

## Characterization of Acute Undifferentiated Leukemia by Combined Analysis of Plasma Membrane-Associated $\gamma$ -Glutamyltransferase and Soluble Terminal Transferase

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$\gamma$ -Glutamyltransferase ( $\gamma$ -GT) is a plasma membrane-associated enzyme present in blasts of certain acute leukemias. We analyzed 90 cases of undifferentiated and differentiated acute leukemias for  $\gamma$ -GT, using a colorimetric assay. Blasts of all patients with common acute lymphoblastic leukemia (ALL) and T-ALL were negative for  $\gamma$ -GT (<5 units). In contrast,  $\gamma$ -GT was significantly elevated in acute myeloblastic or monoblastic leukemia blasts ( $P < .001$ ). In 16 cases of acute undifferentiated leukemia (AUL) studied, the levels of  $\gamma$ -GT ranged from 0 to 93 units; in eight cases,  $\gamma$ -GT was positive (>5 units), and six of these had 2% to 5% Sudan black-positive leukemic cells in the

blast-enriched suspension. Combined  $\gamma$ -GT/TdT analysis revealed that both enzyme markers were mutually exclusive in 75% of AUL cases, suggesting that  $\gamma$ -GT<sup>+</sup>/TdT<sup>-</sup> blasts are of nonlymphoid origin, and  $\gamma$ -GT<sup>-</sup>/TdT<sup>+</sup> blasts are of lymphoid origin. Two cases were devoid of both enzyme activities and could represent truly undifferentiated leukemia. Thus, combined  $\gamma$ -GT/TdT analysis underlines the heterogeneity of AUL and appears to be useful in defining the lineage commitment of undifferentiated leukemic blasts.

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ACUTE unclassified leukemias are neoplasms in which the malignant cells have not yet been identified by the usual multiparameter analyses, including morphological, cytochemical, and immunologic studies. According to several general reports, an average of 5% leukemias remain unclassified.<sup>1</sup> Within this group, many cases have a "lymphoid-like" morphology and do not express myeloid or monocytic cytochemical markers. These leukemias, which are negative for B and T cell markers and which do not express the common acute lymphoblastic leukemia (ALL) antigen CALLA are generally referred to as acute undifferentiated leukemias (AUL), and most of these cases express Ia antigens.<sup>2-4</sup> A further improvement in the characterization of AUL has been achieved by the determination of terminal deoxynucleotidyl transferase (TdT), leading to the recognition of the so-called null-ALL subgroup, identified by a CALLA<sup>-</sup>/T<sup>-</sup>/Ia<sup>+</sup>/TdT<sup>+</sup> blast phenotype.<sup>5-8</sup> Nevertheless, a certain number of Ia<sup>+</sup>/TdT<sup>-</sup> AUL types still cannot be accurately classified. Within this subgroup, it is particularly difficult to assign a lineage commitment to the blast population. According to the French-American-British (FAB) classification,<sup>9</sup> the presence of 3% or more peroxidase-positive blasts is considered as indicative of acute myeloblastic leukemia. It is obviously unsatisfactory to decide on lineage commitment of the entire blast population when a marker is present on such a small percentage of cells.

In previous studies on human leukemias aimed at defining the distribution of plasma membrane-associated enzymes, it was found that the  $\gamma$ -glutamyl transferase ( $\gamma$ -GT) activity levels were significantly higher on AML than on ALL blasts.<sup>10,11</sup> Hence, we measured  $\gamma$ -GT activity levels in AUL

to examine whether such cases could be assigned to a lineage commitment by this method. Our results show that the combined analyses of TdT and  $\gamma$ -GT activities allows the distinction between lymphoid and nonlymphoid leukemias within Ia-positive AUL.

### MATERIALS AND METHODS

*Normal blood leukocytes.* Peripheral blood T cells and enriched B cells from healthy volunteers were isolated using conventional methods; monocytes were recovered after plastic adherence and granulocytes were obtained by dextran sedimentation.<sup>12</sup>

*Leukemic cells.* All patients were studied at presentation. Peripheral blood and/or bone marrow samples were obtained from 90 patients with a hematologic diagnosis of acute leukemia. The cases were classified according to FAB criteria on whole marrow smears by the referring centers (Table 1).<sup>9</sup> Mononuclear cells were separated by means of Ficoll-Hypaque gradient centrifugation at 400 g for 30 minutes and were first reanalyzed on cytocentrifuged preparations both by morphology (May-Grünwald-Giemsa stain) and cytochemistry using Sudan black, naphthol AS-D chloroacetate

