Determination of Ultrastructural Peroxidases and Immunologic Membrane Markers in the Diagnosis of Acute Leukemias

By Jean Pierre Marie, Jean Yves Perrot, Claude Boucheix, Jacqueline Zittoun, Marie Claire Martyre, Michèle Kayibanda, Claude Rosenfeld, Zohar Mishal, and Robert Zittoun

Detection of membrane markers and ultrastructural peroxidase activity was carried out on the blasts of 16 apparently nonmyeloid adult acute leukemias. These patients were selected from 73 adult leukemic patients by the negativity of their routine cytochemical myeloid markers: i.e., myeloperoxidase, chloroesterase activity, and Sudan Black B staining. B and T acute lymphoid leukemias (ALL) were excluded from the study. After concurrent testing from human T lymphocyte antigen (HuTLA), common ALL antigen (cALL), la-like antigens, and peroxidase activity at the electron microscopic level (POEM), only two patients remained undifferentiated (cALL; POEM−). The other cases were classified as following: 6 common ALL (cALL+; POEM+), 1 pre-T-ALL (cALL+, HuTLA−; POEM+), 5 very poorly differentiated acute myelogenous leukemia (AML) (cALL−, POEM+), and 2 mixed leukemias (cALL−, POEM+). Terminal deoxynucleotidyl transferase activity (TdT) was measured in 7 cases and was found to be present at high levels in 4 cases of cALL and in the 2 cases of acute undifferentiated leukemias (AUL); it was absent in two cases of AML. Cytogenetic analysis had showed that 2 of the cALLs, 3 of the AMLs, and the 2 mixed leukemias were Ph1+ positive. We conclude that POEM detection is useful in apparently nonmyeloid leukemias with negative immunologic lymphoid markers, and that the existence of a Ph1 chromosome should be investigated, particularly in the unusual case of mixed (lymphoid-myeloid) acute leukemia.

Two methods are actually used to classify acute leukemias. The FAB classification1 is based essentially on morphological criteria, with little cytochemical staining. The second classification, using immunologic methods and enzyme assays, has led to the division on nonmyeloid leukemia into subgroups.2 In addition to the classical markers for B and T lymphocytes, which include surface membrane immunoglobulins (SmIg), sheep red blood cell receptors (SRBC), and human T lymphocyte antigen (HuTLA), new antisera have been raised to characterize leukemia cells. Greaves et al.3 have shown the presence of a glycoprotein common to the majority of non-T, non-B acute lymphoblastic leukemias (ALL) and to the lymphoblastic cells of the blast crisis in chronic myeloid leukemia (BC-CML), i.e., the common ALL antigen (cALL). The significance of this cALL antigen remains to be determined, but its presence on some pre-B-ALL4 suggests that it is involved at least in B-cell differentiation. Furthermore, cALL, T-ALL, some pre-B-ALL, and BC-CML are linked by the presence of the terminal-deoxynucleotidyl-transferase enzyme (TdT), which suggests a common pathway of differentiation.5 Despite all these techniques, between 10% and 20% of adult nonmyeloid leukemias remain unclassified.2 This subgroup does not appear in the FAB classification, but the L1 and L2 denominations can include some acute leukemias without immunologic markers.6 There is no immunologic method or enzyme assay available at present for the detection of poorly differentiated myeloid leukemia; however, detection of peroxidases by transmission electron microscopy (POEM) can reveal myeloblastic, monoblastic, basophilic, or megakaryoblastic subpopulations.7 We have combined these different approaches (the POEM, the immunologic methods, and the TdT assay) to achieve a finer analysis of acute leukemias seemingly lacking evidence of myeloid differentiation.

MATERIALS AND METHODS

Patients

Seventy-three adult patients with acute leukemia were studied between June 1979 and March 1980. Sixty-seven were in their first active phase, 6 were in relapse; 12 were BC-CML and 4 others were diagnosed as Ph1-positive primitive acute leukemias.

