Two Monoclonal Antiplatelet Antibodies as Markers of Human Megakaryocyte Maturation: Immunofluorescent Staining and Platelet Peroxidase Detection in Megakaryocyte Colonies and in In Vivo Cells From Normal and Leukemic Patients


Two monoclonal anti-human platelet antibodies have been used in an immunofluorescent assay to study megakaryocyte maturation. The two antibodies were specific for platelets and megakaryocytes. AN 51 recognizes an antigen on platelet glycoprotein Ib, the antigen detected by J 15 is on the glycoprotein Ib/IIa complex, since it does not bind to platelets from patients with Glanzmann's thrombasthenia. AN 51 did not stain small cells of normal bone marrow, but it labeled most of the megakaryocytes and all of the platelets. In megakaryocyte colonies, megakaryocytes that had reached full maturity (at day 12 of culture) were stained specifically. AN 51 also labeled micromegakaryocytes (small mature megakaryocytes) in fetal or neonatal cultures and in leukemias, but could not be applied to identification of most leukemic promegakaryoblasts. In contrast, J 15 labeled all megakaryocytes in bone marrow as well as some rare small cells. These cells appear to represent an early cell of the megakaryocyte lineage, since their number was increased in autoimmune thrombocytopenia and they were present as early as the sixth day of culture of megakaryocyte colonies. The size of these cells progressively increased as the culture aged, probably due to endoreplication. In the bone marrow of three patients with leukemias in which promegakaryoblasts were identified ultrastructurally by the presence of platelet peroxidase, J 15 labeled blast cells. Thus, glycoprotein Ib is progressively expressed during megakaryocyte maturation, but its expression is independent of megakaryocyte ploidy, while the glycoprotein Ib/IIa complex is an early antigenic marker of megakaryocyte maturation that may be useful in isolation of megakaryocyte precursors and diagnosis of megakaryoblastic leukemia.

In contrast to rodent megakaryocytes, human megakaryocytes lack this specific cytochemical marker. Therefore, human megakaryocytes possess another cytochemical marker, i.e., platelet peroxidase (PPO), which is detectable by ultrastructural cytochemistry in the endoplasmic reticulum. This enzyme is already present in small cells that do not otherwise possess any specific organelles typical of the megakaryocyte. Unfortunately, this enzyme is only detectable by electron microscopy and is thus difficult to use in the study of the regulation of megakaryopoiesis in vivo and in vitro. The detection of this enzyme has been useful in the study of human megakaryocyte-colony formation in vitro and in the demonstration that the megakaryocyte lineage may be much more frequently involved in blast crisis of chronic myelogenous leukemia (CML) and acute leukemia than previously presumed. For these various reasons, the discovery of differentiation markers for the megakaryocyte series is of considerable importance.

It has been demonstrated that polyclonal antiplatelet antibodies as well as antibodies against platelet glycoproteins or platelet factor react with large megakaryocytes and some small cells. However, their use is restricted by problems of availability, in part because some of them are of human origin. Monoclonal antibodies provide an immunologic tool particularly effective in identifying antigens on the surface of human cells and in studying their relationship with the differentiation process.

We report the use of two monoclonal antiplatelet antibodies, AN 51 and J 15, in the study of in vivo and
in vitro differentiation of the megakaryocyte series and in the characterization of some leukemias. AN 51 is a well characterized antibody that specifically binds to glycoprotein Ib.22 The antigen recognized by J 15, which is still under investigation, appears to be on the glycoprotein IIb-IIIa complex.23 Our results indicate that AN 51 recognizes the mature megakaryocyte found during all phases of ontogenesis and malignant megakaryocytes, whatever their ploidy; in contrast, J 15 labels, in addition to the large megakaryocyte, small immature cells that could be equivalent to small Ach-E-positive cells in rodents. These cells probably correspond to the human promegakaryoblasts identified by the platelet peroxidase.

MATERIALS AND METHODS

Samples

Bone marrow cells for culture or fluorescence assays were obtained by sternal puncture from hematologically normal individuals and from two patients with idiopathic thrombocytopenic purpura (ITP). For the isolation of megakaryocytes, bone marrow was obtained from the ribs of patients undergoing thoracotomy. The blood of 15 patients with acute leukemias or blast crisis of myeloproliferative disease was studied. In these cases, low density cells in the blood were separated by density centrifugation in Ficoll metrizoate (Lymphoprep, Nyegaard, Oslo, d = 1.077). The leukemias were classified according to the FAB criteria24 by May-Grünwald-Giemsa smears and optical cytochemistry.

