Prognostic Significance of Terminal Transferase and Adenosine Deaminase in Acute and Chronic Myeloid Leukemia

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We have analyzed the distribution and prognostic significance of terminal deoxynucleotidyl transferase (TdT) and adenosine deaminase (ADA) in connection with conventional cytology, cytogentic, response to therapy, and survival. The study population consisted of 78 patients with AML, 44 patients with Ph'- CML in chronic phase, and 35 adult patients with Ph'- CML in blastic phase, among which 5 cases presented as Ph'- acute leukemia. Nine percent of the AML cases were positive for TdT and were characterized by a high percentage of blast cells in bone marrow, myeloid features by cytochemistry and absence of the Philadelphia chromosome. The median ADA values of the TdT AML cases were several times higher than those obtained for the TdT- cases. The survival calculated for the two groups of AML cases subdivided according to ADA levels was significantly longer (p < 0.025) for the patients with low levels of ADA (<250 U/10^6 cells). In chronic phase of CML, TdT was absent and ADA values were increased over normal controls only in cases with early signs of transformation. In blastic phase, 31% of the 35 cases were positive for TdT, and ADA values were significantly higher (p < 0.001) in TdT- than TdT+ cases. The survival calculated from the onset of transformation was significantly longer for the TdT- acute phase (10.4 mo) compared to the TdT+ patients (4.8 mo; p < 0.025). Four cases presenting as Ph'- acute leukemia were TdT- and had elevated levels of ADA; 3 of them responded to ALL therapy, reverting to a stable phase of CML.

Cytologic and cytochemical criteria have proved to be useful in the classification of leukemia. An additional important tool in the characterization of the leukemic blasts has been recently provided by the analysis of specific enzymes. Among these, terminal transferase (TdT) is considered a very selective marker for immature lymphoid cells. The analysis of individual cells in bone marrow has been greatly facilitated by the availability of purified antibodies to TdT, which has also allowed the identification of extramedullary leukemic cells. The presence of TdT has been well documented in blast cells of ALL, in lymphoblastic lymphomas, in about 30% of CML patients in blastic transformation, as well as in several T+ and one pre-B-cell line. The enzyme has also been described in a low proportion of cases of AML, and in two cases of Ph'-negative acute leukemia with mixed lymphomyelomonoblastic morphology.

An additional potentially useful enzyme marker appears to be adenosine deaminase (ADA) which, like TdT, is involved in nucleic acid metabolism and is predominantly found in cortical thymocytes. Peripheral lymphoblasts from patients with ALL contain higher levels of ADA than normal blood lymphocytes, whereas levels of enzyme lower than normal controls have been reported in CLL. However, not many data are available about the distribution of ADA in AML and in Ph'-positive leukemia.

In the present study, TdT and ADA activities were measured in AML and Ph'- leukemias, and the observations were evaluated in connection with conventional cytology, cytogentic, response to therapy, and survival. The purpose of this study was to develop independent biochemical analyses for obtaining prognostic information in AML, in the chronic and in the blastic phases of CML, and to characterize the cases presenting as Ph'-positive acute leukemias.

Materials and Methods

Patient Selection

The study population consisted of 72 adults and 6 children affected by acute myeloid leukemia at presentation, 44 adult patients with Ph'- CML in chronic phase, 30 adult patients with Ph'- CML in blastic phase, and 5 Ph' cases presenting as acute leukemia as well as 16 non-neoplastic human controls. All adult patients involved in this study were admitted to the Division of Hematology of Policlinico S. Matteo in Pavia between January 1979 and October 1981. The six children with AML were patients from the De Marchi Pediatric Clinic of the University of Milan.

Analysis of results was blind, i.e., clinical features, cytochemistry, surface markers, and enzyme assay were analyzed independently by different investigators.

