Acute Lymphocytic Leukemia with Prominent Azurophilic Granulation and Punctate Acidic Nonspecific Esterase and Phosphatase Activity

THOMAS M. GROGAN, M.D., SAMUEL J. INSALACO, M.D., RICHARD A. SAVAGE, M.D., AND MICHAEL L. VAIL, M.T.

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Grogan, Thomas M., Insalaeo, Samuel J., Savage, Richard, A., and Vail, Michael L.: Acute lymphocytic leukemia with prominent azurophilic granulation and punctate acidic nonspecific esterase and phosphatase activity. Am J Clin Pathol 75: 716-722, 1981. Cytochemical, ultrastructural, and terminal deoxynucleotidyl transferase determinations in three cases of acute lymphocytic leukemia with azurophilic granules and punctate alpha-naphthyl acetate esterase (ANAE), alpha-naphthyl butyrate esterase (ANBE), and acid phosphatase (AP) activities further described a cytochemical and morphologic variant of acute lymphocytic leukemia. The ANAE, ANBE, and AP activities appeared specifically localized within cytoplasmic granules. Electron microscopy substantiated a prominence of membrane-bound dense-core cytoplasmic granules. The variegated appearance of these dense-core granules and the cytochemical profile suggest that the aberrant granules are lysosomal in nature. Both the prominent cytoplasmic granules and the older age distribution led to early misdiagnosis of myelocytic leukemia. The distinctive cytochemical/morphologic profile and the ease of misdiagnosis suggest an important morphologic variant of acute lymphocytic leukemia. (Key words: Acute lymphocytic leukemia; nonspecific esterase activity; Lysosomal acute lymphocytic leukemia.)

Reports of Three Cases

Case 1

A 38-year-old white woman was evaluated for sore throat, fever, and pallor. Physical examination detected retinal flame hemorrhages. Peripheral blood count, platelet count, and bone marrow findings (Table 1) indicated an anemic, thrombocytopenic patient with circulating blasts and a hyperplastic, effaced marrow. The observed prominence of azurophilic granules and the patient's age suggested an initial diagnosis of acute myelocytic leukemia. The patient was referred to Walter Reed Army Medical Center for treatment. The cytochemical studies (Table 2) and Tdt determinations (see Results) then suggested a diagnosis of acute lymphocytic leukemia. The patient was treated with vincristine sulfate, prednisone, and L-asparaginase, with partial response. She died 40 days after admission of fungal septicemia and pneumonia with a hypoplastic bone marrow.

Case 2

A 14-year-old Oriental girl was admitted to the Walter Reed Army Medical Center for evaluation of swelling of the wrists and fever. Admission peripheral
Table 1. Initial Hematologic Data

<table>
<thead>
<tr>
<th>Patient's Age/Sex</th>
<th>Hematocrit (%)</th>
<th>Hemoglobin (g/dl)</th>
<th>Leukocytes (µl)</th>
<th>blasts (%)</th>
<th>Platelets (µl)</th>
<th>Bone Marrow</th>
<th>Cellularity (%)</th>
<th>Blasts (%)</th>
<th>Blast Morphology*</th>
<th>Initial Diagnosis†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Case 1 38/F</td>
<td>19</td>
<td>6.5</td>
<td>8,300</td>
<td>95</td>
<td>50,000</td>
<td>High N/C ratio; 2–3 nucleoli; prominent &quot;azurophilic&quot; granules</td>
<td>95</td>
<td>95</td>
<td>AML</td>
<td></td>
</tr>
<tr>
<td>Case 2 14/F</td>
<td>30</td>
<td>11.1</td>
<td>40,600</td>
<td>20</td>
<td>90,000</td>
<td>High N/C ratio; 1–2 nucleoli; prominent &quot;azurophilic&quot; granules</td>
<td>95</td>
<td>85</td>
<td>AML</td>
<td></td>
</tr>
<tr>
<td>Case 3 33/M</td>
<td>35</td>
<td>11.6</td>
<td>94,000</td>
<td>5</td>
<td>225,000</td>
<td>High N/C ratio; 2–3 nucleoli; prominent &quot;azurophilic&quot; granules</td>
<td>90</td>
<td>95</td>
<td>CML with blast crisis</td>
<td></td>
</tr>
</tbody>
</table>

