DNA Nucleotidylexotransferase of Normal Persons and Leukemic Patients

Walid G. Yasmineh, Barbara M. Smith, and Clara D. Bloomfield

We describe a relatively simple and rapid assay for DNA nucleotidylexotransferase (EC 2.7.7.31) activity in normal lymphocytes and leukemic cells from blood and bone marrow of patients with various types of leukemia. We followed the method of Beutler and Kuhl (Am. J. Clin. Pathol. 70: 733, 1978) but separated the product of the reaction by precipitation on filter-paper disks instead of by centrifugation. Normal lymphocytes had a mean activity of 13.5 (SD = 9.21; range 3 to 35) pU/10^6 cells. Leukemic cells from the peripheral blood of patients with acute myelogenous leukemia had a mean activity slightly greater than normal (48 pU/10^6 cells); those from patients with acute lymphoblastic leukemia had a mean activity of 863 pU/10^6 cells, or 62-fold the normal mean. Similarly, cells from patients with chronic myelogenous leukemia in acute phase had a normal activity when the cell proliferation was myelogenous, but much higher activities when the cell proliferation was lymphoblastic. Cells from patients with chronic lymphocytic leukemia had normal activity. In leukemic patients, approximately similar results were obtained with cells isolated from bone marrow.

Additional Keyphrases: activity in lymphocytes and bone marrow cells in various forms of leukemia - cancer - radioassay - terminal transferase - TdT

DNA nucleotidylexotransferase (nucleosidetriphosphate: DNA nucleotidylexotransferase, EC 2.7.7.31; also known as terminal deoxyribonucleotidyl transferase, TdT), a nuclear enzyme first described in the early sixties by Bollum (1-3) and Krakowet et al. (4) catalyzes the addition of deoxynucleotidyl triphosphates onto the 3'-OH end of a polydeoxynucleotide primer:

\[ d(pX)_m + n \text{d(YTP)} \rightarrow d(pX)_m \text{d(Y)}_n + n \text{PP} \]

During the last several years, the enzyme has assumed clinical significance as a useful marker in (a) the diagnosis of acute lymphoblastic leukemia, (b) the differentiation between lymphoblastic and myelogenous cell proliferation in the acute phase of chronic myelogenous leukemia, (c) the diagnosis of certain lymphomas, and (d) the prediction of the response of patients with chronic myelogenous leukemia in acute phase to treatment with vincristine and prednisone (5-17). TdT activity is usually determined by incubating the enzyme with a radiolabeled deoxynucleosidetriphosphate ([3H]- or [14C]-dXTP), a divalent cation, and an appropriate primer, in a suitable buffer system. At various intervals, the extent of polymerization is determined by isolating the polymer as an acid-insoluble precipitate and measuring its radioactivity.

Despite this seemingly simple approach, the methods currently used for TdT assay involve different reaction conditions, because the reaction rate is significantly affected by several factors: kind and length of primer (1-11), ratio of monomer to primer concentration (7), kind of divalent cation activator (5-8, 18, 19), kind of deoxynucleoside triphosphate (3-8, 18) and kind of buffer (7, 19). Consequently, the interlaboratory comparison of results has been difficult.

We describe a method for TdT assay that is simple and relatively rapid, and can be performed on as many as 14 samples at a time. We have determined TdT activities of leukemic cells from patients with various types of leukemia and correlated them with the activities obtained by other methods.

Materials and Methods

Reagents and Materials

Ficoll–Hypaque, relative density 1.078: Combine 24 parts of 90 g/L Ficoll (Sigma Chemical Co., St. Louis, MO 63178) with 10 parts of 500 g/L Hypaque (Winthrop Laboratories, New York, NY 10016). Adjust to a relative density of 1.078 by adding small increments of water or the Ficoll solution.

Cacodylate buffer, 1 mol/L, pH 6.8: Made up in distilled water, 1 L of buffer contains 1 mol of potassium cacodylate, 40 mmol of MgCl2, 2 mmol of ZnSO4, and 25 g of bovine serum albumin. The reagent is stable for at least six months when divided into aliquots and stored at -20 °C.

