrane GPIb. Three-quarters of these cells reacted positively.

Cell size. The mean FSC level of the total megakaryocytic cells was fourfold that of the major cell population. Using standard polystyrene beads, fixed human RBCs, and peripheral blood lymphocytes to estimate the size of the small megakaryocytes together with direct microscopy to measure the size of the large ones, the mean diameter of the total megakaryocytic population was 28.1 ± 12.3 μm (range, 10.4 to 46.0 μm; n = 4,180). The size of fixed total megakaryocytic cells sorted directly by the flow cytometer on slides was studied by an anchored cell analyzer (ACAS) as described in Materials and Methods. The distribution of 417 megakaryocytes by size is shown in Fig 2. The mean cell diameter was 32.6 ± 21.8 μm with a range of 12.0 to 50.3 μm.

Cell internal fine structure. Plotting the fluorescence intensity of the cells shown in Fig 1A against SSC (reflecting primarily cell internal fine structure and granularity) revealed a discrete highly fluorescent with high SSC cell population (Fig 3). The number of cells in the selected population (Fig 3-1) is identical to that gated (using the same FL1 gate) in Fig 1A, indicating that the majority of...
megakaryocytes are intensely fluorescent, large, and have complex cytoplasmic structure. The mean SSC level of the megakaryocytes was sevenfold that of the major cell population (Fig 3-2).

Correlation among the cellular characteristics. The relationship among FL1, FSC, and SSC of the total megakaryocytic population is shown in Fig 4. The results show a correlation between size and total cell membrane fluorescence (Fig 4A; $r = 0.97$), SSC and total cell membrane fluorescence (Fig 4B; $r = 0.99$), and size and SSC (Fig 4C; $r = 1$).

To verify the relationship between FL1 and FSC as assessed by flow cytometry, the size and the total membrane fluorescence of sorted and fixed megakaryocytes was measured directly using an ACAS. Figure 5 shows the bidimensional correlated distribution of total membrane fluorescence $\nu$ size ($r = 0.85$), confirming the correlation determined by flow cytometry.

GP\(\text{IIb/IIIa}\) density. The surface density of the GP\(\text{IIb/IIIa}\) complex was estimated indirectly by dividing the total cell membrane fluorescence, FL1, by the FSC (see Materials and Methods). Figure 6 shows that the fluorescence density is inversely related to FSC ($r = -0.98$), suggesting that small cells have a higher surface GP\(\text{IIb/IIIa}\) epitope density than the large ones, although the total membrane fluorescence of the large megakaryocytes is higher. It is noteworthy that in fluorescence micrographs of isolated pure megakaryocytes smaller cells frequently fluoresce more intensely than the larger ones (Fig 7).

Characterization of Megakaryocytes Within Ploidy Classes

Ploidy distribution. To analyze the ploidy distribution of the megakaryocytic population, the fractionated marrow cells were doubly stained, first with the fluoresceinated

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**Fig 3.** Distribution of megakaryocyte-enriched marrow cells by side scatter. Bidimensional contour plot of FL1 v 90° SSC of the cell population shown in Fig 1A with the identical fluorescence gate setting (horizontal line).

**Fig 4.** Relationship between FL1, FSC, and SSC of megakaryocytes. The mean levels of the parameters were calculated as described in Materials and Methods, and were plotted against one another. Both abscissas and ordinate represent relative levels. The error bars indicate ±1 SD. The curves show a representative experiment selected from five similar experiments. Twenty thousand megakaryocytes were analyzed.

**Fig 5.** Relationship between membrane fluorescence and size of isolated, fixed megakaryocytes. The histogram represents the identical ACAS analyzed cell population as shown in Fig 2. The oblique line indicates the linear regression curve ($r = 0.86$). The abscissa represents cellular area in pixels. Cell diameter (μm) in this setting equals $2.256 \times (\text{pixels})^{1/2}$.
monoclonal antibody to GPIIb/IIIa (which emits green fluorescence—FL1) and then with the fluorescent DNA-intercalating agent propidium iodide (which emits red fluorescence—FL2). The intensity of fluorescence of the latter is correlated with the DNA content.

