Megakaryoblastic Acute Leukemia: Identification by the Ultrastructural Demonstration of Platelet Peroxidase

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In some acute leukemias, blast cells may lack morphologic and cytochemical characteristics indicating their original cell line. Whether these cells are in fact undifferentiated or derived from early precursors of lymphocytes or of other cell lines remains open to question. Leukemias with megakaryoblast (MKB) predominance have been considered rare. In these cases, the recognition of the MKB has been based on the large size of the cells and on their morphologic characteristics. Until now however, the identification of early small MKBs has been uncertain when conventional staining and ultrastructural methods have been employed. It has previously been shown that platelet peroxidase (PPO), which is distinct from granulocytic peroxidases, can be employed as a marker of normal small MKB. A new, sensitive cytochemical method for the demonstration of PPO has been applied to the study of a case of acute leukemia with thrombopenia. The majority of marrow and circulating small blasts that exhibit an undifferentiated or lymphoid appearance upon light and electron microscopic examination have been shown to possess PPO. The morphology of PPO-positive blasts is quite variable. They show no granule or demarcation membrane production usually associated with the beginning of normal megakaryocytic maturation. The presence of PPO alone has permitted us to classify this case as a pure MKB leukemia.

The classification of acute leukemias is based mainly on the appearance of marrow and blood cells in stained smears. In addition, some cytochemical methods and the detection of membrane markers may be used to define more precisely the cellular origin of some so-called “poorly differentiated” leukemias. At the present time however, some acute leukemias lack any identifiable morphologic, cytochemical, or membrane markers. They are considered unclassifiable, “undifferentiated,” or “lymphoblastic,” leukemias. As previously reported, the combined technique of electron microscopy and peroxidase cytochemistry has permitted the identification as myeloblasts of cells unclassifiable by light microscopic methods.

In spite of earlier reports on the occurrence of acute leukemias involving megakaryocytic (MK) precursors, leukemias with megakaryoblastic predominance are not at the present time considered as constituting an isolated malignant transformation of megakaryoblasts (MKB), but rather as accompanying the involvement of myeloid cell lines. In these reports, the recognition of blast cells as members of the MK cell line has been based entirely on morphologic features as seen by light microscopy.

A platelet peroxidase (PPO), which is distinct from the granulocytic peroxidases, is synthesized early during MK maturation and is therefore considered as...
a specific enzymatic marker of this cell line.\textsuperscript{19,20} The detection of PPO has proved useful in the recognition of blasts present in patients with abnormal megakaryocytopoiesis.\textsuperscript{21,22} However, in a systematic study, we have been unable to detect reproducibly PPO in either immature precursors or in normal platelets. These difficulties can now be ascribed to an inhibition of the enzyme by the glutaraldehyde fixation of the cells.\textsuperscript{19,23} Improved methods for the cytochemical detection of cell peroxidases have recently been proposed.\textsuperscript{23,24} With these procedures, an intense and reproducible PPO staining is regularly obtained in human and animal platelets.\textsuperscript{29}

In the present report, we describe a case of acute leukemia in which blast cells had an “undifferentiated” morphology at both the optical and the ultrastructural levels. Ultrastructural cytochemistry facilitated the detection of PPO and thus their identification as MKB.

\textbf{MATERIALS AND METHODS}

\textbf{Case Report}

An 80-yr-old man was admitted to the hospital in March 1976 with pancytopenia; his condition had deteriorated a few weeks prior to admission with dyspnea, weakness, and pallor. Upon physical examination numerous ecchymoses were discovered. Neither his lymph nodes, spleen, nor liver were palpable.

