HUMAN MEGAKARYOCYTES

I. Characterization of the Membrane and Cytoplasmic Components of Isolated Marrow Megakaryocytes*

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Platelets are blood elements that play a key role in coagulation and participate directly in the processes of thrombosis and atherosclerosis (1-3). However, the physiologic mechanisms governing platelet formation and release from megakaryocytes remain largely unknown (4). Platelets are nonnucleated cells with only a vestigial protein synthetic apparatus, and therefore most of the structural and functional components associated with platelets are believed to be derived from megakaryocytes. Thus, a better understanding of megakaryocyte biology should provide important new insights into the mechanisms of platelet production and into the expression of the various aspects of platelet function during thrombopoiesis. Since Wright first hypothesized the megakaryocyte-platelet relationship over 70 yr ago (5), most of the information concerning megakaryocytes has been obtained indirectly from studies of patients with platelet-associated bleeding disorders or experimental animals with artificially altered platelet levels (1, 6-8). Recently, techniques have been reported to obtain partially purified populations of megakaryocytes from mouse, rat, and guinea pig marrow tissue (9-11). With these preparations, various platelet-associated components have been identified in nonprimate megakaryocytes (12, 13). Moreover, culture systems have now been devised to grow and examine murine megakaryocytes in vitro (14, 15).

In studies of the differentiation of other types of hematopoietic cells, cell component analysis has proven a valuable tool both for monitoring the degree of differentiation and for identifying the distinct types of progenitor cells (16-18). It is known that individual platelets are heterogenous in their density, size, and function (19, 20), but it has yet to be determined whether these diversities arise as a result of platelet formation occurring at different stages of megakaryocyte maturation or, alternatively, whether they arise because of the existence of separate and distinct committed progenitor cells that generate different platelet subsets. By analogy to other hemo-

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poietic cell lineages, analysis of megakaryocyte cell components may elucidate the mechanisms of megakaryocyte maturation and the development of platelet functions.

In this report, studies were directed toward defining several of the membrane and cytoplasmic elements of human megakaryocytes. After a method was devised for isolating pure populations of human megakaryocytes with high yield, the presence of various platelet-associated components was examined by immunofluorescence. Fibrinogen, plasma factor VIII antigen (factor VIII:AGN), platelet myosin, and platelet glycoproteins I and III were detected in over 90% of human marrow megakaryocytes. Furthermore, parallel analysis of both human and murine megakaryocytes and platelets for Fc receptor, complement receptors, and Ia antigens revealed that the expression of these cell components may be useful markers for monitoring megakaryocyte maturation.

Materials and Methods

Preparation of Human Bone Marrow Cell Suspension. Bone marrow was obtained from ribs routinely removed from individuals undergoing thoracotomy. Written consent from each individual studied was obtained according to the regulations established by the National Institutes of Health, and protocols were approved by the Committee on Human Rights. Ribs, rather than bone marrow aspirates, were chosen as the source of marrow tissue because ribs contained a larger number of marrow cells and little, if any, blood cell contamination. Marrow cells were harvested promptly after the ribs were removed. Ribs cleaned of adherent tissues were cut into fragments of 2–4 cm in length with a bone cutting forceps. Marrow cells were harvested by injecting medium very gently into the central cavity of the bone, forcing out the marrow tissue. Subsequently, the rib fragments were opened with a bone rongeur and additional tissue was flushed out from the inner face of the bone walls. A single cell suspension was obtained by either gently forcing the cells through a 23-gauge needle or by gently pipetting with a Pasteur pipette. Each 2- to 5-cm section obtained from the anterior third of a rib usually provided 1–2 × 10⁸ cells/cm for a total of 5–13 × 10⁸ marrow cells per rib sample. Selective cell loss during this procedure was ruled out by careful histological examination of the rib segments before and after harvesting the marrow. The medium (CATCH) used to harvest marrow cells was derived from the formula described by Levine and Fedorko (9): Calcium and magnesium-free Hanks' balanced salt solution (Grand Island Biological Co., Grand Island, N. Y.) containing 10⁻⁸ M adenosine, 2 × 10⁻³ M theophylline, 3.8% sodium citrate, 3.5% bovine serum albumin (BSA), 20–40 U/ml of highly purified DNase, and 25 mM Hepes buffer, pH 7.0 ± 0.2 (all from Sigma Chemical Co., St. Louis, Mo.). The modification consisted of the addition of DNase and Hepes buffer. Cells were washed once and then resuspended for determination of cell number, differential, and cell viability. Cell viability was estimated by trypan blue exclusion and structural examination by phase contrast microscopy.