Oligodeoxyadenylate primer, oligo d(PA)_16, purchased from P-L Biochemicals, Inc., Milwaukee, WI 53205. The reagent is diluted with water to give an absorbance of 10 at 260 nm (or 0.8 mmol of nucleotide per liter) and stored at -20 °C.

Deoxyadenosine triphosphate, dATP, purchased from P-L Biochemicals. A 5.55 mmol/L solution in distilled water is stable for at least six months when divided into aliquots and stored at -20 °C.

Tritiated adenosine triphosphate, [8-3H]dATP(N), purchased from New England Nuclear Corp., Boston, MA 02118. This reagent, supplied at a specific activity of 10-20 KCl/mmol and a concentration of 1 Cl/L in ethanol/water (1/1 by vol), is stable at -20 °C for at least three months.

[3H]dATP (0.1 Cl/L)/dATP solution: Mix one part of the [3H]dATP reagent with nine parts of the 5.55 mmol/L dATP solution; prepare the mixture fresh at the start of each experiment.

Aquasol-2 liquid-scintillation cocktail was purchased from New England Nuclear Corp.

Filter paper disks. 1.1 cm in diameter, were cut out from stacks of no. 1 filter paper (Whatman, Inc., Clifton, NJ 07014) with an electric cork borer.

Patients

We collected 59 specimens of blood or bone marrow from patients with acute myelogenous leukemia, acute lymphoblastic leukemia, chronic myelogenous leukemia, or chronic lymphocytic leukemia, before therapy was begun (see Table 3).

Preparation of Cell Suspensions

Separate normal lymphocytes or leukemic cells from
Table 1. Components of TdT assay

<table>
<thead>
<tr>
<th></th>
<th>Blank</th>
<th>Standard</th>
<th>Unknown</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cacodylate buffer reagent</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>[3H]dATP/dATP</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Cell extract</td>
<td>20</td>
<td>—</td>
<td>20</td>
</tr>
<tr>
<td>TdT standard*</td>
<td>—</td>
<td>10</td>
<td>—</td>
</tr>
<tr>
<td>Physiologic saline</td>
<td>—</td>
<td>10</td>
<td>—</td>
</tr>
<tr>
<td>Water</td>
<td>10</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Primer solution</td>
<td>—</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Total volume</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
</tbody>
</table>

* The unit of activity of the commercial TdT preparation is defined as the amount of enzyme that catalyzes the addition of 1 nmol of dATP onto dGTP as primer per hour at 37 °C. This unit is equivalent to 16.7 pU of activity as defined in the present study (see Calculation of TdT Activity).

heparinized blood or bone marrow by the Ficoll–Hypaque method described by Boyum (20). Because at least 10⁶ cells are required for the assay, the volume of blood or bone marrow needed varies from 2 to 30 mL, depending upon the cell count. Wash the cells three times in physiological saline, then suspend them in saline at a concentration of 1 × 10⁸ to 3 × 10⁹ cells/mL. In our hands, Wright–Giemsa-stained smears of the cell suspensions showed monocyte contamination to be less than 5% in blood from normal controls and less than 1% in the blood or bone marrow of patients with hematologic malignancies. Keep cell suspensions frozen at −20 °C, at which temperature TdT activity is stable for more than 12 months.

Before assay of TdT activity, rupture the cells by thawing and freezing three times, then centrifuge at 100 000 × g for 60 min. Assay the supernatant fluid for TdT activity.