On admission, the hemoglobin was 9 g/dl with a hematocrit of 27\%, and a mean corpuscular volume of 100 cu $\mu$m; the leukocyte count was 2600/$\mu$l with 48\% neutrophil and 23\% eosinophil segmented forms, 4\% monocytes, 38\% lymphocytes, and 8\% blast cells. The platelet count was 12,000/$\mu$l. Subsequent blood counts are shown in Table 1. The patient had to be transfused weekly. Reticulocyte counts were always below 10,000/$\mu$l. Coagulation data were within the normal range. Hemoglobin electrophoresis disclosed 21% HbF. A sternal marrow aspiration yielded specimens with low cellularity containing 35\% lymphocytes and 35\% blast cells. A marrow biopsy disclosed a low cellular density, but contained a majority of blast cells (see Results).

Despite a vincristine and cytoxan regimen (2 mg and 800 mg weekly) together with supportive therapy, the patient’s condition progressively deteriorated and he died 2 mo after admission. His karyotype was not determined.

At autopsy, the weight of the spleen was 120 g; its architecture was normal although a discrete myeloid metaplasia was found in the red pulp including MK; the liver weighed 1500 g and no abnormal cells were seen.

\textbf{Collection of Cells}

Heparinized venous blood (20 ml) was collected. At the time of this study, the differential count showed 70\% blast cells, 5\% polymorphonuclear leukocytes, and 25\% lymphocytes. Marrow was aspirated from the sternum: because of fibrosis (see below), only a single marrow fragment could be removed for electron microscopic studies. A marrow biopsy was also performed for histologic study.

\begin{table}[h]
\centering
\caption{Blood Cell Counts}
\begin{tabular}{|c|c|c|c|c|c|c|}
\hline
Date & RBC/$\mu$l & HB (g/dl) & WBC/$\mu$l & PMN* (%) & Lymph (%) & Blasts (%) & Platelets/$\mu$l \\
\hline
3/11/76 & 2,890,000 & 9 & 2,600 & 50 & 42 & 8 & 12,000 \\
3/29/76 & 2,610,000 & 7.8 & 3,800 & 44 & 48 & 18 & 15,000 \\
4/3/76 & 4,200,000 & 11.3 & 3,900 & 10 & 40 & 50 & 15,000 \\
4/20/76 & 2,820,000 & 8.3 & 8,300 & 3 & 23 & 74 & 30,000 \\
5/15/76 & 2,100,000 & 6 & 2,200 & 0 & 20 & 80 & 35,000 \\
\hline
\end{tabular}
\end{table}

*Polymorphonuclear leukocytes.
Blood and marrow smears were routinely stained by the May-Grünwald-Giemsa method and were processed for the periodic acid Schiff (PAS) and peroxidase reactions.30

Ultrastructural Cytochemistry

The buffy coat was pipetted off after blood sedimentation at room temperature. Cell peroxidases were detected by two separate procedures. The buffy coat, therefore, was fractionated into two samples that, prior to incubation in diaminobenzidine medium (DAB),31 were treated as follows.

1. One sample was fixed with 1.25%, glutaraldehyde in phosphate buffer, 0.1 M.
2. The other sample was fixed in a tannic acid-aldehyde mixture,28 modified for the study of platelets.29 Prior to fixation, the buffy coat was centrifuged at low speed for 10 min and then washed twice in Hanks' balanced salt solution in order to eliminate plasma proteins that precipitate with the fixative. The fixative consisted of 1%, tannic acid (E. Merck, Darmstadt), 2%, paraformaldehyde, and 0.5% distilled glutaraldehyde (25%, biologic grade, TAAB, England) in 0.1 M phosphate buffer at pH 7.2. The fixative was prepared just prior to use, filtered and chilled at 4°C. Cells were fixed for 1 hr at 4°C, washed in phosphate buffer, and stored for prolonged periods (24 hr) before incubation in the DAB medium; this medium contained 20 mg of 3,3'-diaminobenzidine-tetra-HCl (Sigma Chemicals, St. Louis, Mo.) and 0.1 ml H2O2 at 1%, in 10 ml 0.05 M Tris buffer. The pH was readjusted to 7.6 with a 1 N solution of sodium hydroxide. Incubation was carried out in a dark room at room temperature for 1 hour. The cells were then rinsed in several changes of phosphate buffer (0.1 M pH 7.2) and postfixed for 30 min in 1%, osmium tetroxide in phosphate buffer. After postfixation, the cells were dehydrated in ethanol water solutions and embedded in epon. Sections cut with a diamond knife on an LKB ultratome III were examined either unstained or lightly stained with lead citrate under a Philips EM 300 electron microscope. Cells serving as controls were incubated in medium without DAB or

