Megakaryocyte Maturation Indicated by Methanol Inhibition of an Acid Phosphatase Shared by Megakaryocytes and Platelets

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Optimal conditions necessary for the cytochemical demonstration of megakaryocyte (Mk) and platelet acid phosphatase (AP) were determined. Methanol, a constituent of fixatives commonly used in AP cytochemistry, was found to inhibit MkAP, and the degree of inhibition varied with Mk maturity. Immature Mk contained predominantly methanol-resistant AP, and mature Mk, predominantly methanol-sensitive AP. Platelets contained methanol-sensitive AP similar to mature Mk, suggesting that this enzyme provides an index of platelet formation by Mk. Soluble platelet AP showed three bands on polyacrylamide gel electrophoresis, visualized by the same reactions applied cytochemically. Two bands, accounting for 98% of the platelet AP activity, were slow moving and methanol sensitive; and one fast moving band accounting for 2% of activity was methanol resistant. Measurement of Mk and platelet AP isoenzymes may prove to have applications in evaluating Mk function.

Although different stages of megakaryocyte (Mk) maturation can be evaluated morphologically after conventional bone marrow staining, the criteria are not precise, and pathologic conditions may alter the accuracy of the interpretation. In order to define maturation more precisely, histochemical techniques have recently been applied in attempts to correlate Mk maturation with one or another chemical reaction. Measurements of glycogen or DNA have not been rewarding, for all stages of Mk contain PAS-positive material and may have the same degree of polyploidy. Although acid phosphatase (AP) has been shown to be present in the cytoplasm of Mk, systematic studies of changes in AP with Mk maturation in human beings have not been done, and MkAP isoenzymes have not been noted.

During application of cytochemical techniques for AP we have found that different stages of Mk, identified by conventional morphologic criteria, show differences in their AP activity and that peripheral blood platelets have AP characteristics similar to the cytoplasm of mature Mk. This study is concerned with the basis for observed differences in MkAP activity and for the correlation between MkAP activity and platelet formation.

Materials and Methods

Cell Fixation

In the standard technique, fixation of cells on glass slides was performed in a mixture of 10% formalin in absolute methanol for 20 sec at 0°-4°C. Other conditions of fixation tried were as...
follows: 60% citrate-buffered acetone, pH 3.0, for 20 sec at room temperature; 10% formalin in 0.1 M acetate buffer, pH 5.0, for 20 sec at 0°-4°C; 90% methanol in 0.1 M acetate buffer, pH 5.0, for 20 sec at 0°-4°C; 3% glutaraldehyde in 0.1 M phosphate buffer, pH 7.0, for 20 sec at 0°-4°C. Only smears fixed within 2 hr of obtaining bone marrow were suitable for AP reactions; after fixation smears could be stored in the frozen state for as long as 2 days. It was also necessary to use unfixed smears within 2 hr for AP reactions. Observations on the effects of different fixatives are presented in the Results.

Acid Phosphatase Reaction

The standard method for cytochemical investigation of MkAP or platelet AP (PAP) was a simultaneous azo-dye coupling technique with the α-naphthol AS-MX phosphate fast blue RR system, according to Burstone,11,12 and Markovic.13,14 Substrates and diazonium salt were purchased from Sigma Chemical Co., St. Louis, Mo. Incubation mixtures were freshly prepared by adding 2 ml concentrated substrate (5 mg α-naphthol AS-MX phosphate dissolved in 0.25 ml N-N-dimethylformamide added per ml 2.5 M acetate buffer, pH 5.2) to 48 ml of distilled water containing 25 mg of fast blue RR diazonium salt.

Other cytochemical reactions that were tried included the use of substrates α- and β-naphthol phosphate, α- and β-glycerophosphate, α-naphthol AS-TR phosphate, α-naphthol AS-BI phosphate, and 6-benzoyl 2-naphthol phosphate in combination with fast blue RR, fast red violet LB, and fast garnet GBC diazonium salts.15-19 None of these reactions appeared to visualize MkAP or PAP as well as the chosen standard technique. Control smears were inactivated by heating at 90°C for 3 min before reaction with the complete incubation mixture, or were treated with incubation mixtures in which the substrate or the diazonium salt was omitted.

Counterstaining

After the AP reaction, smears were counterstained with a 1% solution of methylene blue. Counterstaining was performed over the pH range 6.0-7.5 in 0.1 M phosphate buffer for 5-15-min periods at room temperature. Best results were obtained at pH 7.0 for 10 min, which was used routinely.

May-Grunwald-Giemsa (MGG) and hematoxylin staining were also used for confirmation of the results with methylene blue.