Methods

Bone marrow smears were obtained by sternal aspiration. Two-hundred cells were counted for determination of percent of blast cells. All cases were classified according to the FAB classification1 and underwent a conventional cytochemical study2 including: peroxidase,1,8 Sudan Black B, periodic acid Schiff (PAS), beta-glucuronidase, nonspecific esterase combined with inhibition by sodium fluoride, and acid phosphatase. Serum and urine lysozyme were assayed in each case; the levels were consistently increased in acute monocytic and myelomonocytic leukemias, and decreased in all the apparently nonmyeloid cases. The cytologic classification of the 51

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patients with an obvious acute myelogenous leukemia was, according to the FAB classification: 14 M1, characterized with more than

patients with an obvious acute myelogenous leukemia was, according to the FAB classification: 14 M1, characterized with more than 3% of peroxidase-positive blast cells; 20 M2, 2 M3; 13 M4, 1 M5; and 1 M6. Following these classical investigations (Table 1), 22 patients, including 7 BC-CML, remained in the group of apparently nonmyeloid leukemia (peroxidase and Sudan B negative reaction, absence of monoblastic differentiation). When the morphology contradicted the cytochemistry, the results of the cytochemical stains determined the final diagnosis. In addition, these patients were submitted to further analysis.

Optical Peroxidase Methods

Two techniques were used: the Kaplow’s technique using benzidine (B), or a modified Graham’s technique, using alphanaphthol pyronin as substrate (which is not known to be a carcinogen) as described by Piette. A comparable percentage of positive cells and scores were found in 12 normal blood and bone marrow samples, in 8 AML (6 M2, 2 M4), and in 5 ALL or AUL (negative score) studied with the two methods.

POEM Methods

The detection of peroxidase at the ultrastructural level has been extensively described in previous reports. Briefly, three separate procedures can be employed. (1) Incubation before fixation in 3-3'-diaminobenzidine (DAB) medium. (2) Fixation in a tannic acid mixture followed by incubation in DAB medium. (3) Fixation in glutaraldehyde followed by incubation in DAB medium (Graham and Karnovsky method).

The cytochemical controls for these methods have been previously described.

Aliquots from the buffy coat were treated by the three methods and bone marrow samples were treated by methods 2 and 3. All preparations were postfixed in osmium tetroxide, washed, dehydrated, and embedded in epon. Semithin sections (1 μm) were first examined using a phase-contrast microscope. Myeloperoxidase-positive cells were easily recognizable and were counted. When positivity was uncertain, thin sections were examined. Unstained and positive cells were counted in a Phillips EM 300 (INSERM U 91, Laboratory of Dr Breton-Gorius).

Immunologic Methods

Cell suspensions were obtained from a Ficoll-Hypaque-separated fraction of bone marrow and peripheral blood. The techniques used for the detection of SRBC receptors, SmIg, and HuTIA have been previously described. The Reh cell line, established from an ALL patient and possessing the cALL, was employed for preparation of the cALL antibody.

The anti-cALL serum was prepared by two injections (2 wk apart) of 2 × 10⁹ Reh cells coated with antibodies to tonsil lymphocytes and to the lymphoblastoid B-cell line LHN 13 into rabbit. The rabbits were bled out on day 21. Absorptions were carried out consecutively on AB erythrocytes, tonsil lymphocytes, normal bone marrow, LA acute myeloblastic leukemia cells, thymocytes, and the T-cell line JM. The specificity of the antiserum was assessed on a panel of fresh normal and leukemic cells and on several normal and leukemic cell lines.

A rabbit anti-29-34 (1a-like molecule) serum was kindly provided by Dr. Frade. Fluorescein-conjugated goat anti-rabbit IgG was used for the cALL and 1a-like antigen detection.