Density Separation of Human Bone Marrow Cells. The cell density profile of megakaryocytes and other human marrow cell types was determined by centrifugation in density gradients of either BSA (preservative-free 35% BSA wt/wt) or colloidal silica coated with polyvinylpyrrolidone, Percoll (Pharmacia Fine Chemical, Piscataway, N. J.) (21). Solutions were diluted in CATCH medium buffered with 25 mM Hepes, pH 7.0. All fractions had a final pH of 7.0 and osmolality of 290 mosmo. Cell density determinations were performed either with a series of several different tubes, each containing a single density step, or by discontinuous gradient
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For the first procedure, up to $8 \times 10^7$ cells were resuspended in 5 ml of medium of the appropriate density and placed into a 17 x 100-mm polypropylene tube (Falcon Plastics, Div. of BioQuest Oxnard, Calif.). The cell suspension was then underlayered with 5 ml of medium of higher density and centrifuged at 1,000 g for either 20, 30, or 45 min at $20^\circ \pm 2^\circ$C using a swing-out rotor. As it was found that the time of centrifugation did not influence the degree of separation, 20 min was used for all experiments. Cells recovered from the supernate and pellet were analyzed for both number and differential.

For discontinuous gradient centrifugation, a similar type of analysis was performed by layering marrow cells on top of multiple density layers of either BSA or Percoll. Marrow cell suspensions were distributed into various groups of 17 x 100-mm siliconized glass tubes, 5 ml/tube. Cells were underlayered in succession with three 5-ml layers of medium of increasing density. Several groups of tubes received layers that covered a wide range of densities (1.020-1.12 g/cm$^3$ BSA or Percoll). Gradients were pumped beneath the cell suspension with a syringe pump (model 351, Sage Instruments Div., Orion Research Inc., Cambridge, Mass.) connected to capillary tubing that extended to the bottom of each tube. The specific gravity of the BSA and Percoll media was determined both by measuring the refractive index with an Abbe 3-L refractometer (Baush & Lomb Inc., Rochester, N.Y.) and direct weighing 100-ml vol of the various gradient media at $20^\circ \pm 0.5^\circ$C. After preparation, the density media were passed through 0.45-μm filters and stored at 4°C.

Isolation of Megakaryocytes

**STEP 1. PREPARATIVE DENSITY CENTRIFUGATION.** Megakaryocytes were initially separated from the majority of marrow cells by a BSA or Percoll buoyant density centrifugation step performed as follows: (a) $7-8 \times 10^7$ marrow cells were resuspended in 3 ml CATCH medium containing either 1.062 g BSA/cm$^3$ or 1.050 g Percoll/cm$^3$ and placed in 17 x 100-mm polypropylene tubes; (b) cells were then underlayered with 3 ml CATCH medium containing 1.085 g/cm$^3$ BSA or Percoll and overlayered with 3 ml of medium containing 1.040 g/cm$^3$ BSA or Percoll, respectively. After centrifugation at 1,000 g for 20 min at $20^\circ$C, cells were harvested into three fractions. Fraction I comprised all the cells from the top gradient layer and the upper interface. Fraction II consisted of the cells from the intermediate gradient layer, and fraction III was made up of cells from the bottom gradient layer and the pellet.

**STEP 2. VELOCITY SEDIMENTATION.** Megakaryocytes were further purified by processing density gradient-derived fraction I by velocity sedimentation. A 12-ml continuous gradient of 2-4% Ficoll (Pharmacia Fine Chemicals) in CATCH medium pH 7.0 containing 5% fetal calf serum (FCS) was formed in 17 x 100-cm polypropylene tubes with a gradient maker. 10-40 x 10$^6$ cells from fraction I, resuspended in 1 ml CATCH medium containing 5% FCS, were layered on top of the Ficoll gradient. To minimize any gradient interface effects, the cells were gently mixed with the uppermost region of the Ficoll gradient. After centrifugation at 100 g for 5 min at $20^\circ$C, megakaryocytes were harvested from the pellet fraction, whereas the other marrow cells were spread throughout the gradient primarily according to cell size. The pellet was then processed in a second cycle of velocity sedimentation in a similar manner. The final megakaryocyte preparation with the highest purity was obtained from the second cycle pellet.