The classification of myeloid leukemia was based on conventional morphology and cytochemical findings, as recommended in 1976 by an international panel (FAB classification).
Reagents

Monospecific rabbit antibodies to homogeneous calf TdT (R-anti-TdT) and purified goat anti-rabbit IgG antibody coupled to fluorescein isothiocyanate (FITC) were donated by Prof. F. J. Bollum. Goat anti-human immunoglobulins conjugated to FITC were obtained from Behringwerke (Marsburg). Poly d(pA) was a gift of P. Plevani. 3H-dGTP was purchased from Radiochemical Centre (Amersham), and GF/C and DE 8 1 papers were obtained from Whatman (Springfield). Nucleosides and nucleotides were from Boehringer (Mannheim).

The TdT immunofluorescence (IF) assay was performed essentially as described by Stass et al.24 Testing was made on smears of bone marrow aspirates, bone marrow touch preparations, cytocentrifugal spreads of cerebrospinal fluid, which were air-dried at room temperature for no longer than 10 days.

TdT and ADA Biochemical Assays

Mononuclear cells, separated on Ficoll-Hypaque gradient, were washed with phosphate-buffered saline (PBS), counted, and frozen at −20°C. The extraction was made by resuspending the cells at a density of 1–2 × 10^6 in 0.25 M K-phosphate, 1 mM mercaptoethanol, and sonicating 2–4 times for 15 sec with an MSE microtip sonicator set at 10–20 mA. The cellular debris was removed by centrifugation at 40,000 rpm for 60 min at 2°C, and the supernatant was used as enzymatic extract.

The reaction mixture for TdT contained in a volume of 0.10 ml: 0.2 M K-cacodylate, pH 7.5, 0.5 mM 3H-dGTP (100 cpm/pmol), 4 mM MgCl₂, 0.01 mM poly d(pA)s, 1 mM mercaptoethanol, and enzyme extract. At different time intervals, aliquots of the reaction were spotted on GF/C and treated for acid insoluble radioactivity. One unit of TdT activity catalyzes the incorporation of 1 nmole of nucleotide in 1 hr.

The enzymatic extract used for the assay of ADA was the same as that prepared for TdT from peripheral blood (see above). The assay measures the conversion of adenosine to inosine and is essentially as described by Coleman and Hutton.25 The reaction mixture contained in a volume of 0.050 ml: 0.05 M K-phosphate, pH 7.5, 0.6 mM [14C]-adenosine (6000 cpm/pmol), 1 mM 2-mercaptoethanol, and enzyme extract. At fixed time intervals, 15-µl aliquots of the reaction were placed on strips of DE 81 chromatography paper previously spotted at the origins with 5 µl of 100 mM unlabeled inosine. The spots were immediately dried and developed by ascending liquid chromatography on 1 mM ammonium formate for about 90 min. After careful drying, the inosine spot was localized with the aid of an uv lamp, cut out, and counted. One unit of enzyme activity corresponds to 1 nmole of inosine produced in 1 min; the mean value obtained in mononucleated cells of the peripheral blood of 10 adult controls was 113 ± 16 U/10^9 cells.

Cytogenetics

Chromosomal analyses were performed using Q-, GT-, or RB-bandng techniques, either on direct preparations of bone marrow and/or on blood cultures with and without phytohemagglutinin.26 Analyses were performed at the Istituto di Biologia Generale e Genetica Medica, University of Pavia.

Statistics

The actuarial survival curves were calculated according to Berkson and Gage.27 Means are given with standard errors. Student's t test was used to test the significance of the differences between means after log transformation of the variables. Significance of the differences between actuarial survival curves was calculated by the method of F distribution.

RESULTS

TdT and ADA in Acute Myeloid Leukemia (AML)

We studied 72 adults and 6 children affected with acute leukemia classified as myeloid before the initiation of chemotherapy. The mean age of the adult patients was 51 yr and that of the children 6.5 yr. Bone marrow myeloblasts varied from 30% to 100%, peripheral blood myeloblasts from 0% to 100%, with the absolute white blood cell counts varying from 1 × 10^9 to 299 × 10^9/liter.

