A New Solid-Phase Immunoassay for Terminal Deoxynucleotidyl Transferase: Analysis of TdT Antigen in Cells, Plasma, and Serum

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A solid-phase immunoassay for terminal deoxynucleotidyl transferase (TdT), a normal developmental enzyme that synthesizes DNA without template instruction, is expressed in cortical thymocytes and a small population of normal bone marrow cells. TdT is elevated in leukemic cells from patients with T cell and common acute lymphoblastic leukemia (ALL), in some patients with pre-B ALL and lymphoblastic lymphoma, in tumor cells from 30% of patients with chronic myelogenous leukemia (CML) in blast transformation, and in 50% of patients with acute undifferentiated leukemia (AUL). Treatment strategies are often modified when TdT is detected in neoplastic cells because cells containing TdT are sensitive to chemotherapeutic treatment protocols that include vincristine and prednisone.

Our assay for TdT measures enzyme activity using a radioactive substrate. However, the number of nucleated cells required for the enzyme assay is high (2 × 10^7 cells) and often difficult to obtain in leukemic patients. Antigen detection by immunofluorescence and immunoperoxidase methods can be carried out on a glass slide with fewer cells (500 to 1,000), but this test is limited by the fact that unprocessed slides have a short shelf life and cannot be reliably preserved for shipping to central laboratories.

Immunodetection methods for TdT were advanced with the development of an enzyme-linked immunoassay. Both neoplastic cells and plasma of patients with leukemia were reported to contain high levels of TdT antigen using this assay system. Kaneda et al reported a modification in a solid-phase immunoassay with improved sensitivity (0.2 to 0.4 ng/mL); however, TdT antigen was not consistently detected in sera samples of patients whose neoplastic cells contained high levels of TdT.

In this study, we report the development of a solid-phase immunoassay for TdT that is sensitive (≤0.2 ng/mL) and specific. The immunoassay is compared with enzymatic and immunofluorescence methods positive by other tests. However, the level of detection of terminal transferase antigen in plasma or serum of patients with leukemia did not reflect accurately the level of terminal transferase in neoplastic cells. The solid-phase immunoassay was greater than 100-fold more sensitive than conventional assays for enzyme activity, rendering it potentially useful for quantitatively monitoring terminal transferase in patients with leukemia.

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MATERIALS AND METHODS

All chemicals were of reagent grade or higher from standard commercial sources. H-deoxyguanosine-5′-triphosphate was from New England Nuclear Corp (Boston). The enzyme initiator and affinity-purified rabbit anti-cal TdT were prepared in our laboratory. Goat anti-calf TdT was from P-L Biochemicals (Milwaukee). Goat anti-rabbit IgG (affinity-purified–FITC) was from Kirkegaard and Perry (Gaithersburg, Md).

Clinical materials (blood or marrow) were obtained from patients with ALL (at diagnosis, remission, and relapse), AUL, acute myelogenous leukemia (AML), CML, chronic lymphocytic leukemia (CLL), and normal volunteers. Plasma was obtained by centrifuging the whole blood sample collected in EDTA and was stored immediately at –70°C. Serum was obtained from a 2-mL sample of whole blood collected with no anticoagulant. The protocol for obtaining specimens was approved by the Human Studies Committee at the University of Kentucky. Samples were stored up to a period of two years, with no detectable loss in enzyme activity or antigen after thawing. Smears of peripheral blood or marrow were stained for TdT immunofluorescence. The specimen was defined as positive when <5% of cells showed a stippled nuclear pattern.

Blood and marrow samples were collected and processed as previously described. The washed cell pellets were extracted and analyzed for TdT enzymatic activity. Specimens yielding ≥ 10 TdT U/10^6 nucleated cells in peripheral blood or ≥ 20 U/10^6 nucleated cells in marrow were defined as positive, as previously

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established in our laboratory. For the TdT enzyme-linked immunoassay, the cell extracts were diluted 20-fold in a mixture of 30% goat serum, 0.025% Tween 20, and 10 \( \mu g/mL \) gentamicin in phosphate-buffered saline.

