Quantitation of Human In Vitro Megakaryocytopoiesis by Radioimmunoassay

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The isolation and characterization of human megakaryocyte growth factors has been hampered because evaluation of megakaryocyte growth in semisolid medium requires both lengthy incubation and visual quantitation. In addition, colony formation requires cell division, while most regulation of platelet production may involve individual, nonproliferating differentiating megakaryocytes. We have developed a radioimmunoassay (RIA) that makes use of an iodinated murine monoclonal antibody (MoAb) specific for platelet/megakaryocyte glycoprotein IIb/IIIa (GPIIb/IIIa) to measure megakaryocyte production in liquid marrow culture. This assay is sensitive to $3 \times 10^7$ platelets (roughly 30 megakaryocytes) and linear up to $1 \times 10^8$ platelets, and thus it provides a useful range for quantitating megakaryocyte production in in vitro marrow culture. Significant differences (threefold to fivefold) in megakaryocyte/platelet-specific GPIIb/IIa complex are detected between stimulated and unstimulated marrow cultures by day 7, although antigen accrual in stimulated cultures continues through at least day 16. Conditions that promote megakaryocyte growth in semisolid medium (i.e., aplastic plasma and PHA-LCM) have also been facilitory in liquid culture. This rapid and sensitive assay for cell-bound GPIIb/IIIa should facilitate recognition and isolation of megakaryocyte and platelet growth factors.

A ccumulating evidence suggests that the regulation of megakaryocyte production in mammals is exerted at least two different steps in the megakaryocyte-platelet differentiation pathway. Increasingly sensitive assays for megakaryocyte colony formation have facilitated the study of the early (proliferative) phase of megakaryocytopoiesis. This phase of platelet production is, however, the least sensitive to acute changes in platelet count and thus arguably less important in day-to-day platelet number homeostasis. Because most of the control of platelet production may be exerted upon single cells that ultimately become multinucleate, expand their membrane, and mature cytoplasmically to produce platelets, it seems appropriate to develop an assay that would be sensitive to megakaryocyte maturation. In addition, colony assays for megakaryocytopoiesis require 11 to 15 days of incubation before colonies (CFU-M) can be accurately counted by visual inspection. A less time-consuming and labor-intensive assay for use as a screening test for growth factors was therefore most desirable.

The glycoprotein IIb/IIIa (GPIIb/IIIa) complex is present on all recognizable human megakaryocytes and platelets from normal donors. Fluorescent antibodies against this complex have been extremely useful as specific markers for megakaryocytic colonies and in identification of earlier, morphologically unrecognizable megakaryocytic cells from both cultures and patient materials. As Jenkins et al have shown using biosynthetic labeling of isolated CFU-M-derived human megakaryocytes, the GPIIb/IIIa complex is made by megakaryocytes but is not synthesized in detectable amounts by monocyte/macrophage or erythroid colonies. Thus the cell-bound GPIIb/IIIa in a heterogeneous marrow culture cell population specifically reflects megakaryocytic lineage cells present, whether they are platelets, morphologically recognizable megakaryocytes, or early antigen-bearing small round cells. Similar increases in cell surface membrane GPIIb/IIIa would be observed in cultured marrow when more small cells are present, or when a few uninduced cells mature and enlarge, or with development of both kinds of cells.

We have developed a radioimmunoassay (RIA) to detect cell-bound GPIIb/IIIa that allows rapid quantitative assessment of in vitro megakaryocytopoiesis. Because the assay is relatively easy to perform, it should be useful in screening for megakaryocyte growth factors. Because accumulation of GPIIb/IIIa is associated with cytoplasmic maturation as well as with megakaryocyte proliferation, the assay ought to detect growth factors active in the terminal events of megakaryocytopoiesis or platelet production, in addition to factors that influence megakaryocyte proliferation.

MATERIALS AND METHODS

Marrow culture and cell preparation. Nucleated bone marrow cells were obtained from normal volunteers who had given informed consent consistent with institutional guidelines. Marrows were collected into preservative-free heparin (GIBCO Laboratories, Grand Island, NY), diluted one to three in phosphate-buffered saline (PBS), and spun at 120 g for 15 minutes to remove platelet-rich plasma. Red blood cells (RBCs) and granulocytes were separated from marrow mononuclear cells by density gradient separation (Histopaque-1077, Sigma Diagnostics, St. Louis), and another slow spin was done. Adherent cells and additional platelets were depleted by incubation of mononuclear cells for 60 minutes in 10% fetal calf serum in plastic flasks. Assessment of residual platelet/megakaryocytic lineage cells during marrow preparation using the RIA described below reveals 30% to 40% depletion of antigen by each slow spin. Thus 15% to 20% of starting activity remained before adherence, 5% to 10% more antigen was removed by the adherence step, and plated cells cultured contained about 10% of starting cell-bound GPIIb/IIIa.

