HUMAN MEGAKARYOCYTES

II. Expression of Platelet Proteins in Early Marrow Megakaryocytes*

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With the development of methods to isolate human megakaryocytes, it is now possible to study marrow megakaryocytes for various biological properties (1–3). Megakaryocytes are unique cells in that they undergo nuclear endoreplication and cytoplasmic demarcation, giving rise to blood platelets (4). Platelets are essential components in hemostasis and participate in the pathophysiology of thrombosis and atherosclerosis (5–7). Platelets are nonnucleated cells and carry only a vestigial protein synthetic apparatus (8, 9). Previous studies have demonstrated that the majority of human megakaryocytes contain fibrinogen, platelet myosin, plasma factor VIII antigen (factor VIII:AGN),[1] and platelet membrane glycoproteins (1, 2). Growth-promoting activity, or megakaryocyte-derived growth factor(s), similar to platelet-derived growth factor(s) has been demonstrated in human megakaryocytes (3).

Studies on megakaryocytopoiesis in the murine system have been greatly facilitated by the use of a selective cytochemical marker, acetylcholinesterase activity (10). A population of early, differentiated, immature megakaryocytes that contained acetylcholinesterase activity was described in mouse and rat marrow (10–12). Analysis of early megakaryocytes in these systems has brought new insight into the regulatory mechanisms of platelet production (13–14). Similar markers, however, have not yet been established for human megakaryocytes. Recently, Mazur et al. (15) have reported the expression of platelet glycoproteins in colony megakaryocytes grown in vitro using the plasma clot technique.

In this communication, analysis of several platelet proteins by immunofluorescence revealed that platelet glycoproteins Ib, IIb, and IIIa, as well as factor VIII:AGN, and

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[1] *Abbreviations used in this paper:* BSA, bovine serum albumin; HBSS$^{=}$/CAT, calcium- and magnesium-free Hanks' balanced salt solution containing 10^{-3} M adenosine, 2×10^{-3} M theophylline, 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; HBSS$^{=}$/CAT-NaN_3-BSA, HBSS$^{=}$/CAT containing 0.02% sodium azide and 1% BSA; FcR, Fc receptor; factor VIII:AGN, plasma factor VIII antigen; FITC, fluorescein isothiocyanate; TRITC, tetramethylrhodamine isothiocyanate.

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platelet factor 4 are present in virtually all of marrow megakaryocytes and can be considered to be distinct protein markers of this cell line. These same proteins were detected in a small proportion of lymphoid-like mononuclear marrow cells regarded as immature, differentiated megakaryocytes or early megakaryocytes. Early megakaryocytes bear Fc receptors for IgG but lack expression of Ia antigens. Characterization of megakaryocytic cells using platelet protein markers may be helpful for a better understanding of altered megakaryocyte maturation in various disorders.

Materials and Methods

Preparation of Human Bone Marrow Cell Suspensions. Suspensions of human marrow cells enriched for megakaryocytes were prepared using density centrifugation gradients as described previously (1, 2). Marrow tissue was obtained from rib fragments routinely removed from patients undergoing thoracotomy. Written consent was procured from all participating patients before surgery in compliance with regulations established by the National Institutes of Health, Bethesda, Md. Experimental protocols were approved by The Committee on Human Rights of New York Hospital-Cornell Medical Center and Memorial Sloan-Kettering Cancer Center, New York. To obtain single-cell suspension of marrow cells, rib specimens were debrided, cut into 2–4-cm sections, and the marrow tissue promptly harvested with medium as described in more detail elsewhere (1, 2). The medium used throughout the procedures consisted of calcium- and magnesium-free Hanks' balanced salt solution (Grand Island Biological Co., Grand Island, N. Y.) containing 10^{-3} M adenosine, 2×10^{-3} M theophylline, 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 of DNAse I (all from Sigma Chemical Co., St. Louis, Mo.) at a final pH of 7.0 ± 0.05 and 295 ± 5 mosM ($HBSS^{=}$/CAT).