Figure 8A shows the DNA histogram of the megakaryocyte-enriched marrow cells. The major DNA peaks are those of 2N and 4N cells. The peaks of higher ploidy classes are barely visible. To analyze the ploidy distribution of megakaryocytes selectively, an electronic gate was set at an FL1 level to include the entire highly green fluorescent cell population as described above. This setting resulted in a marked shift toward higher ploidy classes (Fig 8B). The major ploidy peak is 16N with additional peaks corresponding to 32N and 64N. The distribution of the FL2 into discrete peaks with successive doubling of the fluorescence intensity confirms that the histogram represents individual ploidy classes rather than randomly formed cell aggregates. The shift toward the higher ploidy classes as a result of increasing the green gate level (FL1) also confirms the specificity of the labeling since the only marrow-derived cells known to exhibit a DNA content of >4N (with the very rare exception of osteoclasts) are megakaryocytes.

The ploidy distribution derived from such DNA histograms is shown in Table 1. While cells of >4N constitute only 2.2% of the total cells, approximately 80% of the megakaryocytic population are 8N cells and higher with a modal ploidy of 16N. It is noteworthy that occasionally an additional two peaks greater than 64N were seen. However, the numbers of cells in these ploidy classes were inadequate for statistical analysis.

To determine whether megakaryocytes were lost selectively during the labeling procedure with the monoclonal antibody to GPIIb/IIIa, ungated analysis was performed on labeled and unlabeled marrow samples stained with PI. No significant difference was found in the frequency of ≥8N cells in the analyzed samples.

No significant difference was observed in the ploidy distribution of the paraformaldehyde-fixed cells vs the hypotonic citrate and RNase-treated cells. The former procedure was ultimately used to permit size measurement and to prevent possible internalization and processing of the fluorescent membrane immune complexes.

Cellular characteristics of megakaryocytic endoreduplication. To identify cellular characteristics that might correlate with DNA content a multiparameter analysis of FSC, SSC, and FL1 levels of each ploidy class was carried out. Figure 9 shows the correlation between the mean values of these parameters and the ploidy level. While the mean values increase with increasing ploidy, a considerable overlap among the FL1, the FSC, and the SSC ranges of adjacent ploidy classes exists as reflected by the wide standard deviation that progressively increases with increasing ploidy level.

The mean FL1 levels of the 2N and 4N cells are similar, although the mean FSC of the 4N cells is higher than that of the 2N cells. Thus, according to the selection criteria used,
Table 1. Ploidy Distribution of Megakaryocytes and Fractionated Total Marrow Cells

<table>
<thead>
<tr>
<th>Cells Analyzed*</th>
<th>Ploidy Class</th>
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<tbody>
<tr>
<td></td>
<td>2N</td>
</tr>
<tr>
<td>Megakaryocytes</td>
<td></td>
</tr>
<tr>
<td>(n = 20,000)</td>
<td>10.0 ± 4.1†</td>
</tr>
<tr>
<td>Fractionated marrow cells</td>
<td>74.0 ± 18.2</td>
</tr>
<tr>
<td>(n = 50,000)</td>
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*Marrow cells were fractionated by Percoll density gradient centrifugation resulting in the depletion of 96% of the total nucleated cells and a 26-fold enrichment in megakaryocytes. Megakaryocytes were selected by the green fluorescence (FL 1) gate setting as described in the text.
†Percentage of total ± 1SD (n = 5).

Fig 9. Relationship between FL1, FSC, SSC, and ploidy. The open squares represent the mean level of the measured parameter for each ploidy class ± 1SD. The ordinate represents relative levels. The curves show a representative experiment (n = 5).

2N cells express equal levels of GPIIb/IIIa although they are smaller than 4N cells.

Megakaryocytes exhibit higher SSC than other cells of fractionated marrow. Figure 10 shows the SSC levels of the individual ploidy classes within the megakaryocytic and the total enriched marrow cell population. The SSC levels of the 2N and 4N megakaryocytes are approximately twofold higher than that of the corresponding total marrow cells. As might be expected, the SSC levels of the 8N and higher ploidy classes are virtually identical, since cells of these ploidy classes comprise megakaryocytes in both populations. However, since the selection of megakaryocytes is based on a high FL1 level, there could be preferential selection of the highly fluorescent 2N and 4N megakaryocytes with possibly a more mature and granular cytoplasm than the average marrow cells of the same ploidy class.

The correlations among the FL1, the FSC, and the SSC levels of the various ploidy classes are shown in Fig 11. As expected, all parameters are well correlated (r = 0.98-1.0) with the exception of the similar FL1 levels of 2N and 4N cells.