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Table 1. Subclassification of Leukemias

Type	No. of Patients	FAB Type	Surface Markers
Common-ALL	22	L1, L2	CALLA <sup>+</sup> , Ia <sup>+</sup> , panT <sup>-</sup>
T-ALL	15		CALLA <sup>+/+</sup> , Ia <sup>-/+</sup> , panT <sup>+</sup>
AUL	16		CALLA <sup>-</sup> , Ia <sup>+</sup> , panT <sup>-</sup>
Myeloid	25	M1, M2, M3	Ia <sup>+/-</sup>
Myelomonocytic	5	M4	Ia <sup>+</sup>
Monocytic	7	M5	Ia <sup>+</sup>

esterase, and nonspecific esterase.<sup>13</sup> Only suspensions containing >90% leukemic cells were selected for enzymological studies.

**Surface marker analysis.** HLA-DR antigen (Ia), CALLA, and pan-T antigen (LAU-A1) were identified by previously described monoclonal antibodies and xenoserum using both complement-dependent cytotoxicity and indirect immunofluorescence by flow cytometry.<sup>14-17</sup> T cells were identified by rosette formation with neuraminidase-treated sheep erythrocytes<sup>18</sup> and by the presence of pan T antigen.<sup>17</sup> B cells were analyzed by direct immunofluorescence after staining of surface immunoglobulins with F(ab')<sub>2</sub> fragments of goat anti-human immunoglobulins.

**Subclassification of ALL (Table 1).** c-ALL was defined by the presence of CALLA and Ia, the absence of pan-T antigen, and immunoglobulins on the surface of the blasts. T-ALL included rosette-positive and rosette-negative cases, both types of leukemia expressing pan-T antigen.<sup>17</sup> Sixteen patients who were classified on whole marrow smears as FAB type L2 or L1 and expressed only Ia were subclassified as having AUL.

**Enzyme assays.** The  $\gamma$ -glutamyltranspeptidase ( $\gamma$ -GT, EC.2.3.2.1) was determined according to Meister<sup>19</sup> with slight modifications.<sup>20</sup> Incubations were performed on intact cells in duplicate at 37 °C in a total reaction volume of 1 mL containing 60 mmol/L glycylglycine 0.3 mol/L NaCl, 20 mmol/L Tris HCl buffer (pH 8.0) and 2.5 mmol/L of  $\gamma$ -glutamyl-paranitroanilide. The reaction was stopped by the addition of 2 mL of ice-cold 1.5 N acetic acid. After centrifugation, the absorbance of the liberated *p*-nitroaniline was measured at 410 nm. The TdT EC.2.7.7.31 was measured in cytosol fractions obtained by ultracentrifugation (100,000 g/30 min/4 °C) of cell homogenates.<sup>10</sup> Assays were performed at 37 °C in a total reaction volume of 50  $\mu$ L containing 0.2 mol/L of K-cacodylate, pH 6.8; 5 nmol/L MnCl<sub>2</sub>; 2.5% bovine serum albumin; <sup>3</sup>H-dGTP (1 mCi/mL, 0.5 mmol/L); 10  $\mu$ L of p(dA)<sub>12-18</sub> (10 OD U/mL) initiator; and 10  $\mu$ L of the cytosol fraction containing the enzyme. After incubation (15 minutes, 37 °C), 20  $\mu$ L of the suspension was added to a tube containing 20  $\mu$ L of yeast RNA and 3 mL of ice-cold 5% trichloroacetic acid. The precipitates were sedimented by centrifugation (1,000 g for ten minutes at 4 °C), rinsed with 5% trichloroacetic acid, and dissolved in 0.3 mL of 0.1 N NaOH. The "blank" assays contained the same reagents except for the substitution of the initiator with 10  $\mu$ L of water. The total activity was measured by placing into a counting vial 5  $\mu$ L of the remaining unprecipitated reaction mixture. All samples were counted for ten minutes with 10 mL of Aqualuma. The enzyme activities were expressed in units, ie, nmol/h per 10<sup>6</sup> cells or pmol/min/mg protein for  $\gamma$ -GT and TdT, respectively.

