

## Comparison of myeloperoxidase detection by flow cytometry using two different clones of monoclonal antibodies

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### Abstract

**Introduction:** Myeloperoxidase (MPO) is present in azurophilic granules which appear in the promyelocyte stage of differentiation and is expressed in granulomonocytic cells. MPO is usually detected by cytochemistry. The demonstration of peroxidase in at least 3% of bone marrow blasts defines an acute leukaemia as acute myeloblastic leukaemia (AML). MPO is important in distinguishing acute myeloblastic leukaemia (AML) from acute lymphoblastic leukaemia (ALL). It is difficult to diagnose AML with minimal evidence of myeloid differentiation (AML-M0) by conventional light microscopy. However, these AML-M0 blasts can be detected by monoclonal antibodies. Anti-MPO recognizes the enzymatically inactive precursor forms of MPO. There are a few commercially available monoclonal antibodies against MPO. In this study, we evaluated two monoclonal antibodies against MPO from different commercial sources. **Methods:** Anti-MPO were purchased from Dako (Denmark) and Becton Dickinson, BD (California, USA). MPO detection was done using the permeabilisation-staining technique, followed by analysis with flow cytometer (FASCalibur, California, USA). **Results:** 63 cases of acute leukaemias (38 ALL and 25 AML) were studied. Anti-MPO by Dako showed that 12/38 (31.6%) of ALL cases were positive, but all these cases were clear-cut negative for anti-MPO from BD. 24/38 (63.2%) of these ALL cases were associated with aberrant expression of myeloid antigens. However, only 8/24 (33.3%) cases with aberrant myeloid antigen expression showed positive reaction to anti-MPO (Dako). 23/25 (92%) of AML showed concordance results for both anti-MPO by Dako and BD. **Conclusion:** Anti-MPO is a useful and reliable marker for the diagnosis of AML. However, this study had demonstrated that results vary with the monoclonal antibody used in ALL cases. Anti-MPO (Dako) had shown false positive result in 31.6% of ALL cases whereas anti-MPO (BD) had shown consistent negative result in ALL cases.

**Keywords:** Flow cytometry, myeloperoxidase (MPO), acute lymphoblastic leukaemia (ALL).

### INTRODUCTION

Acute leukaemia may be classified on the basis of morphology, cytochemistry, immunological markers or cytogenetics or by combinations of these characteristics. All classifications necessarily have an element of arbitrariness, especially when they incorporate continuous variables such as the percentage of cells falling into a defined morphological category, positivity to a certain cytochemical reaction, or the presence of a certain immunological marker. Myeloperoxidase (MPO) is an enzyme present in azurophilic granules which appear in the promyelocyte stage of differentiation, and is the most common functional protein of myeloid cells. MPO is considered to be selectively expressed in cells committed to granulomonocytic differentiation, accounting for

up to 5% of the dry weight of the cell. The main value of the myeloperoxidase reaction is in the distinction between acute myeloid and lymphoblastic leukaemias.<sup>1,2,3</sup> An early myeloblast with negative myeloperoxidase reaction by light microscopy but expressing myeloid antigens is seen in the FAB M0 type of acute myeloblastic leukemias. Not infrequently, it is difficult however to demonstrate MPO activity in blast cells with conventional light microscopy methods.

The detection of MPO precursor protein by flow cytometric analysis with monoclonal antibodies is essential for the determination of the lineage and precise diagnosis of acute unclassifiable leukaemia that accounts for less than 5% of acute leukaemias. Blasts MPO negative by cytochemical assay may be positive

for MPO monoclonal antibodies in 20-40% of AML-M0 cases, suggesting that anti-MPO antibodies might recognize the enzymatically inactive precursor forms of MPO.<sup>4,5</sup>

We have observed that a few of our acute lymphoblastic leukemia cases which were confirmed by morphology, cytochemistry and immunophenotyping, showed positivity for MPO monoclonal antibody by Dako. Further study with MPO monoclonal antibody by BD yield a clear-cut negative result. This prompted us to carry out a comparison study of these two clones of MPO monoclonal antibodies.

## MATERIALS AND METHODS

This is a descriptive cross-sectional study conducted over a duration of two years (September 1999 to September 2001). The study population consisted of all newly diagnosed or relapsed acute leukaemia patients admitted to Hospital University Kebangsaan Malaysia (HUKM) in Kuala Lumpur. All the patients had their bone marrow smear stained with May-Grunwald-Giemsa and cytochemical peroxidase stains. Flow cytometry analysis for intracellular myeloperoxidase using monoclonal antibodies from Dako and BD was done on the mononuclear cells extracted from the bone marrow or peripheral blood samples using HISTOPAQUE-1077 (Sigma Diagnostic, San Louis, USA)

### Patients

#### Inclusion Criteria

There were total of 63 cases, 25 with acute myeloblastic leukaemia (AML), and 38 cases with acute lymphoblastic leukaemia (ALL) recruited in this study. These cases were independently reviewed by two haematologists for the diagnosis based on the following:

- i) Bone marrow / peripheral blood morphology according to FAB classification.
- ii) Cytochemistry reaction with peroxidase stain
- iii) Immunophenotypic characteristics.