Tdt Assay

Terminal deoxynucleotidyl transferase (Tdt) activity was measured using the biochemical method of Bollum modified by Hoffbrand et al. Blood or bone marrow cells were suspended at a density of 1–2 × 10⁷ cells/ml in 0.25 M potassium phosphate buffer, pH 7.5, containing 1 mM mercaptoethanol. Then cells were disrupted by freezing and thawing twice and the extract centrifuged. The reaction mixture contained 0.025 poly-deoxyaadenylic acid (Biological Inc.) as primer, 83 μM 3'-deoxyguanosine triphosphate (specific activity 2000 cpm/mole), (Radiochemical Laboratories, Amersham) as substrate, 42 mM Tris-HCl (pH 7.5), 42 mM potassium chloride, 4.2 mM dithiotreitol, 80 μM manganese chloride, and 10 μl cell extract in a final volume of 60 μl. The reaction mixture was placed at 37°C for 1 hr, stopped into ice with 100 μl of a mixture containing 100 mM sodium pyrophosphate, 20 μl of a mixture containing 100 mM sodium pyrophosphate, 40 μM EDTA, 15 g/liter of bovine serum albumin; then 1 ml 10% trichloracetic acid was added and the reaction left for 15 min. The

| Table 1. Hematologic Data of the Apparently Non-B, Non-T Adult Acute Leukemias |

<table>
<thead>
<tr>
<th>Patients</th>
<th>Age</th>
<th>Sex</th>
<th>WBC (10⁹/liter)</th>
<th>Blasts (10⁹/liter)</th>
<th>PMN (10⁹/liter)</th>
<th>Hb/100 ml</th>
<th>Platelets (10⁹/liter)</th>
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<td>F</td>
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<td>22</td>
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<td>9.2</td>
<td>25</td>
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* Cytologic type, according to FAB classification, and resulting from the analysis of two separate cytologists. U, undifferentiated and unclassified.

† B, benzidine technique; P, Piette technique.
Table 2. PAS Reaction, Immunologic Markers, TdT Activity, and Detection of Peroxidases at Ultrastructural Level (POEM) of the Apparently Nonmyeloid, non-T, non-B Adult Leukemias

<table>
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<tr>
<th>Patients</th>
<th>HuTLA</th>
<th>PAS</th>
<th>Ia-like Antigen</th>
<th>cALL A</th>
<th>TdT U/10⁶ c</th>
<th>POEM</th>
<th>Final Classification</th>
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<td>+</td>
<td>+</td>
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<td>-</td>
</tr>
<tr>
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<td>8</td>
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<td>ND</td>
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<tr>
<td>9</td>
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<td>-</td>
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<tr>
<td>14</td>
<td>-</td>
<td>+</td>
<td>ND</td>
<td>-</td>
<td>0</td>
<td>+50%</td>
<td>M “O”-AML†</td>
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<tr>
<td>15</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>1.24</td>
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<td>-</td>
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<tr>
<td>16</td>
<td>-</td>
<td>-</td>
<td>ND</td>
<td>-</td>
<td>0.54</td>
<td>-</td>
<td>2 Undifferentiated ALL</td>
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*ND, not determined.
†M “O”-AML, poorly differentiated AML (Galton's classification, see text).

acid-insoluble fractions were collected on GF/A glass fiber disks (Whatman), dried filters were put into 10 ml of PCS (Beckman) and counted. One unit of TdT activity equals 1 nmole of dGTP polymerized in 1 hr; results were expressed as units per 10⁶ cells. The values of TdT in normal bone marrow cells (<0.05 U/10⁶ cells), as well as in pathologic conditions, were lower than those previously reported; this difference could be due to the length of the primer utilized.

RESULTS

All 22 apparently nonmyeloid patients were tested for SRBC receptors (E rosettes: E), HuTLA, and Smlg. Two patients had a T-ALL phenotype (E+, HuTLA+, Smlg+, focal acid phosphatase). In 4 of the remaining 20 patients, the concurrent study of POEM and cALL antigen was not possible. Ia-like antigen and TdT activity were investigated in 7 of the 16 remaining patients. When results of immunologic, enzymatic (TdT), and ultrastructural markers were compared, four types of acute leukemias were observed (Table 2).

Common (Non-T, Non-B) ALL: Seven Cases

Six patients showed a typical common ALL phenotype (cALL+, HuTLA+, E+, Smlg+). Two of these 6 patients were BC-CML. Ia antigen was found to be present in 4 of these 6 cases of cALL. Typical PAS reaction, with blocks or coarse granules, was found in 4 of 6 cases.