## RESULTS

**$\gamma$ -GT activity in normal blood leukocytes and leukemic cells.** Plasma membrane  $\gamma$ -GT was tested in normal peripheral blood leukocytes from 14 volunteers and in the blast cell suspension from the 90 patients with acute leukemia. Normal values for peripheral blood leukocytes were

9.6  $\pm$  1.4 units in T lymphocytes, 14.1  $\pm$  3.6 units in B lymphocytes, 11.5  $\pm$  3.0 units in granulocytes, and 0.8  $\pm$  0.5 units in monocytes. These values were very different from those found in their leukemic counterparts. With an arbitrary limit of 5 units,  $\gamma$ -GT activity dissected leukemia samples into two groups, ie, lymphoid and myeloid (Fig 1). In lymphoid leukemias, including c-ALL and T-ALL, all values measured in 37 cases were equal or below 5 units. The mean value for c-ALL blasts was 1.5  $\pm$  0.3 units and for T-ALL blasts, 2.1  $\pm$  0.4 units. This was in sharp contrast with myeloid leukemias, in which high  $\gamma$ -GT activity was recorded ( $P < .001$  as calculated by variance analysis). In this group, M4 and M5 samples had values usually exceeding 15 units and the mean values were, respectively, 81.8  $\pm$  91.4 units in M4 and 45.9  $\pm$  50.7 units in M5. Extremely high values (200 to 250 units) were recorded only in M4 and in M5. In myeloid leukemia (FAB M1, M2, M3), the mean value for  $\gamma$ -GT activity was 13.1  $\pm$  10.4 units. Only four of 25 cases had  $\gamma$ -GT levels below 4 units. No significant correlation was found between  $\gamma$ -GT activity and morphological type of leukemia defined according to the FAB phenotype in AML, although a trend associated higher values in M1 than in M3 (data not shown).  $\gamma$ -GT values had a bimodal distribution in patients with AUL: eight patients had values between 0 and 4 units, and eight patients had values between 6 and 93 units.

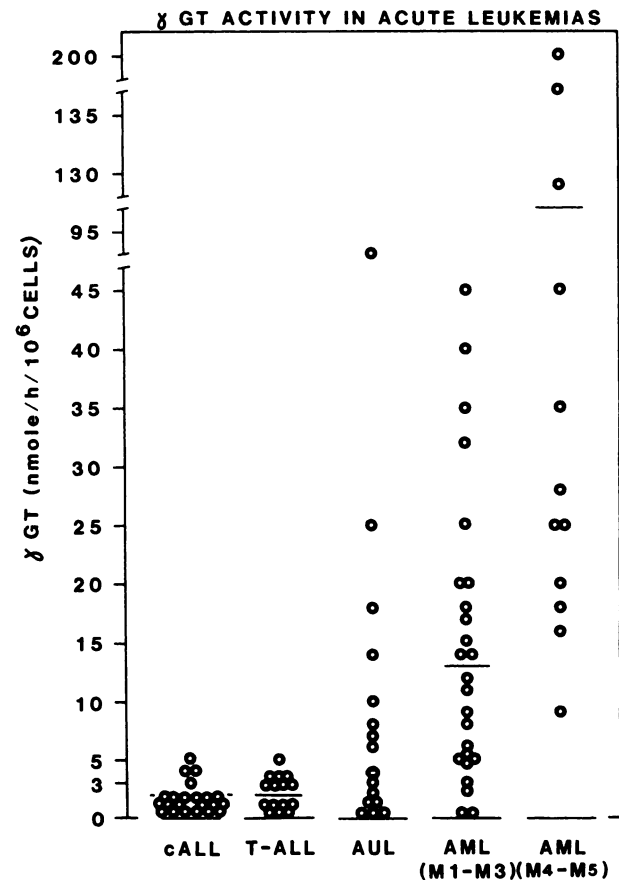


Fig 1.  $\gamma$ -GT activity on blasts from 90 patients with acute leukemia. Horizontal bars indicate means.

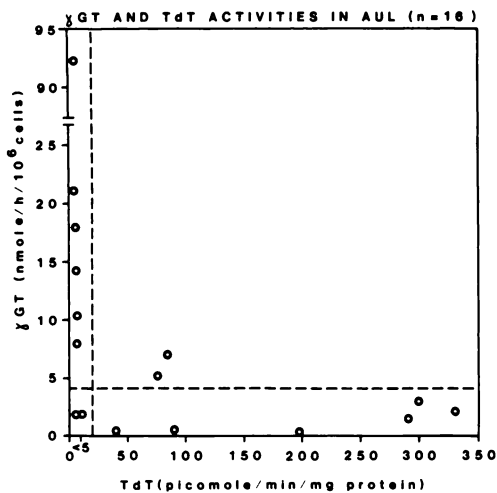


Fig 2. Combined analysis of TdT and  $\gamma$ -GT activities in AUL. Dashed lines indicate arbitrary negativity levels for both enzymes.

