Human megakaryocytes have been shown by immunofluorescent techniques to express platelet glycoprotein IIb/IIIa antigen. We report evidence that megakaryocytes derived from human committed megakaryocytic progenitor cells in vitro (CFU-M) synthesize glycoproteins IIb and IIIa. Nonadherent human bone marrow cells were cultured in human plasma and methylcellulose using conditions that promote large megakaryocytic colonies. On day 13 the megakaryocytic colonies were picked, pooled, and pulsed with 35S-methionine in methionine-free media. Populations of approximately 100,000 cells with >95% viability and containing 70% to 90% megakaryocytes were obtained reliably for study. After the radioactive pulse, the cell suspension was solubilized with nonionic detergent. To reduce nonspecific binding of 35S-labeled proteins to agarose, the lysate was chromatographed sequentially on glycine-quenched Affigel and antihuman factor X-Sepharose. The unbound material from these resins was then chromatographed on an antiglycoprotein IIb/IIIa monoclonal antibody resin (HP1-1D-Sepharose) or on a control monoclonal antibody resin. Bound fractions were eluted and analyzed by polyacrylamide gel electrophoresis and autoradiography. Autoradiograms of diethylamine eluates from HP1-1D-Sepharose revealed two labeled proteins with electrophoretic mobilities identical with those of human platelet membrane glycoproteins IIb and IIIa, isolated using similar conditions. Autoradiograms of material synthesized by control macrophages from the same donors revealed no significant labeling of proteins in the glycoprotein IIb/IIIa molecular weight range, nor were such proteins bound by HP1-1D-Sepharose. Our observations show that protein synthesis by CFU-M-derived human megakaryocytes can be readily studied using a small amount of bone marrow aspirate as starting material. This approach will allow the study of protein synthesis by megakaryocytes from normal subjects or from subjects with clinical disorders, and it will circumvent the need to obtain large amounts of bone marrow to prepare enriched populations of megakaryocytes.

T HE STUDY of protein synthesis by normal human megakaryocytes has been hampered by the difficulty in obtaining sufficiently large populations of cells markedly enriched for megakaryocytes. Other investigators have reported that von Willebrand factor, actin, coagulation factor V, and fibrinogen are synthesized by megakaryocytes enriched from guinea pig bone marrow 1-4 and that platelet factor 4 is synthesized by megakaryocytes enriched from human neoplastic cell lines. 6,7 Also, Rabellino et al. 6 have reported preliminary evidence that megakaryocytes enriched from human marrow and placed in short-term liquid suspension culture synthesize platelet glycoproteins IIb and IIIa.

The aims of this study are to show that (1) large numbers of enriched human megakaryocytes can be obtained from colonies formed in vitro by committed megakaryocytic progenitors (CFU-M) proliferating under conditions that promote large megakaryocytic colonies and (2) these populations of megakaryocytes can be used for the study of protein synthesis. We report here the first evidence that normal human CFU-M-derived megakaryocytes can synthesize platelet glycoproteins IIb and IIIa. We believe that the linkage of in vitro culture studies of human megakaryocytopoiesis and the study of protein synthesis will help set the stage for an increased understanding of normal and disturbed megakaryocytopoiesis in vivo.

MATERIALS AND METHODS

Bone marrow culture. Bone marrow aspirates from consenting normal donors were collected into sterile glass tubes containing preservative-free sodium heparin (O'Neal, Jones and Feldman, St Louis). A mononuclear marrow cell suspension of density less than 1.077 g/mL was isolated, diluted, and plated into a plasma/methylcellulose cell culture system as previously described.6,9

Cells were cultured in 30% plasma from a patient with aplastic anemia and 10% conditioned medium from peripheral blood leukocytes stimulated by phytohemagglutinin (PHA-CLM). Megakaryocytic colonies were identified by inverted microscopy according to previously published criteria.8,11 Moreover, in analyzing the cellular content of 500 picked colonies derived under these culture conditions, approximately 90% of the pooled cells were megakaryocytic as defined by Wright-Giemsa staining and by immunofluorescent staining for human coagulation factor V, platelet factor 4, von Willebrand factor, &thmoglobulin, and platelet glycoprotein IIb/IIIa.12,13 Cells were also cultured in 30% fetal calf serum (Hyclone, Logan, Utah) and 10% PHA-CLM to stimulate macrophage colony growth. Plating densities of 100,000 cells/mL were used to reduce contamination of colonies by surrounding cells.