One patient showed a pre-T phenotype (cALL+, HuTLA+, E- with a PAS-positive reaction.

TdT activity was measured in 4 cases of these non-T, non-B ALL (3 cALL and 1 pre-T-ALL) and was found to be highly positive, albeit to a lesser degree in the pre-T-ALL.

No peroxidases were detected at the ultrastructural level in these 7 patients.

Poorly Differentiated Myeloid Leukemias (M ‘O’-AML): Five Cases

Five patients showed a cytoplasmic peroxidase activity at the TEM level in 20%-50% of their blasts, whereas they were completely peroxidase negative on smears at optical level. Whatever the method used, in 4 cases, the positivity was recognizable on semithin

![Fig. 1. Case 11. Semithin section (1 μm) of blood blast cells (phase contrast) treated for POEM detection by method 2. Some cells (arrows) exhibit few peroxidase-positive granules (×1000). Inset: same cells on stained smears (May-Grünwald-Giemsa technique.)](image-url)
section using the phase-contrast microscope (Fig. 1); whereas, only the TEM was positive in the last patient. In three cases this myeloperoxidase activity was localized in small granules (<0.2 \( \mu \)) (Fig. 2); and in one case the positivity was localized in endoplasmic reticulum without granules (Fig. 3). In the last case, a basophilic peroxidase activity (with typical stippled appearance) was detected in reticulum and in rare granules (Fig. 4). These 5 cases were HuTLA, SmIg, and cALL negative. The Ia-like antigen was positive in one case, negative in one, and not tested in three cases. TdT activity was measured in one case and was found to be completely absent.

**Undifferentiated (or Unclassifiable) Leukemia: Two Cases**

In both cases, B and T markers, cALL antigen, POEM, and PAS were all negative. The Ia-like anti-
gen was present in one case. TdT was assayed in these two cases, and its activity was found to be highly positive.

**Mixed Lymphoid-Myeloid Leukemia: Two Cases**

In these remaining two cases, both myeloid and lymphoid markers were present. HuTLA, SmIg, and PAS were negative, Ia and TdT were not tested. In one case, 80% of the blasts were cALL⁺ and 15% POEM⁺; in the other case, 50% were POEM⁺ and 20% cALL⁺. These two cases were acute leukemias with typical Ph¹ chromosome and without a preceding chronic phase. In the first case, the cALL⁺ population was isolated using a cell sorter (Becton-Dickson, FACS-II): the POEM detection was completely negative in these cells; one can assume consequently that the 15% POEM⁺ cells corresponded to a separate cALL subpopulation.

**Comparison With Cytologic Diagnosis**

In all but one case of non-T, non-B ALLs, the diagnosis of ALL was made by two cytologists (five L2 and one L1). In the following three subgroups, cytologic diagnosis was hesitating between the two observers in 50% of cases. No Auer rods were observed. In only two cases of M "O"-AML, the diagnosis of AML was suspected.

**DISCUSSION**

This study shows the importance of the search for both lymphoid and myeloid markers in the classification of apparently "nonmyeloid" adult leukemias and in particular the value of the cALL antigen, the TdT assay, and the detection of peroxidase using TEM. Of the seven cALL cases, ultrastructural cytochemistry showed a peroxidase activity in 5 cases. In the group of 9 POEM⁻ patients, the leukemic cells of 7 patients were cALL⁺. Six of these 7 cALL were easily recognizable as ALL by the two cytologists.

The absence of overlap between these two markers is probably the consequence of an early myeloid or lymphoid differentiation.

The combination of these two techniques reduced the percentage of so-called "undifferentiated" leukemias to 2.7% instead of the 9.6% of our starting series of 73 cases of adult acute leukemias. If high TdT activity is considered as a lymphoid marker, all of these leukemias could be classified as lymphoid or myeloid.

