Separation of Human Megakaryocytes by State of Differentiation on Continuous Gradients of Percoll: Size and Ploidy Analysis of Cells Identified by Monoclonal Antibody to Glycoprotein IIb/IIIa

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Human bone marrow was separated on continuous Percoll density gradients to analyze the distribution of cells of the megakaryocytic lineage. Megakaryocytes were identified by indirect immunofluorescence using a monoclonal antibody (LJP4) specific to the glycoprotein IIb/IIIa (GPIIb/IIIa) complex of platelets. Neither endothelial cells nor monocytes expressed the epitope identified by this antibody. Simultaneous measurement of size and ploidy were made on 2,359 GPIIb/IIIa-positive cells. The smallest cells were located in the most dense fractions where 81% of all 2N and 66% of 4N cells were found at densities ≥1.050 g/mL. The largest cells were detected in the least dense fractions, with 70% of 16N, 78% of 32N, and 100% of 64N cells found at densities <1.050 g/mL. Ninety-four percent of all GPIIb/IIIa-positive cells <14 μm in diameter were found at densities >1.050 g/mL. An exception to this inverse relationship was observed in the uppermost gradient fractions where an anomalous distribution of size and ploidy was found. Megakaryocytic viability was identified as being greater than 90% in all fractions. The data show that megakaryocytic differentiation as assessed by size and ploidy varies inversely with Percoll density. Separation of marrow on continuous Percoll gradients may be a useful method to separate megakaryocytes at different stages of differentiation.

THROUGHOUT the past decade technical advances in separation technology and in vitro assay methods have led to new insights into the structure and regulation of the megakaryocytic lineage in animals.[1-5] It has only been in the past several years, however, that similar technology has been available for the study of human megakaryocytopoiesis. These technical advances have included in vitro semisolid assays for the human megakaryocytic colony-forming cell,[6-9] identification of immature and mature megakaryocytes by means of specific antisera,[10-17] and separation techniques to enrich for megakaryocytes.[10,18,19]

In mice, the relatively specific marker enzyme acetylcholinesterase has been used to identify early cells of the megakaryocytic lineage that would be otherwise unrecognizable.[20,21] In a recent study using this enzyme marker, we examined the use of continuous Percoll gradients to separate murine megakaryocytes and found that megakaryocytic differentiation as assessed by cell size and ploidy was inversely proportional to density.[22] Since human megakaryocytes are enriched at relatively low density on Percoll gradients whereas megakaryocytic progenitor cells are enriched at higher density, it seems likely that human megakaryocytic differentiation is also inversely related to Percoll density.[9,10] In this study, we have separated human marrow on continuous Percoll gradients using a monoclonal antibody to the platelet-specific glycoprotein IIb/IIIa (GPIIb/IIIa) complex to identify megakaryocytes.[13,14] The data show that low-ploidy cells express GPIIb/IIIa and that human megakaryocytes separate according to their state of differentiation on Percoll gradients.

MATERIALS AND METHODS

Marrow cells. Human bone marrow was aspirated from the posterior iliac crest of normal volunteer donors. Marrow was obtained after informed consent according to the guidelines of the Human Subjects Committee of Scripps Clinic and Research Foundation. The marrow was collected into sterile syringes containing preservative-free heparin (50 U/mL; Sigma Chemical Company, St Louis, Mo). Within 20 minutes of aspiration, the marrow was diluted with 3 vol of suspension medium consisting of 0.129 mol/L NaCl, 8.614 mmol/L Na_2HPO_4, 1.6 mmol/L KH_2PO_4, 13.6 mmol/L Na citrate, 11.1 mmol/L glucose, 1 mmol/L adenosine, 2 mmol/L theophylline, 2.3×10^{-6} mol/L prostaglandin E_1 (PGE_1), and 118.4 KU/mL of DNAase I (Calbiochem-Behring Corp, La Jolla, Calif). The pH of the media was adjusted to 7.0, and the osmolarity was adjusted to 295 ± 3 mosm/L. The marrow was gently pipetted with a siliconized Pasteur pipette.