Initial enrichment of the megakaryocytes and the putative early megakaryocytes was achieved by a slight modification of the density-gradient centrifugation technique previously described (1, 2). As reported, gradients were generated with solutions of colloidal silica particles coated with polyvinyl pyrrolidone (Percoll; Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Piscataway, N. J.), in $HBSS^{=}$/CAT at 295 mosM, pH 7.0. The modification of the technique consisted of (*a*) use of $HBSS^{=}$/CAT (1.007 g/cm^3) instead of 1.040 g/cm^3 Percoll-medium and (*b*) centrifugation of gradients at 725 g for 20 min at 20°C. Density-gradient centrifugation was performed as follows: (*a*) 7–8 $\times 10^7$ marrow cells resuspended in 3 ml $HBSS^{=}$/CAT containing 1.050 g Percoll/cm^3 were placed in 17- × 10-mm polypropylene tubes; (*b*) cells were then underlayered with 3 ml $HBSS^{=}$/CAT medium containing 1.085 g/cm^3 Percoll and (*c*) overlayered with 3 ml of medium $HBSS^{=}$/CAT. After centrifugation, the cells were harvested into three fractions. Fraction I ($d < 1.050$ g/cm^3) comprised all the cells from the top gradient layer and the upper interface; fraction II ($d \cong 1.050$ g/cm^3) consisted of the cells from the intermediate gradient layer; and fraction III ($d > 1.050$ g/cm^3) was made up of cells from the bottom gradient layer and the pellet. After harvesting each fraction, the cells were counted and examined for various protein antigens. Over 86% of megakaryocytes and small lymphoid-like mononuclear cells were found in density fraction I.

Preparation of Blood Leukocytes and Washed Platelets. Blood samples were obtained from normal, healthy volunteers and a patient with Glanzmann's thrombasthenia. Blood was collected in 0.32% sodium citrate in polypropylene tubes. Leukocytes were prepared by resuspending the blood in 3% Dextran T500 (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc.) $HBSS^{=}$/CAT and allowing it to sediment at 20°C for 25 min. The leukocyte-enriched supernate, depleted of most erythrocytes, was washed twice in $HBSS^{=}$/CAT by centrifugation at 225 *g* for 15 min at 20°C. Washed platelets were prepared by centrifugation of whole citrate blood at 225 *g* for 10 min at 20°C. The platelet-rich plasma supernate was then centrifuged at 1,600 *g* for 20 min at 20°C and subsequently washed three times in a 0.12 M sodium chloride solution containing 0.0129 M sodium citrate and 25 mM glucose at pH 6.8 (16). Platelet counts were performed with a Coulter counter (model ZBI; Coulter Electronics Inc., Hialeah, Fla.).

Immunofluorescence Assay. Monospecific rabbit antisera against purified factor VIII:AGN, fibronectin, and isolated platelet glycoproteins Ib, IIb, and IIIa were prepared and tested for specificity as described (17–21). Anti-human platelet factor 4 goat serum was a generous gift

from Dr. Karen Kaplan of Columbia University, New York (21). Polyspecific anti-platelet membrane serum was generated and tested as described (22). After isolation of the IgG fraction from each antiserum by DEAE-cellulose chromatography, each antibody was treated with pepsin (Worthington Biochemical Corp., Freehold, N. J.) to produce $F(ab')_2$ fragments (23). $F(ab')_2$ fragments were separated from trace amounts of uncleaved IgG and pFc fragments by Sephacryl S-200 (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc.) gel filtration. The $F(ab')_2$ fragments were shown to be free of uncleaved IgG and pFc fragments by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and by immunodiffusion analysis with goat anti-rabbit Fc fragment-specific antiserum. Alternatively, intact IgG antibody preparations were treated with soluble *Staphylococcus aureus* protein A (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc.) to functionally block the Fc region of the IgG molecule (24). Effective blockage of the Fc region, without protein precipitation, was obtained by incubating protein A with IgG solutions at a 7:1 wt:wt ratio, first at 37°C for 2 h and then at 4°C overnight. In most instances, direct immunofluorescence was performed using either the IgG $F(ab')_2$ or the protein A-coupled IgG antibody preparation conjugated to fluorescein isothiocyanate (FITC) or tetramethylrhodamine isothiocyanate (TRITC) (both from BBL Microbiology Systems, Becton, Dickinson & Co., Cockeysville, Md.) as described in more detail elsewhere (23). After conjugation to FITC or TRITC, the IgG or IgG $F(ab')_2$ antibody peparations were rechromatographed in DEAE-cellulose, and three fractions of the labeled gamma globulin were eluted sequentially with 0.01, 0.05, and 0.1 M sodium phosphate buffer, pH 7.0. In general, the 0.05 M sodium phosphate antibody preparation with a dye/protein molar ratio of 1 ± 0.3 was found to have the highest specific activity and the least background staining. To study the antiserum specificity by immunofluorescence, serial absorption of each antibody was performed with increasing amounts of the specific and nonrelated antigens such as bovine serum albumin (BSA) at 37°C for 30 min and at 4°C for 12 h. Variations in the fluorescence staining of either megakaryocytes or platelets with eventual extinction with the specific antigen were the criteria for specificity. Specificity of the fibronectin antiserum was further tested using cultured human marrow fibroblasts (25). Anti-platelet glycoproteins IIb and IIIa (1 mg/ml) were tested after incubation at 37°C for 30 min and at 4°C for 12 h with 2×10^9 platelet/ml serum from either a normal donor or a patient with Glanzmann's thrombasthenia.