Marrow culture was performed in 24-well tissue culture plates (GIBCO), at 37°C, 5% CO₂, and humidified atmosphere. Three to 5 x 10⁶ nonadherent, light-density bone marrow cells were cultured
in Iscove's modified Dulbecco's medium (GIBCO) supplemented with 30% serum or plasma, 5 x 10^{-3} mol/L 2-mercaptoethanol, and growth factors as noted, in 1 mL final volume. Plasma and serum samples were obtained from consenting normal volunteers and were filtered through 0.2-μ filters prior to use in cultures. Plasmas were prepared from blood drawn into syringes containing preservative-free heparin. Aplastic plasma was the gift of H. Messner, Toronto, Ontario, and fetal bovine serum (FBS) was purchased (Hyclone Laboratories, Logan, UT). PHA-LCM was prepared as previously described.17

Human umbilical vein endothelial cells cultured to confluence over five days as previously described18 were kindly provided by J. Hoak. After detachment from flasks by a 0.02% EDTA rinse followed by incubation in 0.5% Trypsin (five minutes, 37°C), endothelial cells were washed, counted, and assayed by RIA as above.

Megakaryocytes were quantitated in individual wells by counting nonadherent cells using a hemacytometer and multiplying by the percentage of recognizable megakaryocytes found on a stained cytospin preparation (1,500 cell differentials).

Radioimmunoassay HP1-1D is a murine anti-human glycoprotein IIb/IIIa complex monoclonal antibody (MoAb) of IgG4 isotype.13,15 The antibody is specific for human platelet or megakaryocyte GPIIb/IIIa, and does not recognize cells of other blood or marrow cell lineages.12,15 IgG was isolated from the ascites by protein A-Sepharose11 (Sigma) and iodinated using iodogen-treated tubes (Pierce Chemical Co, Rockford, IL) and Iodine-125 (125I; New England Nuclear, Boston). Platelets were isolated using the Mustard technique16 or were obtained in lyophilized form (Bio/Data, Hatboro, PA). The binding of 125I-HP1-1D to platelets and cultured marrow was examined in phosphate-buffered saline (PBS) containing 20% bovine calf serum to block nonspecific and Fc binding and 0.002% EDTA to prevent clumping. After 30 to 60 minutes of incubation, binding was complete at 4°C and at 22°C, and subsequent RIAs were routinely performed at 4°C with one-hour incubation. On several occasions, however, we did observe small (<15% of total counts bound) increases in 125I-HP1-1D bound between 60 and 180 minutes both to fresh platelets and to cultured marrow. This may have been due to slow access of antibody to antigen in the canalicular system or to possible antigen exposure due to platelet swelling in EDTA. No slow, late increase in binding was observed when marrow cultured 12 days in FCS alone was assayed (the differential included 20% macrophages, <0.1% megakaryocytes). It is possible that by choosing one-hour incubations we did not always maximize binding to platelet lineage cells; however, all tubes for a given assay were stopped within a minute or two of each other (by dilution with cold carrier cells and centrifugation) so that comparisons of binding within sets of tubes are valid.

For the routine assay, bone marrow cells or platelets were suspended in assay buffer (20% heat-inactivated bovine calf serum, 0.002% EDTA in PBS), pelleted, and supernate decanted. The pellet was resuspended in 100 μL chilled assay buffer and 100 μL of iodinated HP1-1D in chilled 0.1 mol/L sodium phosphate buffer, pH 7.4; 1% FBS was added (usually 400,000 cpm or approximately 0.02 μg antibody). The mixture was incubated at 4°C with intermittent shaking for 60 minutes, following which 800 μL chilled buffer containing 4 x 10^6 GPIIb/IIIa-negative carrier cells (usually Jurkat T-lymphoblastoid cells) were added and mixed. After pelleting, the supernate was aspirated, the cells washed once in 1-mL assay buffer, and the pellets counted with a gamma counter. For competition assays, cold antibody was incubated with target cells for 30 minutes at 4°C prior to addition of 125I-HP1-1D. Control MoAbs included anti-TAC, which is specific for interleukin 2 (IL2) receptors (the gift of T. Waldmann), and α-PS, which is specific for the coagulation protein S (the gift of W. Church). Assays from marrow cultures were done in duplicate or triplicate on 200-μL aliquots of suspended cells from each well.

