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Indirect TdT Immunofluorescence Differentiates Hematogones from Lymphoblasts

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ABSTRACT

Background: Bone marrow hematogones or B-lymphocyte precursors may cause problems in diagnosis because of their morphologic and immunophenotypic similarities to neoplastic lymphoblasts. Differentiation between hematogones and lymphoblasts is of crucial importance, especially in precursor B leukemia patients receiving chemotherapy, where regenerative marrow exhibiting increased percentage of hematogones is often confused with hypocellular marrow with residual lymphoblasts. Several studies were done to differentiate between those two cell populations. One dependable technique is the use of multiparameter flow cytometry, which might not be feasible in many centres. Only one previous study used indirect TdT immunofluoresence to discriminate between the two cell types.

Aim of the Study: In this study, we attempted to investigate the utility of indirect TdT immunofluoresence as a new technique to distinguish between hematogones and lymphoblasts to verify its applicability.

Patients and Methods: Bone marrow samples from 35 patients of TdT positive acute precursor B lymphoblastic leukemia and 30 patients with increased numbers of hematogones (patients treated with chemotherapy for NHL and HD) were included in the study. The diagnosis of lymphoblasts and hematogones was based on combined morphology and multiparameter flow cytometry. All samples were examined for indirect TdT immunofluorescence.

Results: Our results showed that all cases of hematogones (100%), showed a particular pattern of coarse granular or specked fluorescence which typically aligned the nuclear membrane, whereas all cases of lymphoblasts (100%), showed a fine granular pattern of fluorescence, uniformly distributed in the nucleus.

Conclusion: In conclusion, our findings showed that indirect TdT immunofluorescence could be regarded as a reliable technique for the differentiation between hematogeones and lymphoblasts.

Key Words: *Hematogones – TdT immunofluorescence – B-lymphocyte precursors.*

INTRODUCTION

Hematogones are the normal B-Lymphocyte precursors which are reported to occur in varying numbers in some healthy infants and young children and with decreasing percentages in adults **[1]**. Hematogones may be particularly prominent in the regeneration phase following chemotherapy or bone marrow transplantation and in patients with lymphoma, autoimmune and congenital cytopenias, metastatic pediatric tumors and acquired immunodeficiency syndrome **[2]**.

Increased numbers of hematogones may cause problems in diagnosis because of the morphologic features they commonly share with lymphoblasts of acute lymphoblastic leukemia (ALL) and lymphoblastic lymphoma **[3]**. Single and two color flow cytometry do not reliably differentiate hematogones from leukemic lymphoblasts **[4]**. Appropriately applied 3 and 4-color multiparameter flow cytometry is reported to distinguish between hematogones and precursor B lymphoblasts in nearly all instances **[5,6]**.

Differentiation between hematogones and lymphoblasts is of particular importance in ALL patients receiving chemotherapy, to distinguish between normal B-cell precursors in the regenerating marrow and residual leukemic blast population **[7]**.

The aim of this study is to investigate the utility of indirect immunofluorescence for terminal deoxynucleotidyl transferase (TdT) in distinguishing between hematogones and lymphoblasts.

PATIENTS AND METHODS

Bone marrow samples from 35 patients of TdT positive acute precursor B lymphoblastic leukemia as well as 30 patients with increased numbers of hematogones (patients treated with chemotherapy from NHL and HD) were included in the study. The diagnosis of lymphoblasts and hematogones was based on combined morphology and multiparameter flow cytometry. The leukemia patients were 19 males and 16 females with age ranging from 3 to 23 years. Those with increased hematogones were 18 males and 12 females with age ranging from 2 to 28 years.

Bone marrow morphologic assessment:

Bone marrow aspirates samples reviewed separately by two different hematopathologists.

Flow cytometric immunophenotyping:

Cell isolation and staining procedures:

Prior to staining, samples were processed using erythrocyte lysis technique. Erythrocytes were lysed using a standard ammonium chloride lysing solution at a ratio of 1 part sample to 5 parts lysing solution and incubated for 20 minutes **[8]**. Samples were washed twice with phosphate buffer saline (PBS), 0.0455% sodium azide, 0.1 bovine serum albumin solution (PAB) and re-suspended in 5% calf serum in RPMI culture medium. Cell counts were adjusted to $5x10^9$/ml and stained with a 4 color combination of antibodies. The amount of antibody added was determined by the manufacturer's recommendations. Specimens were incubated at 2°C to 8°C in the dark for 20 minutes, then washed with PAB and re-suspended in 1% paraformaldehyde in PBS.

Antibodies to the following antigens were used to profile hematogones:

CD10, TdT, CD45 fluorescence isothiocyanate (FITC), CD19, CD22 phycoerythrin (PE), CD20 perdinin chlorophyll protein (PerCP) and CD34 and CD38 allophycocyanin (APC).