#### Samples and preparation

All the patients had their bone marrow stained with May-Grunwald-Giemsa (MGG). Peroxidase staining was also done as part of routine cytochemistry investigation. Additional bone marrow and/or peripheral blood samples were used for immunophenotyping, including the immunochemical MPO study with MPO monoclonal antibodies from Dako and BD.

For the flow cytometry study, the mononuclear layer was extracted using

HISTOPAQUE-1077 and the extracted mononuclear cells were stained with a panel of monoclonal antibodies, i.e. Leucogate (CD14/CD45), Isotype control (IgG1/IgG2), CD2, CD3, CD4, CD5, CD7, CD8, CD10, CD11c, CD13, CD14, CD16+56, CD19, CD33, CD34, HLA-DR and intracellular MPO. Subsequently the cases were subjected to further study to further classify the leukaemic cells into their subtypes: (1) If B lineage was suspected, staining with CD20, CD22, Kappa/Lambda and intracytoplasmic CD22 were done. (2) If CD3 was negative and T cell malignancy was suspected, intracytoplasmic CD3 was done. (3) If M6 was suspected, CD71 and Glycophorin were done and (4) if M7 was suspected, CD41 and CD61 were done. All monoclonal antibodies were obtained from Becton Dickson (Becton Dickson, USA).

For immunochemical myeloperoxidase (MPO) study, the intracellular fixing, permeabilizing and staining technique (FASTIMMUNE intracellular staining system, Becton Dickinson) was used and the cells were analyzed by flow cytometry. According to Picker *et al* in 1995, by incubating the cells *in vitro* with an activating stimulus and the secretion inhibitor Brefeldin A, the antigens or cytokines produced during the activation will be retained inside the cell and subsequently can be stained with the specific monoclonal antibodies and analysed using the flow cytometer.

#### Flow cytometric analysis of myeloperoxidase (MPO)

Four falcon test tubes were labelled as Leucogate (LG)-Simulset LeucoGATE (CD45/14) was included for establishing a lymphocyte acquisition gate that included greater than or equal to 98% of the normal mature lymphocytes in the sample, Simulset Control (Ig G1/ IgG2) control (C), for setting fluorescence markers around a negative population and detecting non-antigen specific antibody binding, myeloperoxidase monoclonal antibody by Dako and myeloperoxidase monoclonal antibody by BD. The fixed/permeabilized cells were resuspended with 200 µL of perm/ wash and 50 µL was added into each of four tubes. 10µL of antibody was added into each tube respectively. The cells were incubated for 30 minutes at 4°C in the dark. Then, it was washed twice with Perm/Wash solution. It was resuspended with phosphate buffer saline before proceeding to flow cytometric analysis.

**Immunochemical myeloperoxidase**

Myeloperoxidase (MPO) is considered positive or negative by using the isotypic control for comparison. A cut off of 20 % or more is considered positive and less than 20 % is considered negative.

**Normal control**

Normal control blood samples from 9 healthy blood donors were used. This was done to get the normal range for the lymphocyte population in healthy blood donors and to use their normal lymphocytes population as control for gating. The geometric mean was obtained using the histogram. A normal curve was plotted and the new cut off point was obtained using the lymphocyte population.

**RESULTS**

**Expression of MPO on acute myeloid leukaemia blast cells**

A total of 63 cases (25 AML cases and 38 ALL cases) were analysed with two different sources of myeloperoxidase monoclonal antibodies from Dako and BD. In AML cases, 18/25(72%) and 20/25 (80%) of the cases expressed MPO detected by anti-MPO (BD) and anti-MPO (Dako) respectively. MPO-negative AML cases consisted of M0 and M5 according to the FAB classification (Table 1). For AML cases, both the MPO (Dako) and MPO (BD) results were comparable in 23/25 cases (92%). There were only two cases that did not agree with each other. One was a case of AML-M0 and another was a case of AML-M5 which were positive for MPO (Dako) but negative for MPO (BD). Fig 1 shows the histograms of a case of AML illustrating the positive staining for MPO (Dako) as well as MPO (BD) as compared to the isotypic control.

