Expression of a Myeloid Marker on TdT-Positive Acute Lymphocytic Leukemic Cells: Evidence by Double-Fluorescence Staining

By P. Bettelheim, E. Paietta, O. Majdic, H. Gadner, J. Schwarzmeier, and W. Knapp

The expression of a myeloid-specific antigen was detected on TdT-positive blast cell populations in two cases of childhood acute lymphocytic leukemia. Double-fluorescence staining by using the monoclonal antibody, VIM-D5, which is specific for cells of myeloid origin, in combination with TdT antisera revealed that a distinct portion of the blast cells carried both markers. The finding represents the first direct demonstration of this specific biphenotype in leukemic cells and was interpreted as the abnormal expression of a myeloid antigen on lymphoid blast cells.

**TERMINAL** deoxynucleotidyl transferase (TdT) has proved to be a valuable biochemical marker for acute lymphocytic leukemia (ALL). In a few cases, acute leukemias with a TdT-positive lymphoblastic predominance and a small population of myeloid blasts have been observed. Conversely, acute myeloid leukemias presenting with elevated TdT activity have been reported. In those cases, the presence of a population of TdT-positive myeloid blasts or the coexistence of lymphoid and myeloid blast cells might be considered.

TdT antisera allow the clear assignment of enzyme activity to certain cells within a whole blast cell population. Using immunofluorescence for TdT detection, an overlap of myeloid markers, e.g., myeloperoxidase, with TdT was rarely found. Since cytochemical staining in combination with immunofluorescence for TdT cannot be performed simultaneously, direct demonstration of both specific myeloid and lymphoid markers on a single cell type had not been possible so far. The recent development of the specific myeloid monoclonal antibody, VIM-D5, prompted us to look for the occurrence of VIM-D5 and TdT positivity in acute leukemia by the immunofluorescent double-label technique. We report the first observation of the expression of TdT and a specific myeloid marker (VIM-D5) on lymphocytic blast cells.

**MATERIALS AND METHODS**

**Patient 1**

In May 1978, the 6-yr-old male patient, H.W., was first admitted to the St. Anna Children Hospital in Vienna. Four years before, the peripheral blood cell count showed 73% of blast cells. The bone marrow was normocellular, with 6-mercaptopurine and a negative reaction in acid phosphatase, peroxidase and α-naphthyl esterase stains. Cytogenetic analysis revealed a normal karyotype.

**Patient 2**

In December 1981, the 3-yr-old female patient, S.Y., was first admitted to the St. Anna Children Hospital in Vienna. The bone marrow smear revealed 98% blast cells of lymphoid appearance (L1 according to the FAB classification), with a negative reaction in acid phosphatase, peroxidase and α-naphthyl esterase stains. Cytogenetic analysis revealed a normal karyotype.

**Cell Preparation**

Heparinized bone marrow specimens were diluted 1:10 in normal saline, layered onto Ficoll-Hypaque gradients, and centrifuged at 400 g for 20 min. Mononuclear cells were collected at the Ficoll-Hypaque and plasma interface and washed twice in phosphate-buffered saline.
MYELOID MARKER ON TdT-POSITIVE CELLS

Terminal Deoxynucleotidyl Transferase (TdT) Assays

Enzyme assay. For the biochemical TdT determination, the micromethod developed by Modak et al.14 was used. Isolated mononuclear cells were suspended at a cell concentration of 1-2 x 10^7/ml in buffer A (50 mM Tris-HCl, pH 7.8, 150 mM KCl, 0.5% Triton X-100, 10% glycerol, 0.5 mM K-EDTA, 0.1 mg/ml bovine serum albumin, and 0.1 mM dithiothreitol) and homogenized in a tight-fitting glass homogenizer. From the cell homogenate supernatants, enzyme activity was purified through binding to phosphocellulose (Whatman P-11). After elution with 0.6 mM KCl buffer, TdT activity was assayed using 1H-deoxyguanosine triphosphate (specific activity 10-12 Ci/m mole) as substrate and a polymer of deoxyadenylic acid with a chain length of 12-18 residues oligo(dA)_{12-18} as primer. Results were calculated from the difference in incorporation in the absence and presence of adenosine triphosphate, a specific inhibitor of TdT, and expressed in units/10^6 cells (1 unit = 1 nmole 1H-deoxyguanosine monophosphate incorporated in 1 hr at 37°C). The 100,000 g supernatant of calf thymus, homogenized in buffer A in a blender, served as standard.

Indirect immunofluorescent staining. Suspensions of living cells were distributed on glass slides in a cytocentrifuge (approximately 5 x 10^7 cells/spot), fixed in absolute methanol (30 min, 4°C), and sequentially incubated with antitransferrase and fluorescein-conjugated F(ab')2-goat anti-rabbit IgG (Behringwerke AG, Marburg, F.R.G.) for 30 min at 30°C. Monospecific rabbit antiserum prepared against glutaraldehyde crosslinked homogenous calf transferrase15 and purified by immunoabsorbent chromatography16 was generously supplied by Prof. Dr. F. J. Bollum, Uniformed Services University of the Health Sciences, School of Medicine, Bethesda, Md.

