Flow cytometric analysis of intracellular myeloperoxidase distinguishes lymphocytes, monocytes and granulocytes

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Abstract

A study was undertaken to evaluate the ability of flow cytometric analysis of intracellular myeloperoxidase (MPO) in differentiating populations of lymphocytes (L), monocytes (M) and granulocytes (G), by means of lysed whole blood method. Anticoagulated blood from 23 normal individuals was lysed with FACS lysing solution and permeabilized with FACS permeabilizing solution before subjected to direct immunofluorescence staining. The geometric means of the fluorescence intensity were measured using FACSCalibur flow cytometer (Becton Dickinson). Populations of L, M and G were gated based on their light scatter characteristics and expression of CD14 and CD45. Then, the fluorescence intensity of MPO expression was studied in these individual cell populations. The results showed that fluorescence intensity of MPO was the strongest in G and weakest in L, whereas M showed intermediate fluorescence intensity. Our findings reveal that discrimination of these three cell types is achievable based upon the sole expression of intracellular MPO.

Key words: lysed whole blood, fixation, permeabilization, intracellular antigens (iAg), myeloperoxidase.

INTRODUCTION

Flow cytometry allows for the simultaneous measurement of multiple correlated parameters on a single cell. The introduction of flow cytometry has improved the analysis of cell surface antigen expression.' Three major populations of normal leukocytes – lymphocytes (L), monocytes (M) and granulocytes (G) – can be analyzed separately by flow cytometry. The size and granularity of the cells result in different kinds of forward (FSC) and side (SSC) scatter properties. Thus, combining the parameters CD14, CD45, FSC and SSC can produce distinct populations of cells on the dot plot.

Myeloperoxidase (MPO) is an azurophilic (pink-blue) granule-associated protein[2] (lysosomal enzyme) localized in the cytoplasm of neutrophilic, eosinophilic and monocytic lineage, but not in lymphocytes. It is widely agreed that MPO is a specific enbyme of myeloid cells which is synthesized in the nuclear membrane and ER of myeloblasts and packaged in the Golgi vesicles and primary (azurophilic) granules.[3] MPO is one of the most abundant proteins in the mature granulocyte, accounting for up to 5% of the dry weight of the cell.[4] It catalyzes the production of hypochlorous acid, a potent microbizidial agent.[5]

Recent advances leading to the development of monoclonal antibodies (McAb) against MPO made it possible to detect not only the active enzyme but also the inactive proenzyme form of MPO, which is undetectable by the conventional cytochemical method.[6] Lately, flow cytometric detection of cytoplasmic antigens has been improved by the development of new fixation and permeabilization reagents.' However, most of the methods are incompatible with whole blood and require isolation of mononuclear cells (blood leukocytes). Cell losses often occurred during this procedure, apparently due to repeated cell washes and centrifugation.[8] In this study, we performed cytoplasmic MPO direct immunofluorescence labeling by means of lysed whole blood method to evaluate the ability of a single parameter intracellular MPO in differentiating populations of lymphocytes, monocytes and granulocytes.

MATERIALS AND METHODS

Subjects

Peripheral blood (PB) from 23 healthy adult

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subjects (14 male, 9 female) were collected in ethylenediaminetetraacetic acid (EDTA) anticoagulated Vacutainer tubes (Becton **Dickinson,** BD). Samples were held at room temperature and processed within six hours of drawing.

Lysis of Red Blood Cells

Whole blood lysis samples were prepared from 100 μl aliquots of normal PB with 2 **ml 1X** FACS lysing solution (BD) and incubated at room temperature for 10 minutes in 12 **x** 75 mm Falcon tubes (BD). The cells were then pelleted for further treatment by centrifuging at 500g for 5 minutes.

Fixation and Permeabilization

The above cell pellet was subjected to fixation and permeabilization by resuspending in 500 μl 1X FACS permeabilizing solution (BD) and vortexing gently. Following incubation for 10 minutes at room temperature in the dark, the cells were washed twice by centrifuging at 500g for 5 minutes in 1 **ml** phosphate buffered saline (PBS) pH7.4 supplemented with 0.5% bovine serum albumin (Sigma) and 0.1% sodium azide (Sigma).

Intracellular Staining

Cytoplasmic labeling was performed by further incubating the fixed cell pellet with 10 μl of the appropriate fluorochrome-conjugated McAb for 30 minutes at room temperature in the dark: CD14/CD45 (Leucogate, BD) and MPO (MPO-

7, Dako). Mouse **IgG** isotypic controls, IgG_1 (BD) was used as negative controls for CD45 and **MPO** whereas IgG_2 (BD) acted as anegative control for **CD14,** to detect nonspecific background fluorescence. Following incubation, cells were washed again twice in the same buffer and resuspended in 1% paraformaldehyde in **1X** PBS.