Separation of marrow cells. The cell suspensions were first layered over a single-density cut of Percoll at 1.080 g/mL. Percoll was diluted to the desired density with suspension medium. No more than 6 mL of cell suspension was layered over 6 mL of Percoll in 15-mL tissue culture tubes. After centrifugation at 400 g for 20 minutes at room temperature, the cells were collected, washed once in suspension medium, and resuspended into 10 mL of the same medium. A 40-mL 30% to 63% continuous Percoll density gradient was formed with a gradient maker (Buchler Density Gradient System, Haake Buchler Instruments Inc, Saddlebrook, NJ) and layered into a 50-ml plastic culture tube (Corning Glass Works, Corning, NY) at a rate of 2.5 mL/min. Ten milliliters of the marrow cell suspension were layered over the gradient, and the cells were centrifuged at 400 g for 20 minutes at room temperature. Subsequently, the bottom of the tube was punctured with an 18-gauge needle, and the gradient was separated into 25 2-ml fractions at 2.5 mL/min using a peristaltic pump and fraction collector (PI and FRAC 100, Pharmacia Fine Chemicals, Piscataway, NJ).

Cell counts and viability. Cell counts were performed with an automated cell counter (Hematology Series 150, Baker Instrument Co, Allentown, Pa) both before and after washing the cells in each fraction. Total cellular viability was determined by trypan blue exclusion and acridine orange inclusion–ethidium bromide exclusion in each fraction.[23]

Megakaryocytic viability was specifically ascertained by the

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Submitted Aug 7, 1985; accepted Dec 16, 1985.

Supported in part by Grants HL29037 and HL31950 from the National Institutes of Health and The Roon Center for Arteriosclerosis and Thrombosis.

Publication number 4010 BCR from the Research Institute of Scripps Clinic, La Jolla, Calif.

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0006-4971/86/6705-0016$03.00/0

fluorescein diacetate (Sigma) method.[24,25] In this technique, viable cells incorporate fluorescein diacetate and metabolize the dye, resulting in intense intracellular fluorescence, whereas nonviable cells do not. Cells from each fraction were first incubated with monoclonal antibody LJP4 for 30 minutes at 0 °C (12.6 μg/mL of the purified IgG diluted in 0.1% ovalbumin). Subsequently, the cells were washed and then incubated with a 1:20 dilution (in 0.1% ovalbumin) of rhodamine-conjugated goat $F(ab')_2$ antimouse IgG (Tago, Inc, Burlingame, Calif) and a fluorescein diacetate solution (0.1 μg/mL) for 20 minutes at 0 °C. After washing, the cells of each fraction were examined with a Zeiss Photoscope 3 (Carl Zeiss, Oberkochen, West Germany) equipped for both fluorescein and rhodamine fluorescence. Megakaryocytes were first identified by using the rhodamine filters; subsequently, the fluorescein filters were used to assess viability. Living cells exhibited bright green fluorescence.

Studies with a monoclonal antibody to monocytes. To determine whether platelet fragments adhere to the surface of monocytes and result in the misidentification of such cells as megakaryocytes, double fluorescence-labeling studies were performed. A 1:100 dilution of both anti–Mac-1 (Hybritech, San Diego), a rat antimouse monoclonal antibody with cross-reactivity to human monocytes and natural killer cells,[26] and LJP4 were applied to separated marrow cells for 30 minutes, washed, and followed by the simultaneous 30-minute incubation with a 1:32 dilution of fluoresceinated goat antirat IgG and rhodamine goat antimouse IgG as previously described. After washing, slides were examined for expression of both antigens.

Studies with endothelial cells. To determine whether human endothelial cells react with a monoclonal antibody to GPIIb/IIIa, one-day-old primary cultures of human umbilical vein endothelial cells grown on glass coverslips were incubated with LJP4 at 12.6, 63, and 126 μg IgG/mL for two hours. After rinsing with phosphate-buffered saline (PBS) the fluoresceinated $F(ab')_2$ fragment of goat antimouse IgG was added for 30 minutes. The coverslips were rinsed and examined for fluorescence. As a positive control, some coverslips were treated with monoclonal antihuman von Willebrand factor (kindly provided by Dr T. Zimmerman, Scripps Clinic and Research Foundation). The expected granular fluorescence pattern was observed.[27]

Studies with a monoclonal antibody to GPIb. To determine whether cells positive for GPIIb/IIIa also express GPIb, 59 GPIIb/IIIa-positive cells identified by rhodamine fluorescence were mapped by recording the microscope stage coordinates of their location. The slides were then incubated for three hours at room temperature with biotinylated LJP3 IgG, a monoclonal antibody produced as will be described. After rinsing with PBS, the slides were incubated with an avidin-biotin-glucose oxidase complex (Vectastain; Vector Laboratories, Burlingame, Calif) for one hour, rinsed, and incubated with the glucose oxidase substrate for 20 minutes. Positive cells were identified by a purple deposit.