* N/C, nuclear/cytoplasmic ratio.
† AML, acute myelocytic leukemia; CML, chronic myelocytic leukemia.

blood count, platelet count, and bone marrow (Table 1) indicated acute leukemia with associated anemia and thrombocytopenia. The observed prominence of azurophilic granules suggested the diagnosis of childhood acute myelocytic leukemia. On this basis, treatment with daunorubicin and cytosine arabinoside was initiated, with initial poor response. When additional cytochemical stains (Table 2) and Tdt (see Results) were performed on pretreatment marrows, a diagnosis of acute lymphocytic leukemia was made. Treatment was changed to vincristine sulfate, prednisone, and adriamycin, which led to complete remission. Maintenance therapy with 6-mercaptopurine and methotrexate has led to persistent remission. The last follow-up date was 12 months after remission.

Case 3

A 33-year-old white man was evaluated for a three-month history of fever, sore throat, and mild bilateral axillary lymphadenopathy. Physical examination revealed an enlarged spleen. Peripheral blood count, platelet count, and bone marrow (Table 1) revealed a mild anemia and an occasional circulating blast. Initially, circulating immature granulated leukocytes were prominent (39% myelocytes, 5% promyelocytes). A marrow cytogenetic analysis showed 50% of the marrow metaphases to be 46XY,22Q−, consistent with the presence of a Philadelphia chromosome in the bone marrow. Leukocyte alkaline phosphatase score was 51. The combined findings suggested an initial diagnosis of chronic myelocytic leukemia. The high marrow blast count indicated blast crisis. Cytochemical studies of the initial marrow (Table 2) coupled with the high Tdt activity suggested a lymphoid blast crisis of chronic granulocytic leukemia, an occurrence previously documented.21,24 The patient was treated with adriamycin, vincristine sulfate, cytosine arabinoside, and prednisone, followed by late intensification therapy utilizing hydroxyurea and 6-mercaptopurine, with consequent partial response. He died 11 months after diagnosis of gram-negative bacterial sepsis and pneumonia. An autopsy was not performed.

Methods

Cytochemical Studies

Bone marrow aspirates and peripheral blood buffy coat preparations were stained before treatment for the presence of alpha-naphthyl butyrate esterase (ANBE)20 with and without sodium fluoride, alpha-naphthyl acetate esterase (ANAE),28 naphthol AS-D chloroacetate esterase NASDE),28 peroxidase (PX),10 acid phosphatase (PA),19 periodic acid-Schiff (PAS) reaction,10 and Sudan black B (SBB),10 along with the routine Wright-Giemsa-stained preparations.

Transmission Electron Microscopy

A buffy coat preparation (Case 2) was layered with 4% glutaraldehyde and fixed overnight. Then 1-mm
cubic sections were washed in phosphate-buffered saline solution for 10 min, postfixed in osmium tetroxide for one hour, dehydrated in graded alcohol, embedded in Epon® 812, and left to polymerize overnight in a 60°C oven. Sections were stained with uranyl acetate for 3 min, washed, dried, and then stained with lead citrate for 3 min. The subsequent grids were then examined under a Zeiss-9® electron microscope.

Terminal Deoxynucleotidyl Transferase Determinations

Immunofluorescence. Immunofluorescence (Cases 1 and 2) was carried out with a double antibody technic as originally described by Bollum and Coons and Kaplan. Bone marrow smears were fixed in absolute methanol at 4°C for 10 min and then air dried. The samples were then rehydrated with phosphate-buffered saline solution, and the column-purified rabbit antitransferase was applied at 100 μg/ml for 30 min at room temperature. Residual rabbit IgG was removed by three washes with phosphate-buffered saline solution, and a second antibody [fluorescein-conjugated goat antirabbit F (ab')₂] was applied. After 30 min of reaction and a subsequent wash in phosphate-buffered saline solution, a coverslip was applied with a drop of 50% glycerol, and fluorescence was observed with the Zeiss photomicroscope. Cells containing nuclear fluorescence were scored as positive.

Assay. Assays were performed on sonicated pretreatment bone marrow aspirates (Case 3) according to the previously described technic of Hutton and Coleman.