TdT Assay

The method is essentially a modification of the method described by Beutler and Kuhl (21). Table 1 lists the components of the incubation mixture at 37 °C. The mixture contains the following concentrations or amounts in a final volume of 50 μL: 0.2 mol of cacodylate buffer (pH 6.8) per liter, 8 mmol of MgCl₂ per liter, 0.4 mmol of ZnSO₄ per liter, 5 g of serum bovine albumin per liter, 1 mmol of dATP per liter, 1 μCi of [3H]dATP, 0.16 mmol of primer nucleotide per liter, and the extract from 2 to 10⁶ to 6 × 10⁶ cells. Pipette the solutions into 100 × 75 mm disposable glass tubes and start the reaction by adding the sample; incubate at 37 °C. With every batch assayed, include a standard in which the cell extract is replaced with 10 μL of TdT standard solution plus 10 μL of physiological saline. Also include with every cell extract, and with the TdT standard, a blank in which the primer is replaced with water.

After starting the reaction, remove 10-μL aliquots of the incubation mixture at 6, 12, 18, and 24 min (or 10, 20, 30, and 40 min for large batches of samples), place them on the filter paper disks, and precipitate the polymer by dropping the disks into flasks containing ice-cold 50 mL/L trichloroacetic acid (about 15 mL per disk). Disks from each of the four incubation intervals are kept in separate containers. After a minimum of 10 min—to allow the precipitated polymer to adhere well to the filter paper—gently swirl the paper disks in the trichloroacetic acid wash, which is then decanted. Wash the disk twice more with cold trichloroacetic acid (to remove excess monomer), then three times with cold ethanol/water (950/50 by vol), and dry them under an infrared lamp for at least 30 min. Transfer the dry disks to scintillation vials containing 1.5 mL of 40 mmol/L NaOH, add 10 mL of Aquasol-2, mix the solutions, and count the radioactivity of each. To monitor the efficiency of excess monomer removal, place blank filter paper disks in the first trichloroacetic acid wash at the end of each sampling interval.

To determine the total amount of radioactivity added in the incubation mixture, pool the incubation mixtures remaining at the end of sampling. Place on filter paper disks duplicate 10-μL aliquots from the pool, then dry and count the radioactivity, as indicated above.

Calculation of TdT Activity

TdT activity in cell extracts is reported as pico-units (IUB units) per 10⁶ cells, where one unit is defined as the amount of enzyme that catalyzes the polymerization of 1 mol of dATP per minute at 37 °C. The activity, in pU/10⁶ cells, is then equal to: (cpm unknown − cpm blank)/total cpm) × 10⁶/t × n, where (cpm unknown − cpm blank)/total cpm is the fraction of the total counts incorporated into polymer, t is the time in minutes for each of the four aliquots, 50 016 is the number of picomoles of dATP in the incubation mixture, including the small amount of tritiated dATP (16 pmol), and n is the number of cells in the incubation mixture.

We calculate TdT activity for each of the four aliquots and report the average.

Results

Kinetics of TdT Assay

Figures 1 and 2 show that the rate of the TdT reaction is linear with time and increasing enzyme activity. Curve 1B represents the rate of an incubation mixture containing 167 pU of TdT standard from calf thymus (see Table 1), and Curve 1A that of a cell extract with increased TdT activity from a patient with chronic myelogenous leukemia in acute phase. In both cases, the rate of the reaction is linear, even after 40 min of incubation. By that time the amounts of dATP incorporated into polymer were 10 400 and 7800 pmol for Curves 1A and 1B, respectively. These amounts represent 20.8 and 15.6%, respectively, of the total amount of monomer initially present in the incubation mixture (50 016 pmol). They also represent an average elongation in the polymer of about
Figure 2. Linearity of TdT assay

13 and 10 residues, respectively, based on the known number of picomoles of p(dA)$_{13}$ primer (and therefore of primer terminal ends) in the incubation mixture (see TdT assay). The slopes of curves 1A and 1B are 259 and 186, respectively, and indicate the TdT activity in pico-units in each incubation mixture. The activity of 186 pU of the standard represents a 111% recovery of the activity (167 pU) that was added to the incubation mixture. Note that this good correlation was obtained despite the fact that the latter activity was determined by the manufacturer, who used a different assay system containing d(pT)$_5$ instead of d(pA)$_{13}$ as primer (see Table 1). Curve 1C represents the reaction rate of an incubation mixture containing the cell extract with increased TdT activity (Curve A) in the presence of 10 mmol/L N-ethylmaleimide and ethanol/water (1/9 by vol), a combination that is known to inhibit TdT activity (6). The nearly complete inhibition of TdT activity indicates that there is no residual activity from DNA polymerase.