DISCUSSION

In this study, several characteristics of normal human megakaryocytes were defined by flow cytometry. This technique was used because of its sensitivity, rapidity, reproducibility, and ability to perform multiparameter measurements on a large number of individual cells. The method was adapted to marrow cells obtained from normal aspirates to obviate the requirement for ribs, and to permit serial studies of patients with disorders of megakaryocytopoiesis.

Enriched marrow was used to facilitate the selection of the megakaryocytic population that could be recognized as a discrete cellular subset. Moreover, the enrichment step reduced flow cytometry analysis time. The density of the single Percoll cut was increased from 1.050 g/mL (usually used for the enrichment step)5,6,8 to 1.055 g/mL. This modification increased the proportion of morphologically recognizable megakaryocytes from 90% to >96% as found in our preliminary studies, and is in agreement with our previous data.11 While significant numbers of large megakaryocytes are not lost by this preliminary enrichment step, that may not necessarily be true for the small megakaryocytes. A direct analysis of the recovery of small (<20 μm) highly fluorescent cells in this study demonstrated that only 10%
were found in the high density (>1.055 g/mL) fraction. Since the majority of 2N and 4N cells are also small, and since these ploidy classes comprise 10% and 11%, respectively, of the megakaryocytic distribution, the 10% loss of small cells would not significantly influence the overall ploidy distribution. However, in pathologic conditions where a large increase in small highly fluorescent megakaryocytes with high density might occur, the extent to which these cells may be excluded must be assessed. If necessary, megakaryocytic analysis can be performed on unfractionated marrow, although increase in sorter time and expense should be considered.

Previous studies by us and others have shown that up to 70% of all small megakaryocytes are found at densities >1.050 g/mL. The discrepancy between the low percentage of cells found at high density in this study and the much higher percentage at high density in previous studies can be reconciled according to the selection criterion used for megakaryocytes. In the present study, only highly fluorescent cells (defined as having fluorescence intensity >50-fold of the control population) were selected to obviate inclusion of cells with nonspecific or autofluorescence, whereas in our previous study11 cells positive for GPIIb/IIIa were chosen by a visual judgment of fluorescence intensity. One implication of this difference is that there may be two categories of small, low ploidy megakaryocytes: (1) those exhibiting high fluorescence meeting our arbitrary defining criterion, and (2) those with less fluorescence, which despite being discernable visually, do not meet the flow cytometric selection criterion. Indeed, the possibility of excluding such cells was also acknowledged by Jackson et al in a flow cytometric study of unfractionated rat marrow. Since the megakaryocytic colony-forming cell may well be devoid of GPIIb/IIIa11 it is likely that there is a continuous acquisition of this protein complex with maturation. This possibility is in agreement with the findings of Jackson et al11 that rat megakaryocytes of density >1.033 bound substantially less antiplatelet antibody than those of the lighter density fraction.

The specificity of megakaryocytic selection depends on the specificity of the cell labeling and the accuracy of the flow cytometric settings. As previously shown, the monoclonal antibody we used reacts only with platelets and megakaryocytes in marrow. The ability to discriminate between megakaryocytes and other marrow cells is demonstrated in Fig 1 where the mean fluorescence level of the megakaryocytes was more than 50-fold that of the control cells. The precision of the selection was evaluated by sorting the large, highly fluorescent cells on slides and examining them by phase contrast microscopy to ascertain that these cells are morphologically recognizable megakaryocytes.

The mean diameter of megakaryocytes in suspension was 28.1 µm and the range 10.4 to 46.0 µm. Levine1 reported a mean diameter of 24 µm and a range of 10 to 48 µm in unseparated marrow, and Berkow et al20 reported a diameter of 33.8 ± 9.6 µm in centrifugal elutriated megakaryocytes. The mean diameter of fixed megakaryocytes examined by the ACAS was 32.6 ± 21.8 µm with a range of 12.0 to 50.3 µm. An increase in diameter of megakaryocytes in marrow smears was previously reported by Levine et al (mean 36 µm)23 and in cytocentrifuged preparations by Berkow et al (50.4 ± 15.5 µm).20 The observation that sorted cells >20 µm in diameter are morphologically recognizable megakaryocytes is in agreement with that of Levine et al.12 Only 2% of the enriched marrow cells (which is similar to the percentage of the megakaryocytes in the preparation) are larger in diameter than 20 µm.