Since the development of these techniques, no systematic analysis using both these lymphoid and myeloid markers has been performed with the exception of the study of Catovsky et al., who used the detection of peroxidases by light and electron microscopy as a criteria for exclusion from their series. In
their study, the proportions of cALL, T-ALL, and Ph<sup>+</sup>-positive ALL in adult patients are similar to those in our series (we have observed 27% cALL, 13% T-ALL and pre-T-ALL, and 27% Ph<sup>+</sup>-ALL out of the 22 apparently nonmyeloid acute leukemia). The paucity of true Ph<sup>+</sup>-cALL in adults must be emphasized (5/73: 7%).

In a recent work, Janossy et al.<sup>16</sup> ran a multiparametric study using immunologic markers and TdT and observed that 7.3% ALL remained unclassified. Using only the cytologic criteria according to the FAB classification, Mertebsmann et al. observed 4% undifferentiated acute leukemia, which were called M "O."<sup>17</sup> The TEM detection of peroxidase in both series would have been extremely useful. In our series of adult patients, 23% of the apparently nonmyeloid leukemias, according to the usual cytologic criteria, were in fact poorly differentiated myeloid leukemias. Such myeloid leukemias were first recognized by Galton and Dacie (M "O"-AML)<sup>18</sup> but were not retained as a separate group in the FASB classification.<sup>1</sup> Our study shows that POEM allows a clear-cut distinction of a M "O" type acute leukemia.

Analogous results were published by Youness et al.<sup>19</sup> in a series of 225 acute leukemias classified after cytologic and cytochemical criteria. Fourteen of the 22 "undifferentiated" cases were recognizable as myeloblastic by the POEM. The small size of the granules and the excellent preservation of the cell structure with the TEM technique explains the discrepancies between the optical and TEM results, but this "time-consuming" technique should be reserved for the apparently nonmyeloid leukemias with negative lymphoid immunologic markers.

Despite the multiplicity of the markers, some rare acute leukemias remain undifferentiated. Our two cases have a high TdT level that could imply a lymphoid origin.<sup>5</sup> The TdT alone cannot classify all AUL: according to Hoffbrand et al.,<sup>14</sup> 50% of the AUL are TdT negative, but these cases were not explored for POEM.

This study confirms the postulated existence of mixed leukemias noted by others.<sup>20</sup> Despite its rarity, the coexistence in the same patient of lymphoid and myeloid markers is of interest. Janossy et al.,<sup>21</sup> using a cell sorter, have separated the two populations in such a case of mixed acute leukemia and have shown that the increased TdT activity was linked to the cALL population. In their cases, as in ours, Ph<sup>+</sup> chromosome was present. Our cell sorter analysis in one case confirms the exclusion of myeloid and lymphoid markers on the same cell. The cell heterogeneity in BC-CML has already been emphasized.<sup>7,21,22</sup> The frequency of Ph<sup>+</sup> CML metamorphosis into cALL<sup>+</sup> has been explained by the involvement of a pluripotent (lymphoid and myeloid) stem cell in the malignant process.<sup>23</sup> The cases of mixed acute leukemia in BC-CML appear to be more frequent than the classical occurrence of two distinct leukemias in the same patient.<sup>24</sup> Consequently, a suspicion of mixed acute leukemia should lead to the search for a Ph<sup>+</sup> chromosome.

In a previous report<sup>7</sup> we have shown the heterogeneity of the blast population in the BC-CML, which exhibited several different peroxidase activities. However, the detection of immunologic markers was lacking in this study, and the peroxidase TEM negative blast cells could not be classified. In the six cases of BC-CML presented in this study, two patients with POEM were cALL negative, two without POEM were cALL positive, and the last two had a mixed population.

In summary, this multiparametric analysis of acute leukemias demonstrates the usefulness of the simultaneous study of lymphoid and myeloid markers. This study, based on immunologic, biochemical, and cytochemical techniques, allows us to reduce markedly the so-called undifferentiated leukemias, with a clear-cut identification of a lymphoid and myeloid type, avoiding in many cases conflicting morphological classifications.

REFERENCES

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