HUMAN MEGAKARYOCYTES

V. Changes in the Phenotypic Profile of Differentiating Megakaryocytes

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Megakaryocytopoiesis involves the commitment, proliferation, and differentiation of hemopoietic progenitor cells in the megakaryocyte/platelet lineage, and the acquisition of functional and structural properties associated with platelet physiology. Megakaryocytes contain an array of alpha granule proteins with membrane receptors involved in platelet adhesion, aggregation, and secretion (1). Recent studies (2–6) characterizing human marrow megakaryocytes have described a number of platelet proteins and cytochemical activities that can be regarded as selective markers for cells of the megakaryocytic lineage. Early immature megakaryocytes (promegakaryoblasts) in normal marrow as well as micromegakaryocytes and atypical megakaryocytes in marrow from patients with megakaryoblastic leukemia and myeloproliferative disorders have been recognized by virtue of these markers (7–12). Moreover, the use of these markers has also been essential to the identification and characterization of human colony megakaryocytes grown in vitro (13–16).[1]

Differentiating hemopoietic cells generally undergo marked structural and functional changes that can be related to different ontogenetic levels (17). Studies of the lymphoid, myeloid, and erythroid lineages have revealed that these changes are paralleled by alterations in their antigenic profiles (18–22). Furthermore, the expression of surface antigens on heterogeneous populations of morphologically unrecognizable hematopoietic progenitors has been demonstrated using complement-mediated cytolytic assays in conjunction with clonogenic assays (23–25). Hybridoma technology, enabling the elaboration of monoclonal antibodies with reactivities restricted to single epitopes, has further enhanced the sensitivity of these bioassays.

This paper reports the recognition of three levels of human megakaryocyte

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[1]Levene, R. B., N. Williams, J. M. Daniel-Lamaziere, L. L. K. Leung, and E. M. Rabellino. Human megakaryocytes. IV. Growth and characterization of colony-forming unit megakaryocytes. Manuscript submitted for publication.

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differentiation based upon the selective expression of cell antigens recognized by a panel of monoclonal antibodies raised against human megakaryocytes and platelets. Using immunofluorescence, an enzyme-linked immunosorbent assay (ELISA)[2] system, and complement-mediated cytolysis, three maturational levels with distinct immunologic phenotypes were identified. These levels were characterized by differentiation antigens that were expressed throughout megakaryocytopoiesis from the clonable megakaryocytic progenitor (megakaryocyte colony-forming unit; CFU-Mk) to either: (*a*) the small, mononuclear, early immature megakaryocyte; (*b*) the classical, mature megakaryocyte with a polylobulated nucleus; or (*c*) the circulating blood platelet. The recognition of discrete phenotypic changes in human marrow megakaryocytes first described in these studies provides the basis for a diagnostically useful classification system of the megakaryocyte/platelet lineage, and enables studies of mechanisms regulating megakaryocytopoiesis in health and disease.

Materials and Methods

Preparation of Human Bone Marrow Cell Suspensions. Normal human bone marrow cells were harvested from rib fragments removed at surgery or, alternatively, from bone marrow aspirates obtained from normal volunteers. Written consent was obtained from all patients before surgery in compliance with regulations established by the National Institutes of Health. Experimental protocols were approved by the Committee on Human Rights of New York Hospital–Cornell Medical Center (New York). Marrow tissue was collected and rendered into single-cell suspensions as described elsewhere (1, 7). The medium used for this procedure was calcium- and magnesium-free Hanks' balanced salt solution (Gibco Laboratories, Grand Island, NY) containing 1.25×10^{-5} M sodium citrate, 2.52×10^{-5} M Hepes buffer, 4.1×10^{-6} M sodium bicarbonate, and 115 U/ml DNase I (all from Sigma Chemical Co., St. Louis, MO) at a final pH of 7.0 ± 0.05 and 295 ± 5 mosM (HBSS/C). Cells were then washed twice in modified McCoy's 5-A medium (Gibco Laboratories) containing 2.62×10^{-2} M $NaHCO_3$ (Sigma Chemical Co.) at pH 7.2 ± 0.05, 295 ± 5 mosM (McCoy's 5-A). Marrow cells were fractionated by buoyant density centrifugation and harvested as reported (1). Gradients were generated with solutions of colloidal silica particles coated with polyvinyl pyrollidone (Percoll; Pharmacia Fine Chemicals, Division of Pharmacia, Inc., Piscataway, NJ) in McCoy's 5-A.

Preparation of Blood Leukocytes and Washed Platelets. Venous blood was collected from normal volunteers in 0.32% sodium citrate in polypropylene test tubes. Blood samples from two patients with Glanzmann's thrombasthenia and one patient with Bernard-Soulier disease were kindly provided by Dr. Margaret Johnson (Wilmington Medical Center, Wilmington, DE). Erythrocyte-depleted suspensions of leukocytes were prepared by sedimentation in solutions of HBSS/C containing 3% dextran T-500 (Pharmacia Fine Chemicals) as previously described (7). Washed platelets were prepared by processing whole citrated blood as reported elsewhere (12, 26).

