Identification of Circulating Micromegakaryocytes in a Case of Refractory Anemia: An Electron Microscopic-Cytochemical Study

By J. Breton Gorius, B. Dreyfus, C. Sultan, A. Basch, and J. G. d'Oliveira

In a case of refractory anemia with an excess of myeloblasts in the bone marrow and with thrombocytopenia, examinations of the blood cells under the electron microscope have revealed nucleated cells similar in size to small lymphocytes. Some of these lymphocytelike cells have scanty cytoplasm identical to that of giant platelets present in the blood, which are pathologic in view of their highly developed system of membranes. In the perinuclear space and in short segments of the smooth endoplasmic reticulum, these blood micromegakaryocytes exhibit a peroxidase activity that is identical to that of mature megakaryocytes of normal bone marrow. They also spread over glass like giant platelets. In the past these nucleated cells could well have been confused with atypical lymphocytes, because on stained slides their atypical appearance makes identification difficult.

Electron microscopic cytochemistry has recently revealed a peroxidase activity in normal bone marrow megakaryocytes and in circulating platelets. This peroxidase activity appears to be confined strictly to the endoplasmic reticulum and perinuclear space of the megakaryocytes, where it persists following platelet release. In the platelet, this peroxidase activity appears localized in the dense tubule system (DTS) described by Behnke. Although the precise nature and function of this enzyme are unknown, its activity differs from that of the peroxidases found in the neutrophils and

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monocytes; the differential sensitivity of this enzyme to several inhibitors permits characterization of the megakaryocyte cell line. In the present case, a similar peroxidase activity was found in highly atypical megakaryocytes in the peripheral blood of a young girl with refractory anemia and an excess of myeloblasts in the bone marrow. Electron microscopic examination of cells from the buffy coat of this patient revealed some mononuclear cells of the size of lymphocytes, but with cytoplasmic characteristics of platelets and, in particular, the peroxidase activity in the perinuclear space and in the DTS.

Phase-contrast microscopic examination established that, like platelets, these cells exhibited spreading on a glass surface. Because these micromegakaryocytes are very difficult to identify by standard techniques of light microscopy, it is likely that in the past they have been mistaken for atypical lymphocytes.

CASE REPORT

The patient (M.B.) is a 22-yr-old unmarried white female who was apparently in good health until February 1971, when severe metrorrhagia began without apparent gynecologic pathology. Severe anemia was discovered shortly thereafter. Following transfusion of 5 U of whole blood, the patient was transferred to the Hematology Service of the Hospital H. Mondor with the diagnosis of acute myeloblastic leukemia.

On admission, the patient was afebrile and, aside from mild pallor, there were no physical abnormalities. There was no purpura, lymphadenopathy, or hepatosplenomegaly. There were a few small retinal hemorrhages.

Additional history revealed frequent headaches for 1 yr prior to admission for which the patient had taken four to six tablets/wk of an analgesic compound, noramidopyrine.

Hematologic Data

Initial blood and bone marrow studies were not entirely consistent with the diagnosis of acute myeloblastic leukemia. In the peripheral blood there were nine myeloblasts and four erythroblasts /100 cells, but the absolute peripheral polymorphonuclear leukocyte count was normal. The leukocyte alkaline phosphatase score was 4. Bone marrow examination showed up to 30% myeloblasts, some of which contained Auer bodies. There were marked erythroblastopenia and numerous megakaryocytes with a little, single or double nucleus. Fifteen days later, a second bone marrow was done, and the differential count was similar. The bone marrow was hypercellular. There were 9% erythroblasts but there were no morphologic abnormalities. There were 31% myeloblasts, 6 promyelocytes, 14 myelocytes, 7 band forms, and 13 mature neutrophils. The number of megakaryocytes was increased. The patient had a normal serum alkaline phosphatase, serum uric acid of 3 mg, blood creatinine of 0.9 mg, a protein of 6.8 g/100 ml (albumin, 4.1; globulin 1 g/100 ml), a serum bilirubin of 0.5 mg/100 ml, fibrinogen of 0.38 g/100 ml and a prothrombin time of 16 sec. The Coombs test was negative. There were no antinuclear antibodies. The karyotype was normal. Since the patient received multiple blood transfusions, the hemoglobin was not studied at this time. However, later (July 1971) a slight increase in fetal hemoglobin was found. In January 1972, studies of six red cell enzymes (hexokinase, glucose-6-phosphate dehydrogenase, glutathione reductase, 6-phosphogluconate dehydrase, acetylcholinesterase, and pyruvate kinase) revealed only a slight decrease in pyruvate kinase activity.