Patient Selection

The three reported cases were the only ones with punctate nonspecific esterase activity among 45 cases of ALL with complete cytochemical profiles at two major leukemia referral centers: the Walter Reed Army Medical Center (WRAMC) (21 cases) and the Cleveland Clinic Foundation (CCF) (24 cases).

Case 2 is one of 18 cases of untreated acute leukemia studied by electron microscopy by one author (T.M.G.) at WRAMC. Among the cases studied, it was the only one with prominent lysosome formation.

Results

Cytopathology and Histopathology

Wright-Giemsa-stained aspirates from all three patients demonstrated a monomorphic population of leukemic blasts. The blasts were characterized by a high nuclear/cytoplasmic ratio, a fine reticular nuclear chromatin pattern, and one to three distinct nucleoli (Fig. 1). The cytoplasm in each case was scant and uniformly basophilic, with many of the blasts (30% to 60%) containing abundant coarse, scattered azurophilic cytoplasmic granules (Fig. 1). The azurophilic granules were scattered throughout the cytoplasm, but were most readily observed in the uropodal region. The peripheral smear Wright-Giemsa preparation in each case showed similar circulating blasts with coarse azurophilic granulation. The prominence of this granulation in the circulating blasts led to the initial clinical impression of myelocytic neoplasia in each case.

The initial bone biopsy specimens taken at admission showed hyperplastic bone marrow (95%) with complete replacement of the marrow by leukemic blasts.

Cytochemistry

Results of cytochemical staining are listed in Table 2. The ANAE (Case 3) and ANBE (Cases 1 and 2) stains showed granular dark-brown staining in more than 40% of the blasts in each case. The activities of both enzymes appeared localized within the previously described cytoplasmic granules (Fig. 1). The most intense activity occurred in a scattered granular pattern, with a very occasional blast showing more diffuse staining of the Golgi region. An occasional diffusely stained histiocyte was apparent in the marrow aspirate. The diffuse activity found in histiocytes was inhibited by incubation with sodium fluoride, whereas the localized punctate activity in lymphoid leukemic cells was not. Acid phosphatase activity was present in a similar scattered localized punctate granular pattern (Fig. 1). The PAS reaction demonstrated coarse, refractile blocklike PAS positivity in greater than 15% of the leukemic cells present. The large azurophilic granules described in the Wright-Giemsa-stained smears had a faint-pink nonrefractile appearance on PAS staining. The leukemic cells showed no activity with NASDE, SBB, and PX stains, indicating an absence of myelocytic enzymatic activity.

Ultrastructure

By transmission electron microscopy, the leukemic cells in the buffy coat (Case 2) demonstrated a high nuclear/cytoplasmic ratio. The nuclei were round to oval, with occasional nuclear envelope indentations. Nucleoli (1–2/nucleus) were generally prominent, and well-organized nucleolemma were occasionally evident. Most nucleoli were associated with the nuclear envelope. The nuclear chromatin was moderately dispersed centrally; clumped heterochromatin was present adjacent to the nuclear envelope.

The cytoplasm contained abundant scattered polyribosomes, a rare strand of rough endoplasmic retic-
Fig. 1. (Upper) Bone marrow aspirate, Case 2. The leukemic cells show scattered prominent azurophilic granules. Wright-Giemsa stain. x1,000. (Lower, right) Bone marrow aspirate, Case 2. Localized, granular cytoplasmic alpha-naphthyl butyrate esterase activity in leukemic cells. Alpha-naphthyl butyrate esterase stain. x1,000. (Lower, left) Buffy coat, Case 2. Localized granular cytoplasmic acid phosphatase activity in leukemic cells. Acid phosphatase stain. x1,000.

ulum, scattered mitochondria, and an occasional distinct Golgi region. Several cells containing nuclear blebs were observed.

Many of the examined lymphoid cells contained numerous scattered electron-dense cytoplasmic granules (Fig. 2). These electron-dense bodies were uniformly membrane bound (Fig. 2, left inset), and considerable heterogeneity was evident. Granules were of variable density and size; several multivesiculate forms were evident (Fig. 2, right inset). Some of the dense granules were seen to occur in association with myelin figures (Fig. 2, left inset). The heterogeneous
variegated appearance of the membrane-bound dense-core granules is suggestive of secondary lysosomal granule formation.

