Ultramicromethod for Measuring the Activity of Terminal Deoxynucleotidyl Transferase

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We describe an ultramicromethod for measurement of terminal deoxynucleotidyl transferase activity. The materials used are as proposed by Hösil (Clin Chem 1977;23:1476–84) for antenatal diagnosis. The technique is based on the methods of Beutler and Kuhl (Am J Clin Pathol 1978;70:733–7) and Yasmineh et al. (Clin Chem 1980;26:891–5). The procedure makes it possible to reduce the blood sample to 2 mL at the most and to reduce the cost of the measurement very considerably. The technique gives results similar to those given by the conventional techniques.

Additional Keyphrases: leukemia • pediatric chemistry

Terminal deoxyribonucleotidyltransferase (TdT, EC 2.7.7.31) is a marker for immature lymphoid cells, so it is used in the subclassification of acute leukemias (1–6) and in the surveillance of the initial phase of chronic myeloid leukemias for the early detection of the occurrence of a blast crisis of lymphoid origin (7–13). Measurement of TdT activity also makes it possible to predict the response to a lympholytic treatment of the vincristine–prednisone type, whatever the morphology of the blast cells (5, 14, 15).

Because TdT is studied in patients from whom multiple blood samples are taken, we propose here a procedure for measuring TdT by an ultramicromethod that requires no more than 2 mL of blood and permits measurement of other markers, such as adenosine deaminase (EC 3.5.4.2), purine nucleoside phosphorylase (EC 2.4.2.1), and 5'-nucleotidase (EC 3.1.3.5) with the same sample. It is based on the methods of Beutler and Kuhl (16) and of Yasmineh et al. (4).

TdT is estimated by measurement of the radioactivity derived from a tritium-labeled deoxynucleotide triphosphate incorporated into an oligodeoxynucleotide chain.

Materials and Methods

Materials

The materials used for the ultramicromethod are as described by Hösil (17) for antenatal diagnosis: perforated plastic plates (and corresponding supports), Teflon mold and rod, a Hamilton® microsyringe (Model no. 7001 N; Bonaduz, Switzerland), and Parafilm®. We also used a sonicator (Braun-Sonic 3005, with microsyringe no. 853811; B. Braun, Melsungen, F.R.G.) and a Tri-Carb beta-counter (Packard, Rungis, France). Reagents

Lymphoprep® (Flobio, Courbevoie, France).

Phosphate buffer, 250 µmol/L, pH 7.5.


Oligo-dA 12-18 (oligodeoxynucleotide 12-18; Eurobio, Paris, France) in distilled water, corresponding to 10 A/mL.

RNA (Sigma Chemical Co., St. Louis, MO 63178), 25 g/L solution in distilled water.

Cacodylate buffer (200 µmol/L, pH 6.8) containing, per liter, 40 mmol of MgCl₂, 2 mmol of ZnSO₄, and 25 g of albumin.

Trichloroacetic acid, 50 g/L.

Ethanol, 95%.

NaOH, 40 mmol/L.

Instagel® (Packard).

Whatman No. 1 filter paper.

Methods

Sample preparation. Mononuclear cells (lymphocytes and blasts) were obtained from 2 mL of heparinized blood by centrifugation on a density gradient (Lymphoprep) according to Boyum's technique (18). (Four milliliters of blood must be used when the leukocyte count is less than 3.5 × 10⁹/L.) They were rinsed and then resuspended in the phosphate buffer at an optimum concentration between 10⁷ and 1.5 × 10⁸ cells per milliliter. The cells were lysed with ultrasound for three 15-s periods at a power of 60 W, then ultracentrifuged (100 000 × g, 1 h, 4 °C). The supernatant fluid (the "leukosyaste") was used directly for measurement of activity, or was stored at −20 °C without loss of activity.

Enzymatic measurement. Microcuvettes were made in the Parafilm by passing the plates under a Teflon mold. The quality of the microcuvettes was checked under a binocular low-power microscope (10× magnification). The following volumes of liquid were deposited with a microsyringe and carefully checked under the microscope: 0.4 µL of an equilume mixture of the solution of oligo-dA and of freshly prepared cacodylate buffer; 0.3 µL of [^3]H]dATP/dADP; and 0.2 µL of leukosyaste.

On each plate of 10 micro-wells there were three controls in which the oligo-dA was replaced with distilled water, six for measurements, and one with 100% radioactivity ([^3]H]dATP/dADP). The plate was covered with a sheet of Parafilm, the wells were sealed hermetically with the rod, and the plate was placed in a 37 °C water-bath for 1 h. The contents of each micro-well were then placed on a strip of filter paper with a rod, and we stopped the reaction by adding 5 µL of RNA solution on each deposit. The strip of filter paper was then placed in 10 mL of cold trichloroacetic acid and kept at 4 °C for 30 min. After two rinses in trichloroacetic acid and three in ice-cold ethanol, the strip of

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filter paper was left at room temperature to dry. Each deposit was cut out and transferred into a counting vial containing 0.5 mL of NaOH solution and 5 mL of Instagel. The counting vials were kept in the dark at 4 °C before scintillation counting.