For the ensuing 3 wk, the hematologic picture remained unchanged. Because of the persistent thrombocytopenia in this young female, chemotherapy with a combination of cytosine-arabinoside and rubidomycin was carried out from March 31, 1971 until April 8, 1971. Prior to the initiation of therapy, bone marrow and peripheral blood specimens were again obtained for examination by phase-contrast and electron microscopy.

Within several weeks after the institution of combination chemotherapy and after a
### Table 1: Blood Examinations Summarized

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<td>9</td>
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<td>Hematocrit (%)</td>
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<td>Platelets (cu mm)</td>
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Fig. 1. Abnormal giant blood platelets. Note abundant clear channels associated with dense tubules presenting enzymatic activity. Mitochondria (M) and granulations (Gr) are seen at periphery. (A). × 17,900. Glycogen particles are rare. (B). × 15,400.
severe aplasia of 3-wk duration, an incomplete hematologic remission occurred slowly with correction of thrombocytopenia. On June 7, 1971 the platelets were 82,000/cu ml and on July 23, 1971 were 158,000/cu ml. The anemia improved without transfusion. The leukocytes count was normal by July 23, 1971. On August 10, 1971, the hemoglobin had dropped to 9 g/100 ml, and the platelets were 56,000/cu ml. On June 7, 1971, bone marrow examination showed 21% myeloblasts with some containing Auer bodies, 19% polymorphs, 39% erythroblasts, and numerous megakaryocytes. Therefore, cytosine-arabinoside (100 mg/day for 5 days) was given on August 10, 1971 with no obvious benefit. The thrombocytopenia increased, and the anemia became severe enough to require repeated transfusions. The numbers of granulocytes, however, were still normal on January 11, 1972 (Table 1). As of May 1972 the situation was stationary.

MATERIALS AND METHODS

Electron Microscopy and Cytochemistry

A heparinized blood specimen was sedimented at room temperature in a 45°-slanted tube. The leukocytes layer was removed and fixed in 1.25% distilled glutaraldehyde in 0.1 M phosphate buffer at pH 7.2 for 30 min. After washing, the fixed buffy coat remained overnight in buffer at 4°C. Some of the cells were directly fixed by 1% osmium tetroxide in 0.1 M phosphate buffer. The remaining cells were prepared for peroxidase staining by transferring them into the Graham-Karnovsky incubation medium5 with 0.2% diamino-benzidine (DAB) and at 4°C for 12-24 hr in order to improve penetration. The cells were then transferred to freshly prepared Graham-Karnovsky medium for 40 min at room temperature. The following controls were carried out: (1) exclusion from the incubation medium of hydrogen peroxide; (2) exclusion of DAB; and (3) addition of each of the following inhibitors aminotriazole (0.001 M), potassium cyanide (0.01 M), and sodium azide (0.1 M) in 0.05 M Tris-HCl buffer for 10 min before incubation in the complete medium. After incubation, the cell specimen was washed in phosphate buffer and fixed in osmium tetroxide. The preparation was then dehydrated in ethanol and embedded in Epon. Thin sections were cut in a Reichert microtome 0.1 m. When cells were incubated in DAB medium, sections were examined without or after lead citrate staining. Preparations were examined in a Philips 300 electron microscope at 40 or 60 kV with a 25 μ objective aperture.

Light Microscopy Study

May-Grünwald-Giemsa-stained blood smears were made as usual. In addition, buffy coat cells were examined with phase-contrast microscopy immediately or after 15 min of spreading on glass at 37°C.