For fluorescence staining of membrane-restricted components, isolated marrow or blood cells ($1–20 \times 10^6$) or platelets ($1–2 \times 10^8$) were incubated with 0.03–0.07 ml of the fluoresceinated antibody in the presence of $HBSS^=$/CAT containing 0.02% sodium azide and 1% BSA ($HBSS^=$/CAT-NaN_3-BSA) at 20°C for 30 min. Cells were then washed twice in $HBSS^=$/CAT-NaN_3-BSA. Fluorescence staining was also carried out by incubating smears of cells with the different antibody preparations after fixing cells in pure methanol for 10 min followed by three washes in phosphate-buffered saline (PBS). Cells were examined with a Leitz Ortholux II microscope (E. Leitz, Inc., Rockleigh, N. J.) equipped with Ploem illuminator and phase-contrast optics. Cells were double-assayed for two proteins by staining cell preparations simultaneously or sequentially with a TRITC-conjugated reagent and/or an FITC-conjugated specific reagent. Double staining of cell preparations was performed using both cell suspensions and fixed cell smears. Combinations of one or two markers on individual cells were separately enumerated by sequential examination of each microscopic field with blue-light fluorescence (FITC), green-light fluorescence (TRITC), and visible-light phase-contrast optics.

Detection of FC Receptors (FcR) and Ia Antigens. Studies of membrane receptors for the Fc portion of IgG and Ia antigens were performed by direct immunofluorescence as previously described (1–2). To detect FcR, $0.05–5.00 \times 10^5$ megakaryocytes or $5–6 \times 10^6$ marrow cells were incubated for 30 min at 20°C with a soluble immune complex, prepared at antibody excess with 25 μl of rabbit IgG anti-ovalbumin conjugated to FITC and 25 μl of ovalbumin (26). Ia antigens were detected with a rabbit $F(ab')_2$ anti-Ia-serum conjugated to FITC prepared and tested as described elsewhere (27). The anti-Ia reagent was a donation of Dr. R. Winchester, The Rockefeller University, New York. After staining for FcR or Ia antigens, cells were smeared, fixed, and stained with antiplatelet glycoprotein IIb or IIIa serum conjugated to TRITC. Cells were examined under fluorescence microscopy as mentioned above.

Results

Expression of Platelet Proteins in Marrow Megakaryocytes. Human megakaryocytes isolated from normal bone marrow tissue were studied for the presence of various platelet proteins using the immunofluorescence technique (Table I). Three of the major platelet glycoproteins (Ib, IIb, and IIIa), factor VIII:AGN, fibronectin, and platelet factor 4 were detected in virtually all isolated megakaryocytes. Analysis of the different platelet proteins in megakaryocyte-enriched marrow preparations were carried out either by staining fixed smears of cells or by incubating suspensions of viable cells with the different monospecific antisera. Megakaryocyte components detected using the fixed cell smears were located primarily intracellularly, while megakaryocyte components stained with cells suspended in the antisera were associated with the cell membrane. The pattern of fluorescence staining for the different antigens was as previously described (1, 2, 28). Intracellular staining was characterized typically by intense homogeneous staining in all areas of the cells except the nucleus (Fig. 1 b). The membrane staining pattern consisted of uniformly distributed flecks throughout the cell (Fig. 1 a). The megakaryocytic morphology of the fluorescent stained cells was confirmed by concomitant examination of the cells under phase-contrast microscopy (Fig. 1 c).

The monospecificity of each antiserum employed was demonstrated by immunodiffusion, immunoprecipitation, and immunofluorescence. In the fluorescence studies, each antiserum was tested for its ability to stain megakaryocytes after absorption with the specific or a nonrelated antigen. Results of a typical immunofluorescence specificity experiment are depicted in Fig. 2. Highly purified plasma factor VIII:AGN, but not BSA, inhibited the detection of megakaryocyte factor VIII:AGN. Similar results were observed in specificity experiments conducted for anti-fibronectin and anti-platelet factor 4 antisera. Antisera against platelet glycoproteins IIb and IIIa were found to be devoid of antibody activity after absorption of each antisera with normal platelets. On the contrary, platelets from a patient with Glanzmann's thrombasthenia proven to be deficient in membrane glycoproteins IIb and IIIa failed to block the activity of both antisera (29, 30). Furthermore, the specificity of the reagents used was determined by analyzing the types of cells stained. With the exception of a small population of low density marrow mononuclear cells (*vide infra*), megakaryocytes and platelets were the only two types of marrow and blood cells that stained with anti-platelet glycoproteins Ib, IIb, and IIIa, anti-factor VIII:AGN, and platelet factor 4