Figure 3 shows a Lineweaver–Burk plot for human TdT with dATP as substrate. The $K_m$ is 0.7 mmol/L, which compares very well with values obtained by other investigators (7, 21). We routinely use in the assay a concentration of dATP (1 mmol/L) that is only slightly higher than the $K_m$. This saves on the amount of dATP used, and consequently on the amounts of $^3$H)dATP and p(dA)$_{13}$ used, because the ratios of dATP to $^3$H)dATP and to p(dA)$_{13}$ affect respectively the sensitivity and optimum activity of the assay (7, 11). As indicated in Figure 1A, however, the rate of the reactions is still linear after 40 min, or after 20.8% incorporation of monomer into polymer.

**Precision and Sensitivity of TdT Assay**

We established the day-to-day variation of the TdT assay by performing 24 replicate determinations on the cell extract represented by Figure 1A, during six months. Mean TdT activity reported as the average of the four intervals of incubation (see Calculation of TdT Activity) was 4208 pU/10$^6$ cells, with an SD of 575 and a CV of 13.7% (Table 2). The CV is higher than values usually obtained for enzymatic determinations, but is reasonably good because of the multiple steps involved in the assay. Table 2 also shows the TdT activities calculated for the individual intervals. Note that for each interval the CV value is somewhat higher than that of the average.

The sensitivity of the assay depends to a large extent on the magnitude of the radioactivity blank, which is usually about 200 cpm or 0.12% of the total cpm (see Calculation of TdT Activity). Greater blank values are sometimes obtained when old dATP reagent is used, as indicated by Beutler and Kuhl (21). In our laboratory, the lower limit of detection is considered to be about twice the blank value, or a net count of 200

**Table 2. Precision of TdT Assay (n = 24)**

<table>
<thead>
<tr>
<th>Incubation, min</th>
<th>TdT, pU/10$^6$ cells</th>
<th>SD</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>3849</td>
<td>647</td>
<td>16.8</td>
</tr>
<tr>
<td>12</td>
<td>4329</td>
<td>757</td>
<td>17.5</td>
</tr>
<tr>
<td>18</td>
<td>4439</td>
<td>664</td>
<td>15.0</td>
</tr>
<tr>
<td>24</td>
<td>4268</td>
<td>646</td>
<td>15.1</td>
</tr>
<tr>
<td>Av.</td>
<td>4208</td>
<td>575</td>
<td>13.7</td>
</tr>
</tbody>
</table>

**Table 3. TdT Activity in Cell Extracts from Patients with Various Types of Leukemia**

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>No. patients</th>
<th>Specimen</th>
<th>Mean</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>16</td>
<td>b.</td>
<td>14</td>
<td>3–35</td>
</tr>
<tr>
<td>AML</td>
<td>6</td>
<td>b.</td>
<td>48</td>
<td>0–145</td>
</tr>
<tr>
<td>ALL</td>
<td>8</td>
<td>b.m.</td>
<td>35</td>
<td>0–108</td>
</tr>
<tr>
<td>CML (myeloid, acute phase)</td>
<td>6</td>
<td>b.</td>
<td>914</td>
<td>186–2888</td>
</tr>
<tr>
<td>CML (lymphoid, acute phase)</td>
<td>9</td>
<td>b.m.</td>
<td>1607</td>
<td>1132–2081</td>
</tr>
<tr>
<td>CLL</td>
<td>5</td>
<td>b.</td>
<td>2123</td>
<td>1625–2620</td>
</tr>
<tr>
<td>CLL</td>
<td>6</td>
<td>b.m.</td>
<td>6</td>
<td>0–16</td>
</tr>
<tr>
<td>CLL</td>
<td>6</td>
<td>b.m.</td>
<td>9</td>
<td>0–24</td>
</tr>
</tbody>
</table>