Monoclonal antibody preparation. Monoclonal antibodies against human platelet membrane glycoproteins were produced by immunizing BALB/c mice with intraperitoneal injections of washed platelet suspensions. Platelets were prepared by gel filtration of platelet-rich plasma through Sepharose CL 2B (Pharmacia) as previously described with the omission of albumin.[28] The first injection consisted of 10^8 platelets in complete Freund's adjuvant. Two boosters of 10^8 and 1.5×10^8 platelets, respectively, in incomplete Freund's adjuvant were given at 1-week intervals. A final booster of 3×10^8 platelets without adjuvant was given four days before fusion. Mouse spleen cells were fused with mouse plasmacytoma cells at a ratio of 6:1. The antibody used for the present study, designated LJP4, is an IgG_1 and was derived from a fusion with P3X63–Ag 8.653 plasmacytoma cells. Fusion and growth of hybridomas were performed using standard procedures.[29] Positive clones producing antiplatelet antibodies were selected by enzyme-linked immunoadsorbent assay.[30] This assay detected mouse IgG reacting with washed platelets bound to plastic microtiter wells by means of poly-L-lysine hydrobromide having a molecular weight of 150,000 to 300,000 (Sigma). Positive clones were subcloned twice by limiting-cell dilution.[31] Monoclonal antibody was then produced in mouse ascites fluid as described.[29]

Purified IgG was obtained from ascites fluid by binding to protein A–Sepharose (Sigma) followed by stepwise elution at pH 6, 4.5, and 3.[32] Subclass specificity of the IgG molecules was defined by Ouchterlony immunodiffusion against the appropriate antisera.[33] LJP4 bound to the GPIIb/IIIa heterodimer complex, but not to isolated GPIIb or GPIIIa following dissociation of the complex by treatment with EDTA at pH 8 at 37 °C for 15 minutes. This was determined by measuring both the binding of ^{125}I-labeled LJP4 IgG to whole platelets and the coprecipitation of the labeled antibody with precipitin arcs formed by membrane glycoproteins reacting with a polyclonal antiplatelet antiserum in a crossed immunoelectrophoretic system.[34]

Monoclonal antibody LJP3 IgG, specific to platelet GPIb, was prepared as previously described. One mg/mL of the IgG was dialyzed against 0.1 mol/L $NaHCO_3$ at 4 °C for six hours. After dialysis, 120 μL of biotin-N-hydroxysuccinimide ester (1 mg/mL in dimethyl sulfoxide) was added per milliliter of protein and incubated at room temperature for four hours. The biotinylated IgG was then dialyzed against PBS overnight.

Number, size, and ploidy measurements of megakaryocytes. Cytocentrifuge preparations of each fraction were performed using centrifuge buckets designed for quantitative purposes.[35] The cells were fixed with acetone and ethanol at a ratio of 3:17 for ten minutes at room temperature, washed with PBS, then rinsed with distilled water and dried. The specimens were first incubated with 12.6 μg/mL of the IgG fraction of monoclonal anti-GPIIb/IIIa antibody for 30 minutes at room temperature. Subsequently, the slides were washed three times with PBS, rinsed with distilled water, and dried. The slides were then incubated with a 1:48 dilution of rhodamine-conjugated goat $F(ab')_2$ antimouse IgG for 30 minutes at room temperature. Following washing three times in PBS and rinsing with distilled water, the slides were then stained with 1.7×10^{-5} mol/L chromomycin A3 (Calbiochem-Behring) for 30 minutes as described previously.[36] Cells in each microscopic field were examined under fluorescein, rhodamine, and phase-contrast optics. For identification of megakarocytes, rhodamine filters were used. Megakaryocytic diameters were established by measuring the geometric mean of two perpendicular diameters of each rhodamine-positive cell. Measurement of megakaryocytic ploidy was determined by fluorescence cytophotometry as described previously.[36] After identification of the megakaryocytic nature of the cell under the rhodamine filter combination, the filters were switched for measurement of ploidy by chromomycin A3 fluorescence emission. Granulocytes obtained from the pellet of the initial 1.080-g/mL density cut were used as the diploid standard. Cells were assigned to discrete ploidy classes by the method of Paulus et al.[37]

RESULTS

Density gradient conditions. The optimal density limits for maximal megakaryocytic separation and recovery was determined after preliminary experiments using initial Percoll concentrations ranging from 20% to 80%. The optimal density limits were ascertained to be 30% at the top and 63% at the bottom. The centrifugation time of 20 minutes was chosen after other experiments had shown that longer cen-

trifugation times did not improve the separation. Similarly, no difference was observed in separation when centrifugation was performed at 4 °C rather than room temperature. The addition of marrow cells directly to the gradient during formation rather than pipetting the cell suspension on top of the gradient also did not affect the final separation.