**Mouse Megakaryocytes.** Mouse megakaryocytes were obtained from three different sources. These included fresh bone marrow tissue, long-term bone marrow cultures, and colony-forming unit megakaryocytes (CFU-M) grown in agar. Marrow megakaryocytes were isolated from (DBA/2 X C57BL/7) F1 mice by the same method described above for human megakaryocytes.

Long-term liquid cultures of bone marrow cells were established by the technique of Williams et al. (15). Briefly, to establish an adherent cell feeder layer, bone marrow cells from a single femur were expressed directly into 25-cm$^2$ plastic flasks (Corning Medical, Corning Glass Works, Medfield, Mass.). The cells were incubated at 33°C in 10 ml of Fisher's medium (Grand Island Biological) containing 20% horse serum (Flow Laboratories, Inc., Rockville, Md.). After either 3 or 4 d, half the medium containing half of the nonadherent cells was removed, and the cultures were fed with an equal volume of fresh medium. After 7 d, all the nonadherent cells were removed, and 3-5 x 10$^5$ freshly isolated bone marrow cells were added in new medium. Each week thereafter, half the cells and medium were removed, and the culture fed with fresh
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medium. The cells harvested at various times up until the 3rd or 4th wk of culture contained 0.5-1% megakaryocytes and CFU-M.

Mouse megakaryocytes were also derived from CFU-M cloned in semisolid agar culture with a modification of the technique of Metcalf et al. (14). Cells from either femoral bone marrow or from liquid cultured marrow cells were plated at concentrations of 75,000 cells/ml in 35-mm Petri dishes (Falcon 3001, Falcon Plastics) in McCoy's 5A modified medium containing 15% FCS and 0.25% Bactoagar (Difco Laboratories, Detroit, Mich.) supplemented with essential and nonessential amino acids, 200 mM glutamine, asparagine, sodium pyruvate, and 10^{-4} M 2-mercaptoethanol. Colonies were grown in the presence of both an obligatory stimulation factor and a potentiating activity. Megakaryocyte colony-stimulating activity was obtained by adding 100 µl of conditioned medium from long-term marrow cultures of a WEHI-3 murine marrow myelomonocytic leukemic cell line (23). Megakaryocyte-potentiating activity was obtained by adding 200 µl of medium conditioned by the long-term culture of bone marrow cells (23). Colonies of megakaryocytes were readily identified by the size of the majority of colony cells. Colonies were generally irregular in shape and had < 50 cells. Cytochemical identification by both large and small megakaryocytes was made on isolated colony cells by staining for the presence of acetylcholine esterase (24). Colonies were individually harvested under microscopic visualization (×40) with a Pasteur pipette, and placed into 400-µl polypropylene tubes (Milian Instruments SA, Geneva, Switzerland) containing CATCH medium.

Preparation of Washed Platelets. Blood samples drawn from normal, healthy volunteers or C57B1/6 mice were mixed with 3.2% sodium citrate in polypropylene tubes. After centrifugation of the blood mixture at 255 g for 10 min at room temperature, the supernatant platelet rich plasma was aspirated, the platelets were pelleted by centrifugation at 1,100 g for 20 min, and then washed three times with a 0.12 M sodium chloride solution containing 0.0129 M sodium citrate and 25 mM glucose pH 6.8 (25). Platelet counts were performed with a Coulter Counter model ZBI (Coulter Electronics Inc., Hialeah, Fla.).