Flow Cytometric Analysis

Stained cells were applied to the modular **benchtop FACSCalibur** flow cytometer (BD) equipped with a 15 **mW** air-cooled 488 nm argon-ion laser. Data acquisition and analysis were performed using **SimulSET** (version 3.1) and **CELLQuest** (version 3.0) softwares. A total event of 15 000 cells were acquired for each sample. The L, M and G were **gated** according to their light scatter characteristics, i.e. forward *vs.* side-angle scatter. Data were expressed as geometric means of fluorescence intensity and as a ratio between the fluorescence emission of sample cells and that of the isotypic control (**P/N** ratio; positive/negative).

RESULTS

Table 1 shows the mean values of the geometric means of fluorescence intensity and the **P/N** ratios for the three cell **types,** that calculated as described by Lanza et. *al.*[9] Intracellular CD14 staining revealed that CD14 was most abundantly expressed in M whereas G were dimly stained (Figure 1, A). Therefore, CD14 expression is not restricted to M, but can also be detected in

TABLE 1: Flow cytometric analysis of intracellular CD14, CD45 and MPO in blood cells from 23 healthy individuals.

Parameter	Cell Type		
	Lymphocytes	Monocytes	Granulocytes
Fluorescence intensity			
(Geometric means)			
Ig G_1	3.49±0.49	6.77±1.11	10.33±4.15
Ig G_2	5.44k1.62	15.71±8.94	13.56±6.07
CD14	2.89±1.03	998.59f881.46	19.97f12.91
CD45	383.19±147.17	291.89±56.92	248.85±122.76
MPO	43.6M24.74	958.19f512.56	3841.97±2782.59
P/N Ratios			
CD14	0.53	63.56	1.47
CD45	109.76	43.13	24.09
MPO	12.52	141.58	371.91

G. The P/N ratio of CD14 expression allowed differentiation of M from L and G, but this was not achievable between L and G. The P/N ratio of CD45 expression enabled the differentiation of L from M and G, but not M from G (Table 1). In practice, employing a histogram plot (Figure 1, B), these three cell types are visually undistinguishable. Nevertheless, by combining CD14 and CD45 expression and light scatter characteristics of the cells, populations of L, M and G can be reliably distinguished and gated.

It was also observed that cells with characteristics of G represent the strongest fluorescence intensity for intracellular MPO. Meanwhile, intermediate fluorescence intensity was shown by cells with characteristics typical for M. On the contrary, L were shown to show low fluorescence intensity. These findings were supported by visually investigating the histogram plot (Figure 1, C). Hence, discrimination among these cell types are achievable based upon the sole expression of MPO antigen.