**Immunofluorescence**

Immunofluorescent terminal transferase determinations in two cases (Cases 1 and 2) indicated that greater than 50% of the bone marrow cells were positive with Tdt nuclear fluorescence (greater than 1% positivity considered pathologic). The Tdt serum assay in Case 3 indicated greater than 300 units of terminal transferase activity per 10⁸ nucleolated marrow cells (>50 units/10⁸ cells usual in leukemic lymphoblasts).

**Discussion**

Lymphocytic leukemia with prominent cytoplasmic granulation has been described to occur in a variety of clinical settings. It may be regarded as a clinically heterogeneous, but morphologically similar, variant of lymphoid leukemia. The morphologic similarity is superficial at best, since forms with inclusions mimicking Auer rods, ribosomal complexes, microtubules, lysosomes, and aberrant mitochondria have been described. However diverse the clinical setting, a number of reported cases, including ours, possess a central feature: the prominent intracytoplasmic granules suggest a diagnosis of myelocytic, not lymphoid, leukemia.

The lymphoid nature of the leukemic blasts in our cases was established by the high degree of Tdt activity and the cytochemical profile. While Tdt activity is not exclusively ascribed to ALL, this degree of activity would be considered unusual in myelocytic leukemia. Furthermore, the combined findings of refractile block PAS positivity (a feature of leukemic lymphoid cells) and absent myelocytic enzyme activity (NASDE, PX, SBB) indicate the leukemic blasts are cytochemically lymphoid.

Our morphologic and cytochemical findings are
similar to those of reported cases of ALL with azurophilic granules.\textsuperscript{1,3,15-26} However, we make the additional observation of scattered punctate ANAE, ANBE, and AP activities in the leukemic blasts. The ANBE, ANAE, and AP activities in our cases appear specifically localized within scattered cytoplasmic granules. Since the nonspecific esterases (NSE) and AP belong to the phosphatase family of enzymes found in lysosomes,\textsuperscript{11,16} the punctate staining in our cases is compatible with a lysosomal location of enzymes. Beta-glucuronidase\textsuperscript{40} and AP\textsuperscript{11-15} activities in previous cases also suggest that ALL with azurophilic granules is lysosomal in nature. However, in these reports,\textsuperscript{1,3,15-26} ANAE and ANBE were not evaluated.

Electron microscopy in our cases substantiated a prominence of membrane-bound dense-core cytoplasmic granules. Furthermore, the size, conformation, and heterogeneous appearance of these dense-core granules in association with autophagic phenomena (i.e., myelin-figure formation) suggest secondary lysosome formation. Collectively, our findings indicate that ALL with azurophilic and dense-core granules and with punctate ANAE, ANBE, and AP activities is a lysosomal variant of ALL.

In previous ultrastructural studies of lymphoid leukemia with cytoplasmic granules, some of the inclusions were crystalline bodies within dilated cisternae analogous to Auer rods\textsuperscript{17}; some were circumscribed arrays of microtubules\textsuperscript{21}; some were composed of ribosomes and tubules\textsuperscript{1}; some were degenerating mitochondria\textsuperscript{16}; others resembled phagolysosomes\textsuperscript{4,5}; some had both ribosomal complexes and autophagosomes.\textsuperscript{15} The granules in our cases correspond to the lysosomal inclusions reported by Bouchieux and associates,\textsuperscript{4} Brunning and associates,\textsuperscript{5} and Komiyama and associates.\textsuperscript{13} Both the lysosomal\textsuperscript{11-15} and mitochondrial\textsuperscript{29} forms have autophagic features suggesting a common denominator of increased catabolism or abnormal metabolism in the leukemic cells.\textsuperscript{5,15,29}

NSE activity is described to occur in both normal and leukemic lymphoid populations. In normal lymphoid populations, both ANBE\textsuperscript{11} and ANAE\textsuperscript{16} are described to occur in a lysosomal location. Indeed, lysosomal esterase may be essential for the normal cytotoxic function of T-cell lymphocytes.\textsuperscript{7} In one T-cell subpopulation (Tg), punctate ANAE activity was associated with azurophilic and dense-core granules, indicating lysosomal origin.\textsuperscript{8} In Wolman's disease, the absolute deficiency of ANAE coincides with aberrant vacuolated lysosomes, further substantiating the lysosomal location of ANAE.\textsuperscript{18} The lysosomal nature of ANAE in neoplastic lymphoid cells appears established in one case besides ours. Prominent focal AP and ANAE activities have been described to occur in ALL complicating Hodgkin's disease.\textsuperscript{4} This enzyme activity is associated with lysosome-like dense bodies at the ultrastructural level.