Megakaryocyte Classification

With methylene blue as the counterstain, Mk were identified according to the MGG criteria for classification, which takes into account cell size, nuclear/cytoplasmic (N/C) ratio, number of nucleoli, nuclear chromatin density, and color of the cytoplasm. Identification of stages of Mk maturation was facilitated by studying sequential bone marrow samples from a patient with a rare form of cyclic thrombocytopenia.20,21 Results on this patient’s Mk were verified by studies of the marrows of 80 cases of acute leukemia at different stages of remission following chemotherapy and of the Mk of two hematologically normal individuals from whom marrow was obtained for other purposes.

The patient with cyclic thrombocytopenia was a 50-yr-old man who developed thrombocytopenia at approximately 30-day intervals with no apparent underlying cause and no abnormalities of white cells or red cells. A series of bone marrow samples, shown by the arrows in Fig. 1, were obtained during various stages of two platelet cycles. At the time platelets were at their lowest level (Fig. 1, day 0), and again when they were falling (Fig. 1, day 28), the bone marrow showed a paucity of Mk but was otherwise normal. Platelet survival determined with 51Cr-labeled autologous platelets was normal during a subsequent fall in platelets.

Megakaryocyte stages identified in bone marrows taken during two platelet cycles were as follows.

Stage I. Following the amegakaryocytic thrombocytopenic phase in patient E.B., the earliest recognizable Mk appearing de novo in the bone marrow (Fig. 1, day 35) were round cells, 15-30 μm in diameter (measured by an ocular micrometer), with an N/C ratio of 1/1 or slightly less.
(estimated). Nuclei were oval or kidney shaped and some cells were binucleated. Nuclear chromatin was a fine reticular network and one or more nucleoli were present. The scanty ring of cytoplasm was dark blue. These cells were considered to be megakaryoblasts according to MGG classification.

Stage II. When platelets were rising (Fig. 1, days 7 and 41), approximately 10% of the Mk were 25–50 μm in diameter with an N/C ratio between 1/1 and 1/2. The nuclei were indented or multilobulated. Nuclear chromatin appeared like that of stage I, except for the presence of some coarse, more heavily stained, occasionally clumped strands. A small proportion of cells contained visible nucleoli. Cytoplasm was lighter blue than in stage I cells. This group of cells was considered to be promegakaryocytic. Along with stage II cells, there were also polylobulated, somewhat larger cells than stage II cells containing no visible nucleoli and more clumped nuclear chromatin. These cells had an N/C ratio of approximately 1/1.5, and were considered to be early mature Mk; they were referred to as Stage IIa cells.

Stage III. The next morphologically distinct group of Mk, when platelets had reached the normal levels (Fig. 1, day 12), were 40–100 μm in diameter, irregular in shape, and invariably had an N/C ratio less than 1/2. These cells were polylobulated and contained coarse chromatin and no nucleoli. There were large amounts of light blue cytoplasm. These cells were considered to be mature Mk. Along with stage III cells, degenerative forms of Mk, essentially naked nuclei, or nuclei with a shred of cytoplasm were seen. The degenerated cells were not considered for classification.

In normal marrow there were 4%–10% stage I Mk, 10%–25% stage II, 50%–70% stages Ila and III, and approximately 10% naked nuclei.

The above stages of Mk maturation, based on conventional staining techniques (MGG and methylene blue), appeared to correspond closely to the same stages defined by criteria based on electron microscopic ultrastructural analysis and autoradiographic measurements of 3H-thymidine incorporation, namely: stage I—megakaryoblasts, characterized by incorporation of 3H-thymidine as evidence of nuclear replication and the presence of relatively few granules and poorly developed platelet demarcation membranes; stage II—promegakaryocytes, characterized by larger size, a decreasing N/C ratio, prominent polyribosomes and granules, and an extensive demarcation system; stage III—granular or mature megakaryocytes, representing the majority of normal bone marrow Mk, distinguished from stage II chiefly by increased cytoplasm, prominent nuclear pyknosis, decreased mitochondrial size, more prominent granules, and more extensive development of pseudopodia as platelet release commenced.

**Estimation of Enzyme Activity**

The intensity of AP was estimated by the size, density, distribution, and color of granules representing the final reaction product of the AP reaction. Degrees of activity could be estimated from 0–4+. Lowest activity, 1+, appeared as an island of up to 10 small discrete, fine light blue granules localized in one part of the cytoplasm. Intermediate activity, 2+, appeared as fine, single light blue-purple granules scattered uniformly through the cytoplasm. Strong activity, 3+, consisted of many coarse single granules, larger than those of 2+ activity, uniformly distributed...
through the cytoplasm. The strongest activity, 4+, appeared as coarse, dense, intensely purple granulation with a tendency for granules to clump together and obscure the nucleus.