The ploidy distribution with a modal value of 16N is in accord with our previous work using chromomycin A3-stained cells. Levine1 indicated the similarity between human megakaryocytes and megakaryocytes of other species with respect to ploidy and some other cellular characteristics. It is of interest therefore, that the ploidy distribution of normal human megakaryocytes in this study is similar to that found by Levine et al11 in guinea pig marrow using Feulgen staining, by Jackson et al12 in unfractionated rat marrow analyzed by two-color flow cytometry, and by Worthington et al10 in elutriation-enriched rat marrow using flow cytometry.

To examine the relationships among the cellular characteristics of size, fine internal structure complexity, expression level of GPIIb/IIIa, and DNA content, a correlative analysis was performed between the corresponding flow cytometric parameters (FSC, SSC, FL1 and FL2) for the total megakaryocytic population as well as for each particular ploidy
classes. Figure 4 shows that a correlation exists among the FSC, SSC, and FL1 of the total megakaryocytes. The correlation between membrane fluorescence level and cell size was also confirmed by the analysis of anchored cells (Fig 5). Nevertheless, as referred to previously, the area of fixed cells may not be identical to the cross-sectional area of cells determined by flow cytometry. It is noteworthy that SSC appears to be a distinct characteristic of megakaryocytes that can be used instead of FSC in bidimensional plots along with FL1 to distinguish this population from other bone marrow cells (Figs 3 and 10).

Although the FL1, the FSC, and the SSC ranges of the three main ploidy classes are a continuum, the mean values of these parameters significantly correlate with the ploidy level (Fig 9). Analysis of the relationships among the mean values of these three parameters showed that they correlate well with one another, with the exception of the similar FL1 levels of the 2N and 4N classes (Fig 11).

A correlation between microscopically determined cell size and ploidy of megakaryocytes in suspension was described by Levine et al who also observed the overlap of size among the various ploidy classes. Worthington et al analyzed elutriated rat megakaryocytes by flow cytometry and found a correlation between ploidy level and both FSC and membrane immunofluorescence. However, no simple correlation was found between membrane immunofluorescence and FSC of the individual ploidy classes. The distribution of FSC and the membrane fluorescence of the major three ploidy classes was broad with substantial overlap. The authors suggested that in addition to the natural complexity involving the relationship between size and FSC, technical considerations such as the proximity of megakaryocytic diameter to that of the nozzle used (70 μm), cytoplasmic fragmentation caused by shear forces, and the use of fragile viable cells may complicate the interpretation of the data. In an attempt to overcome some of these difficulties we used fixed cells as well as a large nozzle (110 μm) and lower sheath pressure (6 lb instead of 11 lb) to decrease cell shape change and possible damage. Nevertheless, as noted previously, the relationship of FSC to cell size (especially for the large cells) is complex and has yet to be characterized adequately.

Despite the finding that total cell membrane fluorescence is correlated with cell size, the estimated fluorescence per unit area decreases with increasing FSC (Fig 6) implying that membrane GPIIb/IIIa epitope density varies inversely with cell size. If corroborated by direct measurements of size in suspension, this finding suggests that the synthetic rate of the GPIIb/IIIa complex decreases during cellular maturation. This possibility is supported by the data of Jackson et al who measured thymidine labeling indices in rat megakaryocytes separated according to different buoyant densities, and of Greenberg et al who measured the amino acid incorporation rate in isolated rat megakaryocytes, showing that the biosynthetic rate of both DNA and protein decrease in mature megakaryocytes. Alternatively, the level of expression rather than synthesis of GPIIb/IIIa may decrease during cellular maturation. Blocking epitope-binding sites by increasing membrane demarcation may, for example, decrease the expression of GPIIb/IIIa.

The data in this report document that flow cytometry is a useful technique for the analysis of several characteristics of human megakaryocytes obtained by routine marrow aspiration, and suggest that the technique will be useful for the examination of these characteristics in pathologic states. Flow cytometry will become increasingly valuable as new probes of megakaryocytic maturation and platelet production are identified.

ACKNOWLEDGMENT

The authors appreciate the excellent technical assistance of Don McQuitty, and statistical advice provided by Dr James Koziol.

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