On day 13 of culture, the megakaryocytic (Fig 1) or macrophage colonies were picked with a finely drawn, sterile Pasteur pipette and pooled into 200 µL of filter-sterilized methionine-free RPMI 1640 (KC-Biological, Lenexa, Kan) containing 1% bovine serum albumin and buffered with 25 mol/L HEPES at pH 7.25 (medium A). Aliquots of the pooled cells were used for cell counts, differentials after Wright-Giemsa staining, and viability determinations by trypan blue exclusion. The remainder of the pooled cells (50,000 to 150,000 cells) were diluted with 800 µL medium A and incubated for 2½ to three days at 37°C in 5% CO2 with 0.5 mCi 35S-methionine supplementation.
(Amer sham Corp, Arlington Heights, III; 1500 Ci/mmol). After the incubation period, the cells and medium were transferred to a glass tube coated with medium A, and Triton X-100 (J.T. Baker, Phillipsburg, N.J.) was added to a final concentration of 1%. The resultant lysates were frozen at -70 °C until immunoisolation was performed.

Preparation of HPI-1D-Sepharose immunoaffinity resin. HPI-1D murine monoclonal antibody has been previously described. The antibody is an IgG and has specificity for an epitope expressed on the human platelet membrane glycoprotein IIb/IIa complex. HPI-1D IgG was isolated from pooled mouse ascites fluid by gel filtration chromatography and was concentrated by ultrafiltration in coupling buffer (0.2 mol/L NaCl, 0.1 mol/L NaHCO3, pH 8.5). CH-Sepharose 4B, activated with N-hydroxysuccinimide, was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Three grams of the resin was washed sequentially with 300 mL 0.001 N HCl, 300 mL water, and 30 mL coupling buffer. The washed resin was stirred for one hour at 22 °C with 13.2 mg HPI-1D IgG in coupling buffer (total volume approximately 30 mL), then 0.1 vol 1 mol/L Tris-Cl (pH 8) was added and the slurry was stirred for an additional hour before washing with approximately 5 volumes of 0.15 M NaCl, 0.02 mol/L Tris-Cl, pH 7.4. The resin was then washed with approximately 5 vol of 50% glycerol, 0.01 mol/L Tris-borate, and 0.001 mol/L CaCl2, pH 8.4, and was stored at -20 °C in this buffer. The efficiency of IgG coupling to activated CH-Sepharose 4B was 88% as determined spectrophotometrically (A280 applied - A280 eluted).