DISCUSSION

Immunoassays using fluorochrome-conjugated McAb to characterize cells have been facilitated by the institution of flow cytometry. However, the methods of cell permeabilization which would permit the detection of intracellular molecules have usually been unsatisfactory. Treatment of cells with various fixatives and detergents has led to morphologic damage, preferential cell loss, cell aggregation or loss of intracellular antigenicity[10]. Techniques have been described that are suitable for *i*Ag labeling
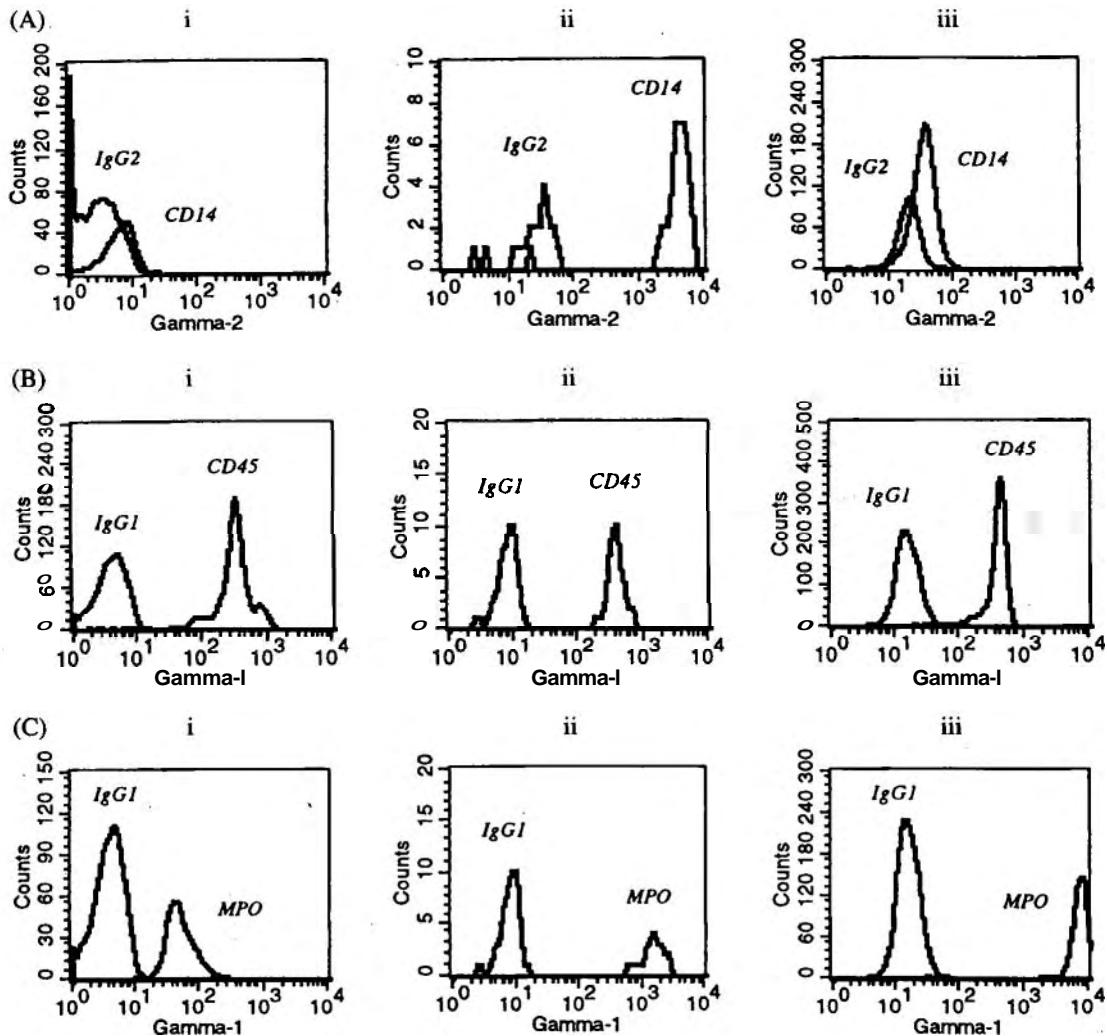

FIG. 1: Fluorescence intensity of (A) CD14, (B) CD45 and (C) MPO expression in (i) lymphocyte, (ii) monocyte and (iii) granulocyte populations.

without altering surface antigen (*s*Ag) expression, employing lysolecithin,[11] buffered formaldehyde acetone,[12] saponin,[13] digitonin,[14] paraformaldehyde and ethanol[15] in the fixation and permeabilization procedures. Hence, formaldehyde plays an important role to preserve *s*Ag for subset evaluation of *i*Ag. Meanwhile, diethylene glycol induces lysis of the red cells and mild permeabilization of the white cells.

We observed that even if the leukocytes were only labeled after penneabilizaiton for *i*Ag without prior *s*Ag labeling, the L, M and G populations still could be analyzed separately by utilizing the FSC/SSC properties of the cells. However, the fixed cells appeared shrunken and the threshold for FSC had to be reduced to accommodate the alteration.

The detection of MPO by flow cytometry with McAb contributes to the precise diagnosis of acute myeloid leukemia in otherwise unclassifiable cases, and thus substantially to the development of effective therapy and cure.[16,17] Nevertheless, before incorporating anti-MP0 in the panel for acute leukemia, the cut-off value has to be established in the respective flow cytometry laboratories to eliminate the effect of high background staining and enable precise interpretation of results.

In this study, a clear-cut P/N ratio for intracellular MPO was observed in L (12.51), which is a cell type typically devoid of myeloid antigens, whereas strong positivity was shown in M and G. Therefore, those cell samples characterized by a P/N ratio greater than 12.51 can be regarded as MPO positive, while cell populations possess a P/N ratio below 12.51 are considered to be MPO negative.

By applying a rapid and simple lysed whole blood method for intracellular staining without rendering alterations in cell morphology and antigenicity, anti-MP0 can be an added value in identifying myeloid cells which are not detectable by other methods or are negative with cytochemical methods.

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