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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 antiserum 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).[1-3] In a few cases, acute leukemias with a TdT-positive lymphoblastic predominance and a small population of myeloid blasts have been observed.[4,5] Conversely, acute myeloid leukemias presenting with elevated TdT activity have been reported.[5-7] 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.[5,7] 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,[8] 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, PAS-positive ALL (L1 according to the FAB classification[9]) had been diagnosed elsewhere, and upon treatment following the Memphis Protocol VII[10] (induction therapy: prednisone, vincristine; CNS prophylaxis: 2400 rad, methotrexate intrathecal; maintenance therapy: 6-mercaptopurine, methotrexate, cyclophosphamide plus pulses prednisone, vincristine), the patient had achieved complete remission. At admittance to the hospital, the boy presented a partial hematologic relapse (20% of lymphoid blast cells in the bone marrow). Induction therapy according to a modified Memphis Protocol VIII[11] (induction therapy: prednisone, vincristine, asparaginase, daunorubicine; CNS prophylaxis: 2400 rad, methotrexate intrathecal; maintenance therapy: 6-mercaptopurine plus pulses prednisone, vincristine) yielded complete remission. In December 1979, despite intensive maintenance therapy, the patient again relapsed partially. For the following 20 mo, maintenance therapy was continued and the patient stayed in excellent physical condition despite blast cell counts constantly elevated between 10% and 30%. In October 1981, the differential cell count showed 50% of blasts in the peripheral blood. Hemoglobin was 12 g/dl, platelets were 64,000/cu mm. Physical examination showed hepatomegaly (2 cm below the costal margin). The bone marrow smear revealed 94% blast cells, 1% promyelocytes, 1% myelocytes, 1% polymorphonuclear leukocytes, 2% lymphocytes, and 1% erythroid cells. In addition to their lymphoid appearance, the majority of blasts had PAS-positive granules and were negative for focal acid phosphatase, peroxidase (modified Graham's technique[12]) and α-naphthyl esterase stains, consistent with the diagnosis of relapse of ALL. Cytogenetic analysis could not be performed, since it was impossible to bring the blast cells into a state of proliferation.

Patient 2

In December 1981, the 3-yr-old female patient, S.Y., was first admitted to the St. Anna Children Hospital in Vienna in bad physical condition, with anemia and thrombocytopenic purpura, pneumonia, hepatosplenomegaly (5 cm below the costal margin) and splenomegaly (4 cm below the costal margin), and general lymphadenopathy. Hemoglobin was 3.7 g/dl, platelets were 6000/cu mm. The peripheral blood cell count showed 73% of blast cells. The bone marrow smear revealed 98% blast cells of lymphoid appearance (L1 according to the FAB classification[9]), positivity in the PAS staining, but 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.[13]

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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 $1-2 \times 10^7$/ml in buffer A (50 m*M* Tris-HCl, pH 7.8, 150 m*M* KCl, 0.5% Triton X-100, 10% glycerol, 0.5 m*M* K-EDTA, 0.1 mg/ml bovine serum albumin, and 0.1 m*M* 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 m*M* KCl buffer, TdT activity was assayed using ^{3}H-deoxyguanosine triphosphate (specific activity 10–12 Ci/mmole) 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/10^8 cells (1 unit = 1 nmole ^{3}H-deoxguanosine 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 5×10^5 cells/spot), fixed in absolute methanol (30 min, 4°C), and sequentially incubated with antitransferase 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 transferase[15] and purified by immunoabsorbent chromatography[16] 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 1. 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 previously[17]).

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

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

*Mean ± SEM.

†Obtained from patients with nonhematologic diseases.

Table 2. Reactivity of VIM-D5 With Leukemic Cells

Diagnosis	No. of Patients Studied	No. of Patients Positive*
Acute lymphoblastic leukemia		
c-ALL†	62	1
B-ALL‡	2	0
T-ALL§	9	0
Null-ALL‖	11	1
Acute myeloid leukemia		
AML	51	43
AMML	32	30
AMol	13	10
Ph^{1+} CML stable phase	12	12
CML-BC-"M"¶	6	3
CML-BC-"L"†	4	0
B-cell malignancies		
Hairy cell leukemia	3	0
Non-Hodgkin's B lymphoma	18	0
B-CLL	40	0
Myeloma	6	0
Plasma cell leukemia	2	0
Sézary (T) leukemia	2	0

*Positive = >20% cell staining.

†Phenotype: $CALL^+$, $SmIg^-$, E^-/T^-.

‡Phenotype: $CALL^-$, $SmIg^+$, E^-/T^-.

§Phenotype: $CALL^-$, $SmIg^-$, E and/or T^+.

‖Phenotype: $CALL^-$, $SmIg^-$, E^-/T^-, TdT^+.

¶Phenotype: $CALL^-$, $SmIg^-$, E^-/T^-, TdT^-.

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 described[18]), 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.