**Fig. 1.** Light micrographs of May–Grünwald–Giemsa stained smears of (A) bone marrow and (B–F) blood. Note the high nucleocytoplasmic ratio, the presence of cytoplasmic blebs (E), and the lymphoid appearance of some cells (F). × 1600.
Fig. 2. Immature large MK in the marrow incubated for the demonstration of PPO. This cell possesses an unlobed nucleus with a nucleolus. Cytoplasm contains some demarcation membranes (DM) and numerous mitochondria (Mi), but granules are rarely seen. Nuclear envelope (arrows) and short segments of endoplasmic reticulum contain the dense product of oxidized DAB. Golgi apparatus (GA) appears unreactive. A platelet (P) shows a positive reaction in the dense tubular system (arrow). At the bottom, note the presence of a smaller MKB with a "lymphoid" aspect, exhibiting a similar although less intense peroxidatic activity in the perinuclear space and in endoplasmic reticulum (arrows). Golgi apparatus at the periphery of the centriole appears unreactive. × 12,600. Inset: MK on stained smears showing a round nucleus.
H$_2$O$_2$. A few samples were preincubated for 30 min at 4°C in $2 \times 10^{-2} \text{ M}$ aminotriazole (AMT) before incubation in complete medium containing the same concentration of AMT. Some cells were also preincubated in $10^{-2} \text{ M}$ KCN for 10 min and subsequently incubated in complete medium containing $10^{-2} \text{ M}$ KCN.

The single marrow fragment was processed according to method 2 of fixation. Some sections of material fixed by method 2 and incubated in DAB medium were treated by a technique for staining glycogen for electron microscopy.32

RESULTS

Light Microscopy Study

Circulating blast cells exhibited a uniform size of 10 μm. These cells had a large nucleus with homogeneous chromatin and one nucleolus (Fig. 1B); the cytoplasm was scanty, basophilic and contained no granules (inset Fig. 8). Thus these blast cells appeared as “undifferentiated,” although some of them displayed a more lymphoid morphology (Fig. 1F); in addition, a few cells showed cytoplasmic blebs (Fig. 1E). In marrow smears, blasts had a grossly similar appearance (Fig. 1A). However, in contrast to circulating cells, marrow blasts varied in size from 10 to 30 μm. In addition, rare dystrophic MK could be recognized, with one or several round and independent nuclei (inset Fig. 2). Blood and marrow blasts were negative for the peroxidase reaction, half of them being slightly PAS-positive.

Blasts constituted the majority of the cells found at histologic examination of the marrow biopsy (Fig. 3). They were variable in size and were characterized by a high nucleocytoplasmic ratio, a conspicuous nucleolus, and a homogeneous cytoplasm. An additional finding was the presence of numerous larger
cells that resembled MK (Fig. 3). Histologic examination also disclosed frequent mitotic figures and a dense reticulin network.

Cytochemical and Electron Microscopic Studies

Bone marrow (Figs. 2–7). Ultrathin sections of tissue fixed by method 2 displayed a rich cellularity and an excess of intercellular fibrils. Erythroblast and granulocyte precursors were not seen. However, some mature granulocytes were present and had a normal appearance: their cytoplasm contained peroxidase-positive (azurophil) and peroxidase-negative (specific) granules. Some lymphocytes, plasma cells, and macrophages could be recognized that had no peroxidatic activity. Erythrocytes were highly reactive as a result of the per-
Fig. 5. Golgi apparatus (GA) of the same cell shown in Fig. 4 sectioned at another level. All cisternae are free of reaction product, while the endoplasmic reticulum (including the nuclear envelope) is strongly reactive (arrows). Numerous monoribosomes are dispersed in the cytoplasm. ×22,300.