The density of each fraction of the continuous Percoll gradient was determined directly by weighing identical volumes of the particular fraction and water. The density of each fraction is shown in Table 1.

Cell recovery. After a single density cut of 1.080 g/mL to rid the marrow sample of red cells and the majority of granulocytes, nucleated cell recovery was 64% ± 3% of the starting marrow cellularity. Final recovery after washing was 58% ± 5%. Megakaryocytic recovery was 82%. To ascertain the percentage of megakaryocytic cells that might have been lost in the pellet in the initial 1.080-g/mL Percoll density cut, 3×10^4 cells in this pellet were also examined for GPIIb/IIIa-positive cells. No positive cells were observed in four separate experiments. Occasional megakaryocytes were observed along the sides of the centrifuge tube. This together with disruption of some of these cells upon washing might account for the 18% loss.

Distribution of GPIIb/IIIa-positive cells. Megakaryocytes identified by indirect immunofluorescence against GPIIb/IIIa averaged 0.04% of the initial starting marrow cells. To determine whether monocytes might also express this antigen,[26] separated marrow was examined for the simultaneous presence of Mac-1 and GPIIb/IIIa. Of greater than 400 Mac-1–positive cells, none expressed GPIIb/IIIa. To preclude the possibility that endothelial cells might express epitopes of GPIIb/IIIa recognized by LJP4, human umbilical vein endothelial cells were examined by indirect immunofluorescence for LJF4 binding. No positive cells were found.

To assess whether GPIIb/IIIa-positive cells also expressed an additional platelet specific antigen, 59 GPIIb/IIIa-positive cells as assessed by rhodamine immunofluorescence were mapped as described and stained for the presence of GPIb by an avidin-biotin immunoenzymatic technique. Forty out of 43 (93%) cells greater than 20 μm in diameter, five out of seven (71%) cells less than 20 but greater than 14 μm in diameter and four out of nine (44%) cells less than 14 μm in diameter were positive for both GPIIb/IIIa and GPIb. Table 1 shows the distribution of glycoprotein-positive cells in each fraction. The number of megakaryocytes identified by monoclonal antibody was inversely proportional to the Percoll density with the exception of the uppermost two fractions. Sixty-two to 114 glycoprotein-positive cells per 10^4 cells were identified in fractions 18 through 24 where megakaryocytes were enriched to 29-fold. Figure 1 shows the density profile of nucleated marrow cells of density <1.080 g/mL together with the megakaryocyte profile. Sixty-one percent of all megakaryocytes are found at densities <1.050 g/mL (fractions ≥16), whereas only 6.2% of nucleated cells are found at these densities.

Table 1. Separation of Human Bone Marrow Cells on Continuous Percoll Gradients

Fraction	Density (g/mL)	Cells/Fraction* (%)	Viability†		Number of MKs‡	Enrichment of MKs
			Total	MKs		
1	1.078	35 ± 4	100 ± 0.1	100 ± 0	0.3 ± 0.04	0.08
2	1.076	9 ± 2	100 ± 0	100 ± 0	0.7 ± 0.2	0.2
3	1.074	5 ± 1	100 ± 0	100 ± 0	0.8 ± 0.2	0.2
4	1.073	4 ± 1	100 ± 0	100 ± 0	1 ± 0.6	0.3
5	1.072	4 ± 1	100 ± 0.2	100 ± 0	1 ± 0.5	0.3
6	1.070	4 ± 1	100 ± 0.4	100 ± 0	2 ± 0	0.5
7	1.067	5 ± 1	100 ± 0.4	100 ± 0	3 ± 0.8	0.8
8	1.065	7 ± 3	100 ± 0	100 ± 0	4 ± 0.8	1
9	1.063	6 ± 2	100 ± 0.2	100 ± 0	4 ± 1	1
10	1.061	5 ± 1	100 ± 0.5	100 ± 0	5 ± 2	1
11	1.058	3 ± 1	100 ± 0.7	100 ± 0	5 ± 1	1
12	1.056	3 ± 1	100 ± 0.4	99 ± 1	13 ± 3	3
13	1.054	2 ± 0.6	100 ± 0.7	99 ± 1	17 ± 2	4
14	1.052	1 ± 0.6	99 ± 0.7	99 ± 2	21 ± 2	5
15	1.050	0.9 ± 0.4	99 ± 1	99 ± 0	24 ± 5	6
16	1.046	1 ± 0.5	99 ± 2	99 ± 0	31 ± 6	8
17	1.040	0.8 ± 0.2	98 ± 3	99 ± 1	46 ± 16	13
18	1.036	0.5 ± 0.2	96 ± 3	98 ± 2	62 ± 23	16
19	1.033	0.4 ± 0.2	96 ± 1	98 ± 1	74 ± 17	19
20	1.030	0.4 ± 0.2	94 ± 3	96 ± 2	101 ± 28	25
21	1.027	0.4 ± 0.2	90 ± 2	97 ± 2	114 ± 27	29
22	1.024	0.5 ± 0.2	87 ± 5	97 ± 2	104 ± 37	26
23	1.022	0.6 ± 0.2	83 ± 5	95 ± 1	108 ± 32	27
24	1.019	0.6 ± 0.2	87 ± 7	90 ± 3	80 ± 14	20
25	1.016	1 ± 0.6	95 ± 5	90 ± 2	30 ± 9	8