Imunoaffinity isolation of human platelet glycoproteins IIb and IIa. Platelet membrane vesicles were prepared from fresh platelet-rich plasma obtained from the Mayo Clinic Blood Bank, using a modified glycerol-hypotonic lysis technique. Pelleted platelet membrane vesicles (4.7 mg protein) were suspended at 22 °C in 2 mL 0.15 mol/L NaCl and 0.01 mol/L Tris-Cl, pH 7.4 (Tris-saline), which contained 1% CHAPS detergent [3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate: Calbiochem-Behring Corp, La Jolla, Calif). Aliquots were removed from the solubilized membrane preparation before and after centrifugation (20,000 g, 15 minutes, 22 °C) for protein quantitation as described. The remainder of the solubilized, centrifuged supernatant (extracted membranes that contained 72% of the membrane protein originally present) was diluted with 1 vol of Tris-saline buffer, then applied at 22 °C to a column of HPI-1D-Sepharose (plastic disposable 12-mL syringe with a porous polyethylene frit, containing 5 mL resin washed with Tris-saline and 0.1% CHAPS, pH 7.4). Following incubation (60 minutes, 22 °C), elution fractions of 2 mL were collected, using a flow rate of 1 mL/min, as the column was washed sequentially at 22 °C with approximately 20-mL vol of the following buffers: (1) Tris-saline and 0.1% CHAPS, pH 7.4; (2) Tris-saline, 0.1% CHAPS, and 5 mmol/L sodium EDTA, pH 7.4; (3) 1.5 mol/L NaCl, 0.01 mol/L Tris-Cl, and 0.1% CHAPS, pH 7.4; (4) 1.5 mol/L NaCl, 0.1 mol/L glycine-Cl, and 0.1% CHAPS, pH 3.0 (each fraction collected in 1-mL of 1 mol/L Tris-Cl, pH 8.0); (5) 3.0 mol/L NaSCN, Tris-saline, and 0.1% CHAPS, pH 7.2; (6) Tris-saline and 0.1% CHAPS, pH 7.4; and (7) 0.05 mol/L diethylamine and 0.1% CHAPS, pH 11.5 (each fraction collected in 2-mL fractions of 1 mol/L Tris-Cl, pH 8.0). Fractions were monitored spectrophotometrically (A280) and by polyacrylamide gradient gel electrophoretic analysis in the presence of sodium dodecyl sulfate (SDS). Following the latter analysis, pooled elution fractions were dialyzed into Tris-saline and 0.01% CHAPS and, if necessary, concentrated by ultrafiltration. Nonreduced protein samples were prepared by incubation (30 minutes, 37 °C) following the addition of N-ethylmaleimide to a concentration of 5 mmol/L. Prior to electrophoresis, sample preparation buffer was added to yield final concentrations of 2.5% dodecyl sulfate, 10% glycerol, 0.01% bromphenol blue, and twofold-diluted stacking gel buffer with or without 5% β-mercaptoethanol. After additional incubation (60 minutes, 37 °C), samples were electrophoresed in polyacrylamide linear gradient slab gels, and proteins were detected with Coomassie brilliant blue R-250 staining as described except that (1) 1.5-mm thick slabs were used, (2) the acrylamide gradient was 4% to 12%, and (3) the lower electrode buffer consisted of 0.04 mol/L Tris-borate, pH 8.6. Gels were calibrated with reduced protein molecular weight standards obtained from Bio-Rad Laboratories (Richmond, Calif): myosin heavy chain (210,000), β-galactosidase (116,000), phosphorylase b (92,500), bovine serum albumin (66,000), ovalbumin (43,000), carbonic anhydrase (30,000), and cytochrome c (12,500). An addi-
tional standard consisted of platelet membrane vesicle proteins treated at pH 7.4 with 5 mmol/L N-ethylmaleimide (30 minutes, 37 °C) prior to sedimentation in Tris-saline and solubilization (60 minutes, 37 °C) in electrophoresis sample preparation buffer (± 5% β-mercaptoethanol).

Immunoaffinity isolation of megakaryocytic glycoproteins IIb and IIIa. The strategy used for the immunoisolation of megakaryocytic glycoproteins IIb and IIIa is outlined. The 35S-labeled proteins. The resultant anti-HFX-Sepharose flow-through material was eluted with approximately 30 mL Imid-CaCl2. Each resin were equilibrated with 0.02 mol/L imidazole, supplied by Dr M.E. Nesheim, Mayo Clinic. One-half milliliter of mouse monoclonal antihuman factor X (HFX)-Sepharose (kindly supplied by Dr W.R. Church, Mayo Clinic) to reduce nonspecific binding of 35S-labeled proteins. The resultant anti–HFX-Sepharose flow-through was divided equally into two aliquots for further immunoisolation chromatography using either HP-1-D-Sepharose (antihuman platelet glycoprotein IIb/IIIa) or control mouse monoclonal antihuman antithrombin III (HAT III) IgG coupled to Sepharose (kindly supplied by Mr G.J. Knutson, Mayo Clinic) for 30 minutes, then placed in Eln’Hancé (New England Nuclear, Boston) for 30 minutes, followed by water rehydration for at least 45 minutes. The treated gels were mounted on chromatography paper (Bio-Rad) and dried on a slab gel drier. Each dried gel was placed inside a wafer rigid-form x-ray cassette lined with Dupont Lightning Plus intensifying screens with Kodak XAR-5 x-ray film for exposure at −70 °C. The time of exposure was adjusted according to the radioactivity of the samples applied to the gel.