Liver cells from a 20-wk-old fetus and cord blood were obtained for culture purposes.

Antibodies

The preparation of one of the monoclonal antibodies, AN 51, has been fully described elsewhere.22 Preparation of J 15 followed a similar protocol. Briefly, BALB/c mice were immunized with a mixture of human platelets and lymphocytes. The spleen cells were fused to cells of the 8-azaguanine mutant murine myeloma cell line P3-NS1/1 Ag 4-1 according to the technique of Kohler and Milstein.22 The fused cells were plated out into tissue culture wells and hybrid cells selected by growth in RPMI 1640 containing hypoxanthine aminopterin, and thymidine. In two separate experiments, antibody in the supernatant of a single well bound to human platelets and not to lymphocytes. Cells from these two cultures were cloned to give the stable cell lines AN 51 and J 15, both of which have been maintained for over 12 mo.

The antigen recognized by AN 51 is glycoprotein Ib22 while J 15 binds to an antigen that was different from that recognized by AN 51.22 Recent results using radioimmunoassay have shown that J 15 bound to all normal platelets, but did not bind to platelets of patients with Glanzmann's thrombasthenia.23 This result suggests that the antigen recognized by J 15 is the glycoprotein IIb-IIIa complex. AN 51 is an IgG, while J 15 is an IgM. The specificity of AN 51 and J 15 for platelets has been demonstrated by the absence of binding to different human tissues, including blood cells (except platelets) liver, brain, exocrine and endocrine glands, heart, skin, and gastrointestinal tract.22

Both antibodies were available as ascitic fluid, obtained from mice in which hybridoma cells were injected intraperitoneally. The concentration of monoclonal antibodies in these samples was about 1 mg/ml. The dilution of the ascitic fluid to concentrations that failed to saturate all the sites on 106 platelets was in the range 10^{-4}-10^{-3} by radioimmunoassay.22

Isolation of Megakaryocytes

The method described by Rabellino et al.13 was used to obtain megakaryocyte preparations of high purity. Briefly, single bone marrow cell suspension, were obtained from the ribs. The first separation step consisted of a Percoll density centrifugation. The second was a velocity sedimentation on a continuous gradient of Ficoll. Two cycles of velocity sedimentation were usually performed.

After the first step of separation, megakaryocytes represented about 1%-1.5% of the total cell population. After the two separation steps, megakaryocytes represented more than 90% of the total cells. Smears of cells from each step were kept at -80°C until use.

Megakaryocyte Colony Cultures

The culture technique for human megakaryocyte colonies was used with slight modification.22 The stimulating factor was conditioned medium from leukocytes stimulated by phytohemagglutinin (PHA).22,23 In all cases (bone marrows, blood cells, cord blood, fetal liver), the light density cells were isolated by Ficoll-metrizoate gradient centrifugation and then seeded by the plasma clot technique. Quantities of 3 X 10⁶ bone marrow or blood cells, 6 X 10⁶ liver cells, and 4 x 10⁶ cord blood cells per milliliter were plated after adherence.22 The cultures were usually studied at day 12. For three bone marrows, cultures were sequentially studied by immunofluorescence at days 3, 5, 6, 7, 8, 10, and 12.

Immunofluorescent Assay

Indirect immunofluorescence was performed using the unlabeled monoclonal ascitic fluid followed by fluoresceinated rabbit anti-mouse Ig antibody (Nordic Immunology, London, England). Two different techniques were used according to the cells studied.

For bone marrow samples, leukemic blast cells, megakaryocyte-enriched preparations or isolated platelets, fresh or 1% paraformaldehyde-fixed cells were incubated with AN 51 or J 15 ascitic fluid at dilutions that varied from 6.4 X 10^{-4} to 5.10 X 10^{-1} for 30 min at 37°C. The concentration of the ascitic fluid was dependent on the batch studied, but the antibody concentration was in all cases in large excess in order to saturate all binding sites on platelets or megakaryocytes.23 The first experiments were performed on fresh cells but later experiments also included paraformaldehyde-fixed cells, since the results were equivalent to those obtained with fresh cells. After two washings, fluoresceinated rabbit anti-mouse Ig was subsequently applied at a 1 X 10^{-4} dilution for 30 min.