Immunofluorescence Assays of Fixed Smears of Human Bone Marrow and Peripheral Blood Cells. Suspensions of either unseparated or low density fractions of human bone marrow cells ($d < 1.050$ g/cm^3), erythrocyte-depleted leukocytes, or washed platelets, were smeared in fetal bovine serum (Flow Laboratories, Inc., Division of Flow General, Inc., McLean, VA) and processed for immunofluorescence staining of membrane and intracel-

[2] *Abbreviations used in this paper:* CFU-GEMM, multipotential progenitor of mixed colonies in vitro, containing neutrophililic granulocytes, erythroblasts, macrophages, and megakaryocytes; CFU-Mk, BFU-E, and CFU-GM, clonable progenitor cells of megakaryocytic, erythroid, and myelomonocytic lineages, respectively; ELISA, enzyme-linked immunosorbent assay; FITC, fluorescein isothiocyanate; HBSS/C, calcium- and magnesium-free Hanks' balanced salt solution containing sodium citrate, Hepes buffer, sodium bicarbonate, and DNase I; KLH, keyhole limpet hemocyanin; SDS-PAGE, polyacrylamide gel electrophoresis; TRITC, tetramethlrhodamine isothiocyanate.

lular components (1, 2, 7). Cells were examined with a Leitz Ortholux II microscope (E. Leitz, Inc., Rockleigh, NJ) equipped with a ploem illuminator and phase contrast optics. Blood platelets and mature marrow megakaryocytes were identified on the basis of their respective prominent morphologic features in concurrent phase contrast microscopic observations (27, 28). Early immature megakaryocytes were recognized in double immunofluorescence assays by simultaneous staining of fixed smears of low density marrow cells with fluorochrome-conjugated monospecific antisera against human platelet glycoprotein IIb or IIIa.

Preparation of Monospecific Rabbit Antisera. Monospecific rabbit antisera against isolated human platelet glycoproteins IIb and IIIa were kindly provided by Dr. Lawrence L. K. Leung (Cornell University Medical College). Heterologous rabbit antisera against ovalbumin and keyhole limpet hemocyanin (KLH) were prepared and tested for specificity as described elsewhere (1, 7, 29, 30). Polyspecific rabbit anti–mouse Fab Ig was a generous gift from Dr. Howard Grey (National Jewish Hospital, Denver, CO). IgG fractions of all rabbit antisera were prepared and subsequently conjugated to fluorescein (FITC) and/or tetramethylrhodamine (TRITC) isothiocyanate (both from BBL Microbiology Systems, Division of Becton, Dickinson & Co., Cockeysville, MD) as described elsewhere (1).

Monoclonal Antibodies. Monoclonal antibodies were raised against pure suspensions of washed human platelets and low density marrow cells enriched for megakaryocytes using standard procedures (31). Briefly, $CB6F_1$ mice (The Jackson Laboratory, Bar Harbor, ME) were immunized twice with either platelets or low density marrow cells. Splenocytes from immunized mice were fused with SPO/2 mouse plasmacytoma cells in the presence of 35% polyethylene glycol 1540 (J. T. Baker Chemical Co., Phillipsburg, NJ). Culture supernatants were tested for the presence of mouse Ig and subsequently screened for reactivity against human platelets by solid phase ELISA. In this screening system, optimal platelet adherence was obtained after coating microtiter plates (Linbro Division, Flow Laboratories, Inc., Hamden, CT) with poly-L-lysine (Sigma Chemical Co.) Binding of antibody to platelets was measured spectrophotometrically after treatment with β-galactosidase-labeled anti–mouse Ig (BRL, Inc., Gaithersburg, MD) and *O*-nitrophenyl-β-D-galactoside (BRL, Inc.). All supernatants were also tested against smeared preparations of fixed platelets and marrow cells by indirect immunofluorescence using TRITC-conjugated rabbit IgG anti–mouse Fab Ig. After cloning by the semisolid agar technique in the presence of thioglycollate-elicited macrophage monolayers, hybrids were reverted to liquid culture and their supernatants retested for reactivity and specificity against marrow and blood cells. To confirm reactivity against early immature and/or classical megakaryocytes, low density marrow cells were stained simultaneously with a monoclonal antibody/TRITC rabbit anti–mouse Fab Ig and a fluoresceinated rabbit anti–platelet glycoprotein IIb or IIIa. The 11 monoclonal antibodies selected for their exclusive reactivity to elements within megakaryocyte/platelet lineage were characterized by immunoelectrophoresis as IgG1 isotype.

Immunoprecipitation and Biochemical Characterization of Platelet Membrane Antigens Recognized by Monoclonal Antibodies. Washed platelets were radioiodinated with ^{125}I (New England Nuclear, Boston, MA) by the tetrachloro-diphenylglycoluril method (32). Radiolabeled platelets were solubilized with 2% sodium dodecyl sulfate (SDS), which was subsequently displaced by 1.7% Triton X-100 (Sigma Chemical Co.) in the presence of 1 μM pepstatin (The Protein Foundation, Osaka, Japan), 2 mM phenylmethysulfonyl fluoride, and 5 mM *N*-ethylamaleimide, and 10 mM EGTA (all from Sigma Chemical Co.) to minimize proteolysis. Platelet protein lysates were subjected to solid phase immunoadsorption with protein A–Sepharose CL-4B beads (Pharmacia Fine Chemicals) coated with various monoclonal antibodies (33). Specifically bound protein was eluted with 2% SDS and subjected to SDS–polyacrylamide gel electrophoresis (SDS-PAGE) under reducing and nonreducing conditions and autoradiography (34, 35).

Complement-mediated Cytolytic Assay. Unseparated marrow cells were washed twice in McCoy's 5-A medium. Aliquots of 6×10^6 cells were then incubated with 375 μl of ammonium sulfate–precipitated mouse monoclonal antibody preparations for 30 min at room temperature, and subsequently washed twice in McCoy's 5-A. Antibody-treated cells

were then incubated with 375 μl of a second antibody, a rabbit IgM anti-mouse IgG, at room temperature for 30 min, and washed twice in McCoy's 5-A medium. Cells were then resuspended in 375 μl undiluted oxidized normal human AB Rh^{D+} serum, used as source of complement, and incubated at 37°C for 30 min (36–38). After two washes in McCoy's 5-A medium, cells were plated in culture as indicated below. Rabbit IgM anti-mouse IgG was prepared by short immunization of New Zealand White rabbits (Hazelton-Dutchland, Denver, PA) with mouse IgG in complete Freund's adjuvant (Gibco Laboratories). Mouse IgG was prepared from DBA/2A (The Jackson Laboratory) serum, first precipitated in ammonium sulfate, followed by DE-52 chromatography in 0.03 M NAH_2PO_4 at pH 8.3. At day 21 of immunization, rabbits were bled by cardiac puncture and serum was precipitated with 50%-saturated ammonium sulfate. The identity, class, and activity of this antibody preparation was confirmed by immunoelectrophoresis.

