Cytocchemical Markers of Differentiation in Acute Leukemia

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Abstract

Material from 110 adult patients with acute myeloid leukemia, acute lymphoblastic leukemia, and chronic granulocytic leukemia (CGL) in blast crisis (BC) was studied by light microscopy (LM) and transmission electron microscopy cytochemistry in order to examine the sensitivity of the tests used to define myeloid differentiation.

Granulocytic differentiation was best visualized by the myeloperoxidase (MPO) reaction. By LM, the Hanker method using 3,3′-diaminobenzidine at pH 7.6 was the most sensitive in demonstrating MPO and the presence of ϕ bodies in most acute myeloid leukemias and some CGLs in "myeloid" BC. ϕ bodies were more numerous when the reaction was carried out at pH 9.7, suggesting that they originate from catalase-containing microperoxisomes. MPO at transmission electron microscopy level (with 3,3′-diaminobenzidine) was more sensitive than the LM techniques because it demonstrated myeloblasts with few positive granules and reaction product in the endoplasmic reticulum. The platelet-peroxidase technique of Breton-Gorius, using a higher concentration of 3,3′-diaminobenzidine and unfixed material, was of even greater sensitivity for the study of myeloid precursors and allowed the identification of megakaryoblasts in CGL in BC.

Monocytic differentiation was best recognized by LM using an esterase method (naphthol AS-acetate or α-naphthyl acetate) and the acid phosphatase reaction. By transmission electron microscopy, the acid phosphatase reaction was positive in small electron-dense granules which are MPO negative and appear to be characteristic of the early maturation stages of mononoblasts.

The techniques described were of value in the classification of CGL in BC and demonstrating the myeloid component in 8 of 56 (14%) acute myeloid leukemia cases which were positive with the enzyme terminal transferase. Five of the latter were "pure" myeloid cell proliferations, and 3 others were "mixtures" of lymphoblasts and myeloblasts (or monoblasts), but with lymphoid cells predominating in 2 of them.

Introduction

Significant advances have been made in the last 5 years in the characterization of leukemic cells. Most forms of ALL can be defined by their membrane and enzyme phenotype (14, 19), and the various types of AML can be classified on morphological and cytochemical grounds (2, 3). There is also a greater awareness of the fact that the patterns of differentiation into "myeloid" and "lymphoid" lines may not be so apparent in some cases. This is often manifested in cases of CGL in BC and in the minority of AML cases which are positive with TDT (10, 12, 19-21, 28).

We have examined the contribution of cytochemical techniques, by LM and TEM, to the classification of the acute leukemias; in this report, we have looked specifically at the sensitivity of the various techniques used in order to determine which ones are more useful to characterize the early maturation stages of myeloid blast cells.

Materials and Methods

BM and PB materials from 110 adult patients suffering from AML (56 patients), ALL (24 patients), and CGL in BC (30 patients) were the basis of our study. Films were stained with May-Grünwald-Giemsa and with the following cytochemical techniques: (a) MPO with 3 different substrates, benzidine hydrochloride, 2,7-fluorenediamine (1), and DAB. The latter was used according to the simplified technique of Hanker et al. (16) for the demonstration of HPO at pH 7.6, but using Giemsa as counterstain. Some experiments were carried out at pH 9.7 for 10 min in the presence of KCN, in order to demonstrate catalase activity (24). (b) AP according to the method of Goldberg and Barka (11); (c) nonspecific esterases were studied with 2 substrates, NASA and ANAE at pH 6.1 (31), both with and without NaF. Most cases were also tested with Sudan Black B and the periodic acid-Schiff reaction.

Ultrastuctural studies (TEM) were carried out with conventional morphology (lead citrate and uranyl acetate stains) and with the cytochemical reactions of MPO in Graham and Karnovsky (13) DAB-H2O2 medium, AP with an adaptation of Gomori's method (25) and platelet-peroxidase (6, 22). The platelet-peroxidase reaction was tested only in cases of CGL in BC using unfixed material according to the method of Roels et al. (26) using a 4-fold greater concentration of DAB in Ringer-Tris buffer. TDT estimations were performed by a biochemical assay (18, 19) and were part of a larger collaborative study (10). Lysozyme (muramidase) was investigated in selected cases in the serum and urine and by a cytobacterial method (8) on PB and BM cells.

Results

Markers of Granulocytic Differentiation

Granulocytic differentiation was demonstrated by the presence of MPO activity. According to the substrate used and other technical variations, a greater sensitivity for the demonstration of peroxidase activity at LM and TEM can be achieved. These conclusions are summarized in Table 1, and the experimental basis of this will be described below.