**Expression of MPO on acute lymphoblastic leukaemia cells: comparison of the immunochemical staining with anti-MPO (Dako) and anti-MPO (BD)**

When the isotypic controls were used for gating of the lymphoblasts, 36/38 (94.7%) of ALL cases were noted to be positive for monoclonal antibody MPO (Dako) but none was positive for monoclonal antibody MPO (BD). When normal lymphocytes were used as control to regate all the 38 case, 12/38 (31.6%) of these ALL cases persistently showing positive reaction to MPO (Dako). Fig 2a demonstrates a case of ALL showing false positive staining for MPO (Dako) that was corrected by regating using normal lymphocytes as control. Fig 2b shows the histograms of a case of ALL that was persistently positive for MPO (Dako) even with normal lymphocytes control.

Of the 38 ALL cases, we have identified 24/38 cases (63.2%) expressing aberrant myeloid antigens (CD13 and / or CD33). Among them, 8/24 ALL cases (33.3%) displayed positive reaction towards MPO (Dako). Conversely, 4/14 ALL cases (28.6%) that lacked myeloid antigens also showed positive reaction to anti-MPO (Dako). Thus, this indicates no significant relationship between the positive reaction by anti-MPO (Dako) and aberrant myeloid antigens (CD13 and / or CD33) expression (Table 2).

**DISCUSSION**

The detection of MPO by flow cytometry had been reported as a rapid and reliable technique for the diagnosis and classification of acute leukaemias. Since then, different MPO monoclonal antibodies were commercialized to be used such as anti-MPO from Dako, Denmark and BD USA. These monoclonal antibodies have different epitopes specificity and required controls to eliminate invalid results. In our study, we have analyzed a total number of 63 cases of acute leukaemias with monoclonal anti-MPO by Dako and Becton Dickinson. Of the 38 cases of acute lymphoblastic leukaemia that were confirmed by morphology, cytochemistry and immunophenotyping, 94.7% of these cases were showing positive MPO expression by anti-MPO (Dako) when compared with isotypic controls.

**TABLE 1: The MPO expression on AML cells detected by two different monoclonal antibodies**

	Anti-MPO (BD)		Anti-MPO (Dako)	
	Positive	Negative	Positive	Negative
AML n=25	18	7 M0 – 2 cases M5 – 5 cases	20	5 M0 – 1 case M5 – 4 cases

**TABLE 2: Comparison of immunochemical staining with monoclonal antibodies from MPO (Dako) and MPO (BD).**

Diagnosis	n	MPO (BD)		MPO (Dako)	
		Positive	Negative	Positive	Negative
ALL	38	0	38	12	26
Myeloid antigens association	Pos	0	24	8	16
	Neg	0	14	4	10

However, the percentage of positive cases dropped to 31.6% with the use of normal lymphocytes as control. Thus, this study illustrates that isotypic controls used for the staining of intracellular MPO with monoclonal anti-MPO (Dako) may yield high false positive results and adjustment with normal lymphocytes is mandatory. A comparison done between the monoclonal antibodies anti-MPO (Dako) and (BD) had shown a discrepancy of 12 cases where anti-MPO (BD) staining were uniformly negative but anti-MPO (Dako) staining were persistently positive even after adjustment with normal lymphocyte controls. This positive reaction to anti-MPO (Dako) could be genuinely true or falsely positive. In studies done by previous researchers namely Nakase *et al*<sup>6</sup> in 1998, they had shown that 7/23 cases (30.4 %)

B-lineage ALL and 1/6 (16.7%) of T-ALL were positive for MPO (Dako, Denmark) and Arber *et al*<sup>7</sup> in 2001 had documented also 23% of adult ALL demonstrated evidence of MPO immunoreactivity with polyclonal antibody and these cases were precursor B-cell lineage with CD13 or CD15 expression, and a subset of these MPO positive cases were persistently positive with monoclonal antibody anti-MPO.

This positive anti-MPO (Dako) staining in ALL cases had prompt us to associate the MPO positivity with the aberrant myeloid antigens expression in these cases. As reported by Nakase *et al*, all their cases of MPO-positive B-lineage ALL and T-ALL were in children less than 10 years. 10/29 cases (34.5%) of ALL expressed myeloid antigens. Only 2 of 10 (20 %) ALL cases expressing myeloid antigens and 5 of 19