One of two 4 color combinations was used to characterize hematogones, one consisted of CD10, CD20, CD22 and CD34 and the other consisted of CD10, CD20, CD19 and CD38.

These antibodies are also used to characterize lymphoblast with the addition of CD7, CD8, MPO (FITC), CD5, CD14, CD33 (PE), CD3, HLA-DR (PerCP) and CD4 (APC).

All antibodies were provided by Becton Dickinson, except TdT which was provided by Supertech, Betheda, MD.

Data were analysed using FACSCalibur flow cytometer (Becton Dickinson) with Cell Quest software (Becton Dickenson).

Immunofluorescence for TdT:

Cell suspensions of bone marrow aspirates were prepared and ammonium chloride used to remove red blood cells. Cytospin smears were prepared from marrow suspensions and incubated together with positive control samples (provided by Supertech, Bethesda, MD) at room temperature in a humidity chamber with 1:6 dilution of primary rat anti TdT antibody (provided by Supertech, Bethesda, MD) for 30 minutes. Smears were then washed twice with PBS over a 10 minutes period to remove excessive antibody. Negative cytospin smears were incubated with 1:20 dilution of normal rabbit serum for the same period of time and washed with PBS in the same manner as the positive cytospin smears and the patients' smears. Smears were then incubated for 30 minutes in a humid chamber with the secondary antibody [goat $F(ab')_2$ anti-rabbit antibody provided by Supertech, Bethesda, MD], then washed twice with PBS. Slides were counterstained with a solution containing 100ul of Eriochrome Black (provided by Panbio, Columbia) in 6ml PBS for 1.5 minutes and then washed with PBS for 1 minute. Cover slips were applied using buffered PBS-glycerin. Slides were then examined under fluorescence light using a mercury-100 fluorescence generator coupled to an Olympus BH2 microscope.

RESULTS

Morphologic assessment:

Hematogone size was variable; with large and small cells. They showed scant basophilic cytoplasm, devoid of granulations or vaculations, uniform condensed nuclear chromatin with mostly absent and occasionally inconspicuous nucleoli (Fig. 1-A). Blast cells were mostly medium sized, occasionally heterogenous in size. They showed scant basophilic cytoplasm,

fine chromatin and inconpicuous to prominent, occasionally multiple nucleoli (Fig. 1-B).

Flow cytometric immunophenotyping:

Hematogones showed very low right-angle light scatter and low forward light scatter, with slightly dimmer CD45 than normal lymphocytes and variable expression of CD10, CD19, CD20 and TdT. The earliest B-lineage precursors (14 cases) expressed CD34 in combination with CD38, CD19, bright CD10, low levels of CD22 and lack of CD20. The next stage (11 cases) expressed complete CD34 down regulation with progressive up regulation of CD20 and CD22. The last stage (10 cases) expressed complete CD10 down regulation. TdT expression paralleled CD34 in the B-cell maturation sequence.

Lymphoblasts exhibited incomplete maturation and immunophenotypic asynchrony and aberrancy that deviated from the normal, continuous and complete B-lineage maturation spectrum observed for hematogones. They commonly expressed myeloid antigens as well.

Nuclear TdT indirect immunofluorescence pattern:

The nuclear pattern of TdT immunereactivity was assessed separately by two different hematopathologists, in at least 5 high power fields. The appearance of nuclear fluorescence using the fluorescent microscope indicated a positive reaction.

All 30 cases of increased hematogones (100%), demonstrated a characteristic coarsely granular or speckled pattern of TdT immunofluorescence; intensely aligning the nuclear membrane (Fig. 2-A). On the other hand, all the 35 cases of B-cell leukemia (100%), showed a finely granular pattern of TdT immunofluorescence, uniformly distributed in the whole nucleus (Fig. 2-B).

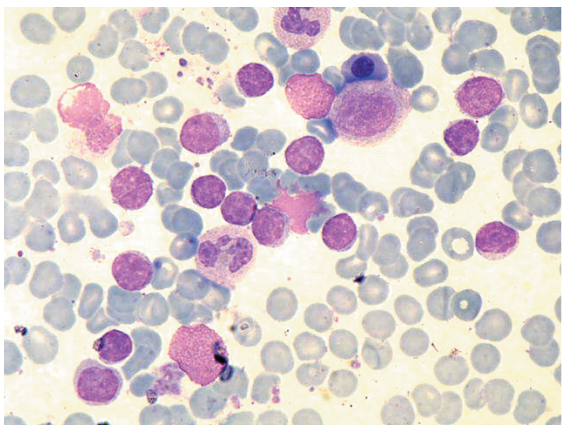

Fig. (1-A): (Left) Bone marrow aspirate smear showing hematogones among normal marrow cells.