Fig. 6. Marrow MKB with a strong peroxidatic activity. ×15,000.
oxidative activity of hemoglobin. The fixation procedure generally allowed good preservation of the cell morphology and peroxidatic activities were reproducibly detected even in sections of deeper zones of the marrow fragment.

Cells reactive for PPO included rare platelets, MK, and 80% of the blast cells (Fig. 2). The intensity of the cytochemical reaction was high in MK and platelets but varied among the remaining blast cells (Figs. 2, 4, 7). In every positive cell, the site of reaction was identical; the dense product that was due to oxidized DAB was located in the perinuclear space and endoplasmic reticulum. Interestingly, the stacks of parallel cisternae of the Golgi complex appeared unstained (Figs. 2, 4, 5), with the exception of a single positive peripheral cisterna (see below).

The large reactive MK were immature. Some of them had demarcation membranes (Fig. 2) and/or large abnormal membrane complexes consisting of both associated demarcation membranes and PPO-positive smooth endoplasmic reticulum; these cells lacked granules. Thrombocytopoietic MK were not seen. Blast cells without any cytoplasmic maturation but with a positive reaction for PPO varied in their morphology: on conventional electron microscopy (without PPO staining), their appearance was that of "undifferentiated" cells, some of them with a lymphoid aspect (Fig. 4). Other rare blast cells contained cytoplasmic vacuoles and myelin figures; in addition, they exhibited cytoplasmic blebs (Fig. 7). A few PPO-positive blasts were larger, and their size was intermediate between that of the remaining blasts and MK.

Examination with the light microscope of thick (1-μm) sections of the DAB-treated and embedded marrow deserves an additional comment. Indeed, in half of the cells, the PPO content was too weak to be visible, while the higher PPO
content of the remaining cells could be seen as a perinuclear brown ring (inset Fig. 4).

_Blood_ (Figs. 8–10). Buffy coat cells treated by method 1 (glutaraldehyde fixation) contained rare mature granulocytes; their azurophilic granules were strongly reactive as a result of their myeloperoxidase (MPO) content. The remaining cells consisted of a majority of blast cells that were not reactive and a few typical small lymphocytes that were also unreactive.

When buffy-coat cells were treated by method 2 (tannic acid-aldehyde fixation), MPO was also detected in rare mature granulocytes; however, in contrast to method 1, the majority (80%) of blasts appeared to be positive for peroxidase staining. The morphology of these PPO-positive blasts was similar to that of marrow blasts, but their size was consistently smaller. The nucleocytoplasmic ratio was often high (Fig. 8), a nucleolus frequently being seen (Figs. 10B, C); the nucleus was round (Fig. 8) or sometimes deeply indented.

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_Fig. 8. Circulating blast cell producing PPO. Without the help of cytochemical data, this cell would have been considered "undifferentiated." Density of the plasma membrane is due to the presence of tannic acid in the fixative. Box corresponds to Fig. 9. ×13,000. Inset: Similar blast observed on blood stained smears. Note the high nucleocytoplasmic ratio._
Fig. 9. Golgi apparatus (GA) of circulating MKB shown in Fig. 8. Cisternae and vesicles of the Golgi appear devoid of PPO in contrast to the positive reaction in the endoplasmic reticulum (arrow). Only one external sacculus adjacent to the Golgi cisternae contains reaction product (double arrows). The nucleus (N) is not deformed by the Golgi zone. ×36,100.

The cytoplasm contained short strands of PPO-positive endoplasmic reticulum, free ribosomes, mitochondria, and a few vacuoles but neither granules nor demarcation membranes. The Golgi apparatus often exhibited an elongated shape, lying between the nucleus and the cytoplasmic membrane. It always appeared to be unreactive for PPO with the exception of one peripheral cisterna located either at its inner or outer face (Fig. 9). The plasma membrane of PPO-positive cells presented rare pseudopods (Fig. 8) or, on some occasions, large blebs (Fig. 10C). The platelets were large, contained PPO in their dense tubular system (Fig. 10A), and had numerous small mitochondria but no granules. In some of them, the surface-connected system was poorly developed.