Immunofluorescence Assay. Monospecific rabbit antisera against human fibrinogen, factor VIII:AGN, α2 macroglobulin, platelet myosin, platelet glycoproteins I and II, as well as human Ia antigen and human and mouse immunoglobulins, were prepared and tested for specificity as described (26-31). Mouse A.TH anti-A.TL (anti-Ia\textsuperscript{b}) serum was a generous gift from Dr. P. Kincade of Sloan-Kettering Institute, New York, and full discussion of its preparation is presented elsewhere (32). After isolation of the IgG fraction from each antiserum by DEAE-cellulose chromatography (31), each antibody was treated with pepsin (Worthington Biochemical Corp., Freehold, N. J.) to produce F(ab')\textsubscript{2} fragments (33). F(ab')\textsubscript{2} fragments were separated from trace amounts of uncleaved IgG and pFc fragments by Sephacryl S-200 (Pharmacia Fine Chemicals) gel filtration. The F(ab')\textsubscript{2} fragments were shown to be free of uncleaved IgG and pFc fragments by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and by immune-diffusion analysis with goat anti-rabbit Fc fragment-specific antiserum. Occasionally, intact IgG antibody preparations were treated with soluble \textit{Staphylococcus aureus} protein A (Pharmacia Fine Chemicals) in order to block functionally the Fc region of the IgG molecule (34). Effective blocking 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. For direct immunofluorescence, the IgG F(ab')\textsubscript{2} fragments were conjugated to either fluorescein isothiocyanate (FTTC) or tetramethylrhodamine isothiocyanate (TRITC) (both from BBL Microbiology Systems, Becton, Dickinson & Co., Cockeysville, Md.) as described in more detail elsewhere (31, 33). In some instances, indirect immunofluorescence was performed, using unlabeled rabbit antibody followed by the fluoresceinated F(ab')\textsubscript{2} fragment of goat anti-rabbit IgG antibody. For fluorescence staining of membrane-restricted components, isolated megakaryocytes (50-150 × 10\textsuperscript{3}) or platelets (1-2 × 10\textsuperscript{8}) were incubated with 0.05-0.1 ml of the fluoresceinated antibody at room temperature for 30 min. Cells were then washed through a 3-ml layer of 20% BSA in CATCH medium. For indirect immunofluorescence, cells were treated with the first reagent, washed twice, and then incubated with 0.05-0.1 ml of the fluoresceinated goat anti-rabbit serum. Staining of cytoplasmic elements was carried out by incubating smears of megakaryocytes with the antibody preparations after fixing in acetone for 15 min followed by two washes in phosphate-buffered saline. Cells were examined with a Leitz
Ortholux II microscope (E. Leitz, Inc., Rockleigh, N. J.) equipped with Ploem illuminator and phase contrast.

**Complement Receptor Assay.** The two types of complement (C) receptors, complement receptor type one, the immune adherence (C4b-C3b) receptor (CR1) and complement receptor type two, the C3d receptor (CR2), were detected by rosette formation with sheep erythrocytes-rabbit IgM antibody-complement complexes (EAC) prepared as described (35, 36).

C receptor-dependent rosettes were formed in 12 × 75-mm polypropylene tubes (Falcon Plastics) by mixing together 0.1 ml of EAC at 2 × 10⁸ with 0.1 ml of either isolated megakaryocytes (5–100 × 10⁶) or platelets (3–5 × 10⁹/ml) resuspended in RPMI-1640 (Microbiological Associates, Bethesda, Md.) followed by incubation on a tube rotator for 15 min at 37°C (35). Rosettes in the cell suspension were enumerated by phase contrast microscopy, or alternatively, differential counts of rosetted cells were made from Wright-Giemsa stained smears. For staining, rosette mixtures were pelleted at 100 g for 2 min, resuspended in 20 µl of FCS and immediately smeared across microscopy slides with a coverslip. The smears were quickly dried with a hair dryer and then stained with Wright-Giemsa.

**Detection of Fc Receptor (FcR).** FcR cell membrane receptors for the Fc portion of IgG, were detected either by rosette formation with sheep and (or) ox erythrocytes sensitized with rabbit IgG antibody (EAgO) or by fluorescence assay with soluble immune complexes conjugated to FITC (37, 38). For the rosette assay, a mixture of 0.05–5.0 × 10⁵ megakaryocytes resuspended in 0.1 ml CATCH and 0.1 ml of EAgO at 2 × 10⁷/ml CATCH was incubated at 37°C for 15 min, centrifuged at 200 g for 5 min, and reincubated at room temperature for 30–60 min. After gently resuspending the pellet, rosettes were enumerated either in suspension by phase contrast microscopy or in Wright-Giemsa stained smears by bright-field microscopy. For the fluorescence assay, 0.05–5.0 × 10⁵ megakaryocytes or 5–10 × 10⁷ platelets were incubated for 30 min at room temperature with soluble immune complexes prepared at antibody excess with either 25 µl of rabbit IgG antikeyhole limpet hemocyanin (KLH) conjugated to FITC and 25 µl of KLH conjugated to FITC, or 25 µl of rabbit IgG antiovalbumin conjugated to FITC and 25 µl of ovalbumin conjugated to FITC. After three washes with CATCH medium, the cells were examined by fluorescence microscopy.