Growth of CFU-Mk in Semisolid Agar. Untreated and treated cells or freshly isolated marrow cells were plated for growth of CFU-Mk using a semisolid agar system, as described in more detail elsewhere.[1] Briefly, cell suspensions of 2×10^5 nucleated marrow cells/ml or cells remaining in this volume after treatment were cultured in a supplemented McCoy's 5-A medium containing 0.26% Bacto-Agar (Difco Laboratories, Inc., Detroit, MI), 17.4% horse serum (Flow Laboratories, Inc.), and 4.4% medium conditioned by the Mo cell line (Dr. David Golde, UCLA School of Medicine, Los Angeles, CA). The supplemented McCoy's 5-A contained 9.3×10^{-2} M sodium pyruvate, 0.74% 50× minimum essential medium (MEM)–essential amino acid mixture, 0.56% 100× MEM–nonessential amino acid mixture, 0.56% 100× MEM-vitamin mixture, 1.12×10^{-3} M L-glutamine, 1.15×10^{-7} M asparagine, 7.59×10^{-8} M serine (all from Gibco Laboratories Co.), 0.93% penicillin (10,000 U/ml)-streptomycin (10,000 U/ml) mixture (Microbiological Associates, Walkersville, MD), 3.53×10^{-2} M NaHCO, and 3.23×10^{-8} M Hepes buffer (both from Sigma Chemical Co.). Growth of megakaryocytic colonies was monitored by staining fixed colony cells by flooding the entire culture dish with either heterologous antisera or monoclonal antibody preparations specific for platelet glycoprotein IIb or IIIa. Colony megakaryocytes were scored on day 7 or 8 by examining stained colonies in situ with a Leitz Diavert phase contrast, inverted microscope equipped with a ploem illuminator and optics for fluorescent microscopy (E. Leitz, Inc.).

Growth of CFU-GEMM and BFU-E. Growth of multipotential hemopoietic progenitors (granulocyte, erythroblast, macrophage, and megakaryocyte colony-forming units; CFU-GEMM) and erythroid bursts (erythroid burst-forming unit; BFU-E) was conducted and scored as previously described (39, 40). Unseparated marrow cells were plated in Iscove's modified Dulbecco's medium (Gibco Laboratories) containing 1% methylcellulose (Callahan Chemical Co., Palmyra, NJ), 30% fetal bovine serum (Biofluids, Inc., Rockville, MD), 5% medium conditioned by leukocytes from patients with hemachromatosis in the presence of 1% phytohemagglutanin (HA-15; Welcome Reagents, Ltd., Welcome Research Laboratories, Detroit, MI), 5×10^{-5} M 2-mercaptoethanol (Sigma Chemical Co.), 1 U of Step III preparation of sheep plasma erythropoietin (Connaught Labs, Ltd., Willowdale, Ontario, Canada), and 0.2 mM Hemin (Eastman Kodak Co., Rochester, NY) (41, 42). Cultures were incubated at 37°C in a humidified atmosphere flushed with 5% CO_2 in air. Colonies were scored with an inverted microscope after 14 d of incubation and identified further after picking out colonies with a fine pipette, transferring them to glass slides, and staining with benzidine and/or Wright's-Giemsa to confirm their mixed nature. Mixed colonies usually contained erythroid, granulocytic, and monocytic cells, and sometimes megakaryocytes. BFU-E were scored from these same plates.

Growth of CFU-GM. The colony assay for granulocyte macrophage colony-forming units (CFU-GM) was performed as described previously (43). Marrow cells were plated in McCoy's 5-A containing 10% heat-inactivated bovine serum, 0.3% agar, and 10% conditioned medium obtained from either the growth of giant cell tumor (GCT) (Gibco Laboratories) or 5637 cell line (44, 45). To monitor the effects of the cytolytic assay on the two subpopulations of CFU-GM, cultures were incubated at 37°C for 7 and 14 d, at which times they were scored for cluster (3–50 cells per aggregate) and colony (>50 cells per aggregate) formation as described (42, 43).

Results

Phenotypic Changes Associated With the Ontogeny of Megakaryocytes/Platelets. Studies of phenotypic changes in differentiating megakaryocytes were conducted using a panel of monoclonal antibodies generated against human megakaryocytes and platelets. Different levels of megakaryocyte maturation were recognized based upon the distribution of specific cell antigens detected by a panel of 11 monoclonal antibodies. These antibodies reacted exclusively with megakaryocytes and platelets in indirect immunofluorescence assays of fixed smears of marrow and blood cell preparations obtained from 25 normal subjects. Furthermore, reactivity against platelets was corroborated by a crosslinked, solid phase ELISA system. Based upon their ability to recognize cell antigens associated with distinct levels of differentiation, monoclonal antibodies were operationally categorized into three groups (Table I). Six different monoclonal antibody preparations categorized in group A recognized epitopes on early immature megakaryocytes, but not on mature megakaryocytes or platelets. Group B monoclonal antibody MkB-1 recognized an antigenic determinant expressed by all early and mature megakaryocytes, but not by platelets. This unique pattern of reactivity is demonstrated in Fig. 1, which depicts isolated marrow megakaryocytes mixed with washed platelets and costained with MkB-1 monoclonal antibody by indirect immunofluorescence (Fig. 1, *a* and *b*). Overlying platelets partially obscure cytologic detail in phase contrast counterpart micrographs (Fig. 1, *a'* and *b'*). Four monoclonal antibodies classified in group C reacted to all early and mature megakaryocytes as well as platelets. Mature marrow megakaryocytes and blood platelets were readily identified on the basis of their respective prominent morphologic features. Early immature megakaryocytes were recognized in double immunofluorescence assays by simultaneous staining of marrow cells with fluorochrome-conjugated monospecific antisera against human platelet glycoproteins IIb and IIIa. The specificity of fluorescence staining was controlled by incubating cells directly with the fluorochrome-conjugated rabbit anti–mouse