*a* AML, acute myelogenous leukemia; ALL, acute lymphoblastic leukemia; CML, chronic myelogenous leukemia; CLL, chronic lymphocytic leukemia.  
*b* = blood; b.m. = bone marrow.
cpm after 40 min of incubation. For an incubation mixture containing the equivalent of 6 × 10^6 cells, this is equal to a TdT activity of about 25 pU/10^6 cells. Sensitivity is also directly proportional to the number of cell equivalents in the extract, and it is therefore desirable to have concentrated extracts when the TdT activity is expected to be low.

Normal Values

A range of normal values was determined on lymphocytes from 18 blood-bank donors, nine men and seven women. Because of the low TdT activity in normal lymphocytes, 50 mL of blood was drawn from each donor and the extracts of the isolated lymphocytes were concentrated so as to contain the equivalent of 2 × 10^6 to 5 × 10^6 cells/mL. The mean TdT value was 13.50 (SD 9.21, range 3 to 35 pU/10^6 cells). This mean activity is about twofold that (6 pU/10^6 cells) reported by Greenwood et al. (11) although the range is about the same (1-27 pU/10^6 cells). It should be noted, however, that they used a different incubation system in which dGTP and poly d(pA) were used as primer and monomer, respectively. Accordingly, in our laboratory, leukemic cells isolated from the blood of patients are considered abnormal when the TdT activity exceeds the mean plus 2 SD, or 32 pU/10^6 cells.

Leukemic Patients

Table 3 shows mean and range of TdT activity in extracts of leukemic cells of patients with acute myelogenous leukemia, acute lymphoblastic leukemia, chronic myelogenous leukemia, and chronic lymphocytic leukemia. Leukemic cells from the blood of patients with acute myelogenous leukemia (n = 6) had a mean activity of 48 pU/10^6 cells, or 3.4-fold the normal mean. Mean activity in bone marrow extracts from patients with acute lymphoblastic leukemia (n = 6) was 863 pU/10^6 cells, or 62-fold the normal mean. In patients with chronic myelogenous leukemia in acute phase, the activity was normal, or slightly below-normal (0-14 pU/10^6 cells; n = 6) when the cell proliferation was myeloid, and very elevated when the cell proliferation was lymphoid (1132 and 2081 pU/10^6 cells; n = 2). Leukemic blood cells from patients with chronic lymphocytic leukemia showed normal or slightly below-normal activity (0-16 pU/10^6 cells; n = 5).

Table 3 also shows the TdT activities of leukemic cells from bone marrow. Since in most cases specimens of both bone marrow and blood were obtained from the same patient, it is not surprising that the mean TdT values and ranges compare favorably with those obtained for the leukemic blood cells. It should be noted, however, that the values for bone marrow cells are in general slightly greater than those for blood cells. This is probably ascribable to the fact that TdT is present predominantly in blasts, of which bone marrow usually contains a slightly greater proportion (11). It should also be noted that the normal range for TdT activity in Table 3 was established on mononuclear cells isolated from normal peripheral blood. In this study, a similar range was not established on mononuclear cells from normal bone marrow. Studies by other investigators (15) have shown, however, that the TdT activity of mononuclear cells isolated from normal bone marrow is about twice that of mononuclear cells from normal peripheral blood.

Discussion

The TdT assay described is simple, relatively rapid, and allows the simultaneous assay of 14 cell extracts. Although the components of the assay mixture are similar to those used by Beutler and Kuhl (21), in our assay method the acid-insoluble product of the reaction is separated from excess monomer by precipitation on filter paper disks rather than by centrifugation. In our experience, centrifugation gave consistently higher blanks, was much more tedious and time consuming, and generally yielded results that were less precise. The use of small filter paper disks that can be washed together in one container has the added advantage of being safer, because it obviates the need for centrifugation and washing of each individual radioactive precipitate. Disks made of filter paper were also found to yield lower blanks than the glass fiber disks used by other investigators (11, 13).