**Results**

**Isolation of Human Megakaryocytes by Density Gradient Centrifugation.** Human megakaryocytes were separated from the majority of other marrow cells by density centrifugation with either BSA or Percoll gradients. The density that maximized this separation was first determined in pilot studies in which the density distributions of the various marrow cell types were analyzed in both density media (Fig. 1). Isolation of megakaryocytes was reproducibly better in Percoll than in BSA. By using BSA as a density medium, megakaryocytes were found in all fractions (1.020–1.085 g/cm³). By contrast, with Percoll gradients, megakaryocytes were found only in low density fractions (1.020–1.050 g/cm³). This difference in density characteristics was restricted to megakaryocytes, because the density distribution of other cell types was not significantly different in either media.

When the cell density distribution of megakaryocytes was compared with that of other marrow cell types, it was found that about 57% of megakaryocytes and only 15% of the other marrow cells contained a density lower than 1.062 g/cm³ BSA. By comparison, a considerably better enrichment of megakaryocytes was obtained in Percoll. Over many experiments, > 90% of all megakaryocytes and ≈ 12% of all other marrow cells were less dense than 1.050 g/cm³.

Based on this distribution analysis, a simplified procedure was devised for using either Percoll or BSA as a separating medium. Because some megakaryocytes were found to be dense in BSA, a procedure involving three densities was established as described in Materials and Methods. With BSA, the fraction with density lower than
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1.062 g/cm³ contained about 90 ± 10 × 10³ megakaryocytes that represented 50% of the starting megakaryocyte population (Table I). Megakaryocytes in this low density fraction contained ≈ 0.4% megakaryocytes representing a 10-fold enrichment over the starting sample. Cells collected from fraction II had 0.08% megakaryocytes, a twofold enrichment over the starting sample. The pellet fraction contained only 0.02% megakaryocytes. A higher degree of purity and recovery of megakaryocytes was obtained with Percoll as compared with BSA. Percoll fraction I, containing cells with a density lower than 1.050 g/cm³, had 1.51% megakaryocytes, representing a 38-fold enrichment over unseparated marrow cells. The megakaryocytes recovered in this fraction accounted for over 90% of the starting sample. Fraction II (d = 1.050 g/cm³) contained only a minor proportion (<5%) of the megakaryocytes.

Among unseparated megakaryocytes, ≈ 90% excluded trypan blue dye. Of those that took up the dye (8–17%), two recognizable groups were observed. In the first group (3–7%), the entire cell, including the nucleus, was deeply stained, and cells were classified as nonviable. In the second group (5–10%), cells were stained with a unique staining pattern. This consisted of a very light, uniform staining of the cytoplasm. The nucleus did not take up the dye, and the viability of this group of cells was difficult to determine. Both groups of dye-stained megakaryocytes were denser than 1.085 g/cm³ BSA and yet were found in the lowest density fraction in Percoll (fraction I).

When cell integrity was assessed by phase microscopy, most of the purified megakaryocytes were observed to have a normal birefringent membrane pattern, and cytoplasmic degranulation was present only in about 3–5% of separated megakaryocytes.
Table I