Abbreviation: MK, megakaryocyte.

*Percentage of total recovered cells in each fraction. The data represent the means ± 1 SD (eight experiments).

†Percentage of viable cells in each fraction.

‡Number of GPIIb/IIIa-positive cells (per 10^4 examined cells in the fraction; four experiments).

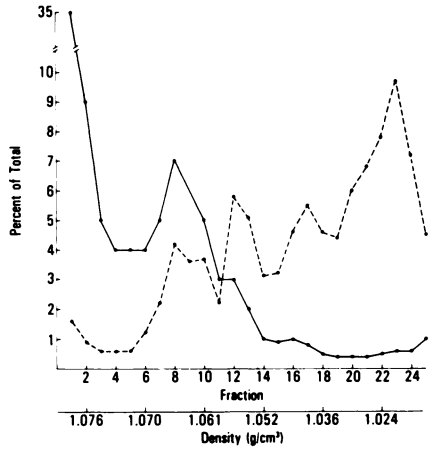

Fig 1. The density distribution of cells of density <1.080 g/mL separated on continuous Percoll gradients. The solid line represents the distribution of total nucleated cells whereas the broken line is the distribution of all GPIIb/IIIa-positive cells.

Viability. Cellular viability was determined by both trypan blue exclusion and acridine orange inclusion–ethidium bromide exclusion (Table 1). In comparison with trypan blue exclusion no significant difference was observed between the two methods except for several of the lower-density fractions in which a 5% to 11% increase in viability was observed with the latter fluorescent method (data not shown). Megakaryocytic viability was determined by simultaneous staining with fluorescein diacetate and indirect immunofluorescence against GPIIb/IIIa using a rhodamine label. Viability in all fractions was ≥90% (Table 1).

Megakaryocytic size. Geometric mean diameters were determined on 2,359 GPIIb/IIIa-positive cells and are shown in Table 2. The mean diameter of unfractionated bone marrow megakaryocytes was 34.1 ± 10.6 μm. The mean diameter of cells of individual fractions was inversely related to the Percoll density. The smallest mean diameters were observed in fractions 1 and 2 (15.3 ± 7.6 μm). The largest mean diameter was observed at fraction 22 (36 ± 8 μm). However, from fraction 23 through 25, an anomalous relationship was observed, with a direct relationship between size and apparent density.

If megakaryocytes are arbitrarily classified by size, 8% of all glycoprotein-positive cells are <14 μm in diameter, 8% are between 14 and 20 μm in diameter, whereas 84% are >20 μm in diameter. If one further classifies these cells according to whether they separate at > or <1.050 g/mL, 94% of all glycoprotein-positive cells <14 μm in diameter are found at densities >1.050 and 70% of intermediate-size cells are found at densities >1.050 g/mL, whereas only 31% of cells >20 μm in diameter are found at these densities. Figure 2 shows the density profiles of cells <14 μm in diameter and those >20 μm in diameter.