To the best of our knowledge, the day-to-day precision studies shown in Table 2 are the only studies in the literature on TdT assay. In our laboratory, a commercial standard and a control are routinely run with every batch (see Figure 1). The control may be an extract of cells with high TdT activity or a pool of cell extracts to which commercial TdT is added. When divided into small aliquots and kept frozen at −20 °C, such controls are stable for use for more than a year.

Our results on leukemic patients are comparable to those obtained by other investigators. In general, TdT activity is highest in patients with chronic myelogenous leukemia with lymphoid cell proliferation (10, 12, 13, 15) and moderately elevated in patients with acute lymphoblastic leukemia (10, 13). It may be slightly increased or normal in patients with acute myelogenous leukemia (6, 10, 13, 15, 16) and is normal in patients with chronic lymphocytic leukemia (10, 12) and acute-phase patients with chronic myelogenous leukemia with myeloid cell proliferation (10, 13).

Wright–Giemsa-stained smears of our mononuclear cell preparations showed that monocyte contamination is <5% in cells from normal controls and <1% in cells from patients with hematologic malignancies. It should be noted, however, that cells isolated from diseases of monoblastic origin, such as acute myelomonocytic and acute monocytic leukemia, show normal TdT activity (14, 15). In one study by Gordon et al. (15), blasts isolated from the peripheral blood of 14 patients with acute myelomonocytic leukemia and seven patients with acute monocytic leukemia stained positively with nonspecific esterase and had normal TdT activity. In the present study, we similarly found normal TdT activity in mononuclear cells isolated from the blood (14 pU/10^6 cells) and bone marrow (7 pU/10^6 cells) of a patient with acute myelomonocytic leukemia.

Table 4 shows direct comparison of the results obtained by the present method and those of three other laboratories, for patients with acute lymphoblastic leukemia. It also lists the types of primer and monomer used in the various methods. Our results compare quite well with those of the three other laboratories, even though different monomers and lengths of primer were used. This is not surprising, however, because the rates of polymerization of dATP and d(pA)₃₀ are approximately equal to those of dGTP and d(pA)₅₀, respectively (7). We have chosen dATP instead of dGTP because the extent

<table>
<thead>
<tr>
<th>Method</th>
<th>Primer</th>
<th>Monomer</th>
<th>Specimen</th>
<th>No. of patients</th>
<th>Mean TdT acyt.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ref. 11</td>
<td>d(pA)₃₀</td>
<td>dATP</td>
<td>b.</td>
<td>6</td>
<td>863</td>
</tr>
<tr>
<td>Ref. 13</td>
<td>d(pA)₅₀</td>
<td>dGTP</td>
<td>b.</td>
<td>18</td>
<td>1219</td>
</tr>
<tr>
<td>Ref. 15</td>
<td>d(pA)₃₀</td>
<td>dGTP</td>
<td>b.m./b.</td>
<td>30</td>
<td>985</td>
</tr>
<tr>
<td>Ref. 17</td>
<td>d(pA)₅₀</td>
<td>dATP</td>
<td>b.m./b.</td>
<td>5</td>
<td>1286</td>
</tr>
</tbody>
</table>

* In refs. 11, 13, and 15, essentially the same system is utilized; cacodylate buffer is used in all four systems.

b = peripheral blood; b.m. = bone marrow; b.m./b. = blood or bone marrow.

The mean activities from refs. 11, 13, and 15 were multiplied by 16.7 to compare them with the activities obtained by our method (see Table 1).
of polymerization with dGTP is limited by the tendency of poly dG to aggregate (6, 7). In the case of the primer, d(pA)$_{35}$ was chosen because it is cheaper (21). Experiments in our laboratory indicate that this cost factor was compounded by the fact that more of the d(pA)$_{35}$ had to be used to achieve a rate comparable to that with d(pA)$_{35}$, presumably because it presents five times fewer 3'-OH terminal ends for chain elongation.

References