Purification of Human Marrow Megakaryocytes by Density Centrifugation and Velocity Sedimentation

| Medium density (g/cm³) | Yield (Number x 10⁹) | Purity (%) | Enrichment* | Viability (%) |
|------------------------|----------------------|------------|-------------|---------------|
| Unseparated marrow cells | 180 ± 80§ | 100 | 0.04 ± 0.02 | 84 ± 10 |
| After BSA gradient centrifugation | | | | |
| Fraction I <1.062 | 90 ± 5 | 50 ± 13 | 0.40 ± 0.16 | 9.5 ± 1.2 | 99 ± 1 |
| Fraction II 1.062 | 22 ± 1 | 12 ± 3 | 0.08 ± 0.06 | 2 ± 0.1 | 99 ± 1 |
| Fraction III >1.062 | 68 ± 4 | 38 ± 5 | 0.02 ± 0.01 | 0.5 ± 0.1 | 35 ± 10 |
| After Percoll gradient centrifugation | | | | |
| Fraction I <1.050 | 169 ± 5 | 94 ± 2 | 1.51 ± 0.32 | 38 ± 10 | 92 ± 5 |
| Fraction II 1.050 | 9 ± 1 | 5 ± 1 | 0.02 ± 0.01 | 0.5 ± 1 | 99 ± 1 |
| Fraction III >1.050 | 0 | 0 | | | |
| After velocity sedimentation of Percoll fraction I | | | | |
| 1 cycle:Pellet | 150 ± 10 | 90 ± 5 | 33.4 ± 10.5 | 835 ± 210 | 93 ± 5 |
| 2 cycle:Pellet | 148 ± 8 | 89 ± 4 | 85 ± 13 | 2125 ± 250 | 93 ± 3 |

* Percent recovery from unseparated marrow cells.
† Fold enrichment over unseparated marrow cells.
§ Mean values and SD of at least 12 experiments for each separation procedure.

Further Purification of Human Marrow Megakaryocytes by Velocity Sedimentation. For greater purification of megakaryocytes, marrow cells harvested from Percoll fraction I were processed by velocity sedimentation. Optimal separation was obtained by centrifuging the cells in a 2-4% Ficoll gradient at 100 g for 5 min (Fig. 2). The vast majority of megakaryocytes were found in the pellet, the fastest sedimenting fraction, together with only 1-3% of the other marrow cells. The results of purification experiments of human megakaryocytes by sequentially processing marrow samples through Percoll gradient centrifugation and velocity sedimentation in Ficoll are given in Table I. After two sedimentation cycles, purity was increased to 2,125-fold (range: 1,500-4,000) over the unseparated sample, and megakaryocytes represented ≈ 85% of the total cells. Overall, ≈ 150,000 megakaryocytes were isolated from each marrow sample, representing over 90% of the starting megakaryocyte population (Fig. 3 and 4).

Identification of Platelet-Associated Components in Isolated Megakaryocytes. Various cell components known to be associated with platelets were investigated in isolated human megakaryocytes by immunofluorescence (Table II). Fibrinogen, factor VIII:AGN, platelet myosin, and platelet glycoproteins I and III were detected on the membrane and in the cytoplasm of most of the marrow megakaryocytes. The pattern of membrane staining for the various antigens consisted of fluorescent spots and patches evenly distributed over the entire cell (Fig. 5). These patterns very much resembled those described on leukocytes for other surface antigens such as surface Ig, H₂ antigens, and leukemic membrane-associated antigens (31, 39, 40). Several technical aspects
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Fig. 2. Sedimentation profile of Percoll fraction I cells after velocity sedimentation in a 2-4% Ficoll gradient at 100 g for 5 min. Columns (shaded: megakaryocytes, open: other types of marrow nucleated cells) represent the mean percent of total cells recovered from a typical experiment in which the yield was > 90% of the starting cells. M, gradient meniscus; B, gradient bottom.

were of importance in showing the specific presence of these membrane antigens on megakaryocytes. First, it was crucial that the megakaryocytes were viable at the time of reaction with the fluoresceinated conjugate. Live megakaryocytes, when stained for membrane components, did not show nonspecific fluorescence and exhibited no “background” reaction. Dead megakaryocytes obtained after hypotonic shock reacted nonspecifically with all fluorescein-conjugated antibodies. The reaction of the dead cells was homogeneous and could thereby be differentiated from the reactions exhibited by the live megakaryocytes. More importantly, the antibodies used to detect megakaryocyte-associated antigens were antibody preparations free of reactive Fc regions, as human megakaryocytes were shown to express membrane receptors for the Fc portion of IgG (see below). The antibody preparations included either pepsin-derived F(ab')2 fragments or whole IgG antibody treated with \textit{S. aureus} protein A that binds to and inactivates the Fc portion of IgG molecules. Except for some monocyte macrophages that stained with antifibrinogen reagents and a few myeloblasts that stained with the anti-Ia serum, megakaryocytes were the only marrow cell type stained with all of the various antisera tested. After acetone fixation of megakaryocytes, the immunofluorescence reagents produced a homogenous staining of cytoplasmic components (Fig. 5).