As already stated, a few of the PPO-negative mononuclear cells were present in the buffy coat treated by method 2. Some had an ultrastructural appearance identical to that of PPO-positive blast cells, while the others possessed a morphology typical of lymphocytes.

Cells incubated in DAB medium without H₂O₂ or without DAB were entirely negative in reaction. Similar results were obtained after incubation in complete medium containing 2 × 10⁻² M AMT. Incubation in medium containing 10⁻² M KCN resulted in a decreased reaction but did not abolish it completely. Sections treated by the periodic acid-thiocarbohydrazide silver proteinate method showed that glycogen particles were distributed throughout the cytoplasm of blasts that either contained or lacked PPO. Their concentration was variable from one blast to another.

DISCUSSION

The present data demonstrate that in a case of acute leukemia, the majority of the marrow and circulating blast cells with an "undifferentiated" or lym-
phoid appearance possessed a PPO, thus allowing their identification as precursors of the MK cell line.

These data have been obtained by use of a simple, improved method for the demonstration of PPO. They confirm earlier observations concerning the value of PPO detection in the identification of nonpolyploid MK precursors, both in pathologic states and in normal human marrow.

Fig. 10. Different morphologic characteristics of blood MKB identified by the presence of PPO (arrows). (A) Nucleus (N) is very deeply indented. At the left a large platelet (P) possesses numerous mitochondria (Mi) but very rare granules. PPO is present in the dense tubular system. x 9200. (B) Nucleus with a large nucleolus (Nu) exhibits some indentations. Plasma membrane shows short and thin pseudopods. x 7650. (C) As in the bone marrow, a few MKB possess large cytoplasmic blebs free of organelles. A nucleolus (Nu) is seen in the nucleus. x 7200.
Employing a similar approach, early cells of the MK series smaller than recognizable MK have been identified by their cholinesterase activity in the rat, guinea pig, and cat, but not in man.

Evidence indicating that the peroxidatic activity detected in the endoplasmic reticulum of normal maturing MK and platelets is due to a peroxidase can be summarized as follows: (1) Enzyme activity is very sensitive to fixation with glutaraldehyde, as is that of several peroxidases from other tissues. (2) Enzyme activity is revealed in unfixed platelets, whereas catalase staining requires prior fixation. (3) The optimal pH of the DAB medium for the staining of endoplasmic reticulum is in the neutral range, while that for catalase is in the alkaline range. (4) The catalase of platelets appears located in the matrix of small particles.

Table 2 indicates the main criteria used to discriminate between PPO and MPO present in neutrophil promyelocytes and promonocytes. It should be emphasized that the most convincing difference is the presence of MPO in granules and Golgi cisternae of promyelocytes; in contrast, PPO is absent from granules and from the Golgi apparatus of MK, except for one peripheral Golgi cisterna (unpublished data). The relationship of this single reactive cisterna to the “Golgi endoplasmic reticulum-lysosome” cisterna (GERL) is questionable since GERL, which is constituted by specialized cisternae of the smooth endoplasmic reticulum, is only located at the inner face of the Golgi apparatus.

PPO has been detected in the endoplasmic reticulum of normal, maturing human MK, including those rare cells that on conventional electron microscopy have a size similar to proerythroblasts and appear “undifferentiated.” These rare immature cells lack any cytoplasmic feature associated with the maturation process of the MK cell line, i.e., granules and demarcation membranes. Thus, when identified by their PPO content, they can be considered as very immature members of the normal MK cell line, i.e., MKB. These MKB, which are often called “immature cells,” have been independently defined by their capacity to synthesize DNA in order to reach the final ploidy of MK varying from 8 N to 64 N. On stained smears only large MKB can be recog-