 Peroxidase and Sudan Black stains were positive in 98% of cases in a major fraction of blasts. Naphthol AS-D acetate esterase was negative in 10 patients, positive in a variable percent of blasts in 28 patients, strongly positive in 8 cases with "monocytic pattern," showing complete inhibition by NaF. Chromosomal analysis was carried out in 48 patients, and anomalies were observed in 8 patients. Two cases presented trisomy 8, two presented translocation 8,21, and 4 had more complex anomalies including loss of 2p, monosomy 7, and variable structural rearrangements.

TdT analysis was performed by using the biochemical assay on peripheral blood and bone marrow of 41 patients (Fig. 1). The AML cases were distributed according to FAB classification and to the TdT analysis, as outlined in Table 1. Six adults and one child (M₀ case) were found to be positive for TdT, representing 9% of the tested population. Immunofluorescence analysis was strictly negative in all other patients studied, except in a single case of childhood AML in relapse showing 5% fluorescent cells in bone marrow. The IF test, performed in 14 cases of AML in complete remission gave less than 2% fluorescent cells in bone marrow.

The mean activity of ADA in AML (626 ± 194 U/10^9 cells) is not significantly different from that obtained in non-T, non-B ALL in our series (607 U/10^9 cells), but significantly lower (p < 0.001) than that obtained in T-ALL (3996 U/10^9 cells). Subdividing the ADA values in patients with AML according to their positivity or negativity for TdT, we observed that the median of ADA activity of TdT-negative cases was higher than that obtained in TdT-
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Epipodophyllotoxin derivative, VP-16 213; CR, complete remission; PR, partial remission; NR, no response; ND, nondetermined.

0.025 than the patients with higher levels of ADA

Groups of AML, subdivided according to the levels of ADA experience a significantly longer survival

The main characteristics of TdT+ AML at presentation are summarized in Table 2. All positive cases were male, had a high percent of blast cells in bone marrow, presented cytochemical features of myeloid origin in a variable percent of blasts, and were Philadelphia chromosome negative. The mean activity of TdT (7.2 U/10^6 cells) was significantly lower that that found in ALL at presentation (87 U/10^8 cells), and the percent of fluorescent cells was usually below 50% of the total nucleated cells, indicating that only a fraction of blasts contained TdT. All adult TdT+ cases had a myelomonocytic or monocytic morphology and were treated according to our protocol for acute myelomonocytic leukemias with a four-drug combination chemotherapy consisting of daunorubicin, cytosine arabinoside, 6-thioguanine, and VPI6.213. One case went into complete remission lasting 8 mo, then relapsed and lost TdT positivity. The TdT- cases were treated with a three-drug combination therapy, including daunorubicin, cytosine arabinoside, and 6-thioguanine. The median survival for the TdT+ and TdT- AML patients was 6.0 and 6.3 mo, respectively.

We have also calculated the survivals for the two groups of AML, subdivided according to the levels of ADA activity: higher or lower than the borderline of 10 U/10^8 cells. The therapy was the same for the two groups. The actuarial survival curves (see Fig. 2) indicate that the patients with low levels of ADA experience a significantly longer survival (p < 0.025) than the patients with higher levels of ADA (10.5 and 2 mo, respectively).

TdT and ADA in Ph+ Positive Leukemias

Morphological and cytogenetic analyses were performed in 44 cases of CML in stable phase showing the presence in all cases of the Philadelphia chromosome and in three cases of additional chromosomal anomalies: three chromosome rearrangements involving, in addition to 9 and 22, chromosomes 2 and 6 (2 cases) and loss of Y (one case).

TdT and ADA were analyzed biochemically in the peripheral blood of 18 and 27 cases, respectively. The TdT IF test was performed in 24 cases. The TdT enzymatic assay was strictly negative, giving values below 0.4 U/10^8 cells, and the IF values in bone marrow were below 1%, with the exception of 3 cases ranging between 2% and 4%.