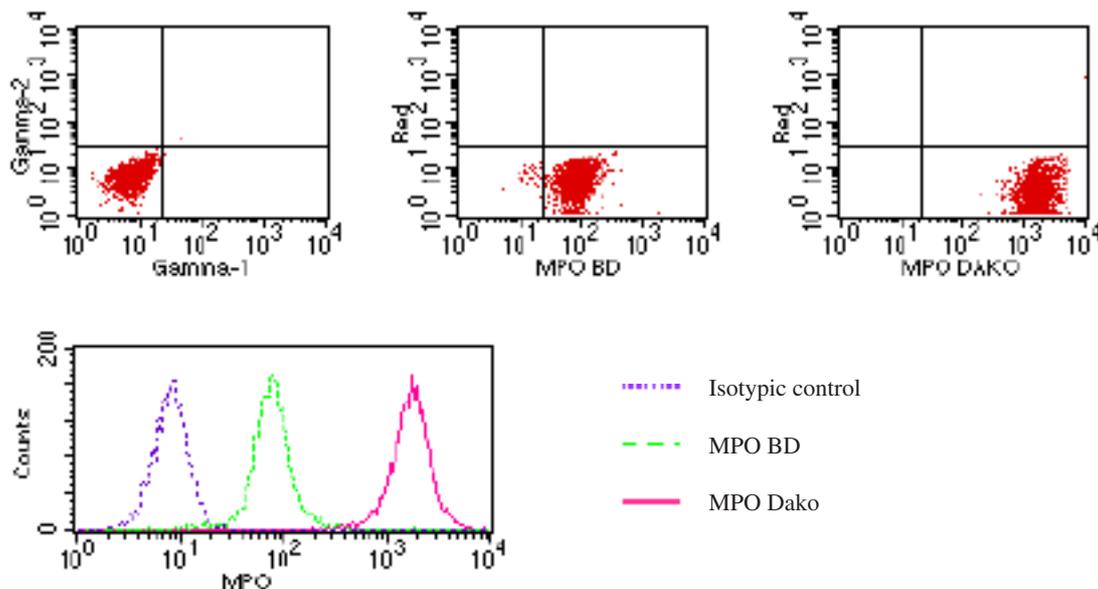


FIG. 1: Dot plots and histogram of the AML blast cells stained positive by anti-MPO (BD) and (Dako) when compared with the isotypic control.

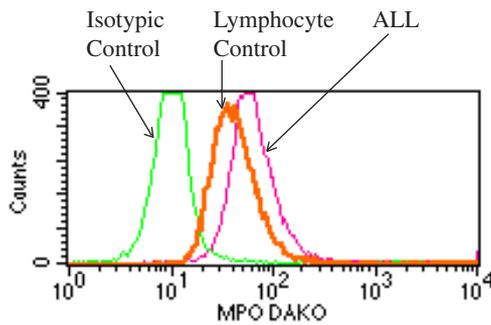


FIG. 2a: A case of ALL showing false positive staining for anti-MPO (Dako) that was corrected by regating using normal lymphocyte as control

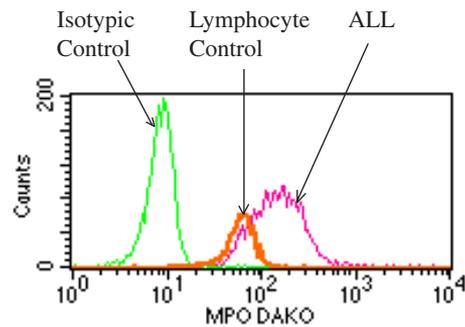


FIG. 2b: Histograms of ALL case that was persistently stained positive for anti-MPO (Dako) using either isotypic controls or normal lymphocytes control

(26 %) ALL cases lacking myeloid antigens were MPO-positive, indicating no significant relationship between the expression of MPO and myeloid antigens (CD13 and / or CD33). In our study, all of our 12 patients with positive reaction to anti-MPO (Dako) were < 12 years old which was comparable with the findings by Nakase et al<sup>6</sup>. However, our patients had a much higher percentage (63.2%) of aberrant myeloid antigens expression. 33.3% of those patients with myeloid antigen expression and 28.6% of those without myeloid antigen expression showed positive reaction to anti-MPO (Dako). Thus, we agree with the previous author that there is no significant relationship between the aberrant expression of myeloid antigen and positive reaction to anti-MPO in these ALL cases.

In our study of the 25 cases of acute myeloblastic leukaemia, concordance results of 92% with positive reaction to both anti-MPO by Dako and BD were documented. There was no problem in detecting myeloperoxidase expression in acute myeloblastic leukaemia by the monoclonal antibodies from both Dako and BD.

The results of our study suggested that there is a difference in the results obtained for immunochemical myeloperoxidase by monoclonal antibodies from different sources i.e Dako and Becton Dickinson in cases of acute lymphoblastic leukaemia. Monoclonal antibody anti-MPO by Dako has shown high false positive results when isotypic controls were used. This may be due to non-specific background staining and the results were improved with the use of normal lymphocytes as control. In the comparison study done with immunochemical myeloperoxidase by anti-MPO (BD), all ALL

cases were uniformly negative. Therefore, the use of anti-MPO (Dako) for evaluation of acute leukaemia cases needs careful evaluation before being used routinely in the laboratory, as falsely positive anti-MPO result may lead to significant alteration in patient management and outcome.

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