TABLE I

Detection of Platelet Protein in Human Megakaryocytes by Immunofluorescence

Antisera	Percent of megakaryocytes stained	
	After cell fixation (intracellular)	In cell suspension (membrane)
Platelet glycoprotein Ib	96 ± 1*	95 ± 2
Platelet glycoprotein IIb	99 ± 1	98 ± 1
Platelet glycoprotein IIIa	98 ± 2	98 ± 1
Factor VIII:AGN	95 ± 3	94 ± 4
Fibronectin	93 ± 4	95 ± 3
Platelet factor 4	85 ± 2	0

* Mean value and 1 SD of at least 12 experiments.

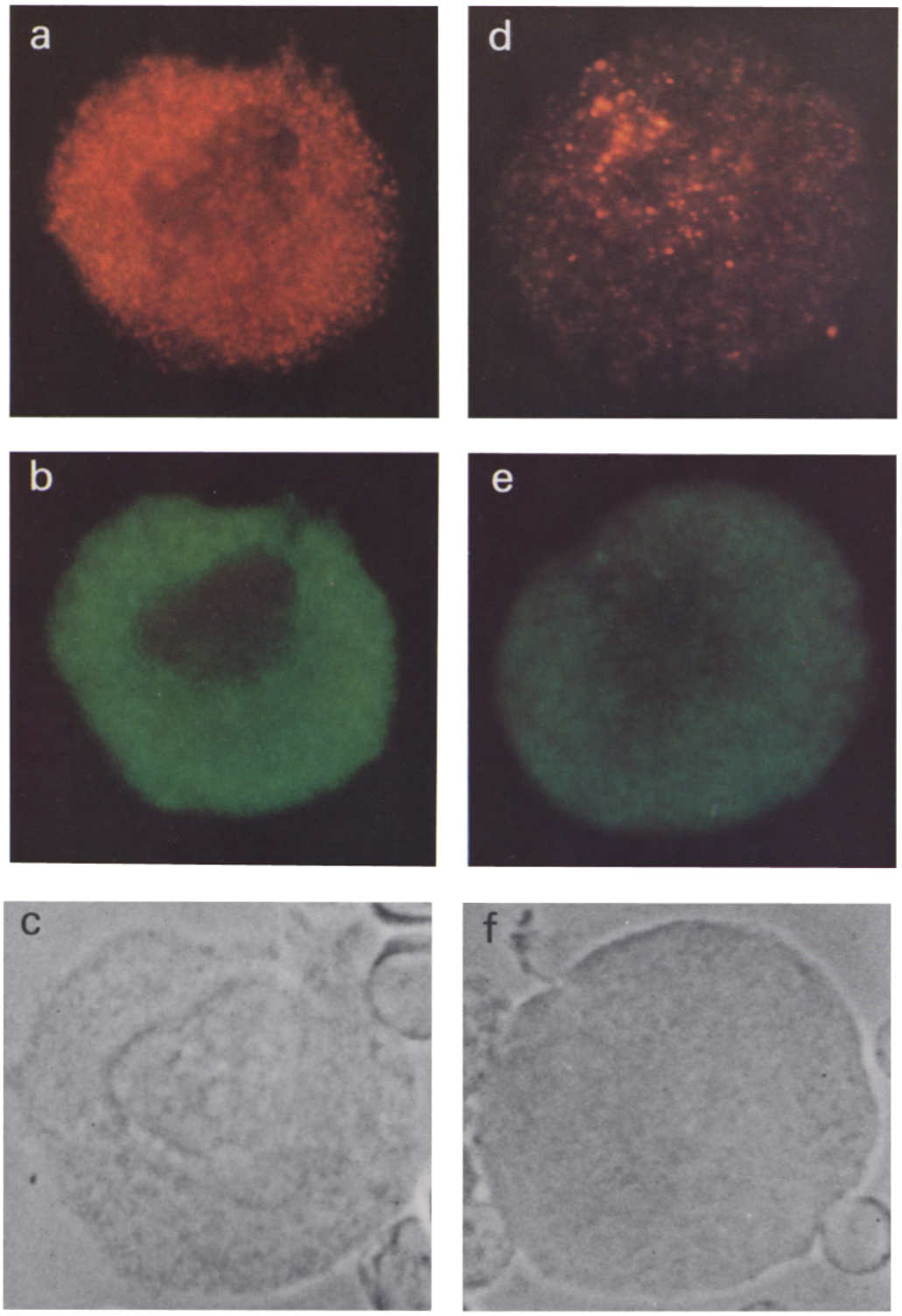

FIG. 1. Immunofluorescent staining of isolated human marrow megakaryocytes. Factor VIII:AGN in megakaryocytes: (a) membrane-associated factor VIII:AGN detected with a TRITC-conjugated antiserum; (b) factor VIII:AGN staining of the same megakaryocyte with a FITC-labeled antiserum after fixation of cells with methanol; (c) same megakaryocyte examined under phase-contrast microscopy. Platelet glycoprotein IIIa in megakaryocytes: (d) membrane-associated platelet glycoprotein IIIa detected with a TRITC-conjugated antiserum; (e) platelet glycoprotein IIIa staining of the same megakaryocyte with an FITC-labeled antiserum after fixation of cells with methanol; and (f) same megakaryocyte examined under phase-contrast microscopy. × 1,200.

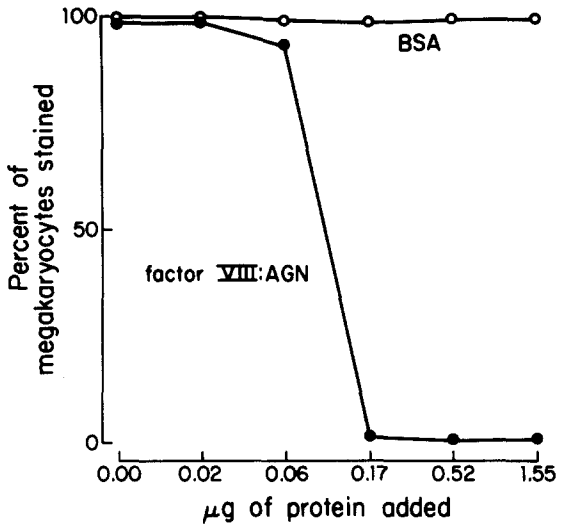

FIG. 2. Effect of purified factor VIII:AGN (●) or BSA (○) on the fluorescent staining of megakaryocytes with anti-factor VIII:AGN serum at 62 μg/ml.

antisera. This firmly established the selectivity of these reagents for cells of the megakaryocytic lineage.

To investigate the topographic distribution of the various antigens within isolated megakaryocytes, cell preparations were subjected to double assay for both membrane and intracellular fluorescence staining. Cells first stained for membrane-associated factor VIII:AGN with TRITC-labeled