RESULTS

Characterization of pooled colonies. To obtain large numbers of human megakaryocytes for the biosynthetic experiments described in the next section, CFU-M-derived megakaryocytic colonies were steriley picked and pooled into a methionine-free serum-free medium. Table 1 summarizes the differential cell counts of pooled colonies picked from culture of marrow cells obtained from three separate normal donors. The megakaryocytic colonies were picked from 30 to 38 tissue culture plates from each donor. The average colony formation from the three donors ranged from 31 to 163 CFU-M–derived colonies per tissue culture plate (100,000 plated cells). Individual megakaryocytic colonies contained from five to 250 cells. The average number of cells per megakaryocytic colony was 105 for experiment 1, 25 for experiment 2, and 92 for experiment 3. One hundred thousand cells with 95% viability by trypan blue exclusion and consisting of 70% to 90% megakaryocytes were typically obtained. Between 5% and 16% of the cells were macrophages. Macrophages are present in the megakaryocytic population because the conditions used to support megakaryocytic colony formation also support some macrophage colony growth and some macrophages are picked accidentally. The variability in differentials between donors also reflects the donor-to-donor variation in the number, type, and density of colony growth in the cultures.

Table 1 also illustrates that macrophage colonies from the same donors can be picked and pooled with minimal megakaryocytic contamination. For control purposes in the biosynthetic experiments, the number of macrophages picked and pooled into methionine-free, serum-free medium was approximately equal to the number of contaminating macro-

<table>
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<tr>
<th>Experiment</th>
<th>Suspension</th>
<th>Cells Picked</th>
<th>Mega</th>
<th>Macro</th>
<th>NRBC</th>
<th>PMN/Other</th>
<th>TCA-Precipitable cpm</th>
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<td>1</td>
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<td>—</td>
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<td>0.4%</td>
<td>95.0%</td>
<td>1.8%</td>
<td>2.8%</td>
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</tr>
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</table>

Abbreviations: Mega, megakaryocyte; Macro, macrophage; NRBC, Nucleated erythroid cell; PMN, polymorphonuclear leukocyte; ND, not determined.

*Erythropoietin (2 U/mL) was present in the medium from which the macrophage colonies were picked.

Approximately equivalent counts were added to each well on the slab gel. 125I-protein molecular weight standards were kindly supplied by Mr G.J. Knutson (Mayo Clinic) and were used to calibrate the gels. Following electrophoresis, the gels were fixed in glacial acetic acid–ethanol–water (3:8:9, vol:vol) for 30 minutes, then placed in Eln’Hancé (New England Nuclear, Boston) for 30 minutes, followed by water rehydration for at least 45 minutes. The treated gels were mounted on chromatography paper (Bio-Rad) and dried on a slab gel drier. Each dried gel was placed inside a wafer rigid-form x-ray cassette lined with Dupont Lightning Plus intensifying screens with Kodak XAR-5 x-ray film for exposure at −70 °C. The time of exposure was adjusted according to the radioactivity of the samples applied to the gel.

**Table 1.** Results of Picking and Radiolabeling Megakaryocytic or Macrophage Colonies

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phages present in the picked and pooled megakaryocytic populations.

The picked and pooled cells from experiments 1 and 2 (Table 1) were incubated with 0.5 mCi $^{35}$S-methionine for 2½ days, then the cells and medium were lysed together, and aliquots of the lysates were treated with 10% trichloroacetic acid (TCA) to determine the amount of incorporated radioactivity (Table 1). The remaining portions of the cell lysates were stored at −70°C until used for immunoaffinity isolation of $^{35}$S-labeled proteins.