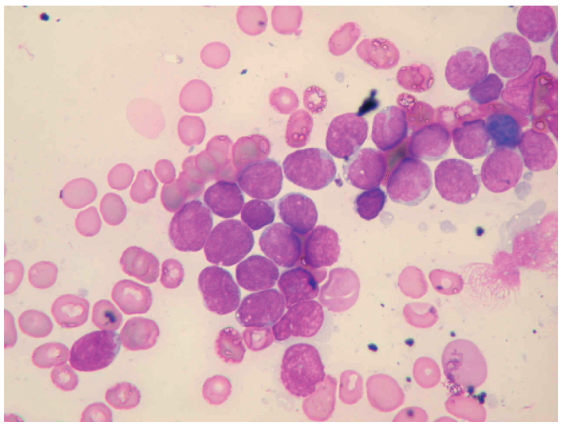

Fig. (1-B): (Right) Bone marrow aspirate smear showing lymphoblasts in a case of precursor B-ALL.

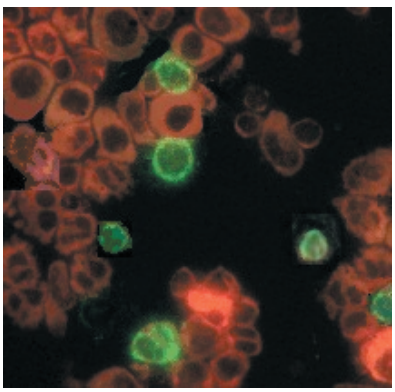

Fig. (2-A): (Left) Indirect TdT immunofluorescence pattern of hematogones (aligning the nucleus).

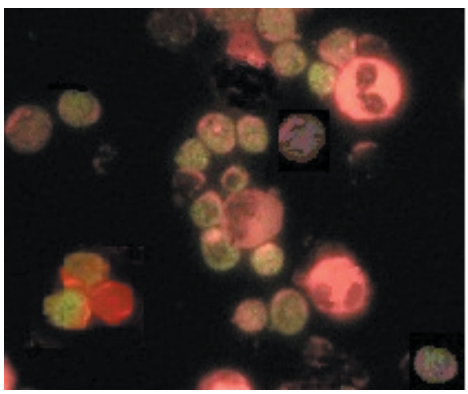

Fig. (2-B): (Right) Indirect TdT immunofluorescence pattern of lymphoblasts (uniformly distributed in the whole nucleus)

DISCUSSION

In patients treated with chemotherapy for precursor B-lymphoblastic leukemia, it is of crucial importance to distinguish between hematogones or normal precursor B-cells which could be increased in percentage and a minimal residual leukemic blast population **[9]**. This could be problematic due to the fact that both cell populations often exhibit morphologic and immunophenotypic similarities **[10,11]**. However, distinction between hematogones and residual lymphoblasts can be readily accomplished in the majority of cases by multiparameter 3 or 4 color flow cytometry **[12,13]**.

Many authors reported various methods for distinguishing hematogones from lymphoblasts. Farahat et al. **[14]**, demonstrated quantitative differences in the number of TdT molecules per cell, with hematogones having a significantly higher TdT level expression compared with lymphoblasts ALL. Weir et al. **[15]**, showed quantitative differences in the light scatter and intensity of antigen expression with clusters of lymphoblasts separated from normal templates of hematogones, using multiparameter flow cytometry. Rimsza et al. **[13]**, used flow cytometry to identify marked heterogeneity of expression of adhesion molecules (CD44 and CD54) in hematogone rich cases compared with ALL cases. Rimsza et al. **[16]**, identified clustering of small numbers of leukemic blasts by immunohistochemistry (1 cluster of 5 or more $CD34^+$, TdT^+ cells) in the bone marrow specimens of patients with ALL in morphologic remission who subsequently experienced relapse.

In the present study, we attempted to investigate indirect TdT immunofluorescence as a reliable technique to differentiate between hematogones and lymphoblasts. In our study, diagnosis of hematogones and lymphoblasts was made according to combined morphologic and 4-color flow cytometer immunophenotypic assessment. Hematogones were found to express a continuous and complete maturation spectrum of normal B-cell development, whereas lymphoblasts typically deviated from the normal maturation pathway. As regards indirect TdT immunofluorescence, 100% of hematogones exhibited a characteristic coarsely granular or speckled pattern of fluorescence, intensely lining the nuclear membrane. On the other hand, 100% of lymphoblasts exhibited a fine granular pattern of fluorescence uniformly distributed in the nucleus. To our knowledge, only Hurford et al. **[7]**, reported indirect immunofluorescence as a technique to distinguish hematogones from lymphoblasts, and we agree in our results with theirs.

We conclude that immunofluorescence against TdT could reliably differentiate normal B-cell precursors or hematogones from lymphoblasts. We recommend its addition in routine follow-up of precursor-B ALL cases in the laboratories that do not have access to multiparameteric flow cytometry.

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