TABLE I

Distribution Profile in Marrow and Blood Cells of Antigens Detected by Monoclonal Antibodies Raised Against Megakaryocytes and Platelets

Monoclonal antibodies	Immunogen*	Cells stained by immunofluorescence‡			
		Immature mega-karyocytes	Mature megakary-ocytes	Platelets	Other cell types
Group A (6)§	Megakaryocytes	+	−	−	−
Group B (1)	Megakaryocytes	+	+	−	−
Group C (4)	Platelets	+	+	+	−

* Monoclonal antibodies were generated against megakaryocyte-enriched, low density marrow cells or washed blood platelets.

‡ Cells were stained by indirect immunofluorescence with monoclonal antibody followed by TRITC rabbit anti–mouse Fab Ig. Immature megakaryocytes were identified as mononuclear lymphoid-like cells also bearing platelet glycoprotein IIb-IIIa. Other cell types included unstained marrow and blood cells, which presumably belong to the pool of mature cells, and are precursors of the myelomoncytic, erythroid, and lymphoid series.

§ Number of different monoclonal antibodies in functional grouping.

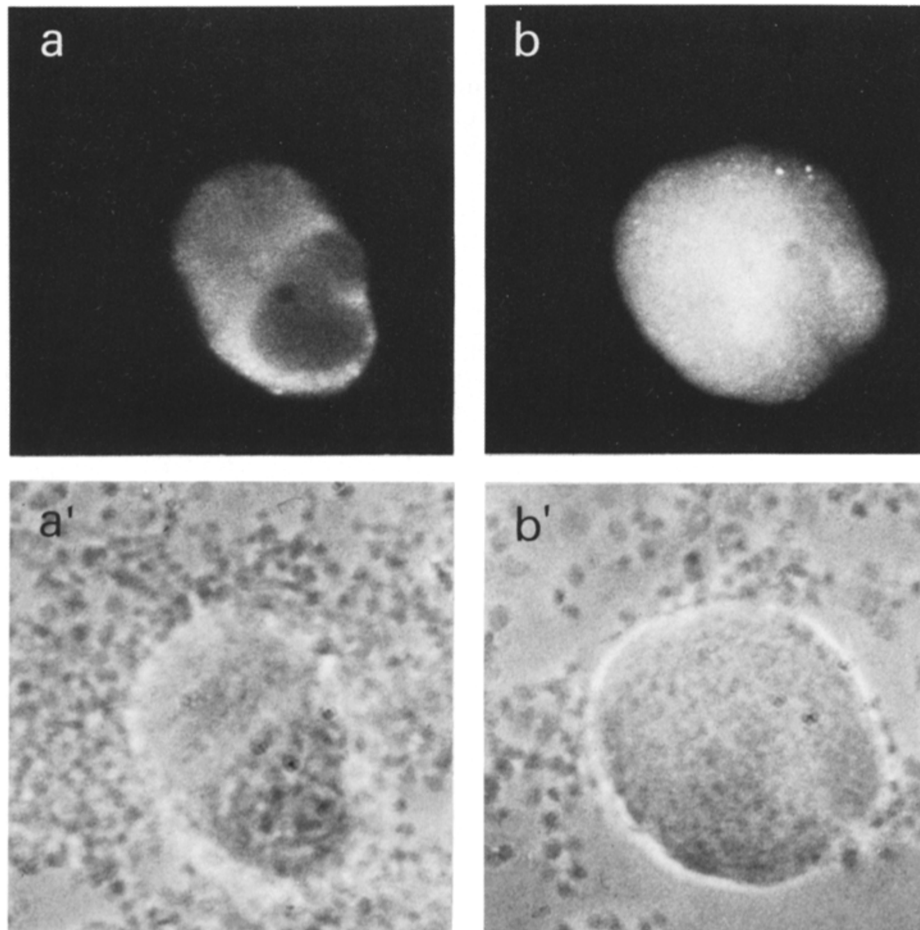

FIGURE 1. Isolated human marrow megakaryocytes and blood platelets costained by immunofluorescence with MkB-1 monoclonal antibody. Washed platelets were mixed with low density marrow cells, smeared, methanol fixed, and (*a* and *b*) stained by indirect immunofluorescence with monoclonal antibody MkB-1, followed by TRITC rabbit anti-mouse Fab Ig. (*a′*,*b′*) Same field under phase contrast microscopy. × 938.

Fab Ig antisera and by preincubating cells with normal mouse IgG or a nonreactive hybridoma secretory IgG. Moreover, no change in the reactivity profile of the different monoclonal antibodies to various cell types was observed when reagents were tested at increased concentrations.