*Correlation between  $\gamma$ -GT, TdT, and cytochemistry in AUL.* TdT analysis revealed two subtypes of AUL. Eight patients had elevated TdT values exceeding 40 units and eight patients had normal values ranging from 0 to 5 units. When  $\gamma$ -GT and TdT activities were correlated (Fig 2), six of eight cases with a high TdT content were nearly devoid of  $\gamma$ -GT activity, and six of eight cases with low TdT activity showed high  $\gamma$ -GT values. Two patients of 16 had blasts showing no increase of both TdT and  $\gamma$ -GT activities, and two had blasts with a concomitant increase of both enzymatic activities. Another interesting finding was the cytochemical profile of the blast-enriched suspensions of patients with AUL processed for the enzymologic study. In the six patients with the enzymatic pattern TdT<sup>+</sup>,  $\gamma$ -GT<sup>-</sup>, no myeloblast was found in the blast cell suspension. In contrast, in the TdT<sup>-</sup>,  $\gamma$ -GT<sup>+</sup> subgroup, the presence of 3% to 5% Sudan black-positive blasts was observed in four of six patients. Furthermore, both patients with simultaneously elevated  $\gamma$ -GT and TdT also had 2% to 5% immature Sudan black-positive cells, but both patients with very low  $\gamma$ -GT and TdT activities had strictly Sudan black-negative blastic populations.

#### DISCUSSION

In acute leukemias, the activity of the plasma membrane-associated enzyme  $\gamma$ -GT is strikingly different in myeloblastic leukemias than it is in lymphoblastic leukemias. In a series of 37 cases of AML, only four cases had less than 5

units  $\gamma$ -GT activity. Alternatively, none of 37 cases with ALL had activity levels above 5 units ( $P < .001$ ). Thus,  $\gamma$ -GT activity in leukemic cells above this level seems to be highly characteristic of nonlymphoblastic leukemias.

This bimodal expression of  $\gamma$ -GT in well-defined acute leukemias allowed us to separate most cases of cytochemically and immunologically AUL into lymphoid and nonlymphoid leukemias. Half of the patients with AUL had elevated  $\gamma$ -GT activity. Moreover, the combined analysis with TdT indicated that both enzymatic markers were mutually exclusive in 12 of 14 cases (activity for both enzymes was absent in two cases). Patients who presented with Ia<sup>+</sup>/TdT<sup>+</sup>/ $\gamma$ -GT<sup>-</sup> blasts were likely to have lymphoblastic leukemia; this is in close agreement with the previously defined null-ALL subgroup.<sup>6-8</sup> In contrast, based on the selective expression of  $\gamma$ -GT in acute nonlymphocytic leukemia, Ia<sup>+</sup>/TdT<sup>-</sup>/ $\gamma$ -GT<sup>+</sup> blasts must be considered as nonlymphoid. This interpretation is strengthened by the presence of 2% to 5% Sudan black-positive myeloblasts in six of eight  $\gamma$ -GT<sup>+</sup> cases. Two further enzymatic subtypes of Ia<sup>+</sup> AUL were identified. One consisted of two patients with TdT<sup>+</sup>/ $\gamma$ -GT<sup>+</sup> blasts. As described above, both had minute amounts of myeloblasts, and these cases could correspond to a very undifferentiated TdT<sup>+</sup> AML or mixed leukemias.<sup>21</sup> The other subtype included two patients, in whom the blasts were devoid of both enzyme activities. The lack of  $\gamma$ -GT would not exclude a myeloproliferation, but Sudan black was strictly negative on these blasts. Thus, in our hands, these two cases remain unclassified. Such cases could represent truly undefined acute leukemias.

In conclusion, it seems that the combined analysis of both soluble TdT and of plasma membrane-associated  $\gamma$ -GT is a valuable method for the characterization of morphologically and immunologically unclassifiable acute leukemias. In addition, it points out that AULs represent a heterogeneous entity. It appears to be particularly helpful in identifying myeloid leukemia patients in whom cytochemical stains (Sudan black in this study) are present in only a very small percentage of blasts. From a therapeutic point of view, it might contribute to the choice of the appropriate treatment for these patients.

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