For semiquantitative scoring the sum of the estimated enzyme activity per 100 cells was used.

**Platelet Acid Phosphatase**

Platelets were prepared from blood anticoagulated with acidified acid-citrate-dextrose and centrifuged at 1100 g for 3 min. Smears of platelet-rich plasma (PRP), prepared in the same manner as peripheral blood smears, were used either unfixed or after fixation in acetone, formalin, methanol, or formalin-methanol with the same cytochemical procedures applied to Mk.

The same standard cytochemical methods were also applied on electrophoretically separated isoenzymes of PAP. To obtain soluble PAP, PRP was centrifuged at 1100 g for 15 min and the button of platelets obtained was washed three times in 0.15 M NaCl to remove plasma proteins. Washed platelets suspended in 0.15 M NaCl at a concentration of 10^6/ml were treated with 5% Triton X-100 in water for 30 min at 4°C, and the disrupted cell suspension was centrifuged at 100,000 g to obtain the supernatant soluble protein fraction. To separate PAP isoenzymes the protein fraction was electrophoresed in 7.5% polyacrylamide gel containing 0.5% Triton X-100 at pH 4.5, using 6-8 mA per tube for 45 min at 4°C. The gels were rinsed in 0.1 M acetate buffer, pH 5.0, and reacted with the same AP substrate described above for Mk, either directly or after fixation in formalin-methanol, formalin, methanol, or acetone at the same concentration used for fixing the cell preparations. Densitometric recordings of the AP reaction in the gels were made with a Gilford densitometer.

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![Fig. 2. Acid phosphatase activity of megakaryocytes from the patient whose clinical course is shown in Fig. 1. Stage I, II, IIIa, and III megakaryocytes as defined by the morphologic criteria described in the text are shown. The dark granules of acid phosphatase reaction are light blue to dark purple. Degree of activity evaluated as described in text. (A) Stage I shows 1+ activity (an island of granules is encircled). (B) Stage II shows 2+ activity. (C) Stage IIIa shows 3+ activity. (D) Stage III shows 4+ activity.](image-url)
RESULTS

Megakaryocyte Acid Phosphatase

In unfixed smears all stages of Mk showed AP activity (Fig. 2), the density of the reaction increasing parallel with the degree of maturation, as shown by examples of individual cells in Fig. 2 and on the basis of AP score in Fig. 4. When smears were fixed in formalin–methanol (Fig. 3B, D), stage III cells showed markedly decreased AP activity (Fig. 3D) compared to the unfixed smears, in which their activity was the highest (Fig. 3C).

Figure 4 shows that Mk in smears stained unfixed or fixed in glutaraldehyde or formalin had essentially the same AP activity, which roughly paralleled the degree of maturity. The activity of cells in smears fixed in acetone was somewhat decreased, but the degree of decrease was similar for all stages of maturity. All Mk in smears fixed in formalin–methanol or buffered methanol showed decreased AP activity, the decrease being most marked in stage III cells, in which activity was essentially absent. Thus methanol appeared to have a marked inhibitory effect specifically on AP of stage III Mk. The inhibitory effect of methanol was time and concentration dependent. The optimal differential effect between stage III and other Mk was obtained by a 20–30-sec exposure to 90% or absolute methanol. Formalin was included in the standard method to improve fixation.

Platelet Acid Phosphatase

In smears of PRP that were used either unfixed or fixed in glutaraldehyde, formalin, or acetone, PAP activity appeared as dense, purple granules in almost all cells. PAP had the same characteristics as AP in stage III Mk. When formalin–methanol or methanol was used to fix PRP smears, PAP was demonstrable in only about 5% of platelets. Figure 3 shows the difference between unfixed (Fig. 3E) and formalin–methanol fixed (Fig. 3F) PRP.

Soluble platelet proteins electrophoresed in acrylamide gels and reacted with AP substrate showed two clearly separated bands of activity (Fig. 5). The fast moving fraction (fraction 1; Fig. 5A-a) was 10.5–13.5 mm from the origin and represented approximately 2% of the total activity. The slow moving fraction, 2.5–7.0 mm from the origin, represented 98% of the total activity (Fig. 5C-a). The slow moving fraction was not homogeneous, for a third fraction close to the origin became visible with prolonged electrophoresis (3 hr) which represented about 10% of the activity (Fig. 5B, D). Since the fast moving fraction became less distinct with prolonged electrophoresis and because fractions 2 and 3 had the same response to inhibitors, a 45-min electrophoresis was optimal for distinguishing methanol sensitivity of PAP.