For the cultures, the immunofluorescent technique was modified slightly in order to stain the megakaryocyte colonies in situ. Fixation by paraformaldehyde was avoided since in some experiments it could detach the plasma clot from the Petri dish. Thus, the antibody was applied without prior fixation. The plasma clot was directly dehydrated in the Petri dishes using filter papers and dried at room temperature. The bottom of each 35-mm Petri dish was removed; one or two squares were demarcated on the plasma clot for each dish, and the ascitic fluid was applied for 1 hr at 37°C. The dilution of the antibody for the cultures was determined as follows. Platelets were isolated and then mixed with the culture medium. A quantity of 5 X 10⁶ platelets were distributed in each 35-mm Petri dish, and the medium subsequently allowed to clot. The clot was dehydrated as above. Several dilutions of the antibodies were tested. The dilution used in further investigations was the one that gave a bright
fluorescence on all platelets. This dilution varied from $6.4 \times 10^{-1}$ to $2.5 \times 10^{-2}$. After two washings, the cultures were usually fixed by applying methanol for 5 min. The fluoresceinated rabbit anti-mouse Ig was subsequently applied at $1 \times 10^{-2}$ dilution for 30 min.

Under these conditions, no background fluorescence was observed. In a few cultures, rare granulocyte colonies could take on a patchy nonspecific fluorescence, already observed in the controls using the Ig alone. This nonspecific fluorescence disappeared after one washing in $10^{-4}$ blue Evans solution. A coverslip was subsequently mounted in glycerol.

Preparations were examined with a Zeiss microscope equipped with phase contrast. Photographs were taken on an Ektachrome ASA 200 film push-pulled to ASA 800.

**Electron Microscopic Studies**

For leukemic cells, the isolated light density cells were centrifuged and the pellet was subsequently treated by two different techniques to reveal platelet peroxidase.\textsuperscript{16,16}

For megakaryocyte cultures, the fibrin network of the plasma clot was digested by pronase $0.1\%$\textsuperscript{12} The cellular pellet was then treated as above.

**RESULTS**

**Normal Bone Marrows**

AN-51- and J-15-labeled large cells were subsequently identified as megakaryocytes by phase contrast microscopy. In addition, J 15 recognized a very low number ($0.8/10^4$ unseparated cells) of small cells that could not be identified by phase contrast or staining.

After partial purification of the megakaryocytes by the Percoll gradient, AN 51 labeled most of the megakaryocytes, but some typical megakaryocytes, identified by their large size and cytologic characteristics, were not labeled. In contrast, J 15 bound to all recognizable megakaryocytes and to the additional small cells that were greatly enriched by the gradient.

After complete purification of the megakaryocytes, J 15 strongly stained all megakaryocytes homogeneously (Fig. 1), while binding of AN 51 was highly heterogeneous and much weaker (Fig. 2).

**Idiopathic Thrombocytic Purpura**

J 15 labeled the large megakaryocytes and also a higher proportion of small cells ($7.5/10^4$ unseparated cells) than in normal bone marrows, while, again, AN 51 only labeled large megakaryocytes.

**Culture of Megakaryocyte Colonies**

Previous studies using morphological criteria for identification of the colonies have shown that the first megakaryocyte colonies appear at day 7 and peak at day 12.\textsuperscript{12,13}