antiserum were subsequently stained for intracellular factor VIII:AGN with a FITC-conjugated antiserum. As shown in Figs. 1 a, b, and c, factor VIII:AGN was demonstrated on the cell membrane as well as in the intracellular compartment of megakaryocytes. Using the same staining system, platelet glycoproteins Ib, IIb, and IIIa, as well as fibronectin, were detected on the membrane and in the intracellular compartment of all isolated megakaryocytes (Fig. 1 d, e, and f). Platelet factor 4, on the other hand, was only found in the cytoplasm of most marrow megakaryocytes (Table I).

In another series of experiments, the relationship between platelet glycoproteins IIb and IIIa in megakaryocytes was studied by immunofluorescence. Platelet glycoproteins IIb and IIIa are known to be closely related functionally and to form a molecular complex in the platelet membrane (31, 32). The expression of platelet glycoproteins IIb and IIIa in megakaryocytes as independent molecular entities was investigated by the capability of one of the monospecific antisera to affect the detection of the other glycoprotein. Detection of platelet glycoprotein IIb or IIIa in smeared, methanol fixed megakaryocytes was not affected by simultaneous or alternate incubation of fixed cells with the two antisera (Fig. 3 a and b).

Identification of Early Human Marrow Megakaryocytes or Megakaryocyte Precursors. Studies were performed to determine whether the platelet marker proteins were also expressed within marrow cells that might represent megakaryocyte precursors or early megakaryocytes (10–14). Analysis of marrow cell preparations by immunofluorescence revealed that a minor proportion of small mononuclear marrow cells were stained for platelet glycoproteins Ib, IIb, IIIa, factor VIII:AGN, and platelet factor 4 (Table II).