**Immunofinity isolation of platelet membrane glycoproteins IIb and IIIa.** Before performing biosynthetic experiments with megakaryocytes, we characterized the conditions required for the immunofractionation of platelet glycoproteins IIb and IIIa on HP1-ID-Sepharose immunoaffinity resin. For this characterization, we used human platelet membrane preparations extracted with CHAPS detergent. Figure 2 shows the results of polyacrylamide gel electrophoresis of detergent-extracted platelet membrane vesicles immunofractionated on HP1-ID-Sepharose. As compared with the protein composition of the material applied to the HP1-ID-Sepharose column (electrophoresis lane 2), the column flow-through material (lane 3) appeared depleted of two proteins with apparent reduced molecular weights of 120,000 and 100,000. This observation suggested that HP1-ID-Sepharose bound most of the glycoprotein IIb/IIIa antigen contained in the material that was applied to the column. Subsequent washing of the column (buffers 2 through 6, Materials and Methods) did not result in the elution of significant protein ($A_{280}$) nor in detectable protein bands upon polyacrylamide gel electrophoresis (data not shown). The platelet membrane proteins specifically bound to HP1-ID-Sepharose were eluted with diethylamine (buffer 7, Materials and Methods). The diethylamine eluate represented approximately 12% of the protein material applied ($A_{280}$). Since one of the unique properties of glycoproteins IIb and IIIa is their characteristic change in electrophoretic migration after reduction of disulfide bonds, we analyzed the eluted proteins before and after disulfide bond reduction. Electrophoresis of the nonreduced diethylamine eluate (lane 5) revealed two major proteins with apparent molecular weights of 135,000 and 80,000, whereas with reduction of disulfide bonds, the two proteins migrated electrophoretically with apparent molecular weights of 120,000 and 100,000, respectively (lane 4). Based upon these observations, similar immunofractionation conditions were used to study $^{35}$S-labeled megakaryocytic proteins that specifically bound to HP1-ID-Sepharose.

**Immunofinity isolation of megakaryocytic glycoproteins IIb and IIIa.** Radiolabeled lysates of picked megakaryocytes were subjected to immunoaffinity chromatography as described in Materials and Methods. The lysates were preechromatographed on glycine-quenched Affigel and anti-H FX-Sepharose to decrease the amount of nonspecific binding by $^{35}$S-labeled proteins to agarose. Half of the anti-HFX-Sepharose flow-through was incubated with HP1-ID-Sepharose and half with control anti–HAT III-Sepharose. Anti–HAT III-Sepharose was used as a control resin because the molecular weight of HAT III is substantially different from that of glycoproteins IIb and IIIa. Bound fractions were eluted as described in Materials and Methods, and pooled fractions were subjected to polyacrylamide gel electrophoresis and autoradiography.

Figure 3A shows the results of polyacrylamide gel electrophoresis of immunofractionated $^{35}$S-labeled proteins synthesized by megakaryocytes from experiment 1. As expected, many proteins are synthesized by picked and pooled cultured megakaryocytes (lanes 1 and 13). Prechromatography on glycine-quenched Affigel and anti–HFX-Sepharose did not significantly change the megakaryocytic protein distribution (lanes 2, 3, and 8) present in the column flow-through fractions. However, two major proteins with apparent molecular weights of 116,000 and 101,000 (reduced) or 131,000 and 87,000 (nonreduced) are depleted from the HP1-ID-Sepharose flow-through (lanes 4 and 9) and are specifically released from HP1-ID-Sepharose by diethylamine (lanes 5 and 10). The apparent molecular weights (under both reducing and nonreducing conditions) of the $^{35}$S-labeled megakaryocytic proteins specifically bound to and eluted from HP1-ID-Sepharose are essentially identical, within experimental error, with those of glycoproteins IIb and IIIa isolated from platelet membranes using similar conditions.