Expression of Megakaryocytic Differentiation Antigens by Colony Megakaryocytes Grown In Vitro. The expression of various epitopes recognized by the panel of monoclonal antibodies was investigated in colony megakaryocytes grown in agar. In a series of double immunofluorescence assays, colony cells were stained sequentially in situ with the different monoclonal antibodies and antiplatelet glycoprotein IIb antiserum was used to identify megakaryocytic cells. All megakaryocytic colony cells harvested at day 7 or 8 were intensely stained by groups A, B, and C monoclonal antibodies. A representative picture of a typical human

megakaryocytic colony grown from unseparated marrow in the semisolid agar system is depicted in Fig. 2. The colony was harvested at day 7, gently dried, fixed, agarase-treated, and stained by indirect immunofluorescence with monoclonal antibody PC-1. In parallel controls for the specificity of the indirect immunofluorescence system, cultures were incubated with only the second antibody preparation or with a nonrelated antibody directly conjugated to a fluorochrome. Colony cells were not stained by either the TRITC rabbit anti–mouse Fab Ig reagent or the FITC rabbit IgG anti-KLH antiserum. Additionally, granulocyte-macrophage colony cells were not stained by group C monoclonal antibody PC-1.

Distribution of Megakaryocytic Differentiation Antigens on CFU-Mk and Other Clonable Hematopoietic Progenitors. Expression of cellular antigens was investigated on clonable hemopoietic progenitors using a complement-mediated cytolytic assay. To ensure maximum complement fixation and to enhance the sensitivity of the cytolytic assay, cells were preincubated with a rabbit IgM anti–mouse IgG preparation before the addition of complement (46). Oxidized human AB Rh^{D+} serum prepared by iodination was used as the source of complement (37). The reactivity of the various monoclonal antibodies against CFU-Mk is expressed as the percent inhibition of colony growth on Table II. These values represent the means resulting from the comparison of quadruplicate experimental conditions to quadruplicate untreated controls. When mean values were derived from multiple experiments, the number of experiments performed is indicated in parentheses. Monoclonal antibodies from group A, which reacted against early megakaryocytes but not mature megakaryocytes or platelets, exhibited 64–100% inhibition of colony growth. Monoclonal antibody MkB-1 from group B, which reacted against early and mature megakaryocytes but not platelets, inhibited 63% of colony growth. Group C monoclonal antibodies, which reacted against early and mature megakaryocytes as well as platelets, had inhibitory effects of 66–100%. It is noteworthy that when various monoclonal

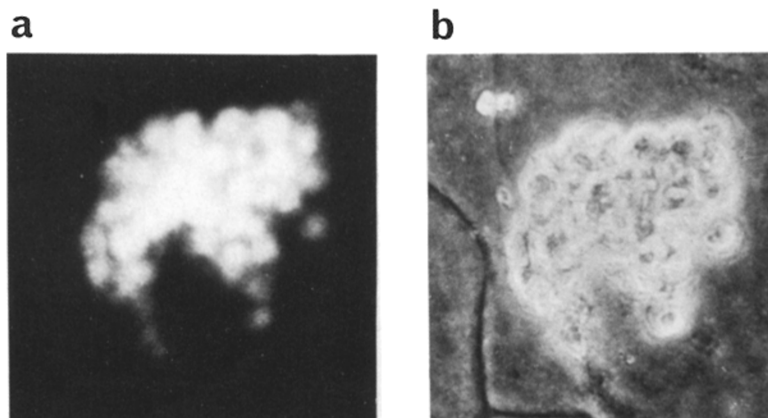

FIGURE 2. Immunofluorescence staining of human megakaryocytic colony harvested at day 7. Culture was dried, methanol fixed, agarase treated, and (*a*) stained by indirect immunofluorescence with monoclonal antibody PC-1, followed by TRITC rabbit anti–mouse Fab Ig. (*b*) Same field under phase contrast microscopy. × 375.

TABLE II
Expression of Membrane Antigens on CFU-Mk Detected by Complement-mediated Cytolysis

Pretreatment of marrow cells			Percent inhibition of CFU-Mk growth‡
Monoclonal antibody	Rabbit IgM anti-mouse IgG*	Complement	
Group A			
MkA-1	+	+	97 ± 4 (4)
MkA-1	−	+	42 ± 0
MkA-2	+	+	82 ± 0
MkA-3	+	+	94 ± 9
MkA-4	+	+	94 ± 9
MkA-5	+	+	100 ± 0
MkA-6	+	+	64 ± 17
Group B			
MkB-1	+	+	63 ± 11 (4)
MkB-1	−	+	31 ± 16
Group C			
PC-1	+	+	77 ± 13 (5)
PC-1	−	+	51 ± 13 (2)
PC-2	+	+	88 ± 9 (4)
PC-2	−	+	52 ± 6
PC-3	+	+	66 ± 4
PC-4	+	+	100 ± 0

* Addition (+) or omission (−) of designated component.
‡ The mean ± SD of percent inhibition was determined from comparison of replicate treated samples with replicate controls not treated with any antibody or complement. Parallel controls of samples treated with various monoclonal antibody preparations, rabbit IgM, or complement, had inhibitory effects <6%. Megakaryocytic colonies grown in agar were identified by immunofluorescence using anti-platelet glycoprotein IIb/IIIa antisera. Number of experiments is indicated in parentheses.

antibody preparations were tested by the cytolytic assay without the rabbit IgM complement fixation amplification step, substantially less inhibition was detected (Table II).