RESULTS

Electron Microscopy Examination Before Treatment (March 3, 1971)

The majority of platelets are large (4–6 μ) with several pathologic characteristics. The membrane system is highly developed with a predominance of clear channels intermixed with the DTS (the peroxidase activity of these tubules makes them easy to identify, (Fig. 1A, B). The microtubules are dispersed rather than organized in normal marginal bands. The glycogen particle content is markedly reduced.

Nucleated cells of 5–6 μ in diameter with the cytoplasmic characteristics of abnormal platelets have been identified. These cells display some big vacuoles (Fig. 2B) and clear channels in close association with the DTS (Fig. 2A).
(See legend on facing page.)
CIRCULATING MICROMEGAKARYOCYTES

Smooth endoplasmic reticulum exhibits peroxidase activity that is also seen in the perinuclear space. The peroxidase activity is inhibited in these cells, as well as in the platelets, by 0.001 M aminotriazole, 0.01 M potassium cyanide, and 0.1 M sodium azide. It is absent when either H₂O₂ or DAB is excluded from the incubation medium. The nucleus of these cells shows numerous folds at the nuclear membrane. Chromatin clumps are abundant and localized along the nuclear membrane. In addition, other cells 6–8 μ in size have been identified with a nucleus surrounded by a narrow rim of cytoplasm. These cells could be identified as megakaryocytes by the presence of rare plateletlike granules, small mitochondria, and large vacuoles. The nuclei show numerous folds, and the perinuclear space contains the characteristic peroxidase activity (Fig. 3). Within the euchromatin, parallel fibers of unknown nature are seen. These “micromegakaryocytes,” constituted 2%–4% of the mononuclear cells in four blood specimens.

In the buffy coat of blood taken after treatment (June 15, July 2, July 23, August 20, October 8, 1971, and February 1972), the number of micromegakaryocytes is the same, (about 100/cu mm) with their size very similar to that of lymphocytes. Without cytochemistry, the distinction between these two categories of cells appears to be possible when the cells have some granules but is very difficult in some cases. However, free ribosomes are absent from circulating micromegakaryocytes. In some of these cells, glycogen particles can be identified.

Examination of Stained Blood Smears

Stained blood smears of the peripheral blood show giant platelets up to 10 μ in size that are often vacuolated. A few myeloblasts are seen, some of which contain Auer bodies. Numerous mononuclear cells, initially classified as atypical lymphocytes, have a diameter varying from 7 to 10 μ. These cells occasionally have an irregular nucleus with dense chromatin clumps. Their cytoplasm is scanty and nonbasophilic and contains large vacuoles; some of these cells have a few small azurophilic, plateletlike granulations. The presence of the micromegakaryocytes, identified cytochemically by electron microscopy, strongly suggests that these small mononuclear cells are in fact megakaryocytes of highly atypical appearance and size.

Fig. 2. Blood micromegakaryocytes. (A). Compare size of lymphocyte (L) and micromegakaryocyte at upper right. Indented perinuclear space has peroxidase activity also present in the cytoplasm in short segments of smooth reticulum. Membrane complex of this micromegakaryocyte is very similar to that of platelet in Fig. 1A. × 12,580. Upper left: a micromegakaryocyte spread on glass in phase contrast. Nucleus is surrounded by a thin zone containing vacuoles and granulations and by a large hyalomere. Note two spread platelets (arrows). (B). Note large vacuoles in this micromegakaryocyte identified by cytochemical reaction in perinuclear space. Note also a few granulations identical to those of platelets. × 12,580.
Fig. 3. Exhausted blood micromegakaryocyte. Nucleus, which presents numerous indentations, is circumscribed by perinuclear envelope containing dense product of enzymatic reaction. Note appearance of these folds due to the plane of section (double arrow). Paracrystalline structure is present in interchromatin space (single arrow). Cytoplasm is reduced to a thin zone in which, besides vacuoles, a granule (Gr) can be identified. × 22,250. Upper left: phase-contrast appearance of a micromegakaryocyte from the buffy coat. Note thin cytoplasmic layer surrounding nucleus, and the dendrites similar to those emerging from the platelets.
Examination In Vitro by Phase-Contrast Microscope

The nuclear folding seen at the ultrastructural level could also be observed in some mononuclear cells by phase-contrast examination.