In cultures of different adult bone marrows, megakaryocytes were stained by both AN 51 and J 15 at day 12. However, the labeling of AN 51 was weak and heterogenous; frequently, large typical megakaryocytes were not labeled. Thus, the staining pattern was studied during the appearance of the colonies. J 15 labeled the first cells at day 5 or 6 of culture. These cells had the size of a lymphocyte, exhibited a very strong staining (Fig. 3), and could be observed as isolated cells at day 5 of culture. From day 5 to day 10, the number of colonies greatly increased. In the same period of time, the size of the colonies and of the megakaryocytes increased (Fig. 4). The colonies that were tight at the beginning became composed of loosely aggregated megakaryocytes (up to 50 cells) after day 10. At this date, cells of the size of lymphocytes could not be observed, the colonies being composed of very large cells and intermediate cells ($\sim 20\mu$). On electron microscopy, the first promegakaryoblasts were also identified at day 6 or 7 of culture and were typically not observed after day 10 of culture. In contrast, the first cells weakly labeled by AN 51 appeared at day 10. The maximum of staining was observed at day 12 of culture (Fig. 5) and was especially strong on the very mature megakaryocytes that were about to lyse or shed platelets. However,
MEGAKARYOCYTE MATURATION

Fig. 3. A small cell is labeled by J 15 at day 6 of culture, while no labeling was observed with AN 51 at this date. Studies on cultures from normal adults. Bone marrow or blood cultures were performed in plasma clot using PHA-LCM as stimulating factor. Megakaryocyte colonies were labeled by the two monoclonal antibodies without prior fixation.

Labeling was much weaker than that observed with J 15.

Cultures of fetal livers and of cord bloods were also performed. At day 12 of culture, AN 51 labeled (heterogeneously) megakaryocyte colonies. Staining was observed in either small or large cells (Fig. 6). In the cultures stimulated by PHA, the staining was much stronger than in the spontaneous megakaryocyte colonies. Studies by electron microscopy have shown that the small megakaryocytes of day 12 in neonatal and fetal cultures were small mature megakaryocytes (micromegakaryocytes) (Fig. 7). This type of cell was not normally observed in cultures from adults. Thus, the cultures mimic findings in vivo. J 15, when applied to neonatal cultures, stained both large and small cells.

In scoring of megakaryocyte colonies in cultures of normal adult bone marrow, J 15 labeling and cytologic techniques gave similar results. However, the fluorescent labeling provided a specific and easily reproducible technique of scoring, while cytologic scoring was tedious and required well stained preparations. At day 12 for the three bone marrows studied, the cloning efficiency was, respectively, 90, 130, and 110/106 plated cells. In contrast to J 15, AN 51 could not be safely used for scoring, since entire colonies may remain unlabeled even at day 12 of culture. In cultures of myeloproliferative diseases, scoring of megakaryocyte colonies required immunofluorescent labeling since most of the colonies were composed of micromegakaryocytes that could not be identified by cytology.

Leukemias

The cells in several types of acute leukemias were tested to evaluate the specificity of the two monoclonal antibodies. Cells from patients with lymphoblastic leukemia of the “null type,” promyelocytic leukemia, acute and subacute myelomonocytic leukemia, monoblastic and myeloblastic leukemias were studied. In all cases, neither antibody stained the blast cells. In addition, 7 patients were studied in whom it was presumed that was megakaryocyte lineage could be...
involved (Table 1). In this study, immunofluorescent labeling was compared to the presence of PPO detected by ultrastructural cytochemistry. AN 51 did not usually label the promegakaryoblasts recognized by PPO (Fig. 8) except in one case (case 7); in contrast it did stain the micromegakaryocytes (Fig. 10) identified by electron microscopy in the acute (Table 1) and chronic phases of myeloproliferative diseases.

In contrast, J 15 stained cells in addition to micromegakaryocytes, as found in cases 2, 6, and 7 (Table 1, Fig. 9). These cells (Fig. 11) may be identical to those identified as promegakaryoblasts (Fig. 8) by electron microscopy, although the fluorescent labeling was less sensitive than the detection of PPO (Table 1, cases 6 and 7).

**DISCUSSION**

This report underlines the value of monoclonal antibodies in studies of differentiation and maturation