 Autoradiography. Cells for autoradiography were labeled with 125I-HP1-1D, as described in the section above, but subjected to one additional wash. Suspended samples were then centrifuged onto clean glass slides by cytocentrifuge, dipped in photographic emulsion (NTB-2, Kodak, Rochester, NY), and developed for approximately two days at 4°C. Following development with D-19 (Kodak), the slides were equilibrated with buffer and stained with Wright's stain.

RESULTS

The RIA, as developed using iodinated HP1-1D, can detect as few as 3 x 10^3 platelets and is linear between 10^4 and 10^6 platelets (Figs 1 and 2). This latter level coincides with calculated antigen excess, if one assumes 40,000 glycoprotein IIb/IIIa complexes per platelet.28 Four separate antibody iodinations and over 50 platelet titrations have yielded completely reproducible results. Approximately 0.02 μg of antibody and 400,000 counts per minute are added to each assay tube. If the average megakaryocyte has approximately 100 times the surface area of a platelet, this assay can detect accurately between 100 and 10,000 megakaryocytes per aliquot.

The specificity of iodinated HP1-1D binding to platelets was ascertained by competition experiments using cold HP1-1D, a murine IgG monoclonal nonreactive with platelets (anti-TAC), and murine polyclonal IgG (Fig 2). Binding of labeled antibody was competitively blocked by increasing concentrations of cold HP1-1D but not by either of the nonspecific antibody preparations. Competition experiments using cultured (day 14) marrow cells similarly demonstrated blocking by cold HP1-1D but not by another murine monoclonal of the same isotype (α-PS is specific for the coagulation protein S and does react with human platelet, Table 1).

*The formula for the surface area of a sphere is 4π r^2. Given an average platelet diameter of 3 μ, and an average megakaryocyte diameter of 30 μ, a ratio of platelet surface area to megakaryocyte surface area of 100 can be calculated.

Fig 1. Binding of 125I-HP1-1D to fresh human platelets. Data shown are the mean of triplicates. Very similar curves were obtained using platelets from different donors or commercially obtained lyophilized platelets.
been cultured 14 days in 30% FCS + 5% PHA-LCM. Cells were reacted '30 minutes at 22°C.

Days in culture, cell-bound GPIIb/IIIa falls, presumably as of a representative experiment are depicted in Fig 3 and 5.

Table 2 gives summary data. During the first four to seven weeks time. However, FBS reproducibly supported a marrow in media supplemented with fetal bovine serum

Table 1. Binding of 125I-HP1-1D to Cultured Bone Marrow Cells is Blocked by Cold HP1-1D but not by Another Monoclonal, α-PS

<table>
<thead>
<tr>
<th>Competing Antibody</th>
<th>CPM Bound ± SEM</th>
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<tbody>
<tr>
<td>None</td>
<td>4221 ± 83.4</td>
</tr>
<tr>
<td>HP1-1D (1 µg)</td>
<td>612 ± 55.2</td>
</tr>
<tr>
<td>α-PS (1 µg)</td>
<td>4559.5 ± 73.9</td>
</tr>
</tbody>
</table>

Target cells were aliquots of marrow cells (10⁶ cells/aliquot) that had been cultured 14 days in 30% FCS + 5% PHA-LCM. Cells were reacted 30 minutes at 22°C with competing antibody prior to addition of 125I-HP1-1D.

Iodinated HP1-1D did not bind to cultured human umbilical vein endothelial cells, with counts bound in a titration from 10⁴ to 10⁶ cells being equivalent to that of the Jurkat T-lymphoblastoid control (<500 cpm). To be sure that trypsin and EDTA used to remove adherent endothelial cells from flasks before the RIA had not destroyed endothelial cell GPIIb/IIIa, platelets were subjected to similar treatment. EDTA-trypsin treatment of platelets caused only a 20% to 30% decrease in binding of 125I-HP1-1D.