Because primate and nonprimate platelets had been previously examined for the expression of immunologic receptors and antigens, human and mouse megakaryocytes were studied in parallel for these surface markers (Table III). IgG-FcR, Ia antigens, and two different types of complement receptors (CR1 and CR2) were detected by immunofluorescence and (or) rosette assay techniques. FcR were found on over 90% of isolated human megakaryocytes with either fluoresceinated antigen-antibody complexes or IgG antibody-sensitized ox erythrocytes. Ia antigens were demonstrated on only 10-15% of isolated human megakaryocytes and, significantly, were not present on platelets. By contrast, FcR and Ia antigens were not detected on freshly isolated or cultured murine megakaryocytes. Human megakaryocytes were devoid of complement receptors, whereas mouse megakaryocytes expressed CR1 but not CR2. Both human
and mouse megakaryocytes did not express detectable surface-bound immunoglobulin (surface Ig). Studies of platelets and their homologous megakaryocytes revealed a parallel expression of FcR, and complement receptors. (Table III).

Discussion

Our studies demonstrate that human megakaryocytes contained several platelet-associated coagulation and membrane proteins that are of paramount importance for the functional role of platelets during coagulation and hemostasis. In addition, studies of certain differentiation markers such as FcR, C receptors, and Ia antigens on megakaryocytes and platelets indicate that these membrane receptors may be useful for monitoring events of megakaryocyte maturation.

These studies were facilitated by the development of a method for isolating highly purified populations of human bone marrow megakaryocytes with almost 90% yield. Highly purified populations of megakaryocytes were obtained by a combination of density centrifugation and velocity sedimentation. Megakaryocytes were heteroge-
Fig. 4. Purified human marrow megakaryocytes stained with Wright-Giemsa. × 788.
TABLE II

Detection of Platelet-Associated Cell Components in Human Megakaryocytes by Immunofluorescence

| Components          | Percent of megakaryocytes stained |
|---------------------|-----------------------------------|
|                     | Membrane                          | Cytoplasm                          |
| Fibrinogen          | 90 ± 5*                           | 91 ± 3                             |
| Factor VIII:AGN     | 80 ± 8                            | 10 ± 4                             |
| α2-Macroglobulin    | 0                                 | 0                                  |
| Platelet-myosin     | 87 ± 2                            | 90 ± 5                             |
| Platelet glycoprotein I | 98 ± 1                      | 98 ± 1                             |
| Platelet glycoprotein III | 97 ± 2                 | 97 ± 2                             |

* Mean value of percent and 1 SD of at least eight experiments.

Fig. 5. Immunofluorescent staining pattern of isolated human megakaryocytes. Membrane-associated fibrinogen, (a) rim pattern of evenly distributed fluorescent spots, (b) patchy staining pattern. Cytoplasmic staining of platelet glycoprotein III, (c) homogeneous staining pattern after fixation of megakaryocyte in acetone.

Diverse in the density properties when BSA was used as the separation medium. This diversity in cell density may reflect the development of increased nuclear ploidy and production of cytoplasmic elements, including formation of dense granules. Alternatively, the density properties may reflect the unique characteristics of the demarcation membrane system that is contiguous with the outer membrane of megakaryocytes (41). With such a system, it might be anticipated that these cells might take up variable amounts of the density medium and thus become differentially sensitive to centrifugation forces.