LM. There was no major difference between the techniques using benzidine hydrochloride and 2,7-fluorenediamine (1). In 17 cases of AML and CGL in BC (myeloid type), a comparison...

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was made between the MPO reaction using benzidine hydrochloride and DAB (HPO) (16) (Table 2). Three features were compared: (a) the overall positivity, which was greater in every case using DAB; (b) the incidence of Auer rods, which were seen with the HPO technique in 4 times as many cases; and (c) the incidence of <j> bodies (16, 17) which were demonstrated in a majority of cases, including 3 of 5 cases of CGL in BC, only with DAB (Table 2). The distinction between Auer rods and <j> bodies was not always easy; the thin, usually fusiform long rods were designated <j> bodies (16) (Fig. 1, A and B) and the shorter, thicker rods were designated Auer rods (Fig. 1C). As discussed below, it may not be essential to make this distinction. Both techniques were negative in ALL, and no <j> bodies were seen in any of the 7 cases studied with the HPO method.

In order to test the possibility that the HPO technique was demonstrating catalase activity, as well as MPO, films of 9 cases were tested simultaneously at pH 7.6 and at pH 9.7. For this experiment, <j> bodies and Auer rods were recorded together. As shown in Table 3, there was a marked increase in the incidence rods at pH 9.7 with or without KCN, which is the optimum pH for the demonstration of catalase (24, 26). In contrast, the overall diffuse positive reaction, other than in the rods, was considerably decreased at pH 9.7. At the alkaline pH, <j> bodies were demonstrated readily in the cases of CGL in BC.

TEM. The majority of cases were studied with the MPO technique. MPO was positive in the primary granules (over 0.2 μm) of myeloblasts and often in the rough endoplasmic reticulum and Golgi membranes (Fig. 2A); less often, the nuclear membranes were positive. The MPO-positive granules in promonocytes were as a rule smaller than in myeloblasts and promyelocytes (Fig. 2B). The reaction in the membranes was seen more consistently when the incubation in the DAB medium together. As shown in Table 3, there was a marked increase in the overall catalase reaction at pH 9.7. For this experiment, <j> bodies and Auer rods were recorded together. As shown in Table 3, there was a marked increase in the incidence rods at pH 9.7 with or without KCN, which is the optimum pH for the demonstration of catalase (24, 26). In contrast, the overall diffuse positive reaction, other than in the rods, was considerably decreased at pH 9.7. At the alkaline pH, <j> bodies were demonstrated readily in the cases of CGL in BC.

The platelet-peroxidase technique was investigated in 12 of 24 cases of CGL in BC. Two main features were observed: (a) in myeloblasts and promonocytes the number of positive granules, the reaction in the rough endoplasmic reticulum, nuclear membranes, and Golgi apparatus, were more readily demonstrated with the platelet-peroxidase technique rather than the MPO technique; (b) megakaryoblasts were characterized by a positive platelet-peroxidase reaction in the rough endoplasmic reticulum and nuclear membranes but not in the granules or Golgi membranes (Fig. 4). Megakaryoblasts were the predominant cell type in 2 cases of CGL in BC and were seen as a minor component in 2 other cases. In none of the latter could they be recognized by LM, although in one of them they were demonstrated by TEM morphology.

Markers of Monocytic Differentiation

In a previous study, we have shown that esterase and AP activities were an early feature of monoblasts while MPO and lysozyme were a relatively late differentiation characteristic (9). We have subsequently studied these techniques in more detail by LM and TEM (25).

LM. We have compared the NASA, ANAE, and AP reactions in 14 AML patients (M4 and M5) and 6 patients with CGL in BC with predominant monocytic differentiation. ANAE was the more consistently positive of the 2 esterase reactions, only one case being falsely negative. NASA was weak or equivocal in 3 cases, which were shown to be monocytic with the other techniques, and was positive in the rest. AP was consistently positive in a diffuse manner in all cases, but in 3 it was weaker than either ANAE or NASA. The most reliable results are obtained when either 2 of the above techniques are used in a patient population.

As in our previous study (9), we have confirmed here that lysozyme measurements are sometimes negative in cases of
AML M5, a poorly differentiated type (2). However, when this test is either positive as high serum levels or positive in a majority of blast cells by the cytochemical method (8), it is specific for monocyte differentiation. This was important in relation to the observation of positive TDT in some AML cases (see below).