![Fig 2. Polyacrylamide gel electrophoresis of immunofractionated platelet membrane proteins (Coomassie blue staining) demonstrating the specificity of HP1-ID monoclonal antibody for glycoproteins IIb and IIIa. Platelet membrane vesicles were detergent extracted and subjected to HP1-ID-Sepharose immunoaffinity chromatography. Fractions were treated with N-ethylmaleimide, solubilized with SDS (+β-mercaptoethanol), and subjected to 4% to 12% linear gradient polyacrylamide gel electrophoresis. The arrows between lanes 4 (reduced proteins) and 5 (nonreduced proteins) depict the electrophoretic mobilities of glycoproteins IIb and IIIa. Lanes: 1 to 4, samples reduced; 5 and 6, samples not reduced. 1, isolated platelet membranes (46 μg protein); 2, CHAPS-extracted platelet membranes (32 μg protein); 3, HP1-ID-Sepharose flow-through fraction (31 μg protein); 4, HP1-ID-Sepharose diethylamine eluate (16 μg protein); 5, HP1-ID-Sepharose diethylamine eluate (16 μg protein); 6, isolated platelet membranes (46 μg protein).](image-url)
Fig 3. Polyacrylamide gel electrophoresis of immunofractionated 35S-labeled proteins synthesized by human megakaryocytes and macrophages (autoradiogram) demonstrating the biosynthesis of glycoproteins IIb and IIIa by megakaryocytes. Metabolically radiolabeled megakaryocyte and macrophage proteins from experiment 1 (Table 1) were solubilized, chromatographed, and analyzed by 5% to 15% linear gradient polyacrylamide gel electrophoresis as described in Materials and Methods. (A) Picked and pooled megakaryocytes. (B) Picked and pooled macrophages. Lanes 1 to 7, samples are reduced; lanes 8 to 13, samples are not reduced. There are approximately 15,000 cpm/lane. The arrows between lanes 7 (reduced proteins) and 8 (nonreduced proteins) depict the electrophoretic mobilities of glycoproteins IIb and IIIa. Lanes: 1 and 13, solubilized cells and culture fluid; 2, Glycine-quenched Affigel flow-through; 3 and 8, anti-HFX-Sepharose flow-through; 4 and 9, HP1-1D (antiglycoprotein IIb/IIIa)-Sepharose flow-through; 5 and 10, HP1-1D-Sepharose diethylamine eluate; 6 and 11, anti-HAT III-Sepharose flow-through; and 7 and 12, anti-HAT III-Sepharose diethylamine eluate.

(Fig 2). Approximately 6.7% of the total counts per minute bound to HP1-1D-Sepharose and was eluted from the column.

Control anti–HAT III-Sepharose did not bind the two major proteins since they eluted in the flow-through fraction (lanes 6 and 11) and were not present in the diethylamine eluate (lanes 7 and 12). A protein of apparent molecular weight 42,000 under reducing conditions, probably actin, nonspecifically bound to both the HP1-1D-Sepharose and the anti–HAT III-Sepharose. This protein also nonspecifically bound to the prechromatography resins (data not shown). Under nonreducing conditions, a minor protein component with an apparent molecular weight of 105,000 was observed to bind to HP1-1D–Sepharose (lane 10). The identity of this protein is not known, but it is also a minor component of the nonreduced platelet membrane proteins that bound to and were eluted from HP1-1D–Sepharose (Fig 2, lane 5).

Since the picked and pooled megakaryocyte colonies contained a significant number of contaminating macrophages, it was important to fractionate the proteins synthesized by picked and pooled macrophages. Approximately 20,000 macrophages were treated in an identical fashion as the megakaryocytic population from the same donor. Figure 3B shows the results of polyacrylamide gel electrophoresis of immunofractionated 35S-labeled proteins synthesized by the control macrophages from experiment 1. The picked and pooled macrophages were able to synthesize significant amounts of protein (Table I and Fig 3B, lanes 1 and 13). However, Fig 3B also shows that significant amounts of proteins with mobilities similar to glycoproteins IIb and IIIa were not synthesized by the macrophages, nor were such proteins bound by HP1-1D–Sepharose (lanes 5 and 10) or by anti–HAT III-Sepharose (lanes 7 and 12). This experiment shows that the amount of glycoproteins IIb and IIIa synthesized by the megakaryocytic population cannot be accounted for by the contaminant macrophages that were present in the megakaryocyte population.

Figure 4 shows that immunofractionation of 35S-labeled proteins synthesized by megakaryocytes and macrophages from experiment 2 yielded essentially the same results as in experiment 1. Again, HP1-1D–Sepharose specifically bound
and released two proteins synthesized by megakaryocytes that have apparent molecular weights (reduced) of 122,000 and 102,000 (lane 6), identical, within experimental error, to those of platelet glycoproteins IIb and IIIa. The proteins were present in the prechromatography lysate and in the anti–HFX-Sepharose flow-through (lanes 3 and 4). They were depleted in the HP1-1D-Sepharose flow-through (lane 5) and were released by diethylamine (lane 6). In experiment 2, approximately 6.1% of the applied counts per minute bound to the HP1-1D resin. Control anti–HAT III–Sepharose did not bind the two proteins since they eluted in the flow-through fraction and were not present in the diethylamine eluate (lanes 7 and 8). Again, a 42,000-dalton protein bound nonspecifically to both the HP1-1D-Sepharose and the anti–HAT III-Sepharose resins.