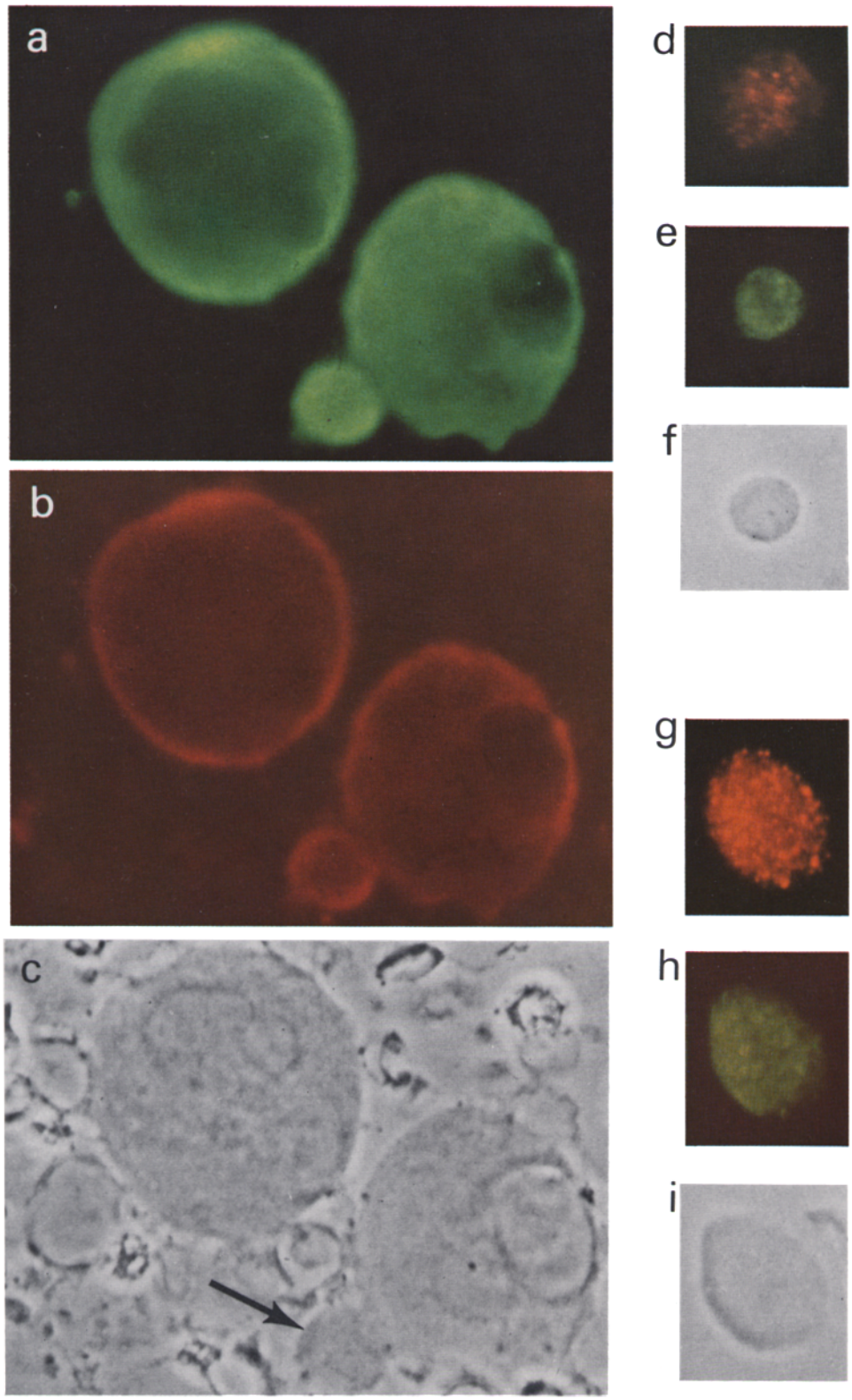

FIG. 3. Immunofluorescence staining of human megakaryocytic cells. Methanol-fixed low density marrow cells (<1.050 g Percoll/cm^3) simultaneously stained with (a) FITC-conjugated anti-platelet glycoprotein IIb serum and (b) TRITC-labeled anti-platelet glycoprotein IIIa serum; (c) same field under phase-contrast microscopy. Arrow indicates small cell positively stained. Small, low density marrow cell double-assayed for (d) membrane platelet glycoprotein IIIa (TRITC) and (e) intracellular platelet glycoprotein IIIa (FITC); (f) phase-contrast microscopy. Small, low density marrow cell double-assayed for (g) membrane factor VIII:AGN (TRITC) and (h) platelet glycoprotein IIb (FITC); (i) phase-contrast microscopy (a–f, × 970; g–i, × 1,200).