Human light density nonadherent bone marrow cells grow well in liquid culture, with formation of recognizable erythroblasts, macrophages, granulocytes, and megakaryocytes depending on the growth conditions. Using the RIA we monitored total cell-bound GPIIb/IIIa present on marrow cells cultured over 15 days in various conditions. The results of a representative experiment are depicted in Fig 3 and Table 2. During the first four to seven days in culture, cell-bound GPIIb/IIIa falls, presumably as residual platelets in the marrow preparation disintegrate, adhere to the plates, or are ingested by other cells. Culture of marrow in media supplemented with fetal bovine serum (FBS) by itself permitted minimal antigen accrual over 2 weeks time. However, FBS reproducibly supported a response to PHA-LCM that was usually significant by day 7 in culture and always two to four times that of FBS alone by day 14. Aplastic human plasma routinely supported the formation of cell-bound glycoprotein IIb/IIIa complex by day 7. Antigen accrual continued through day 15 and was markedly stimulated by the addition of PHA-LCM. Thus although the assay detects a significant difference between marrow cultured in FBS alone and that cultured in aplastic plasma with PHA-LCM by day 7 (mean of fourfold difference), by day 14 this difference is far larger (mean of 30-fold, Table 2).

We found that bone marrow cells cultured with 30% normal human plasma or serum had little cell-bound glycoprotein IIb/IIIa after two weeks in culture (Fig 4). The addition of PHA-LCM to these cultures had variable effects, with some normal samples allowing an increment of twofold or threefold antigen increase over two weeks time, but with the majority of normal plasmas and sera not supporting such megakaryocyte growth (Fig 4). For a given donor, plasma and serum usually had similar growth supporting characteristics (the plasma and serum samples in Fig 4 are from the same donors).

The RIA proved very reliable in measuring megakaryocyte lineage cells in liquid culture in over 40 experiments using different bone marrow samples. Microscopic examination of cytospins from the cultures after day 5 revealed recognizable megakaryocytes proportionate to bound counts (Fig 5). The standard error between aliquots from a given well (duplicates or triplicates) ranged from 0% to 10% of the mean counts bound and was usually less than 5%. Well-to-well variation (ie, duplicate wells set up on day 1 and compared on the day of assay) was occasionally somewhat greater, but statistically significant differences between unstimulated and stimulated wells (for instance fetal bovine serum with and without PHA-LCM) were present by day 11 even in the most unresponsive marrow. Radioimmunoassays were routinely performed only on easily resuspendable cells; however, when adherent cells were removed with a rubber policeman and assayed, no counts above background were observed.

Visualization of the populations of cells binding iodinated HP1-1D was performed using autoradiography on the cultured marrow populations (Fig 6). Activity bound to recog-
nizable megakaryocytes in all stages of maturation and, in addition, was found on rare, small round cells similar to those found in megakaryocyte colonies. Activity was not found on monocytes, macrophages, granulocytes, or erythroblasts.

DISCUSSION

We have developed an accurate, specific, and rapid screening RIA for cell-bound GPIIb/IIIa that is useful for the quantitation of human megakaryocytes grown in liquid marrow culture. GPIIb/IIIa on the surface of cells is quantitated by use of an iodinated MoAb specific for the human platelet or megakaryocyte GPIIb/IIIa complex. Bound antibody is separated from free antibody by centrifugation of cells and the pellet counted in a gamma counter.

The presence of the GPIIb/IIIa complex on megakaryocytic lineage cells ranging from small round morphologically undifferentiated cells to recognizable mature megakaryocytes and platelets has been observed by many investigators as a target for immunofluorescent probes in characterizing cells from cobblestone areas of methylcellulose marrow cultures. Some investigators have questioned the specificity of GPIIb/IIIa or GPIIIa in Western blots (Nichols, unpublished data). We have not found cross-reactivity of GPIIb/IIIa with antigens or cells from outside the human megakaryocyte/platelet lineage. In the present study we examined cell types found in marrow cultures and found no evidence that 125I-HPI-1D bound to monocytes, macrophages, endothelial cells, or fibroblasts (by RIA, autoradiography, or both). We have previously reported the biosynthesis of GPIIb/IIIa by megakaryocyte colonies (CFU-M) plucked from methylcellulose marrow cultures. In those studies our monoclonal against the human GPIIb/IIIa complex, HPI-1D, was used to purify the 35S-methionine-labeled complex from cell lysates, and no detectable GPIIb/IIIa was synthesized by monocyte/macrophage colonies. Thus the sensitivity of the RIA is probably similar to that of colony assays, but since very different products are measured in the different assays, this comparison must be only a general one. Investigators report megakaryocyte colony sizes ranging from three to 500 cells, with mean colony size less