Two important aspects were recognized when assessing ANAE or NASA at LM level. The pattern of reaction in monocytic cells was strong and diffuse and sensitive to NaF. A localized or granular reaction was also seen in megakaryoblasts (also moderately NaF sensitive) and some lymphoblasts. As in normal PB T-lymphocytes (7), the ANAE reaction in lymphoblasts was often, but not always, NaF resistant.

**TEM.** Because techniques for the demonstration of esterases at ultrastructural level were not readily available, we have concentrated our attention on the localization of AP and MPO during monocytic maturation. A positive AP reaction is the most consistent finding by TEM (25). The enzyme activity is localized in very small electron-dense granules (less than 0.1 μm or between 0.1 and 0.2 μm in diameter), which appear to be the earliest differentiation feature seen in monoblasts (Fig. 5). These granules were always MPO negative (25). A summary of the main findings by ultrastructural cytochemistry during monocytic and granulocytic maturation in 20 cases (15 AML and 5 CGL in BC) is given in Table 4.

### CGL in BC

The above techniques, together with immunological marker studies (10, 14) and TDT estimations (19), have allowed us to assess the incidence of the various forms of BC in 30 cases of Philadelphia chromosome-positive CGL. These findings are summarized in Table 5. Mixtures were common, and in one patient (WBC, 215 x 10^9/liter), we have been able to demonstrate 4 lines of differentiation with a different expression in BM and PB: (α) lymphoblasts (70% in BM and 20% in PB) which were TDT and anti-ALL positive, MPO negative (Fig. 6, A and B), and platelet-peroxidase negative (not shown); (β) promonocytes (65% in PB and 25% in BM) (Fig. 2B); (c) myeloblasts (less than 5% in both tissues) (Fig. 2A); and (d) megakaryoblasts (5% in PB and 2% in BM), demonstrated by the platelet-peroxidase reaction (Fig. 4).

### TDT-positive AML

Of 56 cases of AML studied at Hammersmith Hospital, 8 (14%) were found to have a positive TDT, the values ranging from 3.6 to 40 units x 10^8 cells (mean, 17). Levels in nonleukemic BM with this technique are less than 2 units x 10^8 cells (18, 19). The blast cells in 5 cases were found to be predominantly myeloid (60 to 100%). Four were myeloblastic (M1, M2) (Fig. 7), and one was monocytic (M5) with high serum and urine lysozyme levels. Sixty percent of the cells in the latter case were TDT positive by immunofluorescence (performed by Dr. G. Janosy). Three cases had "mixtures" of lymphoblasts and myeloblasts or monoblasts. In 2 of these cases, lymphoblasts predominated, constituting 70 to 80% of the leukemic cells, and 20 to 30% were myeloblasts (Fig. 3). In the third, varying proportions of lymphoblasts (20 to 80%) and monoblasts (30 to 50%) were seen during different stages of evolution. The lymphoblasts had L1 or L2 morphology (2), and 2 cases they were also shown to be TDT positive by immunofluorescence (performed by Drs. M. F. Greaves and G. Janosy) and had a localized ANAE reaction which was resistant to NaF.

### Discussion

This study was aimed at assessing critically the contribution of LM and TEM cytochemical methods in the characterization of blast cells in acute leukemia. A definition of the limits which can be achieved with these techniques is important, so that they can be best correlated with the investigations of immunological and enzyme markers. While the latter are best suited at present to define the various pathways of lymphoid maturation from early precursor cells, shown by other contributors in this volume, cytochemistry is more suitable for assessing the patterns of myeloid differentiation into granulocytic, monocytic, and megakaryocytic lines.

MPO is a well-known marker of granulocytic differentiation. We have compared 2 conventional MPO techniques at LM (1) with the HPO method of Hanker using DAB (16, 17). The latter not only demonstrates the so-called φ bodies in myeloblasts (17) but also proved to be more sensitive for the overall demonstration of MPO activity, including the number of cases with Auer rods (Table 2). The greater sensitivity of this reaction may be due to the use of the DAB medium which is the same substrate used for TEM (13).

We have demonstrated φ bodies in most, but not all, AML and in addition in some CGL in "myeloid" BC. We believe, as does Hanker (15, 17), that φ bodies may represent a crystalline aberration of catalase-containing granules, the microperoxidases of Novikoff et al. (24) known to be present in human promyelocytes and promonocytes and visualized by TEM using DAB in alkaline medium (4). Our experiments with the reaction at pH 9.7 in the presence of KCN (Table 3) strongly support this hypothesis. However, since at pH 9.7 the overall reaction due to MPO decreases, we would recommend using the test.
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routinely at pH 7.6 (16), inasmuch as this will permit the optimum demonstration of MPO together with $ bodies and Auer rods. In problem cases, it may also be worth carrying out the reaction in the alkaline medium.

