**CONCISE REPORT**

**Simultaneous Occurrence of Terminal Deoxynucleotidyl Transferase and Myeloperoxidase in Individual Leukemic Blasts**

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Cases of acute leukemia showing both terminal deoxynucleotidyl transferase (TdT) and myeloperoxidase (MPO) activities are usually classified as acute myelogenous leukemia (AML) regardless of other phenotypic markers. Terminal deoxynucleotidyl transferase (TdT) is a unique nuclear enzyme that catalyzes the polymerization of triphosphates without a template, and is a lymphoblastic-associated marker found in more than 90% of cases of acute lymphoblastic leukemia (ALL) in children and adults. However, TdT+ cells may be found in a small percentage of MPO+ acute leukemias. Recent reports on cases of TdT+ /MPO+ acute leukemia have implied simultaneous expression of TdT and MPO by individual blasts based on percentages of positive cells, and one report using dual staining by immunofluorescence has identified a single cell containing both TdT and MPO activity. Using a new dual staining technique developed in our laboratory that allows simultaneous visualization of MPO and TdT activity by light microscopy, we investigated the potential for leukemic cells to express both MPO and TdT.

**MATERIALS AND METHODS**

**Patients**

Diagnostic bone marrow aspirate smears from three children with AML documented by standard techniques to be MPO+, TdT+, CALLA+ (detected using J5) and HLA-DR were evaluated using the dual staining technique described below. Bachts from patients one and three were classified as M4 in the French-American-British (FAB) system, while those of patient two had M4 features. Marrow aspirate smears from four patients with ALL having MPO, TdT+, CALLA+, and IA+ blasts and rare residual myeloid cells served as TdT+ controls, and aspirate smears from four patients with normal marrows were utilized as TdT+ controls. All clinical specimens were examined within one week to avoid any attenuation of TdT staining. Cytogenetic analyses were performed as previously described.

**Dual MPO/TdT Staining**

Our dual-staining procedure is based on slight modifications of previously published peroxidase-antiperoxidase (PAP) and cytochemical techniques. Briefly, air-dried smears are fixed in 10% neutral, buffered formalin at room temperature for 5 minutes, followed by a 10-minute incubation in a solution composed of 0.15 g of O-tolidine (Fisher Scientific, Pittsburgh, Pa), 6 mL of absolute ethanol, 4 mL of deionized H2O and 10 mL of 3% hydrogen peroxide. This results in yellowish-green to light-brown cytoplasmic staining at sites of MPO activity. The slides are washed for 5 minutes in phosphate-buffered saline, pH 7.4, and subsequently incubated for 5 minutes in 3% hydrogen peroxide. (If inadequate blocking of endogenous MPO activity is apparent from examination of the stained slides, this step may be lengthened to an overnight incubation.) The slides are then washed, incubated for 5 minutes in normal goat serum, and washed again. This is followed by successive 30-minute incubations with rabbit anti-TdT, goat anti-rabbit IgG, and rabbit PAP complex (Molecular Genetic Resources, Tampa, Fla), each with an intervening wash. Finally, the slides are incubated for 10 minutes in 3-amin-9-ethylcarbazole hydrochloride (AEC), resulting in dark reddish-brown intranuclear staining at sites of TdT activity. Diaminobenzidine (DAB) may be substituted for AEC, if desired. Both reagents are potentially carcinogenic and must be handled with caution. Counterstaining with Mayer's hematoxylin for 3 min is performed, and the slides are overslipped in Gelvatol (Monsanto, St Louis, Mo).

**RESULTS**

The four control cases with TdT+ ALL were found to have from 88% to 92% TdT+ blasts and scattered normal MPO+ myeloid cells using the standard techniques for TdT and MPO. After dual staining, 92-95% of the cells were TdT+ and scattered residual...
myeloid cells were again noted to be MPO⁺. No cells stained simultaneously for TdT and MPO. The four normal control marrows showed MPO in the normal myeloid precursors, and either vary rare (<1%) or no TdT⁺ cells using the standard methods. Dual staining yielded the same results, and again there were no cells showing both TdT and MPO activities.