Clonable progenitors cells of other hemopoietic lineages were also studied for the expression of the various antigens recognized by the monoclonal antibodies. For this purpose, marrow cells were processed by the cytolytic assay described above and then cultured, concurrently, for the growth of CFU-GEMM, BFU-E, the bipotential granulocyte-macrophage progenitor (CFU-GM), and CFU-Mk cells. Two distinct subpopulations of CFU-GM were recognized, one forming colonies in 7 d and the second forming colonies in 14 d. Additionally, an operational distinction was made between GM clusters (3–50 cells) and colonies (>50 cells) (43). The effects of PC-2 antibody from group C on colony formation

by CFU-GM, BFU-E, CFU-GEMM, and CFU-Mk was assessed in a series of multilineage cytolytic assays (Table III). In three experiments, the number of colonies scored in entire plates is expressed as the mean of replicate cultures. Percent changes represent comparisons between experimental and control conditions, and reflect reduction or augmentation of colony formation. As shown, monoclonal antibody PC-2 inhibited 87% ($P < 0.001$) of CFU-Mk growth as well as 27% ($P < 0.03$) of BFU-E formation, but showed no significant inhibition of mixed GEMM colonies or granulocyte-macrophage colonies or clusters. The probability of significant differences between samples was determined using Student's t test (47). In similar multilineage assays, monoclonal antibodies MkA-1, MkB-1 and PC-1 inhibited 97% ($P < 0.001$), 58% ($P < 0.05$), and 64% ($P < 0.01$) of megakaryocytic colony formation, respectively (Table IV). Additionally, MkA-1, MkB-1, and PC-1 exhibited no significant inhibition ($P > 0.05$) of GEMM, erythroid burst, or GM colony and/or cluster growth.

Immunochemical Characterization of Group C Monoclonal Antibodies. Cell antigens recognized by group C antibodies were characterized by autoradiographic analysis of immunoprecipitated, radiolabeled platelet membrane proteins after SDS-PAGE. Autoradiograms of eluants obtained from immunobeads coated with PC-1, PC-3, and PC-4 are depicted in Fig. 3. Under nonreducing conditions, all three monoclonal antibodies precipitated proteins with approximate molecular weights of 135,000 and 95,000. After reduction, these antigenic moieties underwent a shift in their electrophoretic mobilities to 125,000 and 115,000 mol wt. The electrophoretic behavior of immunoisolated proteins with respect to molecular weights and shift in mobility after reduction is comparable to that describe

TABLE III

Detection of Membrane Antigen Recognized by Group C Monoclonal Antibody PC-2 on CFU-Mk, CFU-GM, BFU-E, and CFU-GEMM by Complement-mediated Cytolysis

Exp. No.	Pretreatment* of cells			Number of colonies scored						
					CFU-GM‡					
					Day 7		Day 14			
	PC-2	IgM	C	CFU-Mk	Colonies	Colonies + clusters	Colonies	Colonies + clusters	BFU-E	CFU-GEMM
1	–	+	+	10.0 ± 0.0	36 ± 4	116 ± 5	32 ± 2	59 ± 2	53 ± 3	2.0 ± 0.7
	+	+	+	1.5 ± 0.5	39 ± 6	102 ± 12	30 ± 1	59 ± 1	35 ± 7	2.3 ± 0.5
2	–	+	+	7.0 ± 0.0	44 ± 9	122 ± 6	26 ± 4	ND	58 ± 4	6.8 ± 1.3
	+	+	+	1.0 ± 0.8	41 ± 5	117 ± 9	23 ± 5	ND	47 ± 7	4.0 ± 1.4
3	–	+	+	ND	34 ± 2	92 ± 6	17 ± 2	25 ± 2	46 ± 6	3.8 ± 0.8
	+	+	+	ND	37 ± 1	91 ± 1	20 ± 2	27 ± 4	33 ± 3	3.3 ± 1.0
Percent change:				–87.0 ± 8.6§	+3 ± 9	–6 ± 6	–12 ± 6	–4 ± 6	–27 ± 8	–13.0 ± 16

Data represent values ± SD observed for quadruplicate cultures; ND, not done.

* Before plating, marrow cells were pretreated, sequentially, with monoclonal antibody PC-2, rabbit IgM anti-mouse Fab Ig (IgM), and human complement (C).

‡ CFU-GM colonies were aggregates of >50 cells; clusters were aggregates of 3–50 cells.

§ Mean percent change ± 1 SD observed for all experiments; for CFU-Mk, this value also reflects observations reported in Table II. P values of percent change of CFU-Mk and BFU-E colony formation were <0.001 and <0.3, respectively. P values for other colony systems were >0.05.

TABLE IV

Detection of Membrane Antigens Recognized by Groups A, B, and C Monoclonal Antibodies on CFU-Mk, CFU-GM, BFU-E, and CFU-GEMM by Complement-mediated Cytolysis

Monoclonal antibodies	Percent change of colony formation*						
	CFU-Mk	CFU-GM‡				BFU-E	CFU-GEMM
		Day 7		Day 14			
		Colonies	Colonies + clusters	Colonies	Colonies + clusters		
MkA-1	−97 ± 4	−6 ± 13	+4 ± 29	−13 ± 17	−21 ± 13	−15 ± 11	−21 ± 6
MkB-1	−58 ± 11	−21 ± 20	−19 ± 17	−9 ± 10	+1 ± 4	−16 ± 4	−23 ± 20
PC-1	−64 ± 12	−6 ± 8	−7 ± 13	−20 ± 3	−10 ± 3	−17 ± 4	−22 ± 8

Data represent mean percent change ± SD from at least three experiments. *P* values of percent change of CFU-Mk colony formation were: MkA-1, ≤0.001; MkB-1, ≤0.05; PC-1, ≤0.01. *P* values of percent change for other colony systems were >0.05.

* Ranges of mean colony growth for each culture system: CFU-Mk, 7–18; CFU-GM day-7 colonies, 22–44; day-7 colonies + clusters, 92–122; CFU-GM day-14 colonies, 17–34; day-14 colonies + clusters, 25–64; BFU-E, 24–62; CFU-GEMM, 20–7.

‡ CFU-GM colonies were aggregates of >50 cells; CFU-GM clusters were aggregates of 3–50 cells.

for glycoproteins IIb and IIIa (48). To further investigate the nature of the antigen recognized by PC-1, studies were conducted by immunofluorescence on platelets from patients with restricted molecular defects. PC-1 failed to stain platelets from two patients with Glanzmann's thrombasthemia that are deficient in glycoproteins IIb and IIIa. On the other hand, the same antibody stained all platelets from a patient with Bernard-Soulier disease who lacks glycoprotein Ib.