The average value of ADA activity in stable phase was 100± 15 U/10^6 cells, which was very close to that obtained for 10 non-neoplastic controls (113± 16 U/10^6 cells). The values of ADA were increased over the control line only in 2 cases (353 and 267 U/10^6 cells, respectively), showing the early signs of transformation.

The clinical features of 35 adult patients in acute phase of Ph+ leukemia are given in Table 3. The morphology was lymphoid in 9 cases, myeloid in 13 cases, and monocytic in 3 cases. In 10 cases it presented a mixed type, since in peripheral blood and bone marrow, granulated blasts with clear myeloid morphology and agranulated blasts with lymphoid appearance were present.

Cytogenetic analysis showed that 9 cases had, in addition to the standard translocation (9, 22), other single or multiple anomalies in various combinations, including: three-chromosome rearrangements involving chromosomes 2, 3, and 6 (3 cases), duplication of Ph+ (2 cases), isochromosome for the long arm of 17 (1 case), monosomy 7 (1 case), loss of Y (1 case), and a complex rearrangement consisting of the presence of Ph+ chromosome with translocation (5, 22), trisomy 8, and isochromosome for the long arm of 17 (1 case).
Twenty-five patients were analyzed for TdT by the enzymatic assay, 25 patients by the IF test, and 11 by both methods. Eleven of 35 patients (31%) were found positive for TdT. These included 7 patients developing blastic transformation following CML and 4 patients with no history of chronic phase and presenting as Ph+ acute leukemia. The mean value of TdT enzymatic activity obtained in bone marrow and in peripheral blood of TdT+ cases was 96 ± 50 and 53 ± 22 U/10⁸ cells, respectively. These values were close to the mean value found in adult ALL at presentation.²⁸ The percentage of bone marrow cells with nuclear fluorescence ranged between 15% and 60% of total nucleated cells. In the TdT-positive cases, the correlation between morphology by standard methods and TdT assay shows that 6 of 11 cases were clearly lymphoid. The 5 other positive cases had a mixed type morphology in 4 cases and a monocytic morphology in 1 case. The TdT-negative cases (in which no enzyme activity was detectable and with no fluorescent cells in bone marrow) included 23 patients in blastic transformation of Ph¹-positive CML and a single case presenting as Ph¹-positive AML without an apparent chronic phase. The morphology was purely myeloid in 13 cases, monocytic in 2 cases, and mixed in 6 cases. Three cases
showed a "lymphoid" appearance, suggesting a possible micromegakaryocytic origin.30

In cases developing blastic transformation, no difference was observed between the TdT+ and TdT− groups with respect to duration of chronic phases, with medians of 28 mo.

The ADA determinations were of particular interest (see Table 3) since the mean value of the TdT+ group (1331 ± 294 U/10⁸ cells) was significantly higher (p < 0.001) than that found in the TdT− group (290 ± 68 U/10⁸ cells). The correction of the mean activity of ADA for the number of blasts gave a value for the TdT+ group (2426 ± 581 U/10⁸ blasts), which is again significantly higher (p < 0.01) than that found in the TdT− group (744 ± 161 U/10⁸ blasts). An additional observation concerns the distribution of ADA values within the TdT− group. It seems that a positive trend exists between low ADA values and duration of survival. In fact, the median survivals for the cases with ADA higher than 250 U/10⁸ cells and for those with ADA lower than 250 U/10⁸ cells were 4 and 8 mo, respectively.

All TdT− cases were treated according to a protocol including daunorubicin, cytosine arabinoside, and 6-thioguanine. No reversions to chronic phase were observed in this group, with only a partial and short-lasting reduction of blast cells. Eight of the 11 TdT+ patients were treated with a vincristine (V) and prednisone (P) regimen; 7 responded to the therapy and 3 of them reverted to chronic phase of CML.