Results and Discussion

The results are expressed in nanomoles of deoxynucleotide incorporated per hour per 10^8 cells.

Kinetics of the reaction. The enzymatic reaction was linearly related to time up to 120 min (Figure 1). By diluting an active leukolysate we found that, between 0.47 and 1.41 x 10^6 cells per milliliter, the enzymatic activity was directly proportional to the quantity of leukolysate set to incubate (Figure 2).

Reproducibility of the method. In eight months we carried out 11 separate measurements of TdT activity on the stored-frozen leukolysate from a patient (represented by Figure 2) with acute lymphoblastic leukemia. The mean TdT activity was 55.5 nmol/h per 10^8 cells, with a standard deviation of 4.5 and a coefficient of variation of 8.1%.

Usual values. From 15 control subjects we obtained the usual values, which were almost all zero, although we found some values up to 1.9 nmol/h per 10^8 cells. The mean of the values was 0.3 nmol/h per 10^8 cells, and the standard deviation was 0.6 nmol/h per 10^8 cells.

Comparison of the ultramicromethod with the micromethod (Figure 3). The micromethod that we use at present in the laboratory is similar to that of Beutler and Kuhl (16) and requires a 15-mL sample of blood. We performed 27 measurements (25 subjects with leukemia as detailed in Table 1, and two control subjects) of activity by both techniques. The correlation coefficient, r, was 0.93.

Activity of TdT in subjects with leukemia. The technique was tested on some patients. Table 1 shows the results. They resemble those obtained with the usual biochemical technique in our laboratory and in published accounts (5, 7, 10, 13, 16).

TdT is a marker that should be included in the battery of tests used to pinpoint the biology of leukemic cells. This miniaturization of the assay makes it reasonable to envisage considering it even in children. Indeed, the ultramicromethod requires only 2 µL of leukocyte extract, whereas the micromethod requires 100 µL. Thus it is possible to carry

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**Figure 1.** Linearity of TdT assay with time
(C) extract from a patient with acute lymphoblastic leukemia (activity: 55 nmol/h per 10^8 cells)
(♦) extract from a patient with acute lymphoblastic leukemia (activity: 23 nmol/h per 10^8 cells)

**Figure 2.** Linearity of TdT activity with concentration of leukolysate
Points represent the mean of three determinations, error bars represent the standard deviation. The leukolysate studied had an activity of 55 nmol/h per 10^8 cells

**Figure 3.** Comparison of the results obtained by the ultramicromethod and those with the micromethod
13 values are null by the two techniques

**Table 1. TdT Activity in Blood of Patients with Different Leukemias**

<table>
<thead>
<tr>
<th>Leukemia Type</th>
<th>No. cases</th>
<th>Mean nmol/h per 10^8 cells</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chronic myeloid leukemia (myeloid, acute phase)</td>
<td>4</td>
<td>0.8</td>
<td>0-2</td>
</tr>
<tr>
<td>Acute myeloid leukemia</td>
<td>11</td>
<td>3.2</td>
<td>0-16</td>
</tr>
<tr>
<td>Acute lymphoblastic leukemia</td>
<td>7</td>
<td>13.3</td>
<td>0-55</td>
</tr>
<tr>
<td>Chronic lymphoid leukemia</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
out simultaneous measurements of other activities with the ultramicromethod, because 30 μL of leukolysate is generally obtained. In addition, the greater economy of reagents is not negligible, especially with respect to the oligo-da, which is very expensive.

References

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Fluorescence Polarization Immunoassay for Ethosuximide Evaluated and Compared with Two Other Immunoassay Techniques

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We evaluated a new fluorescence polarization immunoassay (FPIA) for ethosuximide in the Abbott TDx® and compared results with those by two other ethosuximide immunoassays, eMIT® (Syva Co.) and aca® (DuPont). The FPIA assay produced within- and between-day CVs of <5% at the low, medium, and high ranges of the standard curve. For the ethosuximide FPIA assay the standard curve was stable during the 47 days of the study. By all three methods, we analyzed 100 serum and plasma samples from patients who were receiving ethosuximide. The coefficient of determination (r²) for TDx versus eMIT was 0.973 (slope, 0.96; intercept, −0.80); for TDx vs aca it was 0.985 (slope, 1.00; intercept, −2.44); both relationships were statistically significant (p <0.05). Values for patient’s specimens were significantly lower by the TDx than by the aca or eMIT methods (p <0.05).

Additional Keyphrases: anticonvulsant drugs · enzyme immunoassay compared

Ethosuximide is a succinimide drug useful in the treatment of absence (petit mal) seizures (1). Because of wide interpatient variability in pharmacokinetic parameters, monitoring the ethosuximide concentration in serum has been recommended, to optimize drug dosage and therapeutic response (2, 3). Based on clinical studies, a therapeutic range of 40 to 100 mg of ethosuximide per liter of serum has been suggested (4).

Advances in immunoassay technology have produced rapid, automated methods for measurement of ethosuximide in serum. We evaluated the comparative performance of a new fluorescence polarization immunoassay (FPIA) in the Abbott TDx® with two other commercially available immunoassays, aca® and eMIT®.