Ploidy. Ploidy measurements were obtained on the identical 2,359 cells on which size measurements were performed. The mean ploidy of each fraction is shown in Table 2, and is inversely related to the Percoll density between fractions 1 and 19, with a subsequent anomalous relationship noted in the least dense fractions similar to that observed

Table 2. Megakaryocyte Diameter and Ploidy of Separated Marrow

Fraction	Mean Diameter ± SD	Mean Ploidy ± SD	No. of Cells
Unfractionated	34.1 ± 10.6	16.9 ± 12.2	76
1	15.5 ± 5.9	3.9 ± 3.4	19
2	15.3 ± 7.6	4.4 ± 4.3	29
3	16.5 ± 5.7	4.4 ± 4.3	10
4	16.1 ± 9.1	4.4 ± 4.4	19
5	16.3 ± 9.3	4.6 ± 4.1	24
6	16.8 ± 9.4	5.3 ± 5.8	45
7	20.8 ± 11.1	7.3 ± 6.2	43
8	21.3 ± 10.1	7.0 ± 7.5	73
9	22.4 ± 10.0	8.6 ± 7.8	50
10	27.6 ± 9.9	10.6 ± 7.1	84
11	30.6 ± 10.7	12.6 ± 8.6	59
12	32.3 ± 9.5	12.8 ± 8.7	93
13	33.3 ± 8.5	14.1 ± 7.4	60
14	33.8 ± 9.1	14.5 ± 8.5	59
15	33.8 ± 8.3	16.1 ± 7.7	59
16	34.2 ± 6.1	16.8 ± 7.8	57
17	34.4 ± 8.1	17.2 ± 9.2	130
18	35.0 ± 7.5	17.7 ± 10.1	150
19	36.4 ± 7.9	19.6 ± 11.7	144
20	35.4 ± 6.9	19.3 ± 11.3	162
21	35.4 ± 7.2	18.6 ± 13.0	213
22	36.0 ± 8.0	17.7 ± 13.1	228
23	34.4 ± 7.5	17.2 ± 12.8	245
24	30.2 ± 8.6	10.9 ± 8.1	188
25	27.5 ± 11.0	10.4 ± 8.4	116

with size. The lowest mean ploidy (3.9) was observed in fraction 1, whereas the highest mean ploidy (19.6) was noted in fraction 19. Table 3 shows the ploidy distribution of megakaryocytes in each fraction. Seventy percent to 80% of the megakaryocytes in the first six fractions were eiher 2N or 4N, with over half of the cells being 2N. A gradual decrease in the 2N and 4N cells is noted with progressively lower density, with these low ploidy cells becoming rare in fractions 15 and greater (1.050 g/mL). Cells of 8N and 16N are observed even at the highest densities, but are most frequent at lower density. The highest ploidy cells are observed in the least dense fractions, with rare 128N cells noted.

The ploidy frequency distribution of the normalized sum of all cells analyzed is also shown in Table 3. The predomi-

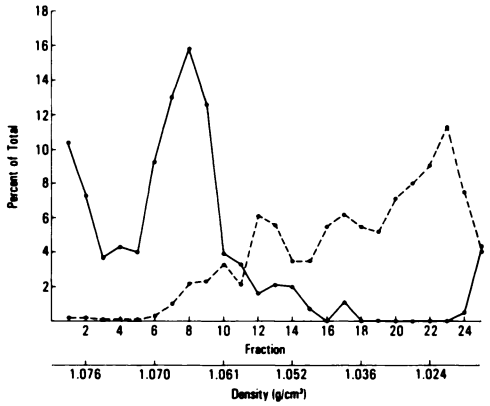

Fig 2. The density distribution of small (<14 μm, solid line) and large (>20 μm, broken line) GPIIb/IIIa-positive cells.

Table 3. Ploidy Distribution of Separated Megakaryocytes

Fraction	2N	4N	8N	16N	32N	64N	128N
Unfractionated	7*	8	21	42	20	2	
1	58	26	11	5			
2	62	18	10	10			
3	60	20	10	10			
4	62	16	11	11			
5	54	21	17	8			
6	55	16	18	9	2		
7	42	14	19	23	2		
8	26	25	33	15	1		
9	28	22	22	22	6		
10	10	14	40	30	6		
11	15	14	19	42	10		
12	9	7	39	32	13		
13	3	5	30	52	10		
14	5	5	32	43	15		
15	2	2	24	57	15		
16	0	2	23	58	17		
17	1	2	22	55	19	1	
18	0	1	23	57	17	2	
19	0	1	18	55	22	4	
20	1	1	23	47	26	3	
21	1	2	22	51	21	2	1
22	2	2	26	49	18	2	1
23	2	3	26	50	16	2	1
24	10	13	41	29	6	1	
25	14	17	39	21	9		
Total	10	8	27	41	13	1	