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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/10^8 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/10^8 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,[28,29] 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.[30] 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 occassionally in typical nonlymphoid leukemias.[5-7,31,34] To explain these findings, several hypotheses had been offered: a defect at the pluripotential stem cell level leading to partial differentiation into two leukemic cell lineages;[31] 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

Clone Designation	Reactive Cells/Antigen	Percent Positive Cells W.H.	Percent Positive Cells S.Y.	References
VIM-D5	Myeloid cells	90	30	8
OKM*	Monocytes, Granulocytes, "Null"-cells	0	0	19
9496SA‖	Monocytes	0	0	20
VIL-A1	Common ALL-antigen	0	>90	21
7.2†	Ia-antigen	50	>90	22
OKT3*	Mature T-Lymphocytes	0	0	23,24
OKT4*	Helper/Inducer T-Cells	0	0	23,24
OKT6*	Corticothymocytes	0	0	23,24
OKT8*	Suppressor/Cytotoxic T-Cells	0	0	23,24
OKT10¶	Precursor and activated cells	80	>90	23,24
OKT11a¶	Pan-T antigen	0	0	23,24
T101‡	T-Lymphocytes, B-CLL	0	0	25
NA1/34§	Thymocytes	0	0	26
9.6 (Lyt3)†	T-Lymphocytes	0	0	27
Rabbit anti-human Immunoglobulin	$F(ab')_2$ (S/Cy)**	0	0	

*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.

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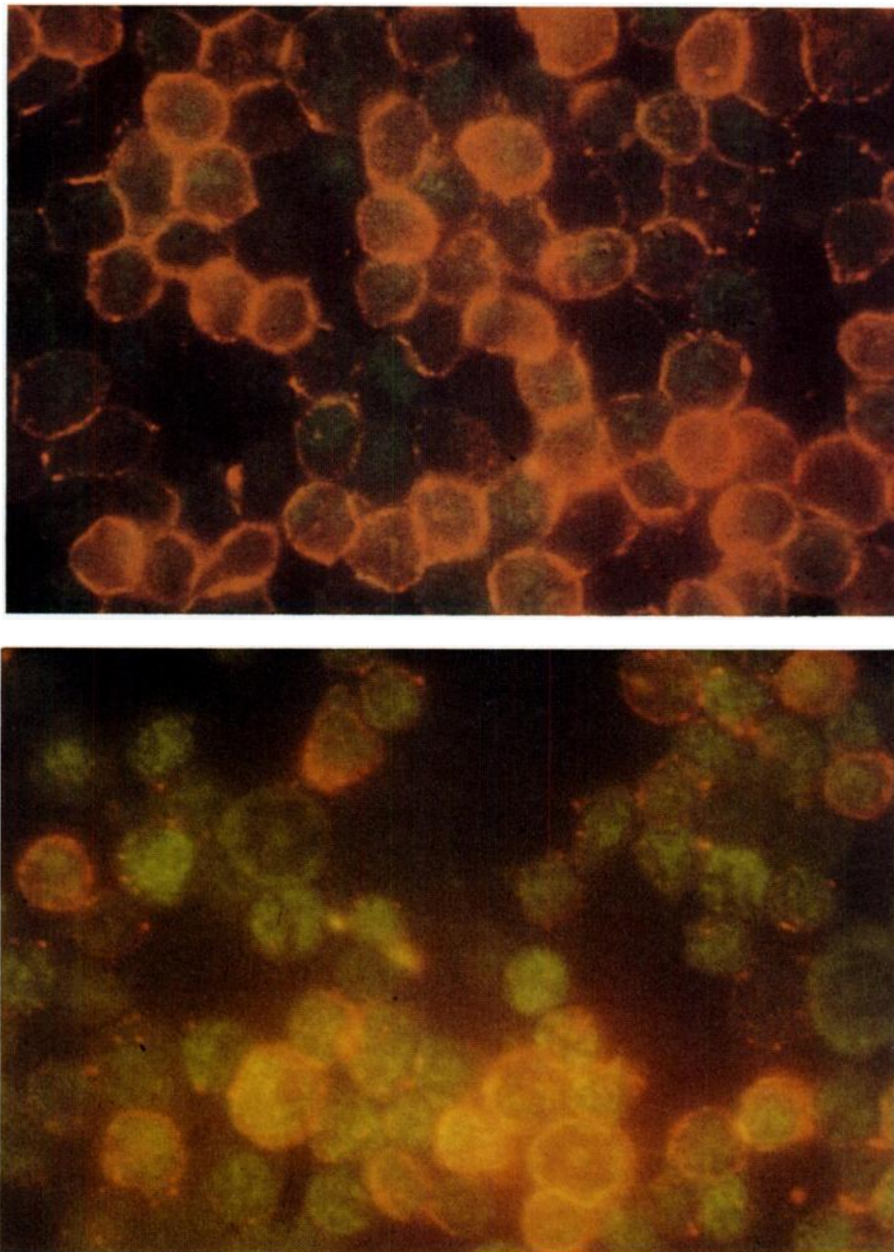

Fig. 1. Double-immunofluorescence with rhodamine-labeled VIM-D5 antibody (surface staining) and fluorescein-labeled TdT antibody (nuclear staining in bone marrow blast cells) from patient H.W. (A) and S.Y. (B).

leukemic cells;[32] or the depression of the TdT genome in the malignant state.[6]

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,[32] 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[8] or CFU-c.[35] 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.[36] 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.

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