Percoll is a separation medium that does not penetrate cells, even after loss of cell membrane permeability (21). Advantage of this property was taken for the isolation of megakaryocytes, as only the salt solution in the separating medium would be taken up and subsequently influence the cell density. When separation was performed in this medium, all megakaryocytes were much less dense than other marrow cells. It is of note that the density distribution of all the other types of marrow cells was not appreciably different when the two different types of density media were used. Because of the special behavior of megakaryocytes in Percoll, it can not be assumed that buoyant density distributions of these cells will be similar with other separation media.
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TABLE III

Membrane Markers on Human and Mouse Megakaryocytes and Platelets

| Marker      | Percent of cells bearing membrane markers |
|-------------|------------------------------------------|
|             | Human | Megakaryocytes | Platelets | Mouse | Megakaryocytes | Platelets |
| FcR         | 95 ± 3* | 85 ± 3 | <0.5 | 0 |<0.5 | 0 |
| Surface Ig  | <0.1 | 0 | <0.5 | 0 |
| CR<sup>+</sup> | <0.1 | 0 | 25 ± 10 | 3 ± 1 |
| CR<sup>+</sup> | <0.1 | 0 | <0.5 | 0 |
| Ia antigen  | 15 ± 8 | 0 | <0.5 | 0 |

* Mean value of percent and 1 SD of at least six experiments.
† CR<sub>1</sub> was assayed with EAC complexes containing purified human C4b and EAC complexes containing mouse C3 in its C3b form on human and mouse cells, respectively.
§ CR<sub>2</sub> was assayed with EAC complexes containing human C3 in its C3d form and EAC complexes containing mouse C3 in its C3d form on human and mouse cells, respectively.

The fact that fibrinogen, factor VIII:AGN, platelet glycoproteins I and III, and platelet myosin were detected on the membrane and in the cytoplasm of over 90% of megakaryocytes suggested that these platelet-related molecules are intrinsically associated with megakaryocytes, and their appearance may occur during the early stages of megakaryocyte development. It is known that various platelet disorders are associated with selective protein abnormalities. These include Bernard Soulier's disease (human platelet glycoprotein Ib and Ia deficiency), thrombasthenia (human platelet glycoprotein IIb and IIIa deficiency), von Willebrand's disease (some types with absent platelet VIII:AGN), and afibrinogenemia (detectable but low platelet fibrinogen) (42-44). Thus, the study of the synthetic function of isolated megakaryocytes from such patients may help elucidate the nature of the cellular abnormalities involved in these various disorders.

Parallel studies of megakaryocytes and platelets from human and mouse sources revealed that there were differences in the expression of FcR and two types of C receptors. A proportion of human megakaryocytes, but not homologous platelets, bore the Ia antigen, indicating that the expression of this marker may be restricted to a subclass of megakaryocytes. This latter type of cell may represent a more primitive megakaryocyte class similar to those early cells expressing Ia antigen in the granulocytic and erythroid series (45, 46). Alternatively, this megakaryocyte class may reflect the development of separate megakaryocyte lineages, with subsequent restricted platelet functions (19, 20). Heterogeneity in the expression of membrane-associated differentiation markers has also been observed with mouse megakaryocytes. Our studies demonstrated that 30% of mouse megakaryocytes expressed CR<sub>1</sub> along with a proportion of blood platelets, indicating that this receptor may be acquired at a late stage of development (17, 47). It is anticipated that analysis of the acquisition and loss of membrane and cytoplasmic components on highly purified megakaryocyte populations will provide further knowledge of the mechanisms of platelet production and the events of platelet release. Furthermore, the availability of highly purified populations of megakaryocytes maintained in culture should help define the microenvironmental conditions that influence the processes of thrombopoiesis.
Human marrow megakaryocytes have been isolated with high purity and yield by processing marrow cells sequentially through density centrifugation and velocity sedimentation. Analysis of the isolated cells for various platelet-associated components by immunofluorescence demonstrated that fibrinogen, plasma factor VIII antigen (factor VIII:AGN) platelet myosin, platelet glycoproteins I and III are present on the membrane and in the cytoplasm of over 90% of marrow megakaryocytes. Parallel studies of human and mouse megakaryocytes and platelets for IgG receptor (FcR), complement receptor type one (CR1) (C3b receptor), complement receptor type two (CR2) (C3d receptor), and Ia antigen by fluorescence and (or) rosette formation methods were performed. FcR were present on most human megakaryocytes and platelets. The Ia antigen was detected on a proportion (10-15%) of human megakaryocytes but it was undetectable on human platelets. CR1 was found on 20-40% of mouse megakaryocytes and also on a proportion of mouse platelets. These differentiation markers may be of use in monitoring megakaryocyte maturation.

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