*Percentage of total.

nant ploidy class is 16N with 41% of the total cells. A significant number of the total cells were of low ploidy (10% 2N and 8% 4N). If one analyzes cells of various ploidies according to density, 81% of all 2N cells are found at densities >1.050 g/mL, whereas 66% of 4N cells are identified at these densities. The majority of higher-ploidy cells are found at densities <1.050 g/mL, with 60% of 8N, 70% of 16N, 78% of 32N, and 100% of 64N cells found at these low densities. Figure 3 shows the relationship between megakaryocyte ploidy and size of all the analyzed cells. The correlation between size and ploidy is fairly close, with a correlation coefficient of .762 ($P < .001$). Of interest is the

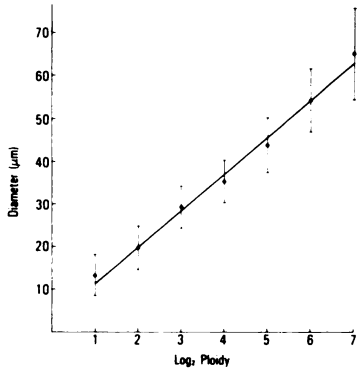

Fig 3. The relationship between mean cell diameter (closed circles) and ploidy. The abscissa represents discrete ploidy categories (log_2) calculated according to the method of Paulus et al.[37] The error bars indicate ±1 SD of the mean. The line represents the computer-generated regression ($r = .762$, $P < .001$).

size distribution of low-ploidy cells. The size distribution of 2N cells ranged from 7 to 46 μm with a mean of 13.3 ± 4.7 μm. The size of nonmegakaryocytes was 10.7 ± 2.8 μm (n = 702). Nineteen of 217 2N cells were between 20 and 30 μm, and one cell was 46 μm. The size range of 4N cells was 14 to 45 μm with a mean of 19.6 ± 5.2 μm. Seven of 166 4N cells were between 30 and 40 μm, and one cell was 45 μm.

DISCUSSION

Previous studies have shown that megakaryocytes are enriched at low densities on Percoll gradients.[10] However, the megakaryocytic colony-forming cell is located at higher densities on Percoll (approximately 1.075 g/mL).[9] These findings suggest that as megakaryocytes mature from their progenitors, their apparent density on Percoll decreases. To test this hypothesis, we separated human marrow over continuous Percoll gradients and defined megakaryocytes as those cells expressing the platelet GPIIb/IIIa complex antigen using indirect immunofluorescence. Consequently, the megakaryocytic nature of the cells was established independently of size, ploidy, or morphology. Although GPIIb/IIIa has been reported to be present on monocytes in addition to platelets and megakaryocytes,[38,39] more recent studies have shown that the antigen is not intrinsic to monocytes.[40] However, the likelihood that the antigen is specific to the megakaryocytic lineage in aspirated marrow does not preclude the possibility that platelet fragments adhere to marrow monocytes[40] or that macrophages ingest effete megakaryocytes and transiently display antigens of that lineage. That no cells examined exhibited simultaneously both Mac-1 and GPIIb/IIIa mitigates against this possibility. Similarly, endothelial cells, a possible contaminant of marrow aspirates, were examined for expression of GPIIb/IIIa as defined by LJP4. Previous studies have suggested that endothelial cells express GPIIb and IIIa[41,42]; however, the present data suggest that not all epitopes of this glycoprotein complex are expressed by endothelial cells. Since it is possible that other monoclonal antibodies may identify such epitopes, endothelial cells should be prescreened with the antibody to rule out inadvertent misidentification.

Double-labeling studies with monoclonal antibody to GPIIb/IIIa and GPIb show that the vast majority of large megakaryocytes colabeled with both antibodies, whereas the smallest and presumably diploid cells labeled only with monoclonal antibody to IIb/IIIa. Studies by Vainchenker et al examining the time of expression of various platelet glycoproteins during in vitro culture have demonstrated that GPIIb/IIIa is the first platelet glycoprotein to be identified.[13] We conclude that the small cells expressing only GPIIb/IIIa are likely to be primitive cells in the megakaryocytic lineage, analogous to those found in day 7 in vitro colonies. Nevertheless, the full cellular range of expression of GPII/IIIa may as yet not be known. A recent report has suggested that monoclonal antibodies to GPIIb/IIIa may react in a cytotoxicity assay with murine nonmegakaryocytic stem cells.[43] It is thus conceivable that a population of nonmegakaryocytic cells may express the epitope of GPIIb/IIIa detected by LJP4. Despite these potential limitations, the use of the monoclonal antibody allows for high specificity with low background fluorescence and the ability to perform

simultaneous size measurements and fluorescence cytophotometry for DNA content on the same cell. The data show that megakaryocytic differentiation is inversely related to the apparent density on Percoll.