**Patients**

The results of the standard and dual stains when applied to the three patients with TdT⁺/MPO⁺ acute leukemia are summarized in Table 1. Briefly, the percentages of positive cells obtained by standard or dual methods were: 10% v 13%, 25% v 32%, and 89% v 84% for MPO; and 83% v 80%, 57% v 62%, and 55% v 39% for TdT. After dual staining, both MPO and TdT were found in 1% of the blasts from patient 1, none from patient 2, and 23% from patient 3 (Fig 1). Cytogenetic studies disclosed a single leukemic stem line in all three patients: a normal karyotype in patient 1, monosomy 7 in patient 2, and an (8;21) translocation in patient 3.

**DISCUSSION**

Although TdT was originally thought to be a specific marker of acute lymphoid leukemia, it is now clear that approximately 10% of MPO⁺-acute leukemias may have greater than 10% TdT⁺ cells and approximately 3% of such cases have greater than 50% TdT⁺ cells. In cases characterized by both TdT and MPO, overlap of activity within individual cells is implied when the sum of the percentage of MPO⁺ and TdT⁺ cells greatly exceeds 100%. Therefore, the implication of overlapping positivity is only useful in cases with a relatively high percentage of both TdT⁺ and MPO⁺ cells; moreover, it does not unequivocally prove the presence of TdT and MPO in the same cell. Results of dual staining have clearly documented that TdT and MPO can be present in the same leukemic blast. The technique also permits the identification of overlapping TdT and MPO activity even in cases where the percentage of one or both is so small that overlap could not be implied, as was true in patient 1. Such overlap of activity is apparently not present in all cases of TdT⁺/MPO⁺-acute leukemia, since patient 2 showed no evidence of overlap. Alternatively, large numbers of cells may contain both TdT and MPO, as was seen in patient 3.

Folds et al. using dual immunofluorescent staining, demonstrated both TdT and MPO activity in a single cell from one case of acute leukemia. However, the immunofluorescence technique is not generally avail-
able, because of the limited supply of antibody to MPO. Further, quenching of the fluorescence makes it difficult to count cells, and hematoxylin staining for simultaneous observation of cellular morphology is not possible. By contrast, with the dual-staining technique one can observe cellular morphology while simultaneously assessing MPO and TdT activity in any given cell, without quenching. In addition, immunoperoxidase techniques are more sensitive than immunofluorescence, allowing detection of lower concentrations of enzyme than those detected by fluorescence. Finally, slides may be overslipped and stored for long periods without any appreciable degradation in staining.

Our finding of TdT+, MPO+, and TdT+/MPO+ blasts in three patients with a single leukemic stem line demonstrates that the leukemic cells in these cases are capable of expressing both TdT and MPO, either independently or simultaneously. There are three possible explanations for the derivation of the TdT+/MPO+ leukemic cells. First, TdT may be expressed normally by a small population of myeloid cells and thus would not be uniquely associated with lymphoblasts. Second, TdT+/MPO+ cells may represent the malignant counterpart of a pluripotential stem cell which expresses both markers and has the potential to differentiate along either myeloid, lymphoid, or mixed lineage. If either of these postulates is correct, one should be able to detect rare cells positive for both TdT and MPO in normal marrows. Although such cells were not apparent in our normal controls, we cannot overlook the possibility that they will be identified when larger numbers of marrows are evaluated.

A third possibility is that the TdT or MPO found in TdT+/MPO+ blasts is the result of aberrant expression of either a myeloid- or a lymphoid-associated enzyme. Such aberrant expression has been referred to as lineage infidelity13 or mixed leukemia,14-16 and is supported by the recent finding of myeloid markers (eg, VIM-D5) on blasts in ALL17 and lymphoid markers [eg, common acute lymphoblastic leukemia antigen (CALLA)] on blasts in AML.15 Such findings support the concept that there is a spectrum of aberrant marker expression in acute leukemia. It is conceivable that the TdT produced by TdT+/MPO+ blasts differs qualitatively from that produced by lymphoblasts and could be selectively identified by the use of monoclonal antibodies. We intend to pursue this possibility, since monoclonal antibodies to TdT are becoming available.18 Further studies of acute leukemia possessing both TdT and MPO are needed to distinguish among the above possibilities and to show whether the presence or absence of overlapping activity has implications for the categorization, cell biology, and treatment of TdT+/MPO+ acute leukemia.

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