TEM techniques for MPO have the advantage that they can identify weak reactions in isolated leukemic cells. This could be very useful to identify a minor or major myeloid component in some TDT-positive acute leukemias, as shown in this study. We have also shown here that the platelet-peroxidase technique (6), in addition to allowing the demonstration of megakaryoblasts, has a greater sensitivity in showing MPO activity in myeloblasts and some monoblasts. As we have not investigated the PPO technique in cases other than GCL in BC, we could not determine the possible incidence of de novo acute megakaryoblastic leukemia (5) in this series. However, we have recently demonstrated by means of this technique the megakaryoblastic nature of the blast cells in 3 cases presenting with features of acute or "malignant" myelofibrosis.4 The main difference with the platelet-peroxidase reaction between myeloblasts and megakaryoblasts is the absence, in the latter cells, of reaction product in granules and Golgi membranes. Megakaryoblasts, megakaryocytes, and platelets are, on the other hand, invariably negative with the conventional MPO reaction (6).

An ultrastructural study of monocytic differentiation with the MPO technique allows only the identification of promonocytes. LM techniques have shown for many years that NaF-sensitive esterase reactions are a characteristic feature of the monocytic series (27). Because TEM techniques for esterase were not fully developed at the time of this study, we have examined another enzyme, AP, which at LM is also consistently positive in monocytic leukemia. The demonstration of AP at ultrastructural level has facilitated the identification of small lysosomal granules, MPO negative and AP positive, which appear to be a unique and characteristic early feature of monoblasts. In this study, we have confirmed our previous observations (25) in a larger number of M5 leukemias. The changes observed during monocytic differentiation are distinct from those seen during the development of myeloblasts (Table 4).

The cytochemical techniques described here, together with immunological and enzyme marker studies described elsewhere (10, 14, 19), have enabled us to classify more objectively a series of 30 consecutive cases of CGL in BC (Table 5). We have confirmed the relatively high incidence of lymphoblastic transformation seen by others, and we have shown a high frequency of cases with monocytic features (M4 and M5 type). Similarly, the presence of mixtures of blasts of various types was relatively common, highlighted by a case described above showing 4 lines of differentiation. These findings further emphasize that CGL is a disorder affecting a common stem cell precursor of myeloid and lymphoid cells (14).

This study has also thrown light on the nature of some acute leukemias which often present difficulties for their correct classification, inasmuch as they simultaneously show features of myeloid and lymphoid differentiation. We have identified 8 cases (14%) in a series of AML patients tested routinely with a biochemical assay for TDT (18, 19). Three cases had mixtures of lymphoid and myeloid blasts. Lymphoblasts demon-

References


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Fig. 1. Light microscopy of myeloblasts, φ bodies (A and B), and Auer rods (C). arrows. HPO technique, x 1,400.
Fig. 2. TEM of a case of CGL in "mixed" BC. A, myeloblasts with large MPO-positive granules and weak reaction in the rough endoplasmic reticulum. × 12,500. B, promonocytes with smaller MPO-positive granules. × 15,000.
Fig. 3. Case of TDT-positive acute leukemia showing 3 MPO-negative blasts and one with numerous MPO-positive granules of varying size. × 6,000.

Fig. 4. TEM of a case of CGL in "mixed" BC showing a megakaryoblast with a positive platelet-peroxidase reaction in rough endoplasmic reticulum and nuclear membrane. Other cells from this case are illustrated in Figs. 2 and 6. × 10,000.
Fig. 5. TEM of a monoblast showing numerous electron-dense granules scattered throughout the cytoplasm. These granules were AP positive and MPO negative (not shown). Lead and uranyl acetate stain, x 10,000.

Fig. 7. TEM of a myeloblast from a TDT-positive AML (M2) showing strongly positive MPO reaction in granules and rough endoplasmic reticulum. All the leukemic cells in this case showed these features. x 9,000.
Fig. 6. Case of CGL in BC with "mixed" blast cell proliferation (also illustrated in Figs. 2 and 4). A and B, TEM of lymphoblasts. A, stained for morphology; B, stained with a negative platelet-peroxidase reaction. × 12,000.