The separation mode of megakaryocytes on Percoll differs from that on gradients of albumin, a phenomenon disparate from other cell types.[10,44] Rabellino has suggested that the differences in separation between Percoll and albumin may be due to the unique characteristics of the megakaryocytic membrane with its extensive demarcation membrane system.[45] Thus, the separation of megakaryocytes on Percoll may not necessarily be an accurate reflection of intrinsic megakaryocytic density. Nevertheless, this characteristic of megakaryocytes has made it possible to enrich megakaryocytes to virtual homogeneity using a single-density cut of Percoll as an initial separation modality.[10,19]

An exception to the inverse relationship between size or ploidy and apparent density is found in the upper several fractions, particularly fractions 24 and 25. In these fractions the mean diameter and ploidy are significantly less than several of the preceding fractions. This anomalous relationship was also observed in studies of murine megakaryocytes,[22] and its explanation is unclear. Dead cells are known to be enriched at low densities of Percoll, and it is conceivable that dead megakaryocytes of any size or ploidy go to the very top of the gradient. However, a direct analysis using a fluorescent probe for viability of GPIIb/IIIa-positive cells has shown that the viability in the uppermost fractions, although slightly less than total, is 90%. It is conceivable that the megakaryocytes in these fractions may differ in some fundamental way from other megakaryocytes, although Wright's stained preparations of these cells showed no obvious differences in cytoplasmic granularity or degree of basophilia.

Of particular interest is the evaluation of 2N and 4N cells, cells not readily evident by morphologic features alone. The presence of GPIIb/IIIa on diploid cells is further evidence that this antigen is expressed very early in the megakaryocytic lineage.[13] It is possible that it is expressed as early as the level of the CFU-megakaryocyte since incubation of murine marrow with monoclonal antibodies specific to the human GPIIb/IIIa complex appears to be cytotoxic to these progenitors.[43]

Other investigators have separated human megakaryocytes on Percoll at a density of 1.050 g/mL and have found that virtually all of the megakaryocytes were found at this or lesser density, whereas no megakaryocytes were found in the pellet.[10] On continuous gradients, however, we find that small and low-ploidy megakaryocytes are mainly found at densities greater than 1.050 g/mL. It is not clear whether this discrepancy reflects (1) a greater ability to identify small megakaryocytes with a monoclonal as opposed to polyclonal antibody or (2) differences in separation characteristics between a single Percoll density cut and a continuous gradient. Nevertheless, the possibility exists that a significant proportion of small, low-ploidy megakaryocytes are lost and undetected with a single 1.050-g/mL density cut. This may not be of importance when the sole objective of a study is to purify megakaryocytes to homogeneity for biochemical studies, but may be of considerable importance when examining the response of early megakaryocytes to stimulators of maturation or proliferation.

As has been observed in previous studies, a close relationship exists between megakaryocytic size and ploidy.[46,47] This relationship also applies to 2N and 4N cells. A few of these low-ploidy cells were larger than might be expected. Conceivably, these large cells may represent nonviable high-ploidy cells whose DNA has degraded. A wide range of size distribution is observed at each ploidy class. If the size range expanded as ploidy increased, it is conceivable that a mechanism that regulates cytoplasmic volume exists that is dependent on ploidy. That is, higher ploidy cells may have a greater capacity to vary their cytoplasmic volumes. Examination of the SD of diameter as a function of ploidy as shown in Fig 3 suggests that this is unlikely since there is no apparent trend in size range irrespective of the ploidy class.

In summary, the data presented herein show that the state of megakaryocytic differentiation is inversely related to Percoll density and that a monoclonal antibody to the GPIIb/IIIa complex identifies small and low-ploidy cells of the megakaryocytic lineage unrecognizable by size criteria. The use of a fluorescence method to both identify cells and ascertain their DNA content allows for the precise identification of diploid and tetraploid megakaryocytes. This methodology may be useful for studies of small, low-ploidy megakaryocytes in various pathologic states and the response of these cells to various physiologic and pharmacologic stimuli.

ACKNOWLEDGMENT

The authors appreciate the secretarial assistance of Sandra Phillips.

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