---

**Table 1.**

<table>
<thead>
<tr>
<th>Type of Leukemia</th>
<th>Light Microscopy</th>
<th>Electron Microscopy</th>
<th>AN 51</th>
<th>J 15</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute leukemia</td>
<td>Promegakaryoblast?</td>
<td>Promegakaryoblasts</td>
<td>ø</td>
<td>ND</td>
</tr>
<tr>
<td>Promegakaryoblast</td>
<td></td>
<td>Basophils (≈60%)</td>
<td>ø</td>
<td>ø</td>
</tr>
<tr>
<td>Blast crisis of CML Undifferentiated blasts</td>
<td>Promegakaryoblasts (≈20%)</td>
<td>ø</td>
<td>ø</td>
<td></td>
</tr>
<tr>
<td>Micromegakaryocytes (≈10%)</td>
<td>ø</td>
<td>ø</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blast crisis of CML Undifferentiated blasts</td>
<td>Undifferentiated blasts (≈80%)</td>
<td>ø</td>
<td>ø</td>
<td></td>
</tr>
<tr>
<td>Micromegakaryocytes (≈20%)</td>
<td>ø</td>
<td>ø</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blast crisis of CML Undifferentiated blasts</td>
<td>Undifferentiated blasts (100%)</td>
<td>ø</td>
<td>ø</td>
<td></td>
</tr>
<tr>
<td>Blast crisis of an idiopathic myelofibrosis Undifferentiated blasts</td>
<td>Proerythroblasts (≈50%)</td>
<td>ø</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Promegakaryoblasts (≈50%)</td>
<td>ø</td>
<td>ø</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;90% peroxidase and small granule myeloblasts or monoblasts</td>
<td>ND</td>
<td>ø</td>
<td>&lt;1%</td>
<td></td>
</tr>
<tr>
<td>&lt;10% promegakaryoblasts</td>
<td>ND</td>
<td>ø</td>
<td>&lt;1%</td>
<td></td>
</tr>
<tr>
<td>Blast crisis of an idiopathic myelofibrosis Undifferentiated blasts</td>
<td>Promegakaryoblasts (≈20%)</td>
<td>ø</td>
<td>ø</td>
<td></td>
</tr>
<tr>
<td>Weak staining 2%</td>
<td>8%</td>
<td>ø</td>
<td>ø</td>
<td></td>
</tr>
</tbody>
</table>
MEGAKARYOCYTE MATURATION

Fig. 9. Micromegakaryocyte from case 3 incubated in DAB medium. The platelet peroxidase reaction is seen in the nuclear envelope (arrow). The cytoplasm contains typical organelles of the megakaryocyte, i.e., α-granules (α-gr.) and demarcation membrane (DM) systems (x 13,300).

Fig. 10. Labeling of a megakaryocyte colony from case 3 by AN 51. Blood cells were cultured; megakaryocyte colonies were obtained. The colonies only contained very small cells labeled either by AN 51 or J 15. Electron microscopy demonstrated that these cells were in fact micromegakaryocytes in vivo as well as in vitro (Fig. 9).

Fig. 11. Labeling of a blast cell from case 6 by J 15. Less than 1% of the blasts were labeled by J 15, while nearly 10% promegakaryoblasts were identified at the electron microscopic level (see Table 1).

of megakaryocytes. The two monoclonal antiplatelet antibodies used recognized the megakaryocytic lineage, but at different stages of maturation.

AN 51 recognizes an antigenic determinant on platelet glycoprotein Ib. This conclusion was based on the observations that AN 51 bound only to platelets and megakaryocytes of several tissues surveyed, that it failed to bind to Bernard-Soulier platelets, and that it precipitated a glycoprotein of 150,000 daltons from platelet membrane,22 which has now been directly identified as glycoprotein Ib (Tobelem, in preparation). This antigen is apparently a late marker of megakaryopoiesis. All normal platelets were labeled by AN 51, while some large megakaryocytes were not stained. In culture, AN 51 bound the megakaryocytes only at a late stage of development (day 12) when they had reached their maximum of maturation, as indicated by ultrastructural studies.12,13,29 The synthesis of this antigen seemed to be linked to the maturation of the cytoplasm but did not depend on the ploidy level of the megakaryocytes. AN 51 labeled the micromegakaryocytes found pathologically in human adults and normally during ontogenesis. The micromegakaryocytes are small cells, fully mature, which can shed platelets.30

In contrast, AN 51 did not usually stain the promegakaryoblast, which is defined as the small cell precursor of the megakaryoblast.31 As the intensity of fluorescence is directly dependent on the number of binding sites, however, the antigen recognized by AN 51 may be synthesized during the entire megakaryocyte maturation phase with a number of binding sites sufficient to be detected only at the end. This hypothesis is further supported by the presence of a faint staining on rare promegakaryoblasts in one case of leukemia. This problem is now under electron microscopic investigation by immunoferritin techniques. It is of importance since Rabellino et al., using a polyclonal antibody against glycoprotein Ib, found that more than 90% of megakaryocytes were labeled.17 This discrepancy may be explained by the high specificity of the monoclonal antibody AN 51, which only recognized a single antigenic site on glycoprotein Ib, as compared to the several determinants seen by a polyclonal reagent.