After 15 min between slide and cover slip at 37°C, these mononuclear cells and platelets exhibit the same spreading. (Fig. 2A). In these spread, nucleated cells the spherical nucleus is in a central position, surrounded by vacuoles, granulations, and a large hyalomere zone. The rare monocytes (0.5%) are distinct from these micromegakaryocytes. In the latter, the centrosphere depressing the nucleus is lacking, and the fine dendritic pseudopodia extending from the cell are characteristic of platelets (Fig. 3).

DISCUSSION

Morphologic and cytochemical studies of blood specimens by electron and light microscopy have revealed in this patient small cells considered to be micromegakaryocytes. The arguments in support of this view are as follows:

1. These cells exhibit a propensity for spreading on a glass surface similar to that of platelets. They display the same cytoplasmic features that the abnormal platelets present in the patient’s blood, i.e., granulations, small mitochondria, vacuoles, membrane complex, and absence of ribosomes.

2. Their perinuclear space contains a specific peroxidase activity identical to that of normal bone marrow megakaryocytes. The nature of the enzymes detected by this technique remains unknown. This megakaryocyte peroxidase activity is seen only under the following conditions—cell fixation by low concentration of glutaraldehyde and long incubation in DAB. Both of these conditions are not necessary to demonstrate peroxidase in granulocytes and monocytes.

When comparing inhibitory effects on neutrophil myeloperoxidase and platelet enzyme with DAB as substrate, it is known that potassium cyanide and aminotriazole strongly inhibit platelet peroxidase activity, while neutrophil granule activity remains almost unchanged. In our experiments as well as in those of Ackerman and Clark, omission of H2O2 does not change peroxidase activity detected in promyelocyte and promonocyte rough endoplasmic reticulum; however, it does inhibit the activity in the endoplasmic reticulum of megakaryocytes and platelets. All these facts would suggest that the enzyme that is detected in megakaryocytes is distinct from peroxidase found in the other cell lines; such a difference could therefore be useful in identifying the megakaryocytes. Under our technical conditions, lymphocytes may exhibit an enzymatic activity located only in the mitochondria. At pH 6, this reaction appears to be constant in mitochondria of all cells, including megakaryocytes. These data would correlate with Novikoff and Goldfischer’s findings on rat liver, suggesting detection of cytochromes in mitochondria.

The small size of these mature megakaryocytes suggests that their ploidy is greatly diminished. The DNA content of these cells is now under study. Reduction in size of megakaryocytes was described in some myeloproliferative diseases. Scarcity or absence of granulations have been previously reported in acquired thrombocytopenic disorders and in some congenital thrombocytopenias.
These micromegakaryocytes were discovered in the blood of the patient whose disease is a matter of discussion. A chronic insufficiency of erythropoiesis is obvious. The leucocyte count was normal, as was the neutrophil count. Excess of monocytes was lacking. On many occasions a few myeloblasts, some of them with Auer bodies, were found in the peripheral blood. Thrombocytopenia was conspicuous. In the bone marrow, neutrophils, metamyelocytes, and myelocytes were well represented, but myeloblasts and promyelocytes were in excess. These bone marrow abnormalities were found on several occasions throughout 14 mo. These clinical and hematologic features have been described as those of atypical leukemia,\textsuperscript{18} smouldering acute leukemia,\textsuperscript{19} and myelomonocytic leukemia.\textsuperscript{20} However, leukemia is not always obvious, even at postmortem examination, when the patient dies of infectious disease, which is not infrequently the case. Therefore, some of these cases could be better named refractory anemia with excess of myeloblasts (RAEM),\textsuperscript{21,22} but the young age of the patient and the presence of Auer bodies are unusual features.

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REFERENCES

13. Undritz, E., and Nusselt-Bohaumilitzky,


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