Monoclonal Antibodies

The monoclonal antibodies and their reactive cell type/antigen used in this study are listed in Table I. The binding of the various antibodies to isolated mononuclear cells was assessed by indirect immunofluorescence with fluoresceinated rabbit F(ab')2 anti-mouse IgG-F(ab')2 antibodies, or in case of the VIM-D5 antigen, by direct immunofluorescence with rhodamine-isolated VIM-D5 hybridoma antibody (method as described previously17).

Table 1. Characterization of VIM-D5 Antibody Specificity on Normal Human Leukocytes

<table>
<thead>
<tr>
<th>Cell Preparations Tested*</th>
<th>No. of Individuals Studied</th>
<th>Percent VIM-D5 Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peripheral blood MNC</td>
<td>10</td>
<td>4 ± 1</td>
</tr>
<tr>
<td>Peripheral blood T lymphocytes (E-RFC)</td>
<td>3</td>
<td>Neg</td>
</tr>
<tr>
<td>Activated T Cells</td>
<td>3</td>
<td>Neg</td>
</tr>
<tr>
<td>Peripheral blood B lymphocytes (SmIg positive)</td>
<td>3</td>
<td>Neg</td>
</tr>
<tr>
<td>Surface la antigen positive MNC</td>
<td>4</td>
<td>Neg</td>
</tr>
<tr>
<td>Plastic adherent MNC</td>
<td>4</td>
<td>5 ± 1</td>
</tr>
<tr>
<td>Peripheral blood basophils</td>
<td>5</td>
<td>Neg</td>
</tr>
<tr>
<td>Peripheral blood granulocytes</td>
<td>7</td>
<td>93 ± 1</td>
</tr>
<tr>
<td>Peripheral blood erythrocytes</td>
<td>8</td>
<td>Neg</td>
</tr>
<tr>
<td>Peripheral blood thrombocytes</td>
<td>5</td>
<td>Neg</td>
</tr>
<tr>
<td>Spleen MNC</td>
<td>3</td>
<td>Neg</td>
</tr>
<tr>
<td>Tonsil MNC</td>
<td>3</td>
<td>Neg</td>
</tr>
<tr>
<td>Thymus MNC</td>
<td>3</td>
<td>Neg</td>
</tr>
<tr>
<td>Lymph node MNC</td>
<td>3</td>
<td>Neg</td>
</tr>
<tr>
<td>Bone marrow MNC†</td>
<td>6</td>
<td>52 ± 13</td>
</tr>
</tbody>
</table>

*Mean ± SEM.
†Obtained from patients with nonhematologic diseases.

VIM-D5 Characterization

The reactivity pattern of VIM-D5 is described in an earlier report.8 Additionally to this report, PHA-activated T lymphocytes (performed as previously described18), suspension of mononuclear cells (MNC) of lymph nodes, and other leukemic cells were tested.

Immunoglobulin Detection

For the detection of immunoglobulins (cytoplasmic and surface Ig), fluorescein-conjugated rabbit F(ab')2 anti-human F(ab')2 antibodies (Behringwerke AG, Marburg) were used.

Double-Fluorescent Staining

Isolated mononuclear cells were first incubated with rhodamine-labeled, isolated VIM-D5 hybridoma antibody. Cytospin preparations of the VIM-D5 labeled cells were then subjected to indirect immunofluorescence staining for TdT as described before.

Fluorescence of the cells was evaluated using a Leitz microscope with incident illumination and equipped for the dual wavelength method.

RESULTS

VIM-D5 Characterization

The characterization of VIM-D5 specificity on normal human leukocytes is given in Table 1. The reactivity pattern of VIM-D5 with leukemia cells is shown in Table 2.
Blast Cell Characteristics of Patients H.W. and S.Y.

When both patients presented with >90% of positive lymphoid blast cells in the bone marrow, the biochemical determination of TdT revealed enzyme levels of 2 and 0.32 U/10⁶ cells in patient H.W. and S.Y., respectively, which were clearly above the activities normally found in the mononuclear bone marrow fraction (<0.1 U/10⁶ cells). Cytologic localization of TdT by indirect immunofluorescence showed that the enzyme was present in 90% of the blast cells from both patients (nuclear staining pattern). The results of further characterization of the leukemic cell phenotypes by monoclonal antibodies are summarized in Table 3. The blast cells from both patients reacted with the monoclonal antibodies VIM-D5, anti-Ia (7,2) and OKT-10, but failed to react with OKM, 9496SA, OKT-3, OKT-4, OKT-6, OKT-8, OKT-11, NA 1/34, T 101, Lyt-3 (9,6), and rabbit anti-human F(ab')₂, heteroantisera (surface as well as cytoplasmic). In contrast to patient H.W., the blast cells from patient S.Y. also showed positive reaction with the anti-cALL antibody VIL-A1. Double-fluorescence staining for TdT and VIM-D5 revealed that in both cases, a distinct portion of the blast cell population (80% for patient H.W. and 30% for patient S.Y.) expressed both TdT and the VIM-D5 antigen (Fig. 1, A and B, respectively).