When the gels were treated with 10% formalin–methanol before the AP reaction, the density of the slow moving fractions decreased by 50%–60% in 30 min, 80%–90% in 45 min (Fig. 5A-b, C-b), and 99% in 75 min (Fig. 5A-c, C-c); after further exposure it could not be detected. The density of the fast moving fraction decreased by only about 10%–20% in 75 min. Buffered methanol produced 70%–80% inhibition of the slow moving fractions in 45 min and did not inhibit the fast moving fraction.
Fig. 3. Continued on next page.
Fig. 3. Acid phosphatase activity in megakaryocytes and platelets on unfixed and formalin-methanol fixed smears. (A) Megakaryocyte stage Ila, unfixed, activity 3+. (B) Megakaryocyte stage Ila, fixed by formalin-methanol, activity 3+. (C) Megakaryocyte stage III, unfixed, activity 4+. (D) Megakaryocyte stage III, fixed by formalin-methanol, activity 0. (E) Platelet-rich plasma smear, unfixed, activity present. (F) Platelet-rich plasma smear, fixed by formalin-methanol, activity absent.

Buffered formalin or buffered acetone produced approximately 15% inhibition of all AP fractions in 45 min, and 0.04 M CuSO₄ completely inhibited all AP activity in the gels in 45 min.

DISCUSSION

We have found that methanol, the usual fixative applied in phosphatase cytochemistry, markedly inhibits an AP component, most likely an isoenzyme, that is present in the cytoplasm of mature Mk and in platelets, but does not sig-

Fig. 4. Degree of activity scored 0–4+ as described in the text. Megakaryocyte stages shown on abscissa. Symbols shown on the graph represent results with formalin–methanol (F/M), unfixed (O), with buffered glutaraldehyde (GA/B), with buffered formalin (F/B), with buffered methanol (M/B), and with acetone. Values are based on scoring a total of 900 stage III cells, 350 stage II and Ila cells, and 60 stage I cells. Content of fixatives described in Materials and Methods.
Fig. 5. Soluble platelet protein prepared, electrophoresed, and reacted with acid phosphatase substrate as described in Materials and Methods. (A) Electrophoretic conditions optimized to increase prominence of band 1 with bands 2 and 3 not separated distinctly. Inhibition of acid phosphatase by formalin-methanol after exposure for 45 min (b) and 75 min (c) compared to untreated gel (a). (B) Electrophoretic time increased to 3 hr for clearer separation of band 3. (C) Densitometric scan of the gels of (A). (D) Scan of the gel of (B).

nificantly affect another AP component, or isoenzyme, present in the cytoplasm of immature Mk. That the inhibition observed is not a form of enzyme latency induced by methanol is indicated by the failure to observe differences in activity when fixed smears were frozen and thawed, by parallel results obtained on cellular and soluble AP, and by the finding that CuSO₄ completely and nonselectively inhibited AP activity in gels under similar conditions of incubation. Since mature Mk and platelets contain a relatively high concentration of methanol-sensitive AP, the presence of this isoenzyme in Mk may be an indication of platelet formation. Comparison of MkAP reactions of smears fixed by methanol and formalin-methanol with those of unfixed smears, or smears fixed by acetone, glutaraldehyde, or formalin, should provide, along with standard
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morphology based on panoptic staining, an indication of the number of mature Mk present. This information may prove to be a useful index of platelet production.

It is conceivable that in certain clinical conditions, for example, those involving rapid platelet turnover, there may be discrepancies between morphologic and biochemical criteria of Mk maturity which would be helpful diagnostically. Comparison of Mk methanol-sensitive and -resistant AP in disorders such as idiopathic thrombocytopenic purpura and other thrombocytopenic syndromes may provide clues to differentiating between defects involving platelet destruction or production. It is also conceivable that, under pathologic conditions, platelets may be formed by less mature Mk, which may be reflected in the relative content of methanol-sensitive and -resistant PAP measurable quantitatively by electrophoresis.

Three bands of PAP were seen on electrophoresis. The two slow moving bands, accounting for 98% of total activity, could be inactivated by methanol, while the fast moving band, accounting for approximately 2% of the total, was resistant to methanol. Since AP that predominates in younger Mk was not sensitive to methanol, methanol-resistant PAP may reflect the amount of that isoenzyme left in Mk when platelets form. The two slow moving PAP bands had the same sensitivity to methanol; it is possible that the slowest moving band, 3, is simply a dimer of band 2.

These findings suggest that there may be other cytochemical approaches to defining Mk function.

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