Fig 4. Megakaryocytocpipoisis in liquid cultures supplemented by normal human plasma or serum as measured by cell-bound 125I-HPI-1D. Three hundred thousand marrow cells per well were cultured with or without added PHA-LCM two weeks prior to assay. (A), Normal human plasma; (B), normal human serum.

Fig 5. Counts bound are proportionate to recognizable megakaryocytes after 12 days in various liquid culture conditions (r = 0.97). Megakaryocytes per fraction were determined by multiplying the number of cells per aliquot (as determined by hemacytometer count) by the percentage of megakaryocytes in a differential of 1,500 cells done on a cytospin from that well. Percentages varied in this representative experiment from 0% to 3%.
than 100 cells. No colonies are routinely formed in unstimulated (normal human plasma) cultures, whereas aplastic plasma and PHA-LCM permit up to 50 colonies per $10^5$ marrow cells. In unstimulated liquid cultures we find 500 to 1,200 CPM bound, which corresponds to about 1 to 2 x $10^4$ platelets or 10 to 20 megakaryocytes. Mature megakaryocytes are found on cytoospin preparations of these cultures, implying that some individual cells may mature in vitro to megakaryocytes under conditions where no colonies are formed. Marrow cultured in aplastic plasma and PHA-LCM binds 4,000 to 30,000 CPM per $10^5$ starting cells by day 14, which by extrapolation from platelet-binding curves corresponds to 1,000 to 10,000 megakaryocytes. We stress that although these calculations demonstrate that colony assays and liquid marrow culture assayed by RIA detect about the same amount of megakaryocyte growth from human marrow preparations, the product measured is very different. The unit of measure in a colony assay is the colony that represents proliferative and differentiative changes occurring among the progeny of one stem cell over time in culture. On the other hand, in the RIA the unit of measure is a GPIIb/IIIa complex expressed on the surface of a cell where it can bind radiolabeled antibody. Cells binding radiolabeled antibody may have differentiated in culture, proliferated in culture, or both.

The GPIIb/IIIa RIA for evaluation of megakaryocyte growth in culture is faster than semisolid culture assays in three respects. The first two respects are technical and involve the time it takes to set up the cultures and to perform the assays. Liquid marrow cultures may be set up much more quickly than semisolid cultures because they require only the addition of measured volumes of media, cells, and variables to 24-well plates. No suspension in methylcellulose, plasma clot or agar, or preparation of individual culture dishes is required. The time for quantitation is much reduced by the RIA as well. We routinely set up 50 to 75 different growth conditions on a given marrow and assay them all in duplicate or triplicate in about four hours. Counting colonies using an inverted microscope or an immunofluorescent microscope takes approximately five to 15 minutes per plate, so that quantitation of the amount of growth for a given condition in triplicate might take 20 to 40 minutes and that for 50 different conditions several days.

The third-time advantage the RIA has over semisolid marrow culture is that it is sensitive to the presence of growth factors after relatively short incubation times. Megakaryocyte colonies under maximally stimulated conditions (aplastic plasma and PHA-LCM) are recognizable by day 8 or 9, with more colonies formed or becoming recognizable through day 14 to 16. Statistically significant differences between stimulated and unstimulated liquid culture wells are detected by the RIA as soon as days 5 to 7, although in liquid culture also antigen accrual continues through day 15.

The formation of megakaryocyte colonies in semisolid medium by definition requires that the committed cells undergo cell division, while much of the regulation of megakaryocyte maturation and platelet shedding probably occurs on cells that undergo few if any further cell divisions. Thus, colony assays, while sensitive for factors that promote or permit proliferation (colony stimulating factors), may not detect regulators of megakaryocyte maturation (megakaryocyte maturation factors). Because this GPIIb/IIIa RIA of liquid marrow culture can measure increases in cell membrane in addition to increases in cell number, we hope that it will be especially useful in recognition and isolation of megakaryocyte maturation factors.

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