The actuarial survival curves (Fig. 3) calculated for the TdT+ patients (CML in blastic phase and Ph1+ ALL) and TdT− patients differed significantly (p < 0.025). The median survivals for the two groups were 10.4 and 4.8 mo, respectively. No patient was surviving at 24 mo.

The clinical features and response to therapy of the 5 cases presenting as Ph1+ acute leukemias are given in Table 4. Three of the 4 TdT+ cases presented a lymphoid morphology and one case had a pure monocytic morphology. All lymphoid cases entered a chronic phase of CML after treatment with V and P; the first reverted to CML after a prolonged period of severe aplasia and subsequently developed a CNS relapse with TdT+ blasts in CNS fluid. The second case experienced a stable phase lasting 4 mo when he developed a TdT− second acute phase. The third case

![Fig. 3. Actuarial survival curves for Ph1-positive leukemia in acute phase, according to TdT. Number of patients is given in parenthesis.](image-url)
Table 4. Patients Presenting as \( \text{Ph}^+ \)-Positive Acute Leukemia

<table>
<thead>
<tr>
<th>Case</th>
<th>Sex</th>
<th>Age (yr)</th>
<th>Cytology</th>
<th>Cytogenetics</th>
<th>TdT (U/10^9)</th>
<th>TdT (IF%)</th>
<th>ADA (U/10^9)</th>
<th>Therapy</th>
<th>Survival (mo)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F</td>
<td>17</td>
<td>Lymphoid</td>
<td>( \text{Ph}^+ )</td>
<td>-</td>
<td>-</td>
<td>1,170</td>
<td>CP</td>
<td>15</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>35</td>
<td>Lymphoid</td>
<td>( \text{Ph}^+ )</td>
<td>106</td>
<td>40</td>
<td>126</td>
<td>CP</td>
<td>12</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>44</td>
<td>Lymphoid</td>
<td>( \text{Ph}^+ ; 7 )</td>
<td>52</td>
<td>-</td>
<td>2,380</td>
<td>CP</td>
<td>12</td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>50</td>
<td>Monocytic</td>
<td>( \text{Ph}^+ ; 21 )</td>
<td>78</td>
<td>60</td>
<td>301</td>
<td>*</td>
<td>7</td>
</tr>
<tr>
<td>5</td>
<td>F</td>
<td>40</td>
<td>Myeloid</td>
<td>( \text{Ph}^+ + t(6;9;22) )</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>*</td>
<td>3</td>
</tr>
</tbody>
</table>

CP, reversion to chronic phase.

*Patients treated with a protocol including daunorubicin, cytosine arabinoside, and 6-thioguanine.

DISCUSSION

The blast cells of most cases of acute leukemias classified as myeloid by conventional methods were TdT- . However, in 7 of the 78 (9%) AML cases, the presence of TdT was assessed by biochemical and IF assays. All positive cases presented a myeloid pattern cytochemically and were Philadelphia chromosome negative. The activity of TdT in the positive cases was significantly lower than in ALL at presentation, and the percent of fluorescent cells was below 50% of the total nucleated cells, indicating that only a fraction of blasts contained TdT. The percent of TdT- cases in AML was close to that reported by other authors. However, recent results from Bradstock et al. using both the biochemical and the IF techniques for detecting the enzyme surprisingly indicate that about 20% of AML cases exhibit fluorescent cells.

The finding of TdT in AML points to the existence of a heterogeneous lymphoid and myeloid cell population, possibly resulting from the evolution of the leukemic progenitor cell into clones having different phenotypes. A mixed type population has also been reported in \( \text{Ph}^- \)-negative acute leukemias other than CML in blastic transformation. In our AML cases, the TdT- cells had the appearance of agranulated blasts and presented, by phase contrast, a larger size than the typical lymphoblasts. The recent finding of TdT fluorescence and myeloperoxidase positivity in the same cell further indicate that TdT could also be expressed in nonlymphoid cells.