Although these reports and ours indicate an association between azurophilic granulation, dense-core lysosomal granules, and punctate NSE activity in lymphoid cells, the association is not universal. Shaw and Ishmael\textsuperscript{25} described cases of ALL with focal ANAE activity but without cytoplasmic granules. Yanagihara and associates\textsuperscript{20} described a case of ALL with ANAE-reactive granules: however, the granules were not azurophilic, and acid phosphatase activity was absent. Furthermore, while the electron-dense structures they described superficially resembled giant lysosomal granules, degenerating mitochondria were suggested as the aberrant granule.\textsuperscript{29}

NSE activity at prescribed \( p \text{H} \), with specific substrates and incubation periods, provides an immunologic marker for circulating normal lymphocytes.\textsuperscript{5,11,14,16} Alkaline ANBE staining (\( p \text{H} \ 8.0 \)) yields single-dot activity in T-lymphocytes and scattered granular activity in "null" lymphocytes.\textsuperscript{11} Localized dot ANAE activity at acidic \( p \text{H} \ (p \text{H} \ 5.8) \) characterizes T-cell lymphocytes.\textsuperscript{14,16} Acidic ANAE (\( p \text{H} \ 5.8 \)) may further discriminate T-cell subpopulations.\textsuperscript{4} Single-dot cytoplasmic activity is present in subpopulation (Tm), while subpopulation (Tg) has dispersed granular activity and azurophilic-granulation.

Recent reports indicate that characteristic patterns of ANAE and ANBE activities also occur in pathologic lymphoid proliferations of T, B, and null cell types,\textsuperscript{4,14,16,22,23,27} indicating that the immunologic lineage may be inferred from the cytochemical profile. On this basis, the scattered NSE activity in our cases suggest leukemic lymphoid cells of either null cell\textsuperscript{11} or T-cell (subset Tg) lineage.\textsuperscript{6} In the two cases of ALL with ANAE-reactive granules previously studied for surface markers, one had 90% E-rosette-positive T-cells; the other had cells of non-B, non-T type.\textsuperscript{29} The Tdt activity in our cases is compatible with either.\textsuperscript{12} This activity definitely excludes a lymphoid neoplasm of B-cell lineage.\textsuperscript{12} However, the absence of surface marker determinations in our cases precludes further immunologic characterization. Furthermore, the short incubation time (40 min) and the \( p \text{H} \ (6.3) \) we employed may have yielded a staining profile different from those of others.\textsuperscript{11,13,16,27} Hence, the inference of immunologic subtype from cytochemical profile is not emphasized.

ALL with ANAE- and ANBE-reactive granules appears clinically, immunologically, and morphologically heterogeneous. This variant is probably the result of abnormal organelle formation, especially of lysosomes\textsuperscript{4,5,15} and mitochondria,\textsuperscript{29} in the leukemic.

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cells. Although heterogeneous in many ways, this variant of ALL has important features in common: the frequent occurrence of cytoplasmic granules of either azurophilic type, as we describe, or non-azurophilic type, as described by others; the frequent occurrence in older children and adults (ten of 13 cases, including ours); older age distribution; or both, has led to erroneous classification as myelocytic leukemia, with consequent inappropriate initial treatment.

For these reasons, the accurate diagnosis of lymphoid leukemia in older patients who have granule-bearing blasts requires corroborative evidence. The combined determination of terminal transferase activity and cytochemical profile is especially critical.

The lack of complete immunologic definition and the clinical diversity of this variant of ALL preclude certain classification, as in the FAB scheme, but distinctive cytochemical/morphologic profile, older age distribution, and ease of misdiagnosis suggest an important morphologic subset. Further immunologic and clinical characterization is necessary to corroborate this view.

References