J 15 also recognizes an antigen restricted exclusively in its tissue distribution to platelets and megakaryocytes (Bastin and McMichael, unpublished). The lack of binding of J 15 to platelets from patients with Glanzmann's thrombasthenia suggests that the antigen is on the glycoprotein IIb/IIIa complex.29

In contrast to our findings with AN 51, J 15 labeled not only all the large megakaryocytes but also small cells. The studies described here indicate that the small
cells are a part of the megakaryocyte series and may represent the human counterpart of the small Ach-E-positive cells of rodents.\textsuperscript{1,2} In fact, the number of small cells stained by J 15 were increased in ITP in contrast to normal bone marrows. In culture, such cells appeared at a date identical to the first promegakaryoblasts identified ultrastructurally by the presence of PPO. These small cells, labeled by J 15, were intermingled with larger labeled cells. As the cultures became older and the megakaryocytes matured, they progressively disappeared and typically could not be observed after day 10 of culture. In addition, such small cells were detected in leukemias only when the megakaryocyte lineage was involved. Thus, it can be assumed that J 15 recognizes an early megakaryocyte precursor. This precursor is still capable of endoreplication since, as mentioned above, these cells progressively disappeared in culture from day 5 to day 10 to be replaced by larger cells. However, it remains uncertain whether this early megakaryocyte is still capable of mitosis; it is certainly a different cell from CFU-MK, since no positive cells were detected in culture before day 5. The cells identified by J 15 in man seem to correspond to the Ach-E-positive cells in rodents, including megakaryocyte and early cells of the megakaryocyte series capable of endoreplication. This promegakaryoblast remains unidentified by morphology, even upon electron microscopic examination,\textsuperscript{11} but can be identified by the presence of PPO in normal marrow\textsuperscript{11} and in megakaryoblastic leukemia.\textsuperscript{16} Three other early markers of the megakaryocyte series have been described: synthesis of factor VIII,\textsuperscript{31,32} serotonin uptake,\textsuperscript{32} and the presence of platelet factor 4.\textsuperscript{31}

Determination of the sequential appearance of these different specific markers of megakaryocyte maturation will be a useful way in which to monitor megakaryocyte maturation, to understand regulation of the late stages of megakaryocyte differentiation in vitro, and to identify and classify megakaryoblastic leukemia more precisely. The preliminary results obtained here clearly indicate that J 15 is useful in the characterization of megakaryoblastic leukemia. In three cases, J 15 recognized cells other than micromegakaryocytes. In these cases, promegakaryoblasts could be detected electron microscopically by the presence of PPO. It remains to be determined whether J 15 labels all or only a part of the blast cells identified by PPO as suggested by study. This problem is now under investigation using both markers at the electron microscopic level.

The study of megakaryocyte maturation by application of monoclonal antibodies also appears to provide a promising approach to the understanding of megakaryopoiesis in vivo and in vitro, as well as in the characterization of some megakaryoblastic leukemias.

ACKNOWLEDGMENT

The authors would like to thank Martine Segear for typing the manuscript and to Ph. Reboul for photographic assistance.

REFERENCES

8. Long MW: Relationship of small acetylcholinesterase (SACHE) positive cells to megakaryocytes and clonable megakaryocyte progenitor cells. Symposium Abstracts, International Society of Hematology, 18th Congress. Montreal, Canada, August 1980, p 143
18. Mazur EM, Hoffman R, Chasis J, Marchesi S, Bruno E:


Two monoclonal antiplatelet antibodies as markers of human megakaryocyte maturation: immunofluorescent staining and platelet peroxidase detection in megakaryocyte colonies and in in vivo cells from normal and leukemic patients

W Vainchenker, JF Deschamps, JM Bastin, J Guichard, M Titeux, J Breton-Gorius and AJ McMichael