The comparison of ADA values found in ALL and in AML indicates that this marker is not useful in distinguishing lymphoid and myeloid leukemias. When ADA values found in AML are subdivided into the two TdT- and TdT+ subgroups, it is evident that the median of the TdT- group is several times higher than that of the TdT+ group. However, the number of TdT-positive patients is, so far, too low to suggest a positive correlation between the two enzymes in AML.

Concerning the prognostic significance of the two markers in AML, the median survivals for the TdT- and TdT+ groups are the same (6.0 and 6.3 mo) though more observations are needed to draw a conclusion. Interesting prognostic information was obtained for ADA by calculating the survival curves for AML patients subdivided into two separate groups according to ADA levels higher or lower than 250 U/10^9 cells. It is evident that the patients with low levels of ADA activity present a significantly longer survival than the patients with high levels of ADA (median 10 mo versus 2 mo, respectively). The low median survivals result from the calculation of the actuarial curves with all tested cases, including early deaths and patients who could not complete induction therapy.

Analysis of TdT and ADA in \( \text{Ph}^+ \)-leukemias provided new information for monitoring the chronic phase of CML, which was characterized by TdT absence and levels of ADA ranging usually within normal controls except in cases showing the early signs of transformation, where an increase in activity over the control was noted.

In the acute phase of \( \text{Ph}^+ \)-leukemias, the TdT assay was positive in 31% of the cases, a value close to that reported by other authors. The levels of TdT enzymatic activity were spread over a large scale and showed no strict correlation with the percent of blasts in bone marrow. Two cases with mixed morphology had a very high percent of blasts in bone marrow and very low levels of TdT. The correlation between lymphoid morphology and TdT positivity was not complete. Two positive cases presented myeloid blasts mixed with agranulated lymphoid cells, while two...
negative cases had a "pure lymphoid" appearance and were possibly of micromegakaryocytic type.30

Clinically, the TdT-positive patients showed a more rapid type of transformation, with an abrupt onset of clinical and hematologic symptoms. Our results confirmed the responsiveness of the TdT+ group to treatment with vincristine and prednisone.12 We also observed that this responsiveness resulted in a significantly longer survival time for the TdT+ group. The median survivals obtained for the two groups were comparable to those reported by Janossy et al.27 on the basis of the reactivity with anti-ALL serum.

The blastastic transformation of CML has been shown to be heterogeneous in respect to the distribution of ADA.23 Of special concern is our observation of a significant increase of ADA values in the TdT+ cases with respect to TdT- transformations. This should permit a better definition of the two groups of patients, TdT+ and TdT-, which are treated with different protocols.12

The presence of elevated levels of ADA in patients with ALL has suggested the utilization of 2'-deoxycoformycin, a specific inhibitor of this enzyme, in the treatment of these disorders.38,39 It would be interesting to study the efficacy of this agent also in AML cases and CML blastastic transformations, which show high levels of this enzyme and have become resistant to conventional therapies.

The characterization of Ph1-positive leukemias presenting as ALL is of particular interest to possibly enlighten the relationships between Ph1+ ALL and Ph1+ CML. Our three cases of Ph1+ ALL were all TdT-positive, had high activity of ADA, and responded to ALL therapy reverting to a stable phase of CML; the Ph1 chromosome was present in all the metaphases at onset and did not disappear after therapy. Altogether, this suggests that these two forms of leukemia are strictly related, arising from a common "target cell."

In conclusion, we have shown that the determination of both TdT and ADA is of particular value to identify subgroups of AML and CML that present significant longer survivals. Work is in progress to define the possible usefulness of ADA for monitoring complete remissions in AML and detecting early transformations of CML.

ACKNOWLEDGMENT

We are indebted to Prof. F. J. Bollum for providing us with the antibody against the terminal transferase. We thank Dr. M. G. Zurlo of the De Marchi Pediatric Clinic of Milan for providing specimens of six cases.

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