Table 2. Distinctions Between PPO and MPO

<table>
<thead>
<tr>
<th>Feature</th>
<th>Reference</th>
<th>PPO in MK</th>
<th>MPO Neutrophil Promyelocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intensity of the peroxidatic activity in endoplasmic reticulum of unfixed cells</td>
<td>29</td>
<td>++</td>
<td>+++++</td>
</tr>
<tr>
<td>Intensity of the peroxidatic activity in endoplasmic reticulum of cells fixed by glutaraldehyde</td>
<td>29</td>
<td>±</td>
<td>+ + +</td>
</tr>
<tr>
<td>Presence of enzymes in Golgi apparatus and granules</td>
<td>20</td>
<td>—</td>
<td>+ + +</td>
</tr>
<tr>
<td>Presence of enzymes in endoplasmic reticulum in congenital MPO deficiency</td>
<td>*</td>
<td>++</td>
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nized by their size depending upon the degree of ploidy (8 N); thus small MKB remained unidentified.

Although there was no marker for DNA replication in the leukemic blast cells, in our study these cells were considered as MKB when PPO was detected by electron microscopic examination. It must be noted that this applied to the majority (80%) of blast cells present in bone marrow and blood. The circulating blast cells were small in size and production of granules or demarcation membranes could not be detected by conventional electron microscopy.

These two cytoplasmic features have enabled us to distinguish the leukemic blasts from the so-called "micromegakaryocytes" (mMK), which have been detected by light and electron microscopy in various chronic myeloproliferative diseases. Such mMK are defined upon conventional electron microscopic examination by their small size, which contrasts with the presence of typical granules and demarcation membranes. The small size of these abnormal cells results from their inability to reach the final ploidy of normal MK.

Whether other criteria exist that favor the megakaryoblastic nature of blast cells is questionable. It must be recognized that the observation in the marrow biopsy of MK of some cells whose size was intermediate between that of small and large blasts is a suggestive finding, but by no means constitutes a definite proof of their MKB nature. Similar comments can be made about the presence of some blast cells with cytoplasmic blebs. These pseudopodial protuberances are frequently observed in the MK cell line, including mMK. They may, however, also be encountered in cells of other series and thus are not a specific morphologic marker. Moreover, the present study clearly shows that round smooth cells without any pseudopodial protuberances are indeed MKB (Figs. 4, 8). Finally, we have demonstrated that circulating and marrow blasts, some of which exhibit a "lymphoid" appearance, are in fact MKB. Such observations emphasize once again the difficulty involved in accurately classifying some cells by light microscopy, despite the use of the recommended routine staining and cytochemical techniques.

At the present time, it must be pointed out that PPO cannot be demonstrated in smears by the benzidine method; this explains the negative peroxidase reaction in blasts. Variations in the PAS reaction of blasts may appear in relation to their glycogen content, as has been demonstrated by cytochemical reactions. The glycogen concentration is independent of the PPO content since we have identified a minor population of blast cells which lacks any detectable PPO but which possesses some glycogen particles. It is tempting to propose that they represent immature MKB that have not as yet started enzyme synthesis. In the absence of any positive characteristic, however, we have been unable to identify them positively.

To date, acute leukemias involving MK precursors have been recognized rarely and termed either "megakaryocytic" or "megakaryoblastic" leukemias. In such cases, the MKB nature of the cells has been tentatively proposed because of the presence of certain morphologic features, such as transitional forms linking more or less typical MK and blast cells or the presence of cells with peripheral blebs.

From the present data it can be predicted that the frequency of acute MKB leukemia will be higher than that reflected by the current literature. It will
therefore be of particular interest to estimate the frequency of its incidence in the future among the so-called “undifferentiated” and “lymphoblastic” leukemias. A similar comment can be made about the identification of cells involved in the blast crisis of some cases of chronic myeloid leukemia.55

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Megakaryoblastic acute leukemia: identification by the ultrastructural demonstration of platelet peroxidase

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