Discussion

Three levels of maturation with distinct immunologic phenotypes were recognized in the megakaryocyte/platelet lineage. The expression of these cellular epitopes was regarded as a differentiation marker for immature/progenitor megakaryocytes, mature megakaryocytes, and platelets. The antigenic modulation underlying the phenotypic changes was characterized by the unidirectional suppression or loss of expression throughout maturation. These differentiation antigens were detected by a panel of 11 monoclonal antibodies, raised against marrow megakaryocytes and blood platelets, which were operationally categorized into three groups, based upon their selective reactivities. Six monoclonal antibodies in group A recognized epitopes expressed on the vast majority of CFU-Mk, on all in vitro grown colony megakaryocytes, and all immature marrow megakaryocytes. This antigenic determinant, however, was not expressed on either mature marrow megakaryocytes or platelets. Furthermore, the group A antibody MkA-1 recognized an epitope that is apparently restricted to the early megakaryocytic compartment and was not detected on clonable progenitors of other hemopoietic lineages. The group B monoclonal antibody MkB-1 recognized an antigenic determinant that is expressed only within the megakaryocytic lineage, by a proportion of CFU-Mk, all immature and mature marrow megakaryocytes, and on in vitro grown colony megakaryocytes. However, this epitope is lost or suppressed during terminal differentiation and is not detectable on

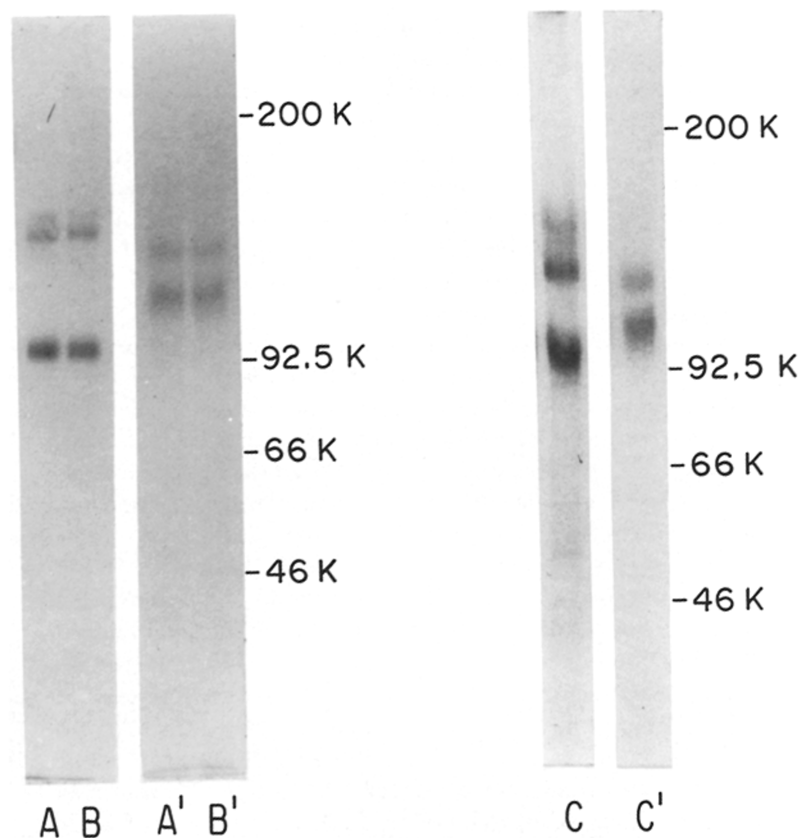

FIGURE 3. Autoradiogram of immunoprecipitated, radiolabeled platelet membrane proteins after SDS-PAGE. Washed platelets were radioiodinated, solubilized, and extracts immunoadsorbed with protein A–Sepharose CL-4B beads coated with different group C monoclonal antibodies. Molecular markers are indicated in kilodaltons on the right: myosin, 200 kD; phospholipase B, 92.5 kD; bovine serum albumin, 69 kD; ovalbumin, 46 kD. Eluants from immunobeads coupled to PC-1 (*A* and *A'*), PC-3 (*B* and *B'*), and PC-4 (*C* and *C'*) were analyzed by 7.5% SDS-PAGE under nonreducing (*A–C*) and reducing (*A'–C'*) conditions.

platelets. The four monoclonal antibodies categorized in group C recognized epitopes expressed on CFU-Mk, in vitro grown colony megakaryocytes, immature and mature marrow megakaryocytes, as well as blood platelets. Furthermore, the group C monoclonal antibody PC-1 had no demonstrable reactivity against any other type of marrow or blood cell, including the clonable progenitors CFU-GEMM, BFU-E and CFU-GM. Consequently, PC-1 may be considered a lineage-specific marker for the entire megakaryocyte/platelet series. Within the same group, PC-2 showed slight significant reactivity against BFU-E, but not against their mature progeny.

From these studies, the reduction of colony growth was interpreted to reflect the expression of the different epitopes on the clonable progenitors as assayed by cytotoxicity. Alternatively, it is possible that the reduction of colony formation is secondary to the elimination of needed accessory cells. Nevertheless, this appears unlikely since these antibodies do not recognize known accessory cells,

such as T or B lymphocytes, or monocytes or any other hematopoietic cells that are not in the megakaryocyte/platelet series.