Approximately 15,000 macrophages from the same donor were also incubated with [35S]methionine, and solubilized aliquots were electrophoresed. A volume of lysed macrophages, equivalent to the volume of lysed megakaryocytes, was electrophoresed in lane 2. No significant amount of labeled protein is seen in this sample. When tenfold more sample was electrophoresed (lane 1), many newly synthesized proteins were observed upon electrophoresis, but no intense bands were seen with mobilities similar to glycoproteins IIb and IIIa. This confirmed that the amount of glycoproteins IIb and IIIa synthesized by the megakaryocytic population in experiment 2 could not be accounted for by the number of contaminating macrophages in that population.

**DISCUSSION**

Our results show that protein synthesis in normal human megakaryocytes can be studied by picking and pooling CFU-M–derived megakaryocytic colonies grown in vitro. We elected to study first the synthesis of glycoproteins IIb and IIIa because of the relatively well-characterized role these proteins play in platelet hemostatic function and because other laboratories and our own have shown that glycoproteins IIb and IIIa are among the first proteins detected by indirect immunofluorescent techniques in the earliest stages of megakaryocytic differentiation.

We demonstrate that approximately 100,000 human megakaryocytes of 70% to 90% purity can be reliably obtained by picking and pooling CFU-M–derived megakaryocytic colonies. Other investigators have studied megakaryocytes enriched from human or nonhuman bone marrow with techniques including density gradient centrifugation and velocity sedimentation or centrifugal elutriation. Both cell sorting and panning techniques have also been used to enrich marrow megakaryocytes. All of these methods either are time consuming or require relatively large amounts of bone marrow, for example, from human ribs, to obtain a significant number of megakaryocytes. Although CFU-M–derived megakaryocytes require 2 weeks of culture, only a small amount of bone marrow aspirate (2 to 3 mL) is required as starting material. In addition, when bone marrow aspiration is not clinically or technically feasible, peripheral blood mononuclear cells can serve as a source of CFU-M–derived megakaryocytes. Thus, it is possible to study protein synthesis in CFU-M–derived megakaryocytes from patients with inherited or acquired disorders of platelet proteins. Our work also shows that enriched populations of other cell types, such as macrophages, can be obtained for use as control cell suspensions for synthetic studies.

Our data confirm that the murine monoclonal antibody HP1-1D reacts with the human platelet membrane glycoprotein IIb/IIIa complex. Furthermore, HP1-1D antibody coupled to Sepharose can be used to purify platelet glycoproteins IIb and IIIa, which have appropriate nonreduced and reduced electrophoretic mobilities in polyacrylamide gels. Recent studies suggest that platelet membrane glycoproteins IIb and IIIa exist as calcium-dependent, noncovalently linked heterodimer complexes and that this association may be required for expression of the functional properties of the proteins. Although HP1-1D antibody is specific for glycoprotein IIb/IIIa antigen and can be used to isolate the two proteins, our studies reported here do not allow us to clearly determine whether HP1-1D antibody has specificity for a heterodimer-dependent epitope of glycoprotein IIb/IIIa or whether the epitope is present on only one of the proteins, both of which remain associated in detergent-solubilized cell preparations and coisolate using HP1-1D–Sepharose.

We find that cultured human megakaryocytes synthesize...
two 35S-labeled proteins that are specifically bound to and released from HP1-1D-Sepharose. Similar 35S-labeled proteins are not specifically bound by control anti–HAT III–Sepharose. The two synthesized proteins specifically bound by HP1-1D-Sepharose have reduced and nonreduced electrophoretic mobilities in polyacrylamide gels identical with those of platelet glycoproteins IIb and IIa.

Our experiments do not eliminate the possibility that macrophages may synthesize small amounts of glycoproteins IIb and IIa. However, the experiments do show that the number of macrophages contaminating the pooled megakaryocytes do not synthesize a detectable amount of glycoproteins IIb and IIa.

The pooled megakaryocytes derived from picked mega-

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CFU-M-derived human megakaryocytes synthesize glycoproteins IIb and IIIa

RB Jenkins, WL Nichols, KG Mann and LA Jr Solberg