Polystyrene beads (6 mm) coated with goat anti-calf TdT and diluted in 10 mmol/L Tris buffer were used. Rabbit anti-calf TdT IgG preparations were conjugated with horseradish peroxidase\(^{21}\) and diluted to approximately 0.8 \( \mu g/mL \) in 10 mmol/L Tris buffer containing 30% calf serum. TdT standards were made by titrating extracts from the RPMI 8402 cell line in the diluent buffer to 0 to 15 ng/mL. Purified human TdT with a specific activity of 133,000 U/mg was the reference standard.\(^{22}\) The positive control consisted of TdT obtained from the RPMI 8402 cell line in diluent buffer containing 10% human blood.

TdT concentrations in individual patient samples were determined by a direct solid-phase (sandwich) enzyme immunoassay. The samples consisted of a frozen cell extract that was thawed rapidly and divided into two aliquots. One was used for assay of enzyme activity, and one was used for antigen detection. We found that this method gave data identical to that obtained by assaying fresh cellular extracts or by assaying frozen cell pellets. Specimens, standards or controls (0.2 mL), were incubated for 90 minutes at 30 °C with the antibody-coated beads. After washing with deionized water to remove unbound material, the beads were incubated with 0.2 mL of horseradish peroxidase-conjugated anti-TdT solution for 90 minutes at 30 °C. Following the incubation period and a second water wash, 0.3 mL of substrate solution (0.3% O-phenylene diamine-2HCl and 0.1 mol/L citrate phosphate buffer, pH 5.5 containing 0.02% \( H_2O_2 \)) was added. The enzymatic reaction was allowed to proceed for 30 minutes at room temperature in the dark. The reaction was terminated by adding 1.0 mL of 5% \( H_2SO_4 \) and the color intensity was quantitated at 492 nm using a Quantum 1 spectrophotometer (Abbott Laboratories). For specimens with TdT concentrations greater than the highest standard, serial dilutions were made.

Standard curves for quantitation of TdT antigen were linear in the region of 0 to 15 ng/mL. Control panels, which have been constructed using lymphoblast cells mixed with 10% blood, illustrate a variability of approximately 5% when the enzyme-linked immunoassay, (EIA) test was performed on successive days (data not shown).

Enzymatic assay data were compared with the enzyme-linked immunoassay data to determine levels of antigen that correlated with elevated TdT activity. Samples were considered to be positive when they contained \( \geq 166 \) ng/10\(^6\) cells in marrow and 86 ng/10\(^6\) cells in peripheral blood.

**RESULTS**

A good correlation between lymphoblast cell extract enzymatic activity and enzyme-linked immunoassay was obtained (Fig 1). All of the samples judged to be positive by enzyme activity were positive by antigen detection. Although a positive correlation between increasing antigen level and increasing enzymatic activity was obtained, it was not linear, particularly at the highest levels of enzymatic activity. The reason for this lack of linearity is not understood, but it is similar to comparisons of enzymatic activity and immunofluorescence detection of antigen.\(^{18}\) Similarly, in all samples judged negative by enzyme assay, negligible levels of antigen were detected.

The TdT enzyme immunoassay was compared with immonofluorescence detection of TdT antigen in blood or marrow smears. The amounts of TdT activity, antigen, and the percentage of TdT-positive cells by immunofluorescence are listed in Table 1. High levels of TdT enzymatic activity correlated well with elevated levels of TdT antigen determined by the enzyme-linked immunoassay and by the immunofluorescence method. When TdT activity was not detected in the sample, TdT antigen was extremely low by both antigen detection procedures.

It has been reported that TdT antigen is detectable in serum and plasma of patients with TdT-positive leukemias, and the hypothesis has been advanced that extracellular fluid might be used to monitor TdT in patients with leukemia.\(^{19}\) We tested this hypothesis with 87 plasma and 64 serum samples from patients undergoing treatment for leukemia. The results with plasma samples are shown in Fig 2. In the 21 cell extracts that were positive for TdT, only six had detectable antigen in plasma. Fifteen of the samples had no TdT antigen. This lack of sensitivity (29%) was clearly unacceptable.

To rule out the possibility that the method of plasma preparation destroyed TdT antigen, we tested the efficacy of monitoring serum samples from three patients (two with ALL and one with CML blast crisis) during a four- to five-month disease course in which several disease relapses occurred. Of the 64 samples tested, the highest serum TdT antigen level was 2.2 ng/mL. In most of the samples, the TdT antigen was undetectable, even though the cell extract exhibited elevated levels of TdT activity (data not shown).
TdT activity in cells

Fig 2. Paired measurements of TdT activity in mononuclear cells and TdT antigen in plasma. Each symbol represents a different patient. Values to the right of the solid and dashed lines on the ordinate are positive for enzyme activity in blood or marrow cells. The solid line parallel to the abscissa is an arbitrary designation for a positive antigen test.