TABLE II

Identification of Small Mononuclear Marrow Cells Bearing Various Platelet Proteins by Immunofluorescence

Antisera	Stained mononuclear cells/10^4 marrow cells		
	Unseparated	After density centrifugation in Percoll	
		<1.050 g/cm^3	>1.050 g/cm^3
Platelet glycoprotein Ib	2.9 ± 1*	32 ± 2	0
Platelet glycoprotein IIb	2.1 ± 1.6	20 ± 1	0
Platelet glycoprotein IIIa	1.4 ± 0.8	30 ± 17	0
Platelet polyspecific anti-membrane	2.0 ± 0.1	46 ± 3	0
Factor VIII:AGN	2.8 ± 0.2	39 ± 2	0
Platelet factor 4	1.5 ± 0.6	36 ± 4	0

* Mean values and 1 SD of five experiments. Incidence of stained cells was established by counting at least 4×10^4 cells in each preparation.

Small cells specifically stained for these proteins represented between 1.4 and 2.9/10^4 unseparated marrow cells. The same incidence was observed with a polyspecific antiplatelet membrane serum. Most of these cells had low cell density and were harvested from the low density fraction of the Percoll density gradient representing ~20–46/10^4 mononuclear cells. Under phase-contrast microscopy, these cells had a lymphoid-like appearance with a round nucleus, an agranular cytoplasm, and a high nuclear cytoplasmic ratio (Fig. 3 c, f, and i). The cell diameter was estimated at ~9–12 μm as compared with erythrocytes of the same cell preparation (6–8 μm).

Studies of the topographic distribution of different platelet protein markers disclosed that the four antigens investigated were detected in the same cell compartments as those described for mature megakaryocytes. Platelet glycoproteins Ib, IIb, and IIIa, as well as factor VIII:AGN, were detected both on the membrane and intracellularly. On the other hand, platelet factor 4 was found only in the intracellular compartment. Small marrow cells stained for membrane and intracellular platelet glycoprotein IIIa are shown in Fig. 3 d, e, and f. Moreover, small low density marrow cells bearing factor VIII:AGN were found to contain platelet glycoprotein IIIa (Fig. 3 g, h, and i). Double-assay experiments performed with all possible combinations of antisera showed that approximately two-thirds of the small marrow cells bearing platelet proteins bore at least two of the antigens tested. Quantitative assessment of individual small marrow cells containing only one or more protein markers was not attempted because of the extremely low incidence of these cells.

FcR and Ia antigen have been described as differentiation markers in cells of the megakaryocytic series. Using the combined-fluorescence assay for membrane and intracellular staining, it was found that FcR were detected in all precursors or early megakaryocytes containing platelet glycoproteins IIb and IIIa. On the contrary, Ia antigen was not detected on any of early megakaryocytes bearing platelet glycoproteins IIb and IIIa.

In an attempt to determine whether small marrow cells bearing platelet proteins formed part of an early megakaryocytic differentiation compartment, the incidence of these cells was correlated with that of mature megakaryocytes and the number of blood platelets (Table III). The incidence of precursor or early megakaryocytes was estimated by the number of small mononuclear cells expressing either platelet

TABLE III

Correlative Analysis of Small, Low Density Marrow Cells Bearing Platelet Proteins with Megakaryocytes and Platelets

Marrow cells	Number of cells bearing platelet protein/10^4 marrow cells*		Small cells: megakaryocytes ratio	Blood platelets ($\times 10^{11}$/liter)
	Small mononuclear cells	Megakaryocytes		
Unseparated	2.24 ± 0.6‡	4.4 ± 0.3	0.51	307 ± 89
After density centrifugation (<1.050 g Percoll/cm^3)	33.4 ± 9.8	73.0 ± 46	0.46	

* Estimated by the number of cells bearing platelet glycoprotein IIb, IIIa, or factor VIII:AGN.
‡ Mean values and 1 SD of at least eight experiments.

glycoproteins IIb, IIIa, or factor VIII:AGN. In normal, unseparated marrow cell suspensions, the ratio of small cells:mature megakaryocytes bearing platelet markers was ~0.5. After density-gradient centrifugation, the ratio was ~0.46. This similar ratio together with high recovery yield of the two types of cells indicated that analysis of megakaryocytic cells in Percoll low-density fractions probably reflects the megakaryocytic population of intact marrow tissue.

Discussion

Our studies demonstrate that human marrow megakaryocytes contain a number of platelet proteins that play key roles in normal platelet physiology. Platelet glycoproteins Ib, IIb, and IIIa, as well as factor VIII:AGN, platelet factor 4, and fibronectin were detected by immunofluorescence in the vast majority of morphologically recognizable megakaryocytes. In addition, a small proportion of lymphoid-like mononuclear marrow cells was found to express these platelet proteins. This population of small mononuclear cells was regarded as early megakaryocytes or megakaryocyte precursors that formed part of an early megakaryocytic differentiation compartment. This population of early megakaryocytes may be analogous to the small acetylcholinesterase-positive cells that have been described in the mouse and rat systems (10–14). Platelet glycoproteins Ib, IIb, and IIIa, as well as platelet factor 4 and factor VIII:AGN, were defined as distinct markers for marrow megakaryocytes and may be helpful for identifying megakaryocytic cells as well as for monitoring sequential events of megakaryocyte differentiation.