Platelet glycoprotein IIb/IIIa contained the epitopes recognized by monoclonal antibodies PC-1, PC-3, and PC-4. This was demonstrated by SDS-PAGE analysis of radioiodinated platelet proteins immunoprecipitated by these reagents. Additionally, in immunofluorescence studies, PC-1 did not stain platelets from two patients with Glanzmann's thrombasthenia deficient in glycoprotein IIb/IIIa but readily stained platelets from a patient with Bernard Soulier disease lacking glycoprotein Ib. Collectively, these results demonstrate that the antigenic determinant recognized by the monoclonal antibody PC-1 is located on the platelet glycoprotein IIb/IIIa complex (49). This is the first report documenting the expression of platelet glycoprotein IIb/IIIa at the level of the committed megakaryocyte progenitor. Previously (7, 8), this antigen has been detected by immunofluorescence in all mature and early immature megakaryocytes, using both heterologous and monoclonal antibody preparations. Furthermore, evidence presented here strengthens the notion that platelet glycoprotein IIb/IIIa is a megakaryocyte/platelet lineage-specific antigen.

Thus far, we have been unable to immunoisolate the PC-2 and MKB-1 antigens. Difficulties in immunoprecipitation of these membrane antigens may be explained by their nature (glycolipids/lipids) or by poor radiolabeling due to the low frequency of tyrosine in the peptides. Because of the restricted reactivity of group A monoclonal antibodies to megakaryocyte precursors and progenitors that occur at extremely low frequency in normal marrow, marrow and blood cells from leukemic patients as well as established cell lines are being screened by flow cytometry, with the expectation that the antigens recognized by these antibodies may be expressed at detectable densities on some of these cells. At this point group A and B monoclonal antibodies have shown no reactivity to leukocytes from 29 patients with various myelo-lymphoproliferative disorders or to cells from a subclone of the K-562 cell line that expresses a multilineage phenotype (R. Levene, E. Rabellino, and B. Koziner, unpublished observations).

Group C monoclonal antibody PC-2's partial reactivity against BFU-E but not its progeny may provide insight into the complex hierarchal interrelationships operating at early levels of hematopoiesis. Various reports have described putative intimate associations between erythropoiesis and megakaryocytopoiesis. Mixed erythro-megakaryocytic colonies have been grown in vitro from mouse bone marrow cells in various systems (50, 51). The clonal origin of these "mega-erythro bursts" from primitive biopotential progenitors has been established by cytogenetic analysis (52). Similar phenotypic relationships between human granulocytes, blood monocytes, and their common bipotential progenitor, CFU-GM, have been detected using monoclonal antibodies. Antigens recognized by 4F2 and MY-7 are expressed on CFU-GM and monocytes (53, 54). Conversely, the antigen detected by MY-1 is recognized on CFU-GM and granulocytes, but not on monocytes (55). Future studies characterizing PC-2^{+} BFU-E may elucidate the exact nature of this interrelationship.

In general, differentiating hemopoietic cells undergo structural and functional changes that are paralleled by alterations in their antigenic profiles (17). These phenotypic changes may involve modifications of membrane glycoprotein or

glycolipid constituents affecting either the acquisition of new antigens, the exposure of cryptic antigens, or conversely, the loss or sequestration of certain antigenic sites (56). These patterns of antigenic modulation have been described for the human lymphocytic, myelomonocytic, and erythroid series (21–25). Furthermore, antigenically distinct subpopulations of precursors within each of these lineages have been recognized (22, 44, 57–60).

Previously, analogous phenotypic characterization of the human megakaryocytic compartment has been limited. Staging of human megakaryocyte maturation traditionally has been achieved using morphological and/or ultrastructural criteria in conjunction with ploidy values (61–63). Assays for cytochemical markers, such as acid phosphatase isozyme analysis have also been used to establish indices of megakaryocyte maturation (64). More recently, analogous studies have been performed using monoclonal antibodies raised against human platelets as well as a crossreactive monoclonal antibody raised against a human schwannoma tumor or monocytic cells (8, 65–67). However, no definitive analysis of the earliest developmental compartments using specific megakaryocytic differentiation markers has been described.

The panel of monoclonal antibodies described in this communication will certainly facilitate a more accurate and rapid staging of megakaryocytes in normal and pathologic marrows. Future studies focused on multiparameter analyses using these maturational markers of the different ontogenetic levels will facilitate recognition and description of patterns of differentiation in vivo and in vitro.

Summary

Human megakaryocytes were studied for phenotypic changes occurring throughout differentiation using a panel of monoclonal antibodies raised against marrow megakaryocytes and blood platelets. 11 monoclonal antibody preparations were selected for restricted specificity against megakaryocytes and/or platelets after screening by immunofluorescence, complement-mediated cytolysis, and solid phase enzyme-linked immunosorbent assay. The expression of the cellular epitopes recognized by these reagents enabled the identification of three levels of megakaryocyte maturation characterized by distinct immunologic phenotypes. Based upon their reactivities against megakaryocytic cells at different ontogenetic levels, monoclonal antibodies were operationally categorized into three groups. Group A consisted of six different monoclonal antibodies that recognized antigens on the colony-forming unit–megakaryocyte (CFU-Mk), in vitro grown colony megakaryocytes, and early immature marrow megakaryocytes, only, and did not detect their respective epitopes on either mature megakaryocytes or platelets. A monoclonal antibody categorized in group B detected a cell antigen expressed by megakaryocytic cells at all maturational levels, but which is lost or suppressed during terminal differentiation and is not expressed on blood platelets. Group C included four different monoclonal antibodies raised against platelets that recognized antigenic determinants expressed on the CFU-Mk, colony megakaryocytes, early and mature megakaryocytes, and platelets. Three group C monoclonal antibodies (PC-1, PC-3, and PC-4) were specific for platelet glycoprotein IIb/IIIa. Additionally, group C monoclonal antibody PC-2 was unique in that it showed partial reactivity against

the clonable progenitor for the erythroid series (BFU-E). Recognition of discrete phenotypic changes in differentiating megakaryocytes will enable multiparameter analyses of these cells as well as the study of factors regulating the dynamics of megakaryocytopoiesis in health and disease.

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