Table 1. Comparison of Enzyme-Linked Immunoassay With Enzyme Activity and Single Cell Immunofluorescence

<table>
<thead>
<tr>
<th>Patient</th>
<th>Tissue</th>
<th>Diagnosis</th>
<th>TdT Antigen (ng/10⁶ Cells)</th>
<th>TdT Immunofluorescence (% Positive)</th>
<th>TdT Activity (IU/10⁶ Cells)</th>
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PB, peripheral blood (mononuclear cells); BM, bone marrow (mononuclear cells); ALL, acute lymphocytic leukemia; ALL-R, ALL in relapse; ALL-r, ALL in remission; AUL, acute undifferentiated leukemia; CML, chronic myelogenous leukemia.

Moreover, we confirmed the instability of TdT antigen in human serum in two in vitro experiments. TdT was extracted from lymphoblasts and diluted 1:50 in a pool of normal human serum. Aliquots were incubated at 37 °C for various time periods, placed on ice, and assayed for residual TdT antigen. As shown in Fig 3 (triangles), 50% of the TdT antigenic activity was lost after 40 minutes at 37 °C. A similar experiment was carried out using the cell line 8402 resuspended in normal human serum and then disrupted. The T/2 of the more concentrated antigen in this experiment was 69 minutes (Fig 3, squares). Since our antibody detects denatured TdT protein, we assume that the antigen is rapidly degraded by proteolytic enzymes present in serum. The instability of TdT at 37 °C was associated only with extracellular antigen. When thymocytes, leukemic cells, or whole blood were maintained at 37 °C for up to 24 hours, little loss (< 10%) of TdT activity was observed in our laboratory (data not shown).

DISCUSSION

TdT determinations have become useful in the diagnosis and management of several types of leukemia.
Heretofore, identification of this enzyme has relied on a relatively cumbersome and technically demanding enzyme assay or immunofluorescence evaluation, which has been associated with problems in reproducibility due to instability of shipped slides, observer error, and atypical antigen distribution, such as that observed in some cases of CML blast crisis. The enzyme-linked immunoassay showed a better than 100-fold improvement in sensitivity over the enzyme assay and required skills and equipment common to laboratory tests utilizing solid-phase enzyme-linked immunoassay technology. The immunoassay described here is ten- to 20-fold more sensitive than an earlier enzyme-linked immunoassay described for TdT and is of comparable sensitivity to a solid-phase enzyme immunoassay described previously. However, this study is the first to analyze large numbers of patient samples by three parameters: enzyme activity, single cell immunofluorescence, and enzyme-linked immunoassay. While it is apparent that an enzyme-linked immunoassay may not supplant slide tests for samples with very small numbers of cells (such as in the cerebrospinal fluid), the sensitivity and specificity of the solid-phase test for TdT antigen were excellent. The advantage of the enzyme-linked immunoassay that may be important in the clinical setting is the utilization of stored, frozen positive and negative control standards, which have been demonstrated to be stable for at least six months in our laboratory. In contrast, techniques have not been found to stabilize TdT antigen on a slide (for extended time periods) for immunofluorescence and immunoperoxidase procedures. This problem has hampered the routine use of these techniques.

The second goal of the study was to evaluate the usefulness of the detection of TdT antigen in serum or plasma samples of patients with TdT-containing leukemic cells. In contrast to Stass et al., in agreement with Kaneda et al., we found that detection of TdT antigen in plasma or serum was an unreliable method of evaluating patients for TdT because 15 of 21 samples with significant levels of TdT detected by cell assay were negative when plasma was studied. It is not apparent why our experimental data with plasma differ from those reported by Stass et al, as no obvious differences in plasma preparation or storage were discerned. As our data agree with those generated by the Kaneda group, the results obtained by Stass et al may simply reflect the small number of samples assayed. As a result of these experiments, we have concluded that serum or plasma samples will be of little use in monitoring TdT in patients during disease course.

The stability of the reagents that were used in the TdT enzyme-linked immunoassay and the day-to-day reproducibility of reagents appeared to be excellent. The good correlation among the TdT enzyme-linked immunoassay, the quantitative enzyme assay, and the single cell immunofluorescence obtained in this preliminary study suggested that further testing of these reagents on a large clinical scale is warranted.

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