These studies were facilitated by the development of a double-fluorescent assay that enabled independent recognition of membrane- and intracellular-associated cell components in isolated marrow megakaryocytes. Using the fluorescent double-assay systems, it was demonstrated that factor VIII:AGN, platelet glycoproteins Ib, IIb, and IIIa, as well as fibronectin, were expressed both on the membrane and in the intracellular compartment of megakaryocytes.

Three major platelet glycoproteins, Ib, IIb, and IIIa, were detected in >90% of isolated megakaryocytes. These glycoproteins were detected on the membrane of living cells as well as in the intracellular compartment of megakaryocytes after fixing cells with methanol, indicating that certain antigenic determinants of the glycoproteins not readily exposed to the outer surface of the cell membrane become accessible to the antibody preparations after fixing the cells. This is in agreement with the

transmembrane location of these glycoproteins in the platelet membrane (31). The intracellular compartment may also reflect storage sites of newly synthesized membrane constituents. In addition, platelet glycoproteins IIb and IIIa were detected as independent molecular entities in megakaryocytes since detection of either glycoprotein was not inhibited when cells were incubated simultaneously or alternately with both reagents. This observation is in agreement with recent studies showing the separate structural identity of these membrane glycoproteins (20, 32).

Factor VIII:AGN has been demonstrated in platelets, endothelial cells, and in megakaryocytes (33–36). Two pools of factor VIII:AGN, one associated with intracellular organelles and the other with the cell membrane, have been detected in >90% of isolated human marrow megakaryocytes. In a previous communication, we reported a similar finding, but only 10% of the isolated megakaryocytes contained intracellular factor VIII:AGN (1). The differences encountered may be a result of the cell fixative used; methanol rather than acetone was used in the current experiments. Synthesis of factor VIII:AGN has been demonstrated in guinea pig megakaryocytes (8). Megakaryocytes appear to be the source of intracellular granule factor VIII:AGN in platelets (37). More controversial is the role of membrane-associated factor VIII:AGN. In platelets, it has been suggested that membrane factor VIII:AGN is involved in the process of platelet adhesion (37). The significance and fate of membrane-associated factor VIII:AGN in megakaryocytes remains to be determined.

FcR and Ia antigen have been recognized as differentiation markers of the B-lymphoid monocytic, myeloid, and erythroid series (38–41). Parallel studies of megakaryocytes and small, low density marrow cells disclosed that FcR were found on all mature megakaryocytes and small marrow cells bearing platelet markers, whereas Ia antigen was only detected on a proportion of mature megakaryocytes, but not on any of the megakaryocytic precursors. The fact that a proportion of mature megakaryocytes and not precursor megakaryocytes bore Ia antigens indicated that the expression of this marker was restricted to a subclass of megakaryocytes. Expression of Ia antigen may take place at an intermediate stage of megakaryocyte differentiation as described in the myeloid series (41). Alternatively, it may reflect the development of a separate megakaryocyte lineage with restricted differentiation and functional properties.

We conclude that at least two major differentiation compartments can be recognized in human megakaryocytopoiesis: one that includes mature megakaryocytes recognizable by their morphology, and the other that comprises a population of small low density lymphoid-like marrow cells that can only be identified by virtue of platelet protein markers. The factors regulating the population dynamics of these two compartments in health and disease remain subjects for future studies.

Summary

Analysis of various platelet proteins by immunofluorescence demonstrated that platelet glycoproteins Ib, IIb, and IIIa, as well as plasma factor VIII antigen (factor VIII:AGN), platelet factor 4, and fibronectin are present in the vast majority of morphologically recognizable megakaryocytes. In addition, a small number of lymphoid-like mononuclear marrow cells, representing ~1.4–2.9/10^4 marrow cells, was found to express the same platelet proteins. This population of early marrow megakaryocytes is analogous to small acetylcholinesterase-positive rat and mouse marrow cells. Fc receptors for IgG were expressed in all megakaryocytes and megakaryocyte

precursors, whereas the Ia antigen was detected only on a proportion of mature megakaryocytes and not on any early or precursor megakaryocytes. Platelet glycoproteins Ib, IIb, and IIIa, as well as factor VIII:AGN, and platelet factor 4 were established as distinct markers for marrow megakaryocytes and may be helpful for identifying megakaryocytic cells as well as for monitoring events of megakaryocyte differentiation.

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