DISCUSSION

Double-fluorescence staining for TdT and the myeloid-specific antigen VIM-D5 on bone marrow blast cells from two patients with childhood ALL revealed that distinct portions of the total leukemic cell populations reacted positively with both antibodies. To our knowledge, this is the first direct demonstration of the expression of both specific lymphoid and myeloid markers on the same leukemic cell.

Even though the double-fluorescence staining technique as such has been applied successfully before, until recently, the lack of a directly labeled monoclonal antibody to myeloid-specific antigens has hampered concomitant staining for lymphoid and myeloid markers. Among all cases of acute myelogenous leukemia with elevated TdT levels examined by us so far, only patients H.W. and S.Y. showed an overlap of TdT and VIM-D5-positive cells, whereas in the other cases, two distinct blast cell populations could be clearly distinguished, one expressing TdT (20%-80% of total leukemic cells), the other carrying the VIM-D5 antigen. Furthermore, in a total of 60 cases of ALL identified, an overlap in the percentages of VIL-A1- and VIM-D5-positive cells, as detected in patient S.Y., has never been observed.

Former reports on a coincident appearance of myeloid and lymphoid markers dealt with elevated TdT activity seen occasionally in typical nonlymphoid leukemias. To explain these findings, several hypotheses had been offered: a defect at the pluripotent stem cell level leading to partial differentiation into two leukemic cell lineages; the possible conservation of TdT from a TdT-positive progenitor cell in myeloid

Table 3. Reactivity Pattern of Blast Cells From Patients H.W. and S.Y. With Monoclonal Antibodies

<table>
<thead>
<tr>
<th>Clone Designation</th>
<th>Reactive Cells/Antigen</th>
<th>Percent Positive Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>W.H.</td>
<td>S.Y.</td>
</tr>
<tr>
<td>VIM-D5</td>
<td>Myeloid cells</td>
<td>90</td>
</tr>
<tr>
<td>OKM*</td>
<td>Monocytes, Granulocytes, &quot;Null&quot;-cells</td>
<td>0</td>
</tr>
<tr>
<td>9496SA†</td>
<td>Monocytes</td>
<td>0</td>
</tr>
<tr>
<td>VIL-A1†</td>
<td>Common ALL-antigen</td>
<td>0</td>
</tr>
<tr>
<td>7.2†</td>
<td>Ia-antigen</td>
<td>50</td>
</tr>
<tr>
<td>OKT3*</td>
<td>Mature T-Lymphocytes</td>
<td>0</td>
</tr>
<tr>
<td>OKT4*</td>
<td>Helper/Inducer T-Cells</td>
<td>0</td>
</tr>
<tr>
<td>OKT6*</td>
<td>Corticothymocytes</td>
<td>0</td>
</tr>
<tr>
<td>OKT8*</td>
<td>Suppressor/Cytotoxic T-Cells</td>
<td>0</td>
</tr>
<tr>
<td>OKT10†</td>
<td>Precursor and activated cells</td>
<td>80</td>
</tr>
<tr>
<td>OKT11a†</td>
<td>Pan-T antigen</td>
<td>0</td>
</tr>
<tr>
<td>T101†</td>
<td>T-Lymphocytes, B-CLL</td>
<td>0</td>
</tr>
<tr>
<td>NA 1/34§</td>
<td>Thymocytes</td>
<td>0</td>
</tr>
<tr>
<td>9.6 Lyt3†</td>
<td>T-Lymphocytes</td>
<td>0</td>
</tr>
<tr>
<td>Rabbit anti-human</td>
<td>F(ab')₂ (S/Cy)***</td>
<td>0</td>
</tr>
</tbody>
</table>

*Ortho Pharmaceutical Co.
†New England Nuclear.
‡Hybritech.
§Sera Lab.
∥Kindly provided by Bethesda Res. Lab.
§§Kindly provided by Dr. P. Kung, Ortho Pharmaceutical.
***S. surface staining; Cy. cytoplasmic staining.
While previous observations suggested a lymphoid marker (TdT) to be present in myeloid cells, in our case, a myeloid antigen (VIM-D5) was found on TdT-positive lymphoid blast cells. Since the existence of a TdT-positive pluripotential stem cell has been discussed, the question arises whether the common expression of TdT and VIM-D5 might originate from the stem cell level. VIM-D5, a differentiation antigen for cells of myeloid origin, reacts positively with progranulocytes but not with early stages, i.e., myeloblasts or CFU-c. Because of this reaction pattern, the assumption of a TdT- and VIM-D5-positive pluripotential stem cell seems unlikely. A possible persistence of TdT during myelocytic differentiation is most likely out of the question, since human bone marrow progenitor cells (CFU-c) have been found to be TdT negative. On the other hand, a derepressed genome in a TdT-positive precursor cell would enable a lymphoid leukemic cell to express abnormally the VIM-D5 antigen. Therefore, we interpret this observation of TdT-positive ALL cells carrying this myeloid marker as the derepression of the genome.
REFERENCES


Expression of a myeloid marker on TdT-positive acute lymphocytic leukemic cells: evidence by double-fluorescence staining

P Bettelheim, E Paietta, O Majdic, H Gadner, J Schwarzmeier and W Knapp