CONCISE REPORT

Histochemical Demonstration of Terminal Deoxynucleotidyl Transferase in Leukemia

By Thomas Hecht, Stephen J. Forman, Ursula S. Winkler, Sally Santos, Karl J. Winkler, Fumiko Carlson, William C. Maslow, William Z. Borer, and Karl G. Blume

White blood cells from 22 patients with leukemia and lymphoma were studied for the presence of terminal deoxynucleotidyl transferase with a peroxidase-antiperoxidase method. The enzyme was detected in leukemic cells of 5 patients with acute lymphoblastic leukemia and 1 patient with chronic myelogenous leukemia, whereas 16 patients with different forms of leukemia or lymphoma were negative for this enzyme. Comparative studies using a biochemical and an indirect immunofluorescence assay revealed complete concordance between these three methods.

Several recently developed techniques have led to new classification of leukemia and lymphoma.1,3 Terminal deoxynucleotidyl transferase (TdT), an intracellular enzyme, that catalyzes the addition of deoxynucleotides to the 3'-OH end of oligonucleotides or polydeoxynucleotides without template instruction is known to be a marker of certain types of primitive lymphocytes.4 Whereas in normal individuals only immature thymic lymphocytes and bone marrow cells express this enzyme, high activity is found in almost all (~94%–96%) patients with acute lymphoblastic leukemia (ALL), in one-third of patients with chronic myelogenous leukemia (CML) in blast crisis, and in some forms of non-Hodgkin's lymphoma.5

The recent availability of antibodies to bovine TdT that cross-react with the human enzyme6 has allowed detection of this enzyme in single cells by immunofluorescence4 and localization of its intracellular distribution by electron microscope techniques.7

We describe here a light-microscopic peroxidase-antiperoxidase (PAP) technique that permits a rapid, specific, and highly sensitive screening for this enzyme in peripheral white blood cells.

MATERIALS AND METHODS

Patients and Clinical Status

Twenty-two patients were studied: ALL at diagnosis (n = 5), ALL in remission (n = 5), acute myeloblastic leukemia (AML) at diagnosis (n = 4), chronic myelogenous leukemia in acute phase (n = 5), and non-Hodgkin's lymphoma (n = 3).

Peroxidase-Antiperoxidase Technique

White blood cells were isolated by density gradient centrifugation and smeared on glass slides using a cytocentrifuge. Following a 45-min fixation with absolute methanol at 4°C cells were rehydrated with 0.1 M phosphate-buffered saline (PBS), pH 7.2, for 15 min.

In a four step procedure cells were incubated at room temperature in high humidity chamber with the following: (1) Rabbit anti-calf TdT (obtained from Bethesda Research Lab., Rockville, Md.) for 30 min. This antibody was used undiluted in the screening procedure; in positive cases it was diluted up to 1:8. (2) Goat anti-rabbit IgG (heavy and light chain specific, dilution 1:10, purchased from Cappel Lab., Cochranville, Pa.) for 30 min. (3) PAP immunocomplex from rabbit (Cappel Lab.), dilution 1:20, for 20 min. (4) Diaminobenzidine tetrahydrochloride (DAB) (Sigma Co., St. Louis, Mo.) 0.3 mg/ml DAB, 0.03% H2O2 in 0.05 M Tris-HCl-0.9% NaCl, pH 7.4, for 10 min.

Between all incubations, cells were washed with 0.1 M PBS extensively. Finally, slides were mounted and evaluated under a light microscope.

Controls were carried out by substituting the primary antibody with nonimmune rabbit IgG and, in additional experiments, omission of the secondary antibody.

In order to test whether this assay is influenced by endogenous peroxidase, all samples were incubated with the staining solution directly after fixation. In addition, two cases of AML were tested for the presence of endogenous peroxidase using the technique of Sato and Sekiya8 with and without prior methanol fixation.

Biochemical and Immunofluorescence Assay

TdT activity was determined biochemically using the method of Beutler et al.8 The immunofluorescence assay was performed according to the method described by Bollum et al.4 The anti-TdT antibody was used undiluted and diluted up to 1:8 in positive cases.

RESULTS

Using the PAP technique, the enzyme TdT could be detected in 6 leukemic blood samples out of 22 tested. Five of those were classified as being ALL and one as CML using various hematologic and cytochemical parameters. TdT-positive cells (for percent see Table 1) showed a brown staining of the nucleus with prominent dark spots in multiple areas, suggesting the presence of high concentrations of TdT antigen (Fig. 1). In 16 blood samples designated as being negative, no cell stained for TdT.

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Peripheral TdT Blast counts (%) Enzyme Activity

<table>
<thead>
<tr>
<th>Patient</th>
<th>Peripheral Blast Counts (%)*</th>
<th>TdT Enzyme Activity†</th>
<th>IF 1:1</th>
<th>1:4</th>
<th>1:8</th>
<th>PAP 1:1</th>
<th>1:4</th>
<th>1:8</th>
</tr>
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<tbody>
<tr>
<td>1 (ALL)</td>
<td>85</td>
<td>181.1</td>
<td>54</td>
<td>49</td>
<td>43</td>
<td>60</td>
<td>61</td>
<td>58</td>
</tr>
<tr>
<td>2 (CML)</td>
<td>59</td>
<td>54.5</td>
<td>41</td>
<td>36</td>
<td>31</td>
<td>56</td>
<td>56</td>
<td>52</td>
</tr>
<tr>
<td>3 (ALL)</td>
<td>NA</td>
<td>80.8</td>
<td>59</td>
<td>48</td>
<td>43</td>
<td>54</td>
<td>48</td>
<td>52</td>
</tr>
<tr>
<td>4 (ALL)</td>
<td>92</td>
<td>87.2</td>
<td>54</td>
<td>54</td>
<td>—</td>
<td>58</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>5 (ALL)</td>
<td>30</td>
<td>156.0</td>
<td>24</td>
<td>15</td>
<td>15</td>
<td>24</td>
<td>20</td>
<td>17</td>
</tr>
<tr>
<td>6 (ALL)</td>
<td>88</td>
<td>105.6†</td>
<td>66</td>
<td>50</td>
<td>46</td>
<td>74</td>
<td>75</td>
<td>70</td>
</tr>
</tbody>
</table>

* Determined by Wright-Giemsa stained blood smears; given in percent of white blood cells.
† Normal range: ≤ 5.2 μU/mg protein.
‡ This value was determined in bone marrow (normal range ≤ 22.6 μU/mg protein).
NA, not available.

The simultaneously performed immunofluorescence method revealed brightly fluorescing cells in the same samples in a generally lower range, with the exception of case 3. No cells were found to be positive in the remaining 16 samples. The biochemical assay was in complete concordance with these results in all 22 patients, i.e., all samples found to be positive in the PAP and IF method showed elevated levels of TdT in this assay (Table 1). No activity (≤ 5 μU/mg protein) was detected in the remaining samples.

Dilutions of the primary antibody in the PAP and IF test in all 6 positive patient samples revealed that the antibody could be diluted at least to 1:8 using peroxidase-antiperoxidase staining without an obvious decrease in the number of stained cells, whereas the percentage of stained cells was obviously lower starting at a dilution of 1:4 in the IF method (Table 1) when compared to the results obtained with undiluted antibody.

The “blast” count of each sample, as determined by Wright-Giemsa stained blood smears, is also given in Table 1.

There was no positive staining in any of the negative controls, either by substitution of the TdT antibody by nonspecific rabbit IgG or by omission of the secondary antibody. Staining was also not detectable if the enzyme reaction was performed directly after fixation. In two cases of AML found to stain for endogenous peroxidase using the method of Sato this enzyme could no longer be detected after a prior 45-min fixation with methanol.

DISCUSSION

The measurement of terminal deoxynucleotidyl transferase is useful for the diagnosis and classification of leukemia. Whereas the biochemical test for TdT is a time-consuming assay, the production of cross-reactive antibodies and the use of immunologic techniques have made it possible to demonstrate TdT-containing malignant cells in the patient’s blood within a few hours. The immunohistochemical PAP method, based on a “sandwich” technique has proved to be more sensitive than immunofluorescence techniques when used for the detection of surface antigens on peripheral blood cells. Our studies demonstrate that the PAP technique allows detection of a higher proportion of TdT-containing cells in five of six specimens than the IF method. Additionally, the antibody could be diluted at least one titer step more suggesting that this method may be somewhat more sensitive.

In all six samples positive for TdT, the enzyme appeared to be restricted to the nucleus of the malignant cells, as described in earlier reports. Recently, TdT has been demonstrated in the cytoplasm of all cells of a T-lymphoblastic cell line, Molt-4, using electron microscopic and immunohistochemical techniques. In the same report, 5%-20% of TdT-positive cells with nuclear and cytoplasmatic distribution could be detected using the same TdT antibody in an immunofluorescence technique. These findings corroborate...
that immunohistochemical techniques are more sensitive than immunofluorescence assays when used to detect this enzyme. The contradictory findings in the subcellular distribution of TdT will require further studies of its distribution at various stages of maturation of immature lymphoid cells.

Control experiments revealed that nonspecific staining did not occur in any preparation and that endogenous peroxidase did not influence the assay. Methanol fixation applied to the cells for 45 min at 4°C was able to block endogenous peroxidase of myelogenous cells as demonstrated in two cases of AML.

Our results demonstrate that the PAP method is a sensitive, cost saving, and simple method for screening and monitoring TdT